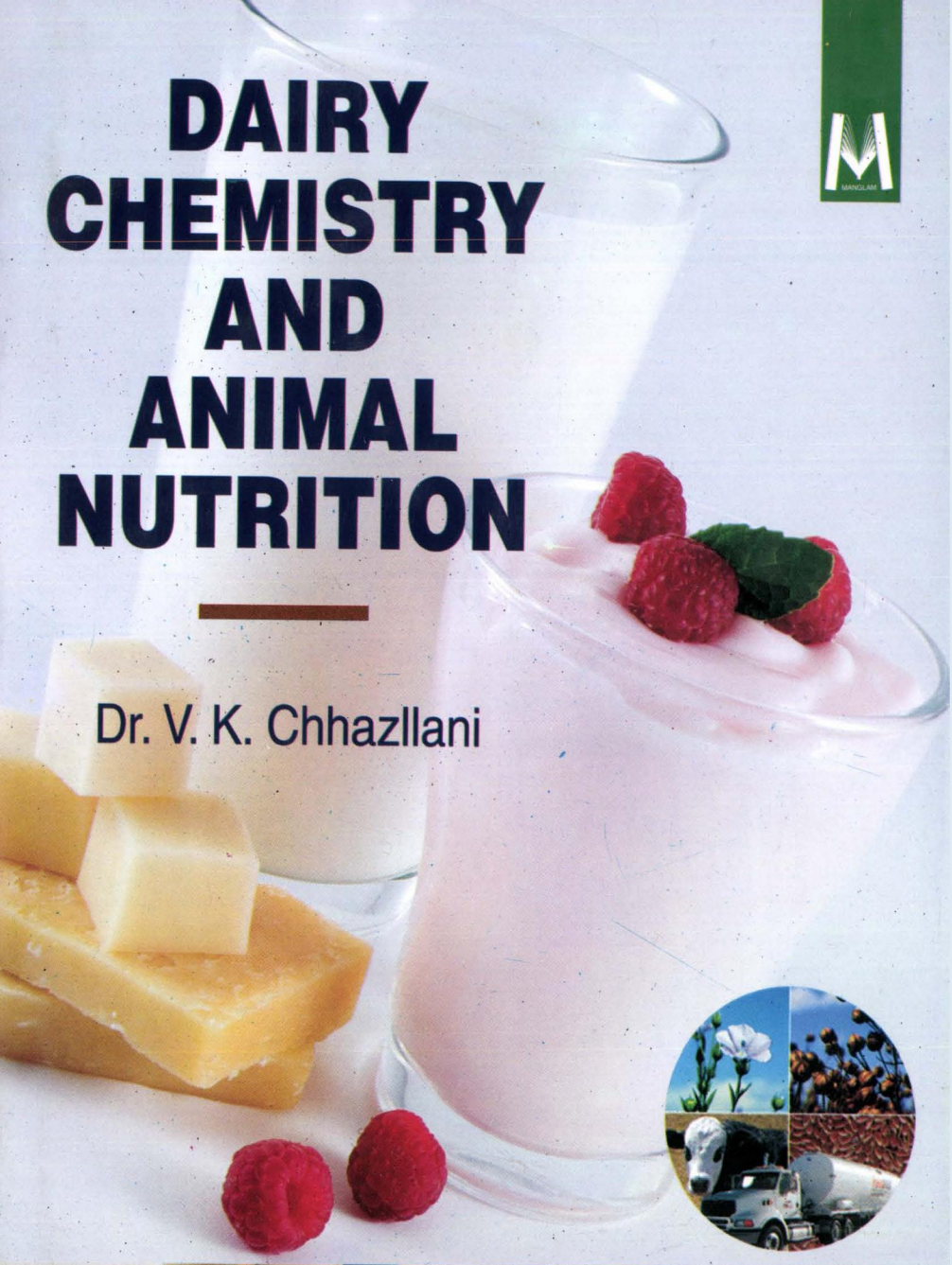
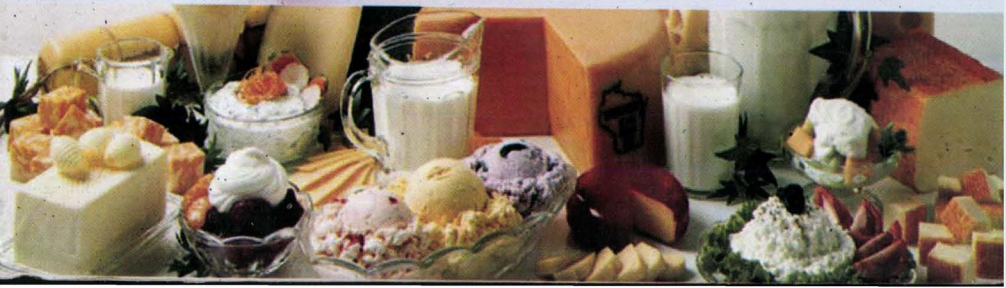
DAIRY CHEMISTRY AND ANIMAL NUTRITION

Dr. V. K. Chhazllani





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Dairy Chemistry And Animal Nutriation

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Preface

The present title presents a thorough and accessible account of Dairy Chemistry and Animal Nutrition has undergone remarkable development in the last there decades. These developments, along with recent widespread application of the newest biomedical technologies, have inbued optimism that new strategies can be developbed for controlling the important diseases that for centuries have been the scourages of mankind, The present text offers a compredium of reviews of the most active areas of research in biochemistry, medicine and related fields of biological sciences. By intent, the chapters are not exhaustive reviews. Special attention has been given to improved and upto date methodologies and techniques which make this work indispensable for the biological research workers.

To make the work more comprehensive and informative, the author has consulted many authoritative books, research journals, abstracts, monographs etc. He is grateful to all those great scholars whose work are cited or substantially reproduced.

There can be no claim to originality except in the manner of treatment and much of the information has been obtained from the books and scientific journals available in the different libraries.

The author expresses his thanks to his friends and colleagues whose continue inspirations have initiated him to bring out this book.

In the mean time, the author will remain sincerely responsible for any shortcomings of the book and be grateful to the readers for their suggestions and constructive criticism for the continuous betterment of the book, He takes this opportunity to appeal to the readers to send their suggestions straightaway to his Publisher.

The author expresses his gratitude to Manglam Publications for their whole hearted co-operation in the publication of this book.

Dr. V.K. Chhazllani

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Milk and Milk Products

Microbiological testing in the dairy plant is critical to ensure that raw milk, other ingredients, and finished products are of high quality. Such testing also serves to verify the adequacy of Hazard Analysis Critical Control Point (HACCP) procedures. Testing for pathogens is normally not done in the dairy plant, but samples are sent to a laboratory located far enough from the plant to preclude introduction of unwanted microorganisms through manipulations in the laboratory.

This chapter lists the chemical, microbiological, and physical tests that might be done on incoming raw milk and considers the specific microbial aspects of raw milk quality. Also discussed are testing of raw milk and raw ingredients, line sampling, and tests for predicting shelf life of products, testing of various types of dairy products, and the future of testing of milk and milk products.

1.1 RAW MILK QUALITY

There are many ways to measure the quality of raw milk. Some of the tests that are done by dairy processing plants either before or after unloading a tanker of milk include the following:

1. Standard plate count (SPC)
2. Direct microscopic count (DMC)
3. Freezing point determination (cryoscope)
4. Presence of inhibitory substances (antibiotic screening test)
5. Sensory evaluation
6. Preliminary incubation (PI–SPC)
7. Direct microscopic somatic cell count (DMSCC)
8. Acid degree value (ADV)

9. Laboratory pasteurization count (LPC)
10. Thermoduric spore count
11. Fat content
12. Total solids content (can also include protein content)
13. Sediment test
14. Presence of aflatoxins
15. Temperature

In addition, the weight (total quantity of milk) of the tanker is obtained to ensure proper payment to dairy farmers and to ensure that the processing plant is receiving all the milk for which it is making payment. However, compositional and chemical quality factors are always important.

Some of the aforementioned tests should be done before unloading the tanker. There is a definite time restraint involved with receiving and unloading a tank load of milk; however, the processor, not the producer, is the customer and should take a reasonable amount of time to obtain satisfactory results from the tests selected. It is recommended that the following tests be done on each tanker load of raw milk before unloading: DMC (until a more definitive test can be done in the same amount of time—bioluminescence may be this test), antibiotic screening test, cryoscope for added water, temperature, and sensory evaluation, which should involve checking the odor of the tanker followed by heating the milk and rapid cooling to taste the sample.

Compositional tests (e.g., tests for fat and total solids) should be done on every tanker of milk, although not necessarily before unloading. If the tanker load of milk is from independent producers, tests for abnormal milk, such as DMSCC, are also needed. Most other tests can be used as troubleshooting tests if there is a shelf life problem.

Some tests are good for troubleshooting purposes. If shelf life problems are of concern, the first step would be to verify the quality of the raw milk. An example would be to use the laboratory pasteurization count (LPC) as a way of determining whether or not there are a significant number of thermoduric bacteria present. As a general rule, if the LPC exceeds 500 cfu/mL, a major thermoduric problem exists in the raw milk supply.

Another problem which still occurs is that of "ropy" milk. *Alcaligenes viscolactis* is considered to be the primary cause of this

defect. Other bacteria can cause varying degrees of ropiness in milk. This particular defect is extremely unpleasant to the consumer and must be detected and prevented by the processor. The major cause of ropiness is improperly cleaned equipment at the dairy farm. This can either be in the milking parlor or in the bulk storage tank. Most of the bacteria causing ropiness are gram negative and are destroyed by pasteurization; however, just as we have concerns with cross contamination (from raw to pasteurized/packaging area) with *Listeria monocytogenes* and other potential milkborne pathogens, if *Alcaligenes* gets into the plant, a major problem can result.

Although the flavor of ropy milk normally is not distinguishable from normal milk, the long threads, or rope, can be pronounced and unforgettable. Johnson described a procedure for testing for ropy milk (if ropiness is a problem, raw milk from every raw tanker should be tested).

1. Incubate sample at 15.5–18.3°C (60–65°F) for 12–24 h. Temperatures as high as 21°C (70°F) may be used, but interference from acid-producing bacteria may be experienced.
2. Following incubation, insert a needle (match stick, small-bore pipette, etc., will do) at several locations on the surface, and slowly withdraw it.
3. Any strings 1/4-inch or longer would be considered to be a positive test for ropiness.

The number of dairy farms has been decreasing steadily to the point where most of the dairy farmers in business (just as with the processors) take their jobs very seriously. As a result, the quality of raw milk is very good. This is not to imply that all raw milk is of excellent quality and cannot be improved. In 1982, Zall et al. summarized results of the SPC, psychrotrophic bacteria count (PBC), and ADV tests of raw milk held at 6.7°C for 0, 3, or 6 days. Asummary of their results follows:

	Day of storage at 6.7°C		
Test (mean)	*0*	*3*	*6*
SPC	4.92	7.36	8.39
PBC	4.45	6.77	8.46
ADV	0.80	1.38	4.89

The above data indicate the practical importance of the legal limit of holding raw milk no more than 72 h. At 3 days' storage,

the PBC had increased to a level which produces significant amounts of heat-stable proteases and lipases. This occurrence can be especially damaging to cheese processors. In addition, the ADV had increased to a point at which rancidity could be detected. This rancid flavor cannot be eliminated; rather the intensity continues to increase. Attempts to camouflage this off-flavor are futile; if the milk with a high ADV was to be added to chocolate ice cream mix, the resulting chocolate ice cream would have a rancid flavor.

There was a substantial increase in bacterial numbers regardless of type whether mesophilic or psychrotrophic. The legal SPC standard for raw milk is 100,000 cfu/mL (individual producer) or 300,000 cfu/mL (commingled) milk. Individual raw milk can consistently be produced with less than 10,000 cfu/mL. Counts in tanker loads of milk vary from less than 10,000 to greater than 1,000,000 cfu/mL. The count in most raw milk (tanker loads) currently being received at fluid milk plants in the United States ranges from 30,000 to 70,000 cfu/mL.

The changes in the standard for the DMSCC from 1,000,000 to 750,000 cells/mL indicate an improvement in raw milk quality. Although there is no rule about increased bacterial numbers with increased somatic cell counts, this correlation does appear to exist. Within the next few years, it is likely that this standard will be reduced even further; for example, to 500,000/mL.

1.1.1 Raw Milk Microflora

According to one recent study, gram-positive bacteria are present in raw milk in much smaller numbers than gram-negative species. These workers reported on numbers of *Pseudomonas* as well as other gram-negative and gram-positive bacteria in both farm bulk tanks and in creamery and plant silos. In farm bulk tanks, regardless of temperature, pseudomonads represented more than 80% of all bacterial isolates. The gram-positive bacteria in milk at the farm bulk tank in this study represented no more than 1% of the total. When the milk was commingled in creamery silos, the pseudomonads represented approximately 70% of the microflora. The gram-positive bacteria increased to 9.0% to 14.1%, depending on temperature. Members of the family Enterobacteriaceae represented up to 15% of the total microflora of milk in the creamery silos.

Celestino et al. (1996) made a most significant conclusion: "As the quality of pasteurized milk improves because of reduction in levels of postpasteurization contamination, the presence of a heat

resistant psychrotrophic bacteria in the milk supply will assume greater importance." Of these, spore-forming microorganisms such as *Bacillus* are the most important. Work in Griffiths' laboratory was reported by these researchers, which indicated that higher heat treatments applied to the milk (70°C rather than 60°C) tended to decrease spore counts, presumably because of the activation of spores, which could subsequently germinate and divide. Using this as evidence, they cautioned that an increase in pasteurization temperature does not necessarily result in an increased shelf life. This has been the tendency of many processors over the past 15 years (since *Listeria* and *Salmonella* became known to the dairy industry).

1.1.2 Spore Formers

It might be concluded that the higher the quality of raw milk, the higher will be the incidence of gram-positive spore-forming bacteria. According to Martin (1974), *Bacillus* species account for 95% of the total spore-forming bacteria in milk, with *Clostridium* species comprising the remaining 5%. He indicated that, in the United States, 43% of *Bacillus* organisms are *B. licheniformis* and 37% are *B. cereus*; however, in other countries, *B. cereus* is predominant. The data indicate that spore-forming bacteria are expected to be present in almost all raw milk supplies. As the dairy processing industry becomes more involved with extended shelf life (ESL) products, the problem with spore-forming bacilli will probably increase. Thus, an aerobic spore count (80°C for 12 min followed by rapid cooling and plating on plate count agar (PCA) with incubation of plates at 32°C for 48 hours) will become a vital microbiological test for raw milk.

1.1.3 Psychrotrophic Bacteria

A simple definition of psychrotrophic bacteria is those bacteria that can grow fairly rapidly at refrigeration temperatures. A psychrotroph is unlike a true psychrophile, which is a bacterium whose optimal growth temperature is 10°C or less. There are not many psychrophiles encountered in the dairy industry. In raw milk, the larger the percentage of psychrotrophic bacteria, the greater the number of problems encountered by the dairy processor using such raw milk. A typical psychrotroph (e.g., a pseudomonad) could conservatively have a generation time (the length of time a bacterial population requires to double in numbers) of 9 h or less at 7°C. Thus, if a load of milk contains 100,000 cfu/mL with 70% of the

microflora being psychrotrophic, then, within 36 h at 7°C, the counts could exceed 1,000,000 cfu/mL. This large number can produce large amounts of proteases and lipases, which can cause serious quality problems for processed products.

In a dated but excellent review of psychrophilic bacteria, Witter (1961) indicated that the choice of the word *psychrophile* was unfortunate, because the root name implied "cold-loving." Many people still use the term *psychrophile* when *psychrotroph* is what is intended. The key to recognizing the difference is in the optimal growth temperature range. Psychrotrophs have an optimal growth temperature in the range of 21°C to 28°C, whereas, as previously discussed, a true psychrophile has a much lower optimal growth temperature. Most of the bacteria that cause problems to the dairy processor are of the psychrotrophic type, which means that, as the temperature is allowed to increase, the generation time is reduced and more psychrotrophs are produced.

Witter (1961) indicated that the natural sources of the predominant psychrophilic (psychrotrophic) bacteria are water and soil. Because water and soil are both present in abundance on dairy farms, it is not surprising to find that these psychrotrophs work their way into the milk supply. Hence, it is incumbent upon all segments of the dairy industry to work at keeping equipment clean (as a means of reducing the number of psychrotrophs gaining entrance into the milk) and temperatures as low as possible to retard growth of the psychrotrophs that do get into milk. Witter (1961) also indicated that, at the lower temperatures, from 7°C to 0°C (their minimum growth temperature), the decrease in growth rate was dramatic. Thus, even though the legal limit for holding milk is 7°C, the closer to 0°C that the milk can be held, the higher will be its quality from the standpoint of growth of psychrotrophic bacteria.

For the reasons just outlined, there is a need to monitor the psychrotrophic population of incoming raw milk. Most measurements are by SPC or DMC, both of which measure total bacterial numbers; those capable of growth at 32°C are measured by the SPC. The PI-SPC (milk is held at 13°C for 18 h before it is plated) is one way of estimating the psychrotrophic nature of the microflora. The milk could be incubated for 24–48 h and then plated (SPC). Regardless, it is very important for the dairy processor to have an idea of the psychrotrophic quality of the raw milk, particularly in cheese making. White and Marshall (1973) found that flavor scores

were significantly lower for Cheddar cheese made from milk containing a protease from a pseudomonad when compared with control cheese. Witter (1961) indicated that pseudomonads (the primary psychrotrophic/psychrophilic group) possess certain characteristics that make them important to milk and other foods. Some of these characteristics are (a) ability to use a wide variety of carbon compounds for energy and inability to use most carbohydrates, (b) ability to produce a variety of products that affect flavor, (c) ability to use simple nitrogenous foods, (d) ability to synthesize their own growth factors or vitamins, and (e) proteolytic and lipolytic activity.

Because a high psychrotrophic load can adversely affect the quality of various dairy products, especially cheese and extended–shelf life products, it behooves the processor to routinely monitor the psychrotrophic population of incoming loads of raw milk.

1.1.4 Proteases

Because psychrotrophic bacteria can produce both lipases and proteases, it is important to understand the activity of the various enzymes that can be liberated into the milk. Many of the proteases tend to be extremely heat stable, which can result in defects during extended refrigerated storage of milk. Adams et al. (1975) studied heat-resistant proteases produced in milk by psychrotrophic bacteria. They found all of the psychrotrophs obtained from raw milk produced proteases that survived at 149°C for 10 s. They reported that 70–90% of raw milk samples contained psychrotrophs capable of producing these heat-resistant proteases. White and Marshall (1973) reported on a heat-stable protease that retained 71% of its original activity after being heated at 71.4°C for 60 min. Also, the enzyme hydrolyzed milk protein at 4°C.

In another study, Adams et al. (1976) isolated 10 gram-negative psychrotrophs from raw milk that readily attacked raw milk proteins. They reported that κ- and β-casein were most susceptible to attack by these psychrotrophs, although they indicated that some of the isolates also attacked whey proteins. They further stated that the proteolysis did not require large populations of psychrotrophs; 10–20% decrease in κ-casein during 2 days at 5°C accompanied growth of one isolate to a population of only 10,000/mL. Guinot-Thomas et al. (1995) studied proteolysis of raw milk during storage at 4°C. They specifically looked at the effect of plasmin and microbial proteinases. Their study demonstrated the greater importance of microbial proteinases than of plasmin at this temperature. Also, they

reported that hydrolysis of caseins by microbial proteinases affected mainly the κ-casein fraction, colloidal calcium, and consequently casein micelles. They concluded that this effect will be noted even more as the number of psychrotrophs becomes higher. Rollema et al. (1989) compared different methods for detecting these bacterial proteolytic enzymes in milk. This was a study in which two fluorescamine assays, a trinitrobenzene sulfonic acid (TNBS) assay, an azocoll assay, a hide powder azure (HPA) assay, and an enzyme-linked immunosorbent assay (ELISA) were tested for their effectiveness in detection of proteolytic enzymes from six strains of psychrotrophic bacteria. These workers concluded that the TCA-soluble tyrosine and the thin-layer caseinate diffusion assay are too insensitive to be used for quality control of dairy products. They stressed that a good correlation between the proteolytic activity determined with an assay and the keeping quality of the product is a prerequisite for applicability of the assay for quality control of dairy products. Their preliminary study indicated that this requirement could be reasonably satisfied by the fluorescamine, TNBS, and azocoll assays.

1.2 MICROBIOLOGICAL TESTING OF RAW MILK AND RAW INGREDIENTS

1.2.1 Raw Milk

Because the microbiological quality of raw milk does not improve during storage, it is critical that the processor evaluate the raw milk to ensure that only high-quality milk is accepted. With regard to microorganisms, the following information must be known:

1.2.1.1 Total count or aerobic plate count

Classically, this is determined by the use of the SPC procedure. In legal matters concerning acceptability of an incoming tanker of milk or milk from an individual producer, the SPC is the standard to which other screening tests are compared.

1.2.1.2 DMC

In this procedure, as outlined in *Standard Methods for the Examination of Dairy Products*, results can be obtained within 15 min by a trained laboratory technician. Dead as well as living cells are counted, so the DMC should result in a slightly higher count than the SPC. The big advantage is that results may be obtained before milk is unloaded into the processing facility. This allows for much better microbiological control over incoming raw fluid dairy

products. The problem that many people encounter when initially using the DMC is that they try to be too "fine" with the results; for example, they may try to distinguish between a count of 40,000 and 45,000 instead of just using the DMC to detect the very high count loads. The DMC was not designed to reflect minor differences in numbers of bacteria; rather, in this instance, the test is strictly used to determine whether a tanker load of milk, cream, or condensed skim milk is of sufficiently high microbiological quality to be unloaded into the plant.

1.2.1.3 Psychrotrophic estimates

There are many types of bacteria in raw milk. It is critical to know what percentage of the population is of a psychrotrophic nature. The standard psychrotrophic bacteria count (PBC) requires incubation of the plate for 10 days at 7°C. This length of time is commercially unacceptable to determine the psychrotrophic population of raw milk. Various elevated incubation temperatures (e.g., incubation of plates at 18°C or 21°C using PCA) give an estimate of the psychrotrophic population. Incubating raw milk (cream or condensed skim milk) for 24–36 h at 7°C followed by SPC incubation also gives some idea as to the number of psychrotrophs present.

1.2.1.4 PI-SPC

Johns (1960) first described this method for evaluating raw milk quality. His method involved incubating raw milk at 12.8°C (55°F) for 18 h. Following this preliminary incubation, a conventional plate count was done. This method was thought to identify milk that had been subjected to less than desirable sanitary conditions at the farm level. Maxcy and Liewen (1989) found that preliminary incubation at the recommended temperature (12.8°C) did not have a selective effect for specific groups of microorganisms. Thus, apparently, the PI-SPC procedure is not extremely reliable as a means of evaluating raw milk quality. Certainly, the time involved for this procedure minimizes its effectiveness in screening raw milk supplies.

1.2.1.5 Coliforms

According to *Standard Methods for the Examination of Dairy Products*, coliforms are a group of bacteria that comprise all aerobic and facultatively anaerobic, gram-negative, non–spore-forming rods able to ferment lactose and produce acid and gas at 32°C or 35°C within 48 h. Typically, coliforms are used as a measure of sanitary conditions in the processing and packaging of pasteurized dairy

products. Coliforms are destroyed by pasteurization; hence, any coliforms found in the pasteurized product indicate postpasteurization contamination.

Coliforms may also be of value in checking raw milk. There is no legal standard for the numbers of coliforms that might be present in raw dairy ingredients. It is suggested that a value of 100 coliforms per milliliter be used as an initial screening tool for raw milk. The procedure used would be the same as that outlined in *Standard Methods*. As with pasteurized milk, coliforms are "indicator organisms." This simply means that if coliforms are present, conditions may be suitable for the presence of enteric pathogens, such as *Salmonella*.

1.2.1.6 Adenosine triphosphate bioluminescence assays

In an excellent overview of how ATP bioluminescence can be used in the food industry, Griffiths (1996) agrees with other researchers that these assays may be used successfully for determination of microbial loads in raw milk within 10 min. Griffiths (1996) described that the milk is incubated in the presence of a somatic cell-lysing agent and then filtered through a bacteria-retaining membrane. The microorganisms retained on the filter are then lysed with the lysate being assayed for ATP activity. He stressed that microbial populations down to 10^4 cfu/mL can be detected with a greater precision than with the SPC.

Griffiths (1996) described the work of Pahuski et al. (1991), which involved a "concentrating" reagent, Enliten, that clarifies milk and allows removal of microorganisms by centrifugation. These workers indicated that a combination of this treatment along with an ATP assay enabled detection of microbial levels down to 2×10^4 cfu/mL within 6–7 min.

1.2.2 Dairy Ingredients

Many dairy ingredients other than raw milk are received by dairy and food processing plants. Some of these products include nonfat dry milk, whey powder, whey protein concentrates and isolates, condensed skim milk, condensed whole milk, sweetened condensed skim milk, and whole milk, cream, and butter. These ingredients must also be tested to ensure their overall quality and that they meet established microbiological criteria. The SPC and the coliform count using violet red bile agar (VRBA) are outlined in *Standard Methods for the Examination of Dairy Products*. This

compilation of accepted methods is descriptive with regard to sampling and the quantity of ingredient required for appropriate analysis. Representative samples of each incoming batch should be tested to ensure acceptability. When receiving dried products, a statistically valid number of samples should be obtained. Various sampling procedures have been used by companies, with the military standard MIL-STD-105D being a well-accepted method for determining the number of samples to be taken. A rough approximation for sampling is based on the following formula (does not take into account degree of severity).

$$\text{Number of samples} = \frac{\sqrt{\text{batch size}}}{10}$$

The number of samples should be randomly drawn to ensure representative sampling and testing of the entire batch.

1.2.3 Nondairy Ingredients

Many ingredients other than dairy products are brought into dairy processing plants. Examples of such products include fruits, nuts, stabilizers, emulsifiers, fat replacers, sucrose and other sweeteners, and spices. The key to ensuring the quality of all ingredients, especially nondairy ingredients, lies with the requirement of a product specification sheet. Each supplier that provides products to a dairy processing plant should provide an individual product specification sheet for each item sold to that company. The specification sheet, which should be updated annually, should contain a description of the product as well as guidelines that the product must meet.

Microbiological testing should be outlined on the product specification sheet. This includes the type of tests to be done and either the method outlined or a reference to the procedure to be followed. The specification should ensure that ingredients have been tested for specified pathogens and are known to be "pathogen free." Again, the SPC and the coliform count are commonly used procedures in evaluating the quality of many of these ingredients. Counts are typically related to the grade of product being received. Samples must be obtained as soon as the products arrive so accurate and prompt microbial analysis can be accomplished.

The following is an outline of microbiological testing that should be done on incoming raw dairy ingredients and nondairy ingredients, as recommended by myself and H. E. Randolph.

Microbiological testing of raw milk

Test	*Suggested standard (cfu/mL)*
1. Direct microscope count—every tanker (before unloading)	200,000
2. Coliform (violet red bile agar)—every tanker (backtrack to individual producer if necessary)	100
3. Standard plate count (PCA)—silos daily	100,000
4. PI-SPC (18 h at 12.8°C)—silos daily (backtrack if necessary)	300,000

The PPC or the PI-SPC is especially critical for cheese operations, because the presence of proteases from psychrotrophic bacteria can adversely affect yield as well as quality of these concentrated products.

1.3 LINE SAMPLING/TESTING

One of the most important aspects of microbiological testing of milk and milk products is line sampling. If only the finished product is tested, then it is only known whether the finished product is "good" or "bad"; however, if the shelf life of the product is less than desirable, it is not known where the postpasteurization contamination occurred. To gain such information is the purpose of line sampling. In a fluid milk operation, line samples should be obtained at the following locations:

1. At or immediately after the high-temperature, short-time pasteurizer. This is done to ensure that neither the regenerative plates nor cooling plates have pinhole leaks.
2. Preceding pasteurized milk storage tanks. This verifies the cleanliness of the pasteurized milk lines leading from the pasteurizer to storage tanks.
3. Line sample leading from the pasteurized milk storage tanks. This is done to ensure cleanliness of the storage tank itself.
4. Immediately preceding entry of the milk into the separate fillers.

By checking each of these locations, postpasteurization contamination can be pinpointed.

Because most dairy processing plants have welded pipelines and do not disassemble all of their piping, the method for obtaining aseptic line samples becomes critical. One very efficient way of obtaining good samples is by use of the QMI Aseptic Sampler. The aseptic samplers are inserted into stainless steel elbows for ease of sample extraction. Even though virtually any size sample can be taken, a minimum of 50 mL and preferably 60 mL should be used.

There is a greater chance of detecting microorganisms that could be detrimental to product shelf life from a larger sample.

Regular grommets can also be inserted and then a syringe and needle can be used to extract samples of similar size. Samples in the syringes can be used for any number of microbiological evaluations. The primary bacterial types of concern in these samples are coliforms and psychrotrophic bacteria. To enhance enumeration of psychrotrophic bacteria, a step commonly used is to incubate the sample (in the syringe) at 21°C for 18 h. Following this preincubation, the sample can either be plated for SPC or for coliforms (VRBA). The preliminary incubation is not absolutely necessary, but it does enhance enumeration of any psychrotrophs or heat-injured coliforms that might be present. A SPC on the fresh milk is virtually meaningless. Thus, the different options to consider with regard to microbiological evaluation of line samples are: (a) fresh milk coliform count—VRBA, (b) PI-VRBA, (c) PI-SPC, and (d) PI plus any other selective media designed to enumerate psychrotrophic bacteria, such as PI + CVT (crystal violet tetrazolium agar).

After counts are obtained (counts should be viewed as the same as for any finished fluid product), gram stains of preparations from colonies on plates can be made to determine whether the microorganisms appearing in "spoiled" products are similar to those observed in line sampling. This can be a direct indication of the presence of bacteria that are reducing shelf life.

1.4 SHELF LIFE-PREDICTING TESTS FOR FLUID MILK-TYPE PRODUCTS AND ESTMATION OF ACTUAL PRODUCT SHELF LIFE

The term *shelf life* can be used interchangeably with the term *keeping quality*, which is defined as the time a product remains acceptable in flavor after packaging. The question then becomes, What is an acceptable shelf life for fluid milk products. Before answering this question, the temperature at which the product is held when shelf life testing is done must be specified. The temperature most commonly used is 7°C (45°F), which is chosen because it approximates the temperature of dairy cases in supermarkets and the home refrigerator. Also, as has previously been pointed out, in all shelf life prediction studies, the "potential" shelf life is actually what is being measured, because the experimental sample stored in a cooler in the laboratory is not subjected to the rigors of distribution and transportation.

Almost all tests that are designed to predict the shelf life of dairy products are based on detection of gram-negative psychrotrophic bacteria (especially the pseudomonads). These microorganisms cause most shelf life problems, especially in fluid milk and cottage cheese. Regardless of the method, the key to predicting the shelf life of milk and milk products is that the method must be rapid—reliable and meaningful results must be obtained within 72 h and ideally within 24 h.

In addition, results of tests to predict shelf life must be compared or correlated with the actual product shelf life. Thus, to determine whether or not a particular test to predict shelf life is effective, the actual product shelf life must be assessed. The actual product shelf life is determined by holding the samples at 7°C and testing them every day until an off-flavor develops. The shelf life is then estimated as the day the off-flavor developed minus 1. To minimize the number of times the container is opened and closed, the products do not need to be tasted until after day 10 (assuming that the product had a shelf life of 10 days or more). It is important in determining basic product shelf life to use the same container, because each filler head (on a gallon filler) can yield significantly different results. In selecting samples from a filler, it is good to rotate the samples obtained so that, over a given period, all filler heads can be sampled.

Correlation between the results of shelf life prediction and actual product shelf life at 7°C can be ranked using the following scale: excellent, >0.90; good, 0.80–0.89; fair, 0.70–0.79. Because of low initial numbers of bacteria in freshly pasteurized milk, most shelf life testing consists of preincubating the product (in its original container) at 21°C for 18 h followed by some rapid bacteria-detection method.

The Moseley Keeping Quality Test consists of incubating the finished product in its original carton at 7°C for 5–7 days followed by doing the SPC. This test has been used for many years by dairy processors as a way of evaluating the "staying power" of their products. The big drawback is the length of time required for results; that is, 7–9 days before actual counts are obtained. As newer tests to predict shelf life are developed, the tendency is for dairy processors using the Moseley Keeping Quality Test to correlate results of the new test with those of their regular test. This is not the way to evaluate a new test. The results of any test to predict shelf life should be correlated with actual product shelf life, not with the results of another test. Erroneous conclusions may be drawn. Thus,

the best testing protocol is a preliminary incubation of the product so any psychrotrophs present can be enumerated rapidly. Many time and temperature combinations have been evaluated, but the one set of conditions that seems to optimize outgrowth and enumeration of the psychrotrophs is incubation for 18 h at 21°C. Therefore, the preliminary incubation (PI) mentioned in the remainder of this chapter represents 18 h at 21°C.

Some of the proven methods to predict shelf life are as follows:

1. Moseley Keeping Quality Test.
2. PI plus various plating methods: PI + SPC (incubation of plates at 32°C for 48 h); PI + mPBC (incubation of plates at 21°C for 25 h) (mPBC = modified psychrotrophic bacteria count on PCA); PI + CVT (1 L of PCA containing 1 mL of a 0.1% crystal violet solution followed by sterilization, cooling, and addition of 2,3,5-triphenyl tetrazolium chloride [TTC]) (plates are incubated at 21°C for 48 h); PI + VRBA (incubation of plate at 32°C for 24 h).
3. Bioluminescence.
4. Catalase detection.
5. Limulus amoebocyte lysate (LAL) assay. This procedure involves detection of endotoxins produced specifically by gram-negative bacteria.
6. Impedance microbiology.
7. Dye reduction (HR1, HR2).
8. Reflectance colorimetry (the LABSMART). This is a tristimulus reflectance colorimeter that monitors dye pigment changes caused by microbial activity.

These methods reflect the most current information about the basics of shelf life prediction techniques. However, no one procedure is ideally suited for every plant application.

Bishop and White (1985) used PI + impedance detection time (IDT) to successfully predict the shelf life of fluid milk. For fluid milk products, the PI + IDT yielded the highest correlation ($r = 0.94$) between test result and actual product shelf life at 7°C. By comparison, the correlation obtained for the Moseley Keeping Quality Test was $r = 0.75$. Because of the 7–9 days required before results are available from the Moseley test and because fluid milk products have a shelf life of approximately 14–21 days at 7°C, there is no question which test would be of more value to the processor. Any of the tests discussed that can give results within 72 h are of more

value not only in predicting shelf life but also in controlling the sanitary operation of the plant. Fung (1994), in an excellent overview of rapid detection methods, described 10 attributes of an ideal rapid or automated microbiological assay system for food:

1. *Accuracy*: especially sensitive for false-negative results
2. *Speed*: accurate results within 4 h
3. *Cost*: designed for each application
4. *Acceptability*: must be "official"
5. *Simplicity*: ideally, "dip-stick" technology
6. *Training*: adequate for test or kit
7. *Reagents and supplies*: stability, consistency, availability
8. *Company reputation*: performance of product is critical
9. *Technical service*: rapid and thorough
10. *Space requirements*: should not take up a whole laboratory

Most of the tests discussed meet most of these criteria.

Another method is described by Bishop (1988) as the Virginia Tech Shelf-Life Method (VTSLM), which involves a preliminary incubation (21°C) followed by simple plating. He describes this method as being reliable, accurate, relatively rapid, economical, and familiar to laboratory personnel. He advocates aseptically transferring 10 mL of a pasteurized fluid milk product into a sterile test tube and incubating the tube and its contents at 21°C for 18 h. The sample is then mixed well and diluted 1:1000 with the diluted sample being plated on PCA and incubated at 21°C for 25–48 h. He indicated that this method provides an estimate of the growth potential of psychrotrophic bacteria that may be present in the sample. The time variation for the plate incubation indicates the difference between agar and 3M-Petrifilm methods. If PCA is used, add 50 ppm of a filter sterilized solution of 2,3,5-triphenyl tetrazolium chloride (TTC) to the melted and cooled (44–46°C) agar before pouring plates. Only the red colonies should be counted. Counts can then be extrapolated to indicate estimated shelf life. Shelf life categorization by VTSLM follows:

Petrifilm/agar count (cfu/plate)	*Total count (cfu/mL)*	*Estimated shelf life (days)*
≤1	≤1,000	≥14
1–200	1,000–200,000	10–14
≥200	≥200,000	≤10

By continuing to do the test to predict shelf life on a regular basis and reacting to the results, confidence can be instilled from quality assurance and production standpoints. Most spoilage of fluid milk–type products occurs from presence of pseudomonads and related gram-negative bacteria. The tests discussed tend to emphasize detection and enumeration of gram-negative rods.

Gutierrez et al. (1997) reported on generating monoclonal antibodies against live cells of *Pseudomonas fluorescens*, which were used in an indirect ELISA format to detect *Pseudomonas* spp. and related psychrotrophic bacteria in refrigerated milk. The researchers indicated that development of an ELISA technique using these specific antibodies would facilitate rapid screening of refrigerated milk for detection of high concentrations of bacterial cells. They reported a good correlation ($r = 0.96$) between the colony numbers of psychrotrophic bacteria from commercial milk samples maintained at 4°C by the SPC method and the ELISA technique. These authors stressed the advantages of the indirect ELISA technique as being its versatility, simplicity, and speed.

There is still somewhat of an art in predicting the shelf life of dairy products. Because there is no one perfect test for all needs, processors must carefully select the one or two tests that best fit into their overall quality assurance program. The key points regarding prediction of shelf life are as follows:

1. Know the actual potential shelf life of the products as measured at 7°C (45°C).
2. Select the test to predict shelf life that best fits the total program.
3. Routinely do the tests and develop a history, categorizing the results.
4. Ensure top management commitment to define a course of action in case product failure is projected by the tests.

1.5 MICROBIOLOGICAL TESTING OF MILK AND NONCULTURED PRODUCTS

1.5.1 Fluid Milk Products

Shelf life becomes critical for fluid milk products. Shelf life of pasteurized milk has been defined as the time between packaging and when the milk becomes unacceptable to consumers. Because the actual product shelf life is between 10 and 21 days at 7°C, rapid shelf life prediction tests, as discussed previously, become

critical. Dairy processors generally do a good job of cleaning and sanitizing; thus the number of contaminating bacteria (psychrotrophs) is so small that some form of preincubation is required to obtain numbers large enough for rapid detection tests to enumerate them.

For the reasons just stated, the following are recommended for microbiological testing of fluid milk–type products:

1.5.1.1 Estimation of coliforms

At a minimum, a coliform (VRBA) test should be done on representative samples of all fresh products. H. E. Randolph and I agree that a better test would be a "stress" coliform test wherein the product is incubated at 21°C for 18 hours followed by coliform estimation on VRBA. According to *Standard Methods for Examination of Dairy Products*, plates are incubated at 32°C and counted after 24 h of incubation. The PI-VRBA allows for outgrowth of heat-injured coliforms, which might not show up on a coliform count made directly on fresh products. Petrifilm or VRBA agar in regular Petri dishes may be used. Whereas VRBA agar is normally used for detection of coliforms, PI allows for detection of some psychrotrophic types that may be present.

1.5.1.2 Shelf life prediction tests

Any of the shelf life prediction tests discussed previously may be used. Specifically, it is recommended to use one of the following: PI + SPC (18 h at 21°C plus 48 h of plate incubation at 32°C); PI + CVT (product incubation for 18 h at 21°C followed by incubation of crystal violet tetrazolium agar for 48 h at 21°C); PI + other rapid detection methods, for example, PI + bioluminescence, PI + impedance detection, and PI + reflectance colorimetry. These other systems can be very effective and accurate in predicting shelf life. Because of the cost of some of the systems, it may be necessary to use them for more than one test, such as for raw milk evaluation, equipment cleanliness, and culture viability in addition to shelf-life prediction. Smithwell and Kailasapathy (1995) described a rapid test for detection of psychrotrophs wherein milk is mixed with a selective agent (benzalkonium chloride) and a bacterial indicator (tetrazolium salt) and incubated at 30°C. The researchers indicated that gram-positive bacterial growth is suppressed by the benzalkonium chloride, and they stipulated that, if gram-negative bacteria are present, they grow and multiply. Once the numbers reached approximately 10^7/mL, the tetrazolium salt is reduced and the color of the milk changes from white to red. This is similar to the HR1-HR2 test described

by H. E. Randolph. The authors caution that the time required for this color reaction to occur depends on the amount of milk examined and the level and activity of bacteria present. These reduction-type tests lack the sophistication of some of the other test methods, but they do have the major benefit of being visible so shelf life tests can be observed by plant employees. This increases the interest by plant personnel in the sanitary processing and packaging of their fluid milk products.

1.5.1.3 Sensory evaluation of representative samples of fresh product

Milk from all fillers and all labels should be tasted fresh. Samples can be combined to minimize the total number of samples that need to be discarded.

1.5.1.4 Sensory evaluation at end of shelf life

Samples need to be tasted at some point at or beyond the code date. This time can be extended as shelf life of the product improves. Many dairies express this type of evaluation in terms of a certain percentage of products that are good (or bad) after a certain number of days at 7°C (45°F). Ideally, 100% of the products would be good when evaluated at day 21. As a rule, the number of days in which 90% or more of the products remain good can be used. Thus, a dairy may start out testing after 10 days at 7°C until success is achieved on a continual basis in 90% or more products being good. Subsequently, the sensory evaluation may be gradually moved to anywhere from 14 to 21 days until continual success is noted. If new shelf life problems occur, evaluations may have to revert to a shorter time to achieve satisfactory results.

The quality of the raw milk, as discussed previously, is still a very important issue. Celestino et al. (1996) indicated that storage of bulk raw milk resulted in increased numbers of lipolytic and proteolytic bacteria. They found that, on the average, the number of psychrotrophs as a proportion of the total plate count increased from 47% to 80% after 2 days of storage at 4°C. Thus, finished product quality can definitely be affected if raw milk is stored for too long a time (legally no more than 72 h, ideally no more than 48 h).

1.5.2 Cottage Cheese—Noncultured Dressing

In evaluating the microbiological quality of cottage cheese, the places where cottage cheese could become contaminated (from a

keeping quality standpoint) must be considered. There are only three things that consistently cause shelf life problems to the cottage cheese industry:

1. *Wash water.* The wash water must have proper pH and chlorine level.
2. *Dressing.* The cream dressing, whether for full-fat, low-fat, or nonfat cottage cheese, affects the quality of the finished product. If the dressing contains many psychrotrophic bacteria, the desired shelf life will not be obtained. This is especially true in dressings to which no culture has been added.
3. *Packaging operation.* The blending of curds and dressing and filling of cottage cheese cartons constitute excellent opportunities during which psychrotrophic bacteria can gain entry into the finished product. Great care must be exercised to ensure that only cleaned and sanitized food contact surfaces are being used.

These three areas hold true whether the cottage cheese operation is very small with all operations other than packaging being handled within the cheese vat or whether the operation is large with separate washer coolers, blenders, and packaging machines. Thus, samples should routinely be taken to ensure the microbial quality of each of these areas. First, daily samples of the wash water should be obtained and plated for coliforms and psychrotrophic bacteria. Second, daily samples should be obtained of the dressing and tested to ensure microbiological quality. Again, both coliform and psychrotrophic testing should be done. Third, a statistically valid number of samples should be used to evaluate finished product quality.

In other words, cottage cheese with noncultured dressing should be handled very similarly to fluid milk products. If cultured dressing is used, the primary test to use is a coliform (VRBA) test on the fresh product.

With regard to how cottage cheese should be sampled, *Standard Methods for Examination of Dairy Products* prescribes the use of a sterile blender-container on a balance and tared to which 11 g of cottage cheese are added aseptically along with 99 mL of warmed (40–45°C), sterile 2% sodium citrate solution. The sample is blended for 2 min, after which the product is diluted (if needed) and plated. Also, a Stomacher might be used (11 g of sample and 99 mL of diluent) to blend the cheese sample.

Another method used by some dairies for microbiological examination of cottage cheese is simply plating the dressing found

in the container of finished product. This works for some freshly dressed cheeses, but many cheeses do not have enough dressing from which separate extractions can be made. In these instances, blending the cheese either in a sterile blender or in a Stomacher is necessary.

1.5.3 Frozen Dairy Desserts

The microbiological evaluation of frozen dairy desserts consists of two basic parts: (a) ingredients and mix samples and (b) finished product. Some of the ingredients used in ice cream that should be tested microbiologically include fluid dairy products, dry dairy products (especially nonfat dry milk and whey powder), fruits, nuts, colors, flavors, stabilizers, and emulsifiers.

Fruits and nuts may be weighed into wide-mouth containers (11-g portions should be used) to which 99 mL of dilution water is added. The mixture is soaked for 5 min, shaken vigorously, and plated. The recommended tests to be used for these type products are:

1. Coliform count on fresh samples.
2. Yeast and mold counts. Probably the most commonly used medium for yeast and mold counts is acidified potato dextrose agar. These plates must be incubated at 25°C for 5 days with counted plates having between 15 and 150 colonies.
3. SPC.

All fluid milk products, including fluid milk, cream, and condensed skim and whole milk, are plated as described previously.

Stabilizers and emulsifiers should be plated using 1 g in 99 mL of dilution water. The sample is shaken vigorously for 15 s and allowed to hydrate at 20–40°C for up to 20 min. The product is then plated for SPC and coliform count (VRBA).

For finished products, a statistically valid number of samples representing each type of product and each label change should be obtained. Finished product samples should be thawed at a temperature of up to 40°C for no more than 15 min. A coliform count on fresh product is a good indication of whether sanitary methods were used in processing and handling the mix and finished product. Psychrotrophs can also be a problem. White and Marshall (1973) indicated that heat-stable enzymes produced by typical psychrotrophs could cause a measurable effect on ice cream mix that approached significance from a sensory evaluation standpoint.

1.5.4 Butter

By definition, butter must contain at least 80% milk fat. It seems, then, that the microbiological quality of butter is not as critical as it is with other dairy products, yet microorganisms can and do survive and grow quite well in butter and related products. White and Marshall (1973) evaluated the effect of heat-stable proteases on several dairy products, including butter. They did not find any significant effect of the proteases. This is not surprising, because butter contains only about 1% protein. Microorganisms with high lipolytic activity would be expected to have a greater effect on high-fat products. *Standard Methods for Examination of Dairy Products* lists the following tests that can be done on butter or margarine-type products: SPC, coliform count (VRBA), proteolytic count, psychrotrophic count, lipolytic count, *Enterococcus* count, and yeast and mold counts. Furthermore, other authors recommend the following tests be done routinely in a creamery operation:

1. SPC (1:1000 dilution as recommended by *Standard Methods for Examination of Dairy Products* [SM]).
2. Coliform count (VRBA-1:2-1:10 dilution-SM).
3. Lipolytic count (1:100 dilution-SM).
4. Yeast and mold count (1:2-1:10 dilution-SM). Wilster (1957) recommended a standard of 50 yeast and molds per gram of melted butter. This seems high for present-day circumstances.

1.5.5 Dry Milk and Whey Products

Dry dairy ingredients are used in a wide variety of products, including other food products as well as dairy products. The quality of the finished products can be affected by the quality of these milk ingredients. Nonfat dry milk adds many desirable properties to dairy foods; however, these desirable properties are minimized when inferior powders are used. The same may be said of the use of sweet whey powder and especially the newer whey protein concentrates and whey protein isolates. These ingredients may be purchased in various amounts, but typically the product arrives in 40- to 50-lb bags or even in totes.

With regard to microbiological analyses, most dairies are performing the SPC and coliform count (VRBA). Three to 5 mL of agar overlay may be used on surfaces of solidified plates before incubation if spreading of colonies is a problem when these products are tested. With a dried product that has obviously been exposed to some heat treatment, the presence of spore-forming bacilli can be

common. Also, the DMC may be used to evaluate incoming samples of nonfat dry milk and whey products. Typically, this analysis is done by making a 1:10 dilution (11 g of product in 99 mL dilution water) of the sample before it is examined microscopically. *Standard Methods for Examination of Dairy Products* recommends the use of 2% sodium citrate solution in making these 1:10 dilutions if certain samples dissolve less readily. The reports would show as DMC/g of NDM or whey powder.

1.5.6 Ultra-High–Temperature Products

With a commercially sterile product, the presence of any microorganisms able to grow under conditions of product storage is considered detrimental to the shelf life of the product. Also, because the product normally is held at ambient temperatures, any slight contamination during the aseptic packaging process will damage the product.

Bockelmann (1989) indicated that, under current circumstances, the reject rate for ultra-high temperature (UHT)–type products is approximately 1 defective (unsterile) unit per 100,000 produced packages. To improve beyond this point, for example, to achieve a reject rate of 1/100,000,000, would be impossible because of construction limits of the equipment. He stated that for UHT plants in use at that time, sterilization effects of between 10 and 12 could be assumed. He said that of 10^{10}–10^{12} bacteria spores fed into the process, 1 spore would survive, and that the microbiological end result of such a process was dependent on (a) the sterilization effect of the UHT process and (b) the bacterial spore count in the raw product.

Thus, the number of bacterial spores present in raw milk is of definite importance when dealing with a "sterile" finished product. According to *Standard Methods for Examination of Dairy Products*, 200 mL of raw milk should be placed in a sterile Erlenmeyer flask with a screwcap lid. The milk should be heated to 80°C for 12 min and then cooled immediately in an ice bath and plated on PCA with added starch and plates incubated at 32°C for 48 h. Even though the plates could be incubated at 7°C for 10 days for psychrotrophic spore counts, the mesophilic spore count as just outlined should provide more meaningful information on UHT-type products.

For finished product testing, *Standard Methods for Examination of Dairy Products* recommends swabbing the outside surface of the finished product container with 70% alcohol. The needle of a sterile,

single-service hypodermic syringe should then be inserted through the package wall and the appropriate amount of sample removed. Because the product is thought to be sterile, precise measurements are normally not needed, because any contamination is considered bad.

Bockelmann (1989) used the sterilization effect and the maximum acceptable defect rate as a means of establishing the following proposed standard for spore counts in raw materials such as raw milk:

Standard spore count in raw materials (UHT sterilization effect: approximately 11; maximum acceptable defect rate: 1:1000)

No. surviving per milliliter	Aim	Action	Limit
10 min, 80°C	<100	–1,000	–10,000
10 min, 100°C	<10	–100	–1,000

With regard to packaging material for UHT products, Bockelmann (1989) indicated the infection rate resulting from the manufacturing process of these packaging materials to be insignificant (i.e., 0.5 microorganism/100 cm^2), about 3–5% of the bacteria were identified as *Bacillus* spores.

Bernard (1983) made several observations on some of the other microbiological considerations for testing aseptic processing and packaging systems. He indicated that, before establishing appropriate times, temperatures, and exposure concentrations to provide for commercial sterility, appropriate test organisms must be determined for each particular sterilization medium. Some of the test organisms for the different sterilization media are as follows:

Sterilization medium	*Bacillus* spp.
Superheated system	*B. stearothermophilus* (1518)
	B. polymyxa (PSO)
H_2O_2 and heat	*B. subtilis* strain A
H_2O_2 and UV	*B. subtilis* strain A

In addition to the sensory and physical/chemical testing done on UHT finished products, microbiological testing is also critical. Edwards (1983) indicated that SPCs and coliform counts, among other tests, of aseptically processed products done immediately after

packaging are ineffective as quality control procedures because of the extremely low number of viable organisms present in an unsterile container and due to the very low numbers of unsterile containers. He said that, to provide a more effective and more rapid method of detecting these low numbers of viable organisms, samples are typically incubated at an elevated temperature (e.g., 35°C [95°F]) to allow for rapid growth of most microorganisms that might be present. He stressed that incubation time may vary depending on product characteristics and types of tests to be used to detect nonsterility. It is necessary to incubate UHT samples at an elevated temperature (e.g., 35°C) for approximately 1–3 days. Even if bacteria have been substantially heat injured, this time-temperature combination allows for outgrowth of any survivors. Also, this combination facilitates early detection of enzymes, especially the proteases.

Edwards (1983) indicated that there are two types of samples that should be obtained: (a) aimed samples and (b) random or timed samples. Aimed samples are obtained when the risk of contamination is greater than during normal operations such as during start-up and splices. Evaluation consists of container integrity tests and product incubation. Random or timed samples are obtained during normal operation. Evaluation of these samples consists of container integrity tests, product incubation, and shelf life monitoring.

These samples should be obtained at different locations such as after the packaging machine, after the downstream equipment, and from the warehouse. If nonsterility is observed, resampling of the product should be done from that period, with evaluation by container integrity tests and product incubation. The defect rate in the aseptic processing and packaging systems, which Edwards (1983) said was the most common, was 1 in 10,000, and some of the sources of nonsterility were inadequate heat treatments of the product, inadequate equipment sterilization, contamination of equipment after sterilization, inadequate package sterilization, contamination of package after sterilization, faulty package material, nonhermetical seal, improper machine adjustment, damage from downstream equipment, and damage from handling and shipping.

1.6 MICROBIOLOGICAL TESTING OF CULTURED DAIRY PRODUCTS

In this discussion, cultured dairy products include cultured milk (buttermilk), cultured or acidified cottage cheese, cultured or acidified sour cream, and yogurt. A total count or SPC is not suitable for

measuring the microbiological quality of these products, because a viable bacterial culture has been added to each of them. Even for noncultured cottage cheese dressings, an SPC on fresh product is meaningless because of the low numbers of microorganisms present after pasteurization. Thus, the coliform count (VRBA) is the primary microbiological test that is used in evaluating cultured dairy products.

For any of the cultured milks (e.g., whole, low-fat, or skim buttermilk), the coliform count may be determined by plating 1:1 on VRBA. With regard to cottage cheese, ideally, the product should be blended in a sterile blender. *Standard Methods for Examination of Dairy Products* recommends the use of a sterile spatula to aseptically transfer 11 g of cottage cheese into the sterile blender, which had been preweighed. Then, 99 mL of warmed (40–45°C), sterile 2% sodium citrate solution is added. The product is then thoroughly mixed for 2 min. The product is then plated with 1 mL of the blended 1:10 dilution being transferred to a VRBA plate.

As discussed previously, an alternative method used by some dairy plant laboratories to test for the presence of coliforms in cottage cheese is simply to plate the dressing directly out of the cottage cheese carton. This can be somewhat difficult, especially if the cottage cheese is relatively dry. Blending yields more consistent results.

Goel et al. (1971) evaluated the duration that coliforms would survive in yogurt, buttermilk, sour cream, and cottage cheese during refrigerated storage. They noted a marked decrease in numbers of most coliforms tested in yogurt, buttermilk, and sour cream after 24 h of storage at 7.2°C. Hence, there is a definite need to test for the presence of coliforms in these type products within 24 h of manufacture and packaging. With cottage cheese, there was less of a decrease in numbers of coliforms than for the other cultured dairy products. Barber and Fram (1955) cautioned that coliform-like colonies on VRBA should be confirmed for yogurt and other products containing fruit or added sweetener.

Also, yeast and mold counts are done by some dairies on some of the cultured dairy products. These counts could be done on buttermilk, cottage cheese, or yogurt. Many times yogurt develops a yeast or mold problem as opposed to any bacterial-related shelf life–ending problems. *Standard Methods for Examination of Dairy Products* lists the following media to be used for yeast and mold enumerations: (a) acidified potato dextrose agar, (b) yeast extract–dextrose–chloramphenicol agar, and (c) dichloran–Rose Bengal–

chloramphenicol (DRBC) agar. In addition, Petrifilm provides a yeast and mold agar that is used by many dairy laboratories.

The most common flavor criticism of cottage cheese, sour cream, and buttermilk-type products is that they "lack flavor" or are "flat." Because the incubation time or temperature has not allowed the culture of bacteria to produce sufficient flavor, the resulting product tends to have a flat flavor. Because of this, the presence of any contaminating microorganisms, especially coliform, or psychrotroph-type bacteria, or yeast and molds, can cause relatively slight off-flavors to become more pronounced because of the absence of competing desirable flavor notes. Extreme effort should be made to enhance bacterial starter (e.g., acid and diacetyl) activity to the point where desirable flavors may be noted in products such as sour cream and buttermilk.

1.7 MICROBIOLOGICAL TESTING OF RIPENED CHEESES

Natural cheeses, regardless of variety, readily support growth of many microorganisms even though moisture content, salt content, pH, and other compositional factors vary from cheese to cheese. Cheeses may contain pathogenic bacteria (e.g., *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella*. This is the exception and not the rule, because cheese is a concentrated dairy product, and if all "make" procedures have been followed and good manufacturing practices adhered to, the probability of foodborne pathogens being present is remote. This is true of Cheddar cheese, for example, as long as the pH in the finished product is controlled (<5.3).

Standard Methods for Examination of Dairy Products recommends any one of three procedures to mix a cheese sample for subsequent microbiological analysis:

1. Transfer 11 g of cheese into 99 mL of sterile aqueous 2% sodium citrate at 40–45°C. The cheese is then blended for 2 minutes and plated either direct (1:10) or with further dilutions.
2. Weigh 1 ± 0.01 g into a presterilized 177-mL Whirl-Pak bag. The cheese is then macerated, after which 9 mL of 2% sodium citrate at 40°C is added. The bag is closed with the contents rolled and then plated.
3. Eleven grams of cheese and 99 mL of diluent are mixed in a Stomacher 400. The cheese is blended for 2 min then plated.

Microbiological tests that are done on hard cheese may vary

from one processor to another; however, the coliform count and the *Staphylococcus* count should be done. *Staphylococcus* counts are especially critical when there is an abnormally high pH value. It is recommended that a *Staphylococcus* count be automatically done on any Cheddar-type cheese with a pH greater than 5.2.

Interpretation of the coliform count is the same as for any dairy product, that is, a high count indicates unsanitary conditions involved in processing and packaging the product. As discussed previously, coliforms are "indicator organisms." This means that the occurrence of coliforms indicates that conditions are suitable for the presence of enteric pathogens. This does not mean that pathogens are definitely present but that the cheese was handled in a manner that allows enteric pathogens to be present. Coliforms are important indicators, and hence this test should not be ignored.

1.8 FUTURE OF MICROBIOLOGICAL TESTING OF DAIRY PRODUCTS

There is a tremendous amount of work being done regarding development of rapid detection methods for total numbers of both bacteria and specific organisms, primarily pathogens. Karwoski (1996) and Fung (1994, 1995) discussed different areas of research in food microbiology, and a summary follows of what these two investigators have reported:

1. Sample preparation: Two useful instruments in this area are the Stomacher and the Gravimetric Diluter.
2. Total viable cell count: Various alternatives include the following: Automated spiral plating method: Isogrid System (all colonies have square shape, reported to be easier to count; Petrifilm; Redigel System; and Direct epifluorescent filter technique (DEFT) slides read by systems such as the Bio-Fos Automated Microbiology System.
3. Differential cell count.
4. Pathogenic organisms.
5. Enzymes and toxins.
6. Metabolites and biomass.

In an article dealing with microbiological testing in the dairy industry, White (1996) summarized some of the methods that Fung had reviewed. Some of these methods are as follows:

1. Microbial ATP detection: Bioluminescence as a screening tool for accepting raw milk shows great promise. Reybroeck and

Schram (1995) outlined a test that took less than 6 min. They described this method as being very useful as a sensitive and rapid semiautomatic method for fast microbiological screening of raw milk on arrival at a dairy plant.

2. Impedance detection in foods.
3. Omnispec Bioactivity Monitor System: A tristimulus reflectance colorimeter monitors dye pigment changes caused by microbial activity. The LABSMART System highlights this use of reflectance colorimetry.
4. Catalase test: This test is very useful in detecting strongly catalase-positive bacteria, such as pseudomonads.
5. Many miniaturized diagnostic kits for identification of microorganisms.
6. Genetic techniques: DNA/RNA probes are a sensitive method for detection of pathogens (e.g., *Listeria* and *Salmonella* detection using The Gene-Trak Assay System). Sensitivity 1×10^5 organisms per milliliter broth. Wolcott (1991) indicated that polymerase chain reaction (PCR) has become the preferred method for amplifying DNA. This enables detection of target microorganisms in hours rather than days. This procedure has tremendous potential in all areas of food microbiology, including dairy microbiology. The BAX System for screening *Salmonella* is one example.
7. Enzyme-linked immunosorbent assay (ELISA), systems produced in the United States by Organon Teknika, use monoclonal antibodies as a diagnostic test, especially for foodborne pathogens. Development of the ELISA technique using monoclonal antibodies specific to *Pseudomonas* and related psychrotrophic bacteria as outlined by Gutierrez et al. (1997) shows great promise.
8. Vitek Immuno Diagnostic Assay System (VIDAS): A multiparametric immunoanalysis system that uses the enzyme-linked fluorescent immunoassay (ELFA) method. All intermediate steps are automated.

There continues to be a need for methods that can rapidly detect the presence of certain types of bacteria. Personnel at a dairy plant must be able to determine whether equipment is clean, to screen rapidly all incoming raw ingredients, and to predict rapidly (<24 h) the shelf life of finished products. By monitoring raw ingredients, monitoring the processing and packaging environment

and providing a more limited testing of finished products, a dairy processor becomes much more proactive in eliminating safety and quality hazards.

Other innovations such as addition of carbon dioxide to milk and other dairy products such as cottage cheese serve to extend the shelf life of the products. Certain questions have been raised that relate to packaging for such products (e.g., high-barrier films being required to retain the CO_2).

Thus, much has changed in the testing of milk and milk products by dairy processors. Environmental samples for pathogens are commonly being sent to commercial testing laboratories, more sophisticated equipment is being found in the laboratories, and many of the laboratories are becoming larger because of consolidation and takeovers of smaller operations. However, one significant fact cannot be forgotten: For the dairy industry continually to provide safe, long-lasting products to the American consumer, rapid, accurate, and reliable testing must be done. It is extremely important for management to react to the data provided by this testing. As confidence is gained by quality assurance personnel and production management, the American consumer will continue to receive dairy products that are as good and safe as products produced anywhere in the world.

2

Fermented Milk

Fermented milks result from the selective growth of specific bacteria in milk. These products have evolved around the world over thousands of years and are believed to have originated in the area that is now the Middle East. These products probably resulted from the need to extend the shelf life of milk in the absence of refrigeration. Storage of raw milk at ambient temperature probably led to growth of lactic acid and other bacteria. This bacterial activity produced desirable flavors, and, importantly, increased the shelf life of milk because of a high acid content. Procedures of fermented milk production were subsequently refined, the products became popular, and gradually spread to Asia, Europe, and other parts of the world. Consumption is now the highest in European countries, but these products form an important component of the diet in many other countries as well.

Today yogurt, buttermilk, and sour cream are probably the most widely consumed fermented milk products, but there are many different types of such products that are either manufactured commercially or produced on a small scale, and sometimes in homes, for local consumption. In addition to being excellent sources of nutrients, these products have become popular because of potential health benefits.

2.1 MICROORGANISMS USED TO MANUFACTURE FERMENT MILK

Microorganisms used to manufacture fermented milk primarily include those that can ferment lactose to lactic acid and may be either of the mesophilic or thermophilic type. Nomenclature for these

organisms has evolved over the years as a greater understanding of their genetics has been acquired. Pure strains of these organisms are readily available from commercial suppliers, but it is not uncommon, especially for small-scale manufacturers, to use product from a previous batch as culture for the next batch. In such instances, there is a potential for quality of the end-product to vary from batch to batch because of changes in culture characteristics that may occur over repeated transfers. This is especially evident in products that normally require a combination of organisms in a specific ratio, such as rods and cocci in a 1:1 ratio for yogurt. Over repeated transfers as a mixed culture, one of the species is likely to dominate and hence alter the characteristics of the fermentation and consequently flavor and texture qualities of the product.

In addition to lactic acid producers, other types of organisms may also be employed to impart desired flavor or therapeutic properties to fermented products. Examples include organisms that produce diacetyl or acetaldehyde for flavor or small amounts of alcohol in products such as kefir. Organisms such as *Bifidobacterium* spp. and *Lactobacillus acidophilus* are added for therapeutic purposes. Leuconostocs are used in products such as cultured buttermilk to produce diacetyl via citrate fermentation.

2.1.1 Enumeration

Legislation in some countries and codex regulations require the presence of viable organisms in yogurt. In the United States, the National Yogurt Association requires the presence of at least 10 million yogurt bacteria per gram at the time of consumption if manufacturers wish to display the "Live and Active Cultures" symbol on yogurt packages. Furthermore, many fermented milk products possess therapeutic properties largely because of the presence of selected viable organisms. These organisms have to be present in specified numbers to impart such therapeutic properties. Therefore, the use of proper enumeration procedures is vital. Lactic agar is used to enumerate lactic acid bacteria, whereas deMan, Rogosa, and Sharp (MRS) and lactobacillus agars are suitable for lactobacilli. Special consideration is given to products that are made with a combination of cultures. An example is yogurt that is manufactured with rods and cocci and sometimes also with bifidobacteria. It is important not only to enumerate but also differentiate these types of organisms. Enumeration procedures such as those that use yogurt

lactic agar are recommended for differentiating between rods and cocci. On this agar, *Streptococcus thermophilus* colonies are small and white and *Lb. delbrueckii* subsp. *bulgaricus* colonies are large and white and have a white cloudy zone.

A critical issue in enumeration of bacteria in cultured products is the occurrence of acid injury to cells, especially during storage of the product. The pH in most fermented milk products drops to below 4.6 and causes sublethal injury to surviving lactic acid bacterial cells. Such sublethally injured cells are not able to multiply in media used in routine counting procedures but require an enriched medium which will help repair the injured cells. Pariente et al. (1987) demonstrated that counts of heat-injured *Lb. casei* were underestimated when *Lactobacillus* selection (LBS) and Rogosa media were used for enumeration. Application of soya trypticase broth to recover injured lactobacilli has been recommended. *Standard Methods for the Examination of Dairy Products* suggests the use of standard methods agar (SMA) for enumerating injured cells, but this agar is not selective. It can be made more selective by adding 0.02% sodium azide, which does not inhibit lactic acid bacteria but does inhibit others such as enteric bacilli. In a direct epifluorescent filter technique for differential determination of sublethally injured bacterial cells, the RNA of viable cells is stained orange by acridine orange, whereas inactive cells and DNA are stained green. This characteristic also is applicable to *Lb. delbrueckii* subsp. *bulgaricus*, *S. thermophilus*, and *Lb. acidophilus*. Working with cells injured by freeze drying, de Valdez et al., (1985) demonstrated that highest recovery was obtained on LAPTg agar for various lactobacilli and lactococci.

2.1.2 Inhibition of Growth

Development of adequate flavor and texture in fermented milk products requires optimal growth of culture organisms. This is readily attained with proper manufacturing conditions and handling of cultures. If a batch starter is used daily, facilities for aseptic culture transfer and maintenance of cultures should be available. Presence of substances in milk such as phages and sanitizers can inhibit cultures. Antibiotics have a static effect on bacteria, but yogurt bacteria, *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*, are particularly sensitive. Penicillin at 0.01 IU/mL of milk will inhibit these organisms, whereas mesophilic lactococci are not as sensitive. It is important, therefore, to test every batch of milk for antibiotics.

Bacteriophages of organisms used to manufacture fermented milks have been identified. Phages of mesophilic lactic acid bacteria are well known to cheese makers but have also been found in buttermilk production facilities. Moineau et al. (1996) isolated 27 different phages from 27 buttermilk plants in the United States. Although not as common as phages of mesophiles, those of thermophiles, such as yogurt bacteria, have also been reported and can arrest the fermentation. Phage control systems have been described and involve culture rotation, the use of phage-inhibitory media, and, most important, proper sanitation at the plant. Phage-inhibitory media are usually rich in phosphates to chelate calcium. Some strains of *S. thermophilus* do not grow well in high-phosphate media. Chlorine as a sanitizer is very effective against phages. Sanitizers must be used with caution, however, because residual sanitizers in fermentation vats, piping, or packaging cups will also inhibit starter organisms. The latter is particularly applicable for products that are fermented in consumer cups. Sanitizers such as quaternary ammonium compounds in particular can be a problem. If a residual film of such sanitizers is left on equipment surfaces, the sanitizers are released slowly over time and inhibit culture organisms that come in contact with them. Sensitivity is strain dependent but thermophiles are generally more sensitive than mesophilic lactococci.

Another mode of inhibition in milk is by the naturally present lactoperoxidase system. This system has to be activated for inhibition to occur and requires the presence of the lactoperoxidase enzyme, H_2O_2, and thiocyanate. Some starter bacteria used to produce fermented products, such as *Lb. acidophilus* and *Lb. delbrueckii* subsp. *bulgaricus*, produce H_2O_2 during fermentation and consequently activate the lactoperoxidase system. Guirguis and Hickey (1987b) concluded that inhibition by this system was strain dependent and that strains most affected were those that produced H_2O_2. *S. thermophilus* was not inhibited by the lactoperoxidase system.

2.2 TYPES OF FERMENTED MILKS

Numerous types of fermented milks exist around the world. Products range from yogurt, which is probably the most widely known, especially in the Western world, to more regional products such as mala (or maziwa lala) of Kenya, which is manufactured using mesophilic cultures, and dahi of India, which is largely made either in the home or by small-scale dairies. There are distinct

differences in characteristics between the different types of products, depending on type of organisms and type of milk used. For example, Bulgarian buttermilk has a very strong acid flavor (2–4% lactic acid), whereas yogurt has a milder acidic and acetaldehyde flavor. On the other hand, koumiss, which is traditionally made from mares' milk, is slightly alcoholic, because yeasts are used in its manufacture. Texture of products also varies from liquid, such as for cultured buttermilk and liquid yogurt, to thick gel as for yogurt and sour cream. Some products such as viili from Scandinavia are characterized by their ropiness, which is intentionally induced by the use of cultures that produce exopolysaccharides to provide a thick body. Such cultures may also be used to manufacture low-fat yogurts to provide adequate body. Milk used for manufacturing fermented products is largely from the cow, but across the world milk of other species is also employed. In India, for example, the water buffalo is a common source. Yogurt-like products in Iran are produced from milk of sheep or goats, and in some parts of Tibet milk of the yak is used. The type of milk affects end-product characteristics partly via influence on growth of culture bacteria.

Thus, fermented milks encompass a wide range of products that possess diverse characteristics and employ a wide range of manufacturing procedures that are designed to promote optimal growth and activity of the chosen culture organisms.

2.2.1 Yogurt

The term *yogurt* (yoghurt) encompasses a wide range of products. Yogurt is a fermented dairy product, which is generally manufactured from pasteurized milk. Its fat content ranges from 0 to over 4% depending on region and legislation. High-temperature pasteurization of the yogurt mix is employed to obtain a smooth and firm body. Nonfat dry milk or stabilizers may also be added to increase the water-holding capacity and therefore improve its body. The latter is particularly applicable to low-fat products.

Several different types of yogurt are commercially available. These include plain (no added flavors), flavored, liquid, carbonated, and low lactose. The flavored yogurts include the sundae-style in which fruit puree is layered at the bottom of the cup and is mixed with the yogurt before consumption. The other type is Swiss-style, in which plain yogurt is gently blended with fruit puree before packaging. Such yogurts require high levels of solids and stabilizer to obtain the desired high viscosity. Liquid yogurts are popular in

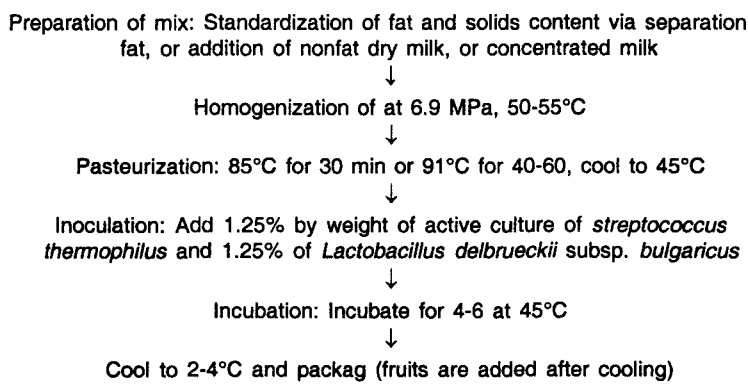

Figure 2.1 Steps for manufacture of yogurt.

Europe, Canada, and Japan, and differ from gel-type yogurt in that they are in a homogeneous, pourable state. No whey separation should occur during storage.

Manufacture of yogurt involves several key steps: standardization of mix, homogenization, heat treatment, cooling to incubation temperature, inoculation with yogurt cultures, incubation, cooling, and packaging.

2.2.1.1 Starter organisms

Many countries have their own standards of identity for yogurt with regard to composition as well as starter bacteria. Most countries and codex regulations define yogurt as the product obtained by fermenting milk with a culture that includes *Lb. delbrueckii* subsp *bulgaricus* and *S. thermophilus*. Some countries permit additional lactic acid bacteria, whereas others, such as Australia, require only *S. thermophilus* and a lactobacillus of choice. The United Kingdom requires *Lb. delbrueckii* subsp. *bulgaricus* to which other lactic acid bacteria can be added.

S. thermophilus (coccus) and *Lb. delbrueckii* subsp. *bulgaricus* (rod) are thermophilic organisms and grow best at approximately 45°C but not above 50°C. They are typically added in a 1:1 ratio. Bulk cultures may be prepared separately from pure strains or frozen concentrates may be added directly to the mix. The latter eliminates the need to maintain culture transfer facilities. Rods and cocci function symbiotically to produce typical yogurt characteristics. Either culture independently is unable to produce the ideal balance of acid and flavor. *S. thermophilus* initiates lactic acid production and lowers the oxygen level, which stimulates growth of *Lb. delbrueckii* subsp.

bulgaricus. The pH is lowered to approximately 5 by the cocci and then to less than 4 by the rods. The rods in turn promote growth of *S. thermophilus* via production of peptides and amino acids.

S. thermophilus is more sensitive to acid than is *Lb. delbrueckii* subsp. *bulgaricus*; hence during extended storage of yogurt, the former (cocci) are likely to be injured by the acid and gradually die off. Therefore, although the initial ratio of rods to cocci may be 1:1, this ratio may change in favor of lactobacilli during storage of the yogurt. As the rate of acid and flavor production is strain dependent, the rod and coccal strains should be selected so there is a balance of acid and acetaldehyde production. Rate of acid production alone should not be the criterion for strain selection. Acetaldehyde is produced by both *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*. Both organisms produce threonine aldolase which helps convert threonine to acetaldehyde but lactose is also a source.

It is now common in the yogurt industry, particularly in Europe, to enhance the body of yogurt by using cultures that produce exopolysaccharide. Some strains of lactic acid bacteria, including the thermophilic yogurt bacteria, can produce exopolysaccharides that act as stabilizers and thicken the body of yogurt. The polysaccharides can be extracellular or in encapsulated form. Some strains of cultures produce polysaccharides that can lead to a ropy texture, whereas others provide a thickening effect without ropiness. This may be important, because criteria for sensory evaluation of yogurt generally view ropiness as a defect.

In recent years bifidobacteria-containing yogurt has become popular in Japan, Canada, France, and Germany. Such yogurt is manufactured either with bifidobacteria singly or as mixed cultures with *Lb. acidophilus* and *S. thermophilus* and provide therapeutic properties to yogurt. Bifidobacteria of human origin are preferred and include *Bifidobacterium breve, Bi. longum, Bi. infantis*, and *Bi. bifidum*. An inoculum rate of >10% has to be used, because bifidobacteria are slow acid producers. Incubation is at 36–42°C for 6–8 h to enable curd formation and provide viable counts of up to 100 million per gram in the final product. An advantage in using bifidobacteria is that over-acidification does not occur in the yogurt during production and storage. Bifidobacteria yogurt therefore has a milder (less acidic) taste. To ensure viability during storage of yogurt, proper strains of bifidobacteria must be selected.

2.2.1.2 Defects

Yogurt by nature is a high-acid (low pH) product and is therefore inherently protected against defects caused by most contaminating organisms. Furthermore, the high pasteurization temperature used in processing the mix eliminates most contaminating bacteria. Nevertheless, certain defects, some microbially induced, may occur. Perhaps the most common defect is high acid and consequently high acetaldehyde flavor. This may develop under improper manufacturing and storage conditions. If the rods and cocci are maintained as a mixed culture, after repeated transfers at high temperature rods will dominate the culture. They then become the primary acid producers when used to make yogurt and produce excessive amounts of acid (over 2%). This can be prevented by maintaining the two cultures separately and adding them in a 1:1 ratio at the time of inoculation of the mix during manufacture of yogurt. Another critical factor is the rapid cooling of yogurt after incubation to prevent continued growth of lactobacilli. Many manufacturers use blast tunnels for cooling to 10°C within 50 min. Excessive acid production may also lead to body and texture defects such as shrinkage of curd and wheying-off. Other texture defects may also occur in yogurt, such as weak or excessively heavy body, which are generally related to improper use of stabilizers. Proper selection and use of ingredients, especially stabilizers in the mix, can address these defects. Yogurt manufacturers often add 2–4% nonfat dry milk to increase the total solids content to over 15%. This helps to develop a firm body, and is especially useful in low-fat and nonfat yogurts. A disadvantage is that the resulting yogurt will have a high lactose content (approximately 6%) that will allow the lactic fermentation to continue. Acidity of such yogurts is therefore high. An alternative is to concentrate milk by ultrafiltration to raise the protein content and lower the lactose level. The protein concentration that can be used with such procedures is <5.6%, since excessive fortification leads to an undesirably firm body.

Another microbially induced defect is bitterness. This occurs if the milk supply contains spore-forming organisms such as *Bacillus subtilis* or *B. cereus*. Spores of these organisms are able to survive high heat treatment. Yeasts and molds are acid tolerant. Therefore, contamination by yeasts and molds can be a problem, particularly in fruit-flavored yogurts if poor-quality contaminated fruit preserves are used.

2.2.2 Cultured Buttermilk

Cultured buttermilk is a lightly salted fermented milk product that is manufactured from nonfat or low-fat milk using mesophilic cultures and flavor-producing organisms. Unlike yogurt, the flavor of buttermilk includes lactic acid, diacetyl, and acetic acid. Diacetyl is obtained from citric acid fermentation during manufacture of buttermilk. Cultured buttermilk should have a smooth thick body, with the correct balance of acid and diacetyl flavor.

Preparation of milk: Standardize milk to desired fat content via separation of fat. Add 0.15% citric acid, if needed, and 0.1% salt

↓

Homogenization at 6.9 MPa, 50-55°C (low-fat and whole milk products only)

↓

Pasteurization: 85° for 30 min or 88°C for 2 min, cool to 22°C

↓

Inoculation: Add 0.5% by weight of active culture of *Lactococcus lactis* subsp. *lactis* or *Lactococcus lactis* subsp. *lactis* (biovar. *diacetylactis*) or *Leuconostoc mesenteroides* subsp. *cremoris*

↓

Incubation: Incubate for 14-16h at 22°C

↓

Break curd by agitation after pH has reached 4.5 and cool to 10°C with gentle agitation

Figure 2.2 Step for the manufacture of cultured buttermilk.

2.2.2.1 Starter organisms

Cultured buttermilk is produced with combinations of mesophilic lactic acid bacteria that will produce lactic acid as well as diacetyl. Species used include *Lactococcus lactis* subsp. *cremoris*, *Leuconostoc mesenteroides* subsp. *cremoris*, and *Lc. lactis* subsp. *lactis* (biovar. *diacetylactis*). The latter two produce diacetyl and small amounts of carbon dioxide. *Lc. lactis* subsp. *lactis* (biovar. *diacetylactis*) also produces acetaldehyde, which is not desirable in buttermilk, and therefore this bacterium should be used with caution. The lactic acid producers thrive on lactose, whereas the flavor producers require the presence of sufficient citric acid to produce diacetyl. The naturally present citric acid in milk should be supplemented by the addition of sodium citrate (0.1–0.15%). The flavor producers do not produce an appreciable amount of lactic acid but do require acidic conditions for proper growth and fermentation of citrate. Therefore, sufficient activity by the lactic acid producers is necessary (pH 5) before flavor producers can

function. Levata-Jovanovic and Sandine (1997) have reported on the use of a *Leuc. mesenteroides* subsp. *cremoris* strain in combination with a ropy *Lc. lactis* subsp. *cremoris* culture for improving the flavor and texture of buttermilk. An important advantage of using leuconostocs is that these organisms are relatively insensitive to phages.

Flavor producers are rather temperature sensitive. If the temperature of incubation is maintained at 27°C instead of the optimum 22°C, they will not produce sufficient diacetyl and consequently acid rather than a balance of acid and diacetyl flavor will dominate the finished product. Diacetyl-producing bacteria also possess an enzyme that converts diacetyl to acetyl methyl carbinol (acetoin). This results in a loss in the quantity of diacetyl in buttermilk. Hence, production of cultured buttermilk requires proper selection of culture bacteria as well as manufacturing conditions that will induce balanced growth of acid and flavor producers.

Cultured buttermilk typically has a thick, homogeneous body. Vedamuthu and Shah (1983) patented a procedure for manufacturing such a product using a mixture of slime-producing *Lc. lactis* subsp. *cremoris* and non–slime producing *Lc. lactis* subsp. *cremoris* and/or *Lc. lactis* subsp. *lactis*. Ropiness occurred only if >80% of the culture mixture was a slime producer.

2.2.2.2 Defects

In many respects, cultured buttermilk is a delicate product that can have defects if proper care is not taken during manufacture. On the other hand, culture characteristics and proper manufacturing conditions have been well documented, and, if employed, good-quality product can be readily obtained. Many defects of cultured buttermilk can be linked to improper culture usage, whereas others are related to manufacturing procedures. Culture-related defects can be flavor defects and may indirectly also lead to body defects. Even buttermilk produced under the best sanitary conditions may lack flavor (flat flavor) if the environment is not optimal for the growth of flavor producers. For example, a high incubation temperature (27°C) discourages growth of flavor producers; therefore insufficient diacetyl will be present. Such defects can be prevented by ensuring that the acid-producing culture is active, because the flavor producers will be activated only after sufficient acid has been produced (0.8–0.85%, pH 5) and incubating at 22°C. Milk should be supplemented

with citrate, and after the curd has been broken at the optimum pH, the product should be rapidly cooled with gentle agitation. This will prevent degradation of diacetyl. If incubation is not monitored and if fermentation is not halted by cooling, acid production will continue and may even exceed 1%. This process is not reversible and produces a highly acidic product with a loss of diacetyl. Excessive acidity will also lead to wheying-off because of a lowered water-holding capacity of the proteins. Such wheying-off may also result from excessive and high-speed agitation during cooling after fermentation is completed. During storage such a product will separate into whey and a heavy protein mass that settles to the bottom.

A weak culture that is contaminated with organisms such as psychrotrophs and coliforms will lead to unclean, and, in extreme conditions, bitter flavors. Contaminating bacteria such as coliforms and *Pseudomonas* spp. possess a relatively high level of diacetyl reductase which degrades diacetyl. One strain of *Enterobacter aerogenes* had an activity of 345 units of enzyme protein per milligram compared with 100 units for *Lc. lactis* subsp. *lactis* (biovar. *diacetylactis*). Such enzyme activity leads to a product that lacks flavor. Good manufacturing and sanitation practices are therefore vital and can easily prevent such defects. Proper starter maintenance, including replacement of the mother culture at regular intervals, is also a good practice to ensure continued high activity of the starter culture.

Some of the aforementioned culture-related defects will eventually lead to body and texture defects. For example, if the culture lacks adequate activity and if the product is cooled at low acidity, the finished product will not have optimum viscosity. In contrast, excessive viscosity can result from cultures such as *Lc. lactis* subsp. *lactis*, which form long chains. Some contaminants produce slime, which results in a highly viscous product.

2.2.3 Sour Cream and Creme Fraiche

The two main fermented cream products are sour cream and creme fraiche. The later originated in France but is now also used in other countries. Because of their high fat content, 18 and 50%, respectively, they are used for dips and toppings rather than for direct consumption. Cultures used for these products and manufacturing procedures are similar to those for cultured buttermilk. The high-fat and solids contents provide these products with a thick

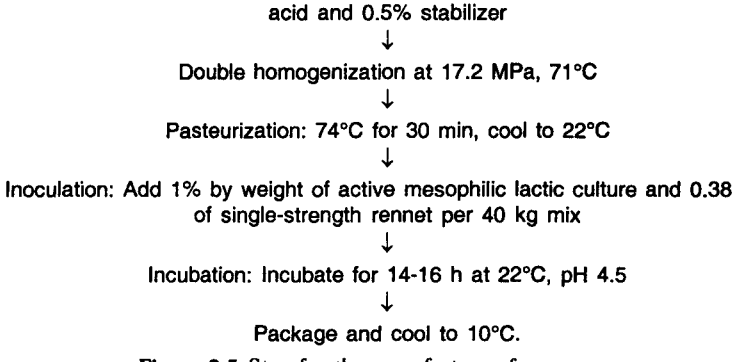

Figure 2.3 Step for the manufacture of sour cream.

and heavy body. The manufacturing procedure for sour cream is especially designed to produce a very thick body. Sour cream typically has a clean acidic flavor with hints of diacetyl. Mesophilic lactic acid and flavor-producing cultures are used along with double homogenizing and a small amount of rennet for developing body. Creme fraiche, on the other hand, is also manufactured with the same cultures but the pH is higher (6.2–6.3).

As sour cream is a high-fat product (approximately 70% fat on dry basis), manufacturing a low-fat, and, particularly a fat-free product, is challenging. Simply replacing the fat with moisture, as is done in most low-fat cheeses, does not provide the required thick and smooth body of sour cream. Thickening agents such as starches, stabilizers, and fat replacers therefore play an important role in these products. Lee and White (1991) demonstrated that good body and texture in sour cream of 5 and 10.5% fat could be obtained with gelatin, modified food starch, or methoxyl pectin. Addition of rennet helps firm the body but also leads to syneresis and proteolytic activity. The use of a starch-based texturizing agent has also been suggested. This agent consists of an insoluble microparticle (titanium dioxide), xanthan gum, and pregelatinized starch. Commercial milk or egg protein–based microparticulated products used as fat replacers have application in reduced-fat sour cream production. The aforementioned procedures provide adequate body to low-fat sour cream, but development of proper balanced flavor is also important. Flavor-delivery systems have been developed that consist of fat globules or polyhydroxyalkanoates in which large amounts of fat-soluble flavor compounds are included. When these systems are incorporated into low-fat and fat-free sour cream, the fat-soluble flavor compounds

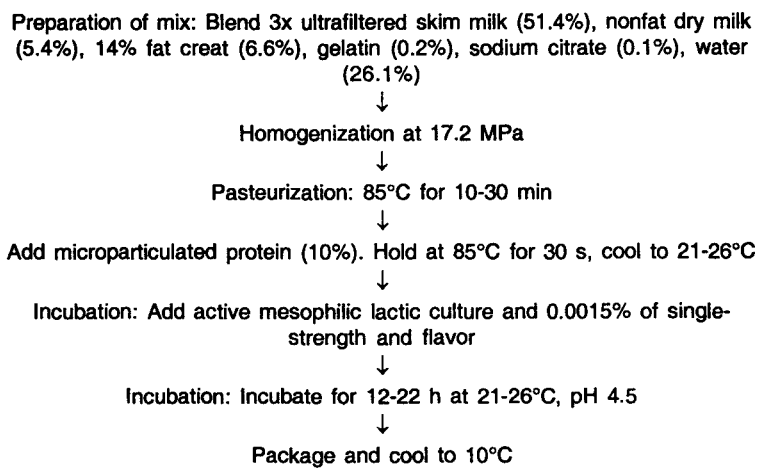

Figure 2.4 Steps for the manufacture of reduced fat sour cream.

are released and complement other compounds that are produced by the starter bacteria.

2.2.3.1 Starter organisms and product defects

Most of the culture issues discussed previously for cultured buttermilk apply to sour cream as well. As with most fermented milk products, good-quality sour cream can keep for a long time (4 weeks) under refrigeration, because the high-acid content prevents growth of contaminants. During extended storage, however, enzymes of bacteria that survived pasteurization may cause development of bitter and unclean flavors via proteolysis. Good manufacturing and sanitation practices should be employed to prevent such defects.

2.2.4 Acidophilus Milk

Acidophilus milk is a fermented milk produced mainly by the use of lactobacilli and is believed to have therapeutic properties. It can have an acid content of up to 2%, which is unpleasant to some, so consumption is limited. Manufacture of this product first involves sterilization of nonfat or low-fat milk followed by inoculation (5%) with an active *Lb. acidophilus* culture. Incubation is for 24 h at 36°C, and this generally results in a titratable acidity of 1%. After incubation, the product is cooled and packaged. In addition to tartness, the product also has a strong cooked flavor from sterilization of milk before fermentation. Because of these qualities, the product is not popular. These drawbacks have been overcome in a product from Finland, which is manufactured by fermenting demineralized,

lactose-hydrolyzed whey with *Lb. casei* GG and then adding fruit flavors.

An alternative for ingestion of *Lb. acidophilus* is sweet acidophilus milk. Initially, this product contained only *Lb. acidophilus* but now also includes bifidobacteria. Pasteurized, low-fat, skim, or whole fluid milk is packaged with added viable *Lb. acidophilus* and bifidobacteria. As the inoculated fluid milk is held refrigerated, growth of these bacteria does not occur during storage but occurs in the intestinal tract after consumption. Such growth depends on strain of *Lb. acidophilus* used. Because these organisms are present, the milk must always be refrigerated. Shelf life under such conditions is 2 weeks. Extended storage and/or storage at high temperatures will lead to curdling of milk from acid produced by the added bacteria. A similar Swedish fluid milk product contains *Lb. reuteri* in addition to *Lb. acidophilus* and bifidobacteria.

2.2.5 Kefir

Kefir is originally a Russian liquid fermented milk product. Approximately equal amounts of lactic acid and alcohol are produced during fermentation. Typical flavor results from a balance between lactic acid, diacetyl, aldehyde, ethanol, and acetone. Fizz is provided by the carbon dioxide that is also produced during fermentation. In the manufacture of kefir, milk is heated to 85°C for 30 min and cooled to an inoculation temperature of 22°C. It is then inoculated with kefir grains and fermentation occurs over 12–16 h. The kefir grains are then filtered out and reused.

2.2.5.1 Starter organisms

Kefir grains consisting of yeasts, bacteria, and polysaccharides are used for kefir production. The yeasts include *Saccharomyces kefir* and *Torula* spp. or *Candida kefir* and bacteria include *Lb. kefir*, leuconostocs, lactococci, and various others. Takizawa et al. (1998) isolated 120 strains of lactobacilli from kefir grains; the most prominent was *Lb. kefirogranum*. The grains require proper care and should be held using routine sanitary practices. Contaminants such as coliforms, micrococci, and bacilli, if present, will lead to a variety of flavor defects.

Kefir-like products with only small amounts of alcohol and with flavors such as strawberry are also manufactured in the United States. Yeasts and various *Lactobacillus* spp. and *Lactococcus* spp. are used.

2.2.6 Koumiss

Koumiss also is a product of Russian origin and is largely used in that country for therapeutic purposes. It is made with a combined acid and alcohol fermentation traditionally from mare's milk but cow's milk also can be used. Even though the acid content of koumiss is high, no curd is visible because of the relatively low protein content of mare's milk (2%). Fermentation is accomplished with a combination of *Lb. delbrueckii* subsp. *bulgaricus* and a lactose-fermenting yeast, *Torula* spp. The finished product contains 1.0–1.8% lactic acid, 1.0–2.5% ethanol, and carbon dioxide. The latter makes for a frothy product.

2.2.7 Fermented Milks of Scandinavia

Scandinavians are among the highest consumers of fermented milk products. It is not surprising, therefore, that some unique fermented products have originated in Scandinavian countries. Examples include viili, langfil, kelder-milk, skyr, ymer, and several others. Some of these products possess unique characteristics such as a heavy, ropy body obtained by the use of specially selected cultures, which, in some instances, includes mold.

Viili is a fermented product of Finland that may be either plain or flavored with fruit. The fat content may vary from 2 to almost 12%, depending on classification (such as low fat, full fat). Milk is heated to a high temperature (83°C for 20–25 min), tempered to the incubation temperature of 20°C, and inoculated with 4% starter culture consisting of *Lc. lactis* subsp. *lactis*, a diacetylactis culture, *Leuc. mesenteroides* subsp. *cremoris*, and *Geotrichum candidum*, a mold. Incubation occurs in consumer cups at 20°C for 24 h (final acidity of 0.9%). The purpose of incubation in consumer cups is to allow fat to rise to the surface during incubation where the geotrichum mold will grow and contribute to the typical musty aroma. Furthermore, complex carbohydrates formed by the organisms used give the product a heavy, ropy characteristic.

Ymer is a fermented product of Denmark that has a high protein content of 5–6%. Current commercial procedures use ultrafiltration technology to concentrate the milk protein before fermentation. Concentration by some of the more traditional procedures involves either allowing curd to drain or applying heat to curd to induce syneresis. Before fermentation, milk receives a high-heat treatment (90–95°C for 3 min). Incubation is at 20–22°C with an inoculum

consisting of *Lc. lactis* subsp. *lactis* (biovar. *diacetilactis*) and *Leuc. mesenteroides* subsp. *cremoris*. Consequently, the product has a pleasant acidic flavor balanced with hints of diacetyl.

Another concentrated fermented product of Scandinavia is skyr. This product is from Iceland and has almost 13% protein. Such a high concentration is achieved commercially with the help of a centrifugal separator similar to one used in the manufacture of quarg. Skim milk is fermented with thermophilic lactic acid bacteria similar to those used for yogurt along with lactose-fermenting yeast. Small amounts of rennet may also be added to obtain proper body. With active cultures, a pH of 4.6 is obtained within 4–6 h at 40°C. After an additional 18 h at 18–20°C, the pH drops to 4, the product is pasteurized, and is then centrifuged at 35–40°C for concentration. Because of the presence of yeast, ethanol occurs in the final product along with lactic acid, diacetyl, acetaldehyde, and acetic acid.

2.2.8 Fermented Milks of India

India, the largest milk-producing country in the world today, has a long history of dairying. Production and consumption of milk and milk products date back many thousands of years. Today, numerous indigenous products are available locally. Of these, fermented milk products such as dahi, lassi, srikhand, and misti doi are important parts of the diet.

Dahi is a product made by fermenting milk of the cow or water buffalo milk with lactic acid bacteria. It has a clean, acidic flavor with slight hints of diacetyl. The texture is similar to that of yogurt. Much of the dahi consumed in India is either made at home or by small dairies. In both instances, the culture usually consists of the previous day's product, but pure cultures are also available. Hence, composition of culture and consequently flavor can vary from batch to batch. The legal standards of identity for dahi that is produced commercially and sold in the market are the same as for milk from which dahi is made. The manufacturing procedure for dahi is simple. Milk of the cow, water buffalo, or a mixture is briefly boiled and cooled to room temperature. It is then inoculated with 0.5–1.0% culture and incubated at room temperature for 12–16 h. With an active culture, the final pH is 4.5–4.7. Because room temperature in tropical countries varies according to the season, it is not uncommon to find thermophilic cultures in dahi. Dahi typically contains a mixture of *S. thermophilus, Lb. delbrueckii* subsp. *bulgaricus, Lc. lactis* subsp. *lactis, Lc. lactis* subsp. *cremoris,*

Lc. lactis subsp. *lactis* (biovar. *diacetylactis*), *Lb. helveticus, Lb. casei*, and *Lb. acidophilus*. The initial boiling step eliminates undesirable organisms from the milk, but it is important to have an active culture. After repeated transfers, the culture may lack activity and, in the absence of adequate acid production, undesirable flavors from growth of yeasts and mold may occur. Because yeasts tolerate acid, it is important to prevent postheating contamination of the milk with these microbes.

Lactic acid bacteria of dahi have antimicrobial effects against pathogenic and spoilage bacteria. Some of these effects come from cell-free extracts and are believed to be associated with production of H_2O_2 by lactobacilli and bacteriocin-like compounds by some lactococci.

Dahi is typically stored at room temperature; hence lactic acid continues to develop rapidly after its manufacture. Researchers have attempted to eliminate this by introducing nisin (25 IU/mL) in dahi after fermentation is completed.

Dahi is consumed as such and is also used as a base for producing other products. Examples include lassi, srikhand, and ghee. Lassi is a liquid product that is manufactured by blending water and dahi and mixing to a uniform consistency. The ratio of dahi to water depends on the consistency desired. The product is lightly salted or sweetened.

Srikhand is a popular product that is manufactured at home and also commercially. Fresh dahi is drained either with a cheesecloth overnight or with the help of a centrifuge. The drained curd is mixed with an equal proportion of sugar and enough cream to adjust the fat content to 5–6%. Additional flavorings such as fruits, nuts, and spices may be added. The final product has 40–45% moisture, 5–6% fat, 40–45% sugar, and a shelf life of at least 30–35 days at 10°C. Post-production acidification is restricted by the presence of a large amount of sugar, but spoilage occurs through growth of yeasts and mold and the presence of heat-stable proteolytic and lipolytic enzymes that cause undesirable flavors. The shelf life can be improved to almost 2 months by pasteurizing the product before packaging. The use of nisin as a preservative has also been suggested. It is also important to ensure that good-quality sugar is used, such as that which is hot-air treated to improve the microbial quality of srikhand. Antibacterial effects of dahi described above also apply to srikhand.

Ghee is clarified milk fat and has been used for cooking in India for thousands of years. Although it is not a fermented product, some procedures to manufacture ghee use dahi as a base. Dahi, when churned, is separated into a fat-rich product (butter) and buttermilk. Butter is then heated to 110–120°C, cooled, and filtered. When cooled, it has a granular texture. Much of the flavor of this product results from metabolites of the lactic fermentation during dahi manufacture.

A fermented product similar to dahi called misti doi is popular in eastern India. The manufacturing procedure is similar to that of dahi except that before boiling 6–6.5% sugar is added to milk. The intense heating concentrates milk and gives it a slight brownish color. Approximately 1% culture (previous day's product) is added and incubation occurs at approximately 40°C for 12–15 h. Thermophilic lactic organisms predominate. For example, in one study, 45% of total isolates were *S. thermophilus*, 35% were *S. lactis*, and 20% were *Enterococcus faecalis*. Although this product is commonly produced at home and in small-scale dairies, standardized commercial procedures for large-scale production have been developed.

2.3 FERMENTED MILKS OF THE MIDDLE EAST

Fermented milk products have a long history in Middle Eastern countries. Popular products include laban rayeb, labneh (concentrated yogurt), kishk, and zabady. Other regional names for some of these products also exist. Laban rayab is traditionally prepared by pouring unhomogenized whole milk in pots and held at room temperature. Fat rises to the surface and is removed. The defatted milk undergoes a natural fermentation and then is ready for consumption. Variations of this product are laban khad and laban zeer. The former is prepared by allowing milk to ferment in a goat pelt, whereas the latter is made in earthenware pots called zeer which are used for incubation. The season, and hence the temperature, will determine the dominating microflora of these products. Generally, lactococci dominate in the cold season and lactobacilli in the warm season. Laban zeer is used to make another highly nutritious product called kishk.

To prepare this product, laban zeer is mixed with wheat grains that have been softened by boiling in water, sun-dried, and ground. The mixture undergoes a 24-h fermentation. The product, now with high viscosity, is divided into small pieces and then sun-dried and stored until consumed. Spices may be added. Kishk, which has

approximately 8% moisture and 1.85% acidity, has a shelf life of several years.

A concentrated fermented product called labneh that has 7–10% fat is produced in several Arabian countries. It is made at home using traditional procedures as well as on a large scale in dairies. The basic procedure for this product involves concentration of milk after fermentation is completed. For commercial production, skim or whole milk is fermented with yogurt cultures, but strains that produce exopolysaccharides are not used because of the difficulty in removing whey after fermentation. The fermented product is then separated with the help of centrifugal separators such as those used in manufacturing quarg. Alternatively, milk is fermented after concentration by ultrafiltration to the desired composition. A traditional product of Egypt similar to labneh is zabady, which is made by fermenting milk that has been concentrated by boiling with thermophilic cultures in porcelain containers.

3

Butter and Related Products

3.1 INTRODUCTION AND DEFINITIONS

3.1.1 Volumes of Butter and Brief History

Worldwide consumption of butter and milkfat products is estimated at 2,420,000 tons in 1993 for countries where data are available. In 1998, the United States produced 1082×10^6 lb of butter with none being purchased by the government as surplus. Butter was one of the first dairy products manufactured by humans and has been traded internationally since the 14th century. All butter manufacture relies on cream as a starting material. From ancient times through the latter part of the 1800s, cream was obtained from milk by gravity separation. In the 1850s, creameries began producing butter on a small scale. Large-scale manufacture only became possible after development of the mechanical cream separator in 1877.

3.1.2 Composition and Types of Butter

Butter is a water-in-oil emulsion, wherein milkfat forms the continuous phase. This is in contrast to cream, which is an emulsion of milkfat globules suspended in an aqueous phase. Thus, an emulsion phase inversion occurs during manufacture of butter. This happens in churning of cream, and, as a result, milkfat is concentrated in the product. Butter contains 80% milkfat (typically 80–81%), 17% moisture, 1% carbohydrates and protein, and 1.2–1.5% sodium chloride (with no salt, the milkfat increases to 82–83%). The pH of sweet cream butter (unfermented) is about 6.4–6.5. Many countries allow sodium chloride and lactic cultures as the

only nonmilk additives in butter. Some countries allow neutralization of cream and addition of natural coloring agents to adjust for seasonal variation in colorant in the cream (e.g., annato, carotene, and turmeric).

There are two kinds of butter: sweet cream, which may or may not be salted, and ripened-cream butter. In ripened cream butter, citrate in cream is fermented by certain lactic acid bacteria to produce acetoin and diacetyl; the latter imparts a characteristic flavor to the product. Ripened-cream butters are more popular in Europe, whereas unripened or sweet-cream butter is preferred in the United States, Ireland, England, Australia, and New Zealand. When whey produced during cheese making is passed through a separator, the result is whey cream. Whey cream is processed into butter, usually as a blend with sweet cream. Butter from a $\leq 20\%$ whey cream and sweet cream blend may be indistinguishable from that made from 100% sweet cream. Butter is also manufactured from neutralized or nonneutralized whey cream, usually as a blend with sweet cream.

3.2 MANUFACTURE OF BUTTER

The manufacture of butter is uniquely characterized by the following three processes:

1. Concentration of the fat phase of milk. This is done by separation or standardization of milk which results in cream.
2. Crystallization of the fat phase. Large numbers of small solid fat crystals in globular form are required, with each globule surrounded by liquid fat. Although pasteurization of cream yields a fully liquefied milkfat, cooling and tempering for at least 4 h at approximately 10°C is necessary to develop an extensive network of stable fat crystals surrounded by liquid milkfat. In making ripened-cream butter, addition of lactic acid bacteria to pasteurized cream cooled to 16°C is followed by incubation until a pH near 5 is attained. Cooling to 3–5°C stops the fermentation followed by warming to 10°C immediately before churning. This technique controls the fermentation while allowing for liquid fat on the globule exterior.
3. Phase separation and formation of a plasticized water-in-oil emulsion. Churning breaks the oil-in-water (o/w) emulsion and results in a plastic, water-in-oil (w/o) emulsion. The phase inversion occurs in both batch and continuous churns. During churning, vigorous agitation is used to disrupt the membrane on each milkfat globule. When the emulsion breaks, milkfat

globules have formed pea-sized granules. Continued aggregation of fat globules forms a continuous matrix at an optimal temperature. The optimal temperature is dependent on triglyceride composition and season of the year; for example, 10°C summer and 11°C winter. Churning is inefficient with homogenized cream or if the milkfat is too liquid or solid (too warm or too cold, respectively). The proper blend of liquid fat surrounding solid fat is necessary. The optimum temperature for continuous churning is from research conducted on batch churns to minimize fat losses. Continuous churn operations require similar cream conditions to those for batch churns to control fat losses to buttermilk. Using batch churns, researchers found cream must "break" or aggregate into pea-sized granules in 45 min to minimize fat losses in buttermilk. These same principles of operation have been used in developing butter manufacturing techniques with the continuous churn. Cream is pasteurized at a minimum temperature of 85°C and held for at least 15 s at that temperature. Research proved that high-temperature pasteurization was necessary to allow for frozen (–30°C) storage of butter for 2 years as with Commodity Credit Corporation (CCC) purchases of surplus product. Lipase native to milk, in particular, may reactivate with lesser thermal treatment resulting in spoilage of butter by hydrolytic rancidity.

Working of butter accomplishes two purposes: first, even distribution of moisture and salt in tiny droplets, and second, to allow for fat crystal growth to increase spreadability and to minimize brittleness of the product. After churning and working, butter is salted. Salting is done near the end of working in a continuous churn and at moisture standardization in a batch churn to prevent loss of salt. Packaging occurs after salting and may be done directly into retail portions or in bulk containers (25 and 31 kg are common). National intervention boards in the European Economic Community stipulate a storage temperature of –15°C; however, a lower temperature is frequently used, particularly for unsalted butter. A temperature of –30°C was effective for storing butter in excess of 1 year. Stored frozen butter is later thawed and microfixed and then packaged into retail containers. Microfixing is a mechanical process that reestablishes the physical structure of butter lost as a result of freezing. Butter from different manufacturers may be blended together during repackaging. Without microfixing, butter will have texture problems (lack of spreadability) and may show

moisture leakage. Thus, butter manufacture involves partial or complete separation of cream from raw milk, pasteurization, possible fermentation by added lactic acid bacteria (when ripened-cream butter is manufactured), churning, working, salting, packaging, storage, and perhaps later repackaging. All of these activities impact on the microflora of the final product.

3.3 MICROBIOLOGICAL CONSIDERATIONS IN BUTTER

The microbiology of butter reflects the microflora present in pasteurized cream from which it is made, water added at the time of salting butter, sanitary conditions of process equipment, manufacturing environment, and conditions under which the product is stored. Intrinsic properties of butter, for example, a_w, pH, salt content, uniformity of moisture distribution and droplet size, all impact microbiological stability.

3.3.1 Cream

The main source of microorganisms in butter made under excellent sanitary conditions is cream. Raw milk may be contaminated with a wide variety of pathogenic and spoilage microorganisms. The microflora of raw milk is related to that found in and on the cow's udder, milk-handling equipment, and storage conditions. Proper handling, pasteurization, and storage conditions should result in a predominantly gram-positive microflora in milk. Psychrotrophic *Bacillus* spp. and *Clostridium* spp. have been found in 25–35% and 8% of raw milk samples, respectively. These organisms survive pasteurization of cream. A review of pathogenic microorganisms in raw milk was prepared by the International Dairy Federation.

3.3.2 Importance of Pasteurization

The Code of Federal Regulations (21 CFR 58.334) stipulates that pasteurization of cream for butter manufacture will be at or above 85°C for 15 s. This thermal treatment minimizes reactivation of lipase native to milk. Further, after 2 years of frozen storage at –30°C, resultant butter will still have a score of 92 or grade A. Moreover, there are further benefits to this process. Many microorganisms are inactivated. However, there is a lack of research data to show destruction of enzymes from psychrotrophic bacteria during this thermal exposure. Because finished butter is stable during frozen storage, it is thought that all enzymes were destroyed. Pasteurization of cream from raw milk is designed to eliminate vegetative microbial pathogens and reduce numbers of potential

spoilage organisms. In the United States, cream must contain not less than 18% fat. However, heatresistant microbes and spores of *Bacillus* and *Clostridium* will survive. Temperatures between 95 and 112°C are commonly used to inactivate them. Cream is also heated to inactivate lipases (which cause hydrolytic rancidity in butter), reduce intensity of undesirable flavors by vacuum treatment (e.g., from feed ingredients), activate sulfydryl compounds (which can reduce autooxidation of butter), and liquefy milkfat for subsequent efficient churning.

3.3.3 Ripening

Many people in western and northern Europe and a few in the United States prefer the flavor of butter manufactured from microbiologically ripened cream. Traditionally, pasteurized cream is adjusted to 21°C and inoculated with lactic cultures composed of pure or mixed strains of *Lactococcus lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris*, *Leuconostoc mesenteroides* subsp. *cremoris*, and *Lc. lactis* subsp. *lactis* biovar *diacetylactis*. Ripening occurs for 4–6 h until a pH of about 5 is achieved, and then cream is cooled to stop the fermentation. In this process, spoilage microorganisms are controlled primarily through the bacteriostatic effect of lactic acid produced by the starter culture.

3.3.4 NIZO Method

The NIZO method for producing a cultured butter is allowed in several countries and is used by many dairies in western Europe. In the NIZO method, starter culture is not added to cream, but instead, a mixture of diacetyl-rich permeate and starter cultures is worked into butter. Fermentation of partly delactosed whey or other suitable media containing milk components by lactic acid bacteria (i.e., *Lactobacillus helveticus*) continues for 2 days at 37°C, and then the medium is ultrafiltered to remove proteins and bacteria and to further concentrate the medium. During ultrafiltration, macromolecules are removed and concentrated in the retentate, whereas low molecular weight solutes pass through into the permeate stream. The pH of butter made with the permeate from this process is more easily adjusted in the desired range of 4.8–5.3. This permeate can be stored at 4°C for more than 4 months under proper conditions. Advantages cited for this process are numerous.

Homofermentative lactic acid bacteria such as *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* are used to produce lactic acid from lactose in dairy products. However, flavor production

requires addition of a heterofermentative organism such as *L. mesenteroides* subsp. *cremoris* or *Lc. lactis* subsp. *lactis* biovar *diacetylactis* to produce diacetyl. Diacetyl, in addition to imparting flavor, inhibits gram-negative bacteria and fungi.

3.3.5 Churning and Working

The bacterial load of buttermilk is typically greater than that of cream or butter. When culture-ripened cream is used to manufacture butter, most starter culture organisms are retained in buttermilk; however, some remain in butter. In several studies, butter made from cultured cream retained 0.5–2.0% of the culture organisms present in cream. Olsen et al. (1988) found numbers of *Listeria monocytogenes* were 6.7–15.0 times higher in pasteurized but subsequently inoculated creams than in butter manufactured from the same cream. In an earlier study, *Staphylococcus aureus* behaved similarly. These organisms are gram positive, and it is unclear how other microorganisms with different cell wall and membrane structures distribute themselves between cream and butter. Diacetyl content of milkfat increases during churning; agitation during churning favors oxidative processes needed for diacetyl production. The pH of salted butter can prohibit formation of diacetyl.

3.3.6 Moisture Distribution during Churning and Working

From 10 to 18 billion droplets of water are dispersed in 1 g of the water-in-oil emulsion that is butter. Given the low microbial load expected in pasteurized sweet cream (less than 20,000 cfu/mL), most of the droplets are sterile. This depends on size and degree of dispersion of droplets and the microbial level in cream. The diameter of water droplets in conventionally made butter has been reported at <1 to >30 μm.

The number of water droplets greater than 30 μm in diameter is inversely proportional to the time of working during conventional (batch churn) butter manufacture. A consequence of uneven distribution of droplets containing microorganisms is a high degree of nonhomogeneity regarding microbial distribution in butter. Inadequate working of the butter in batch churns results in poor dispersion of water droplets and promotes microbial spoilage. Further, this defect can be observed on a trier in the form of moisture droplets. The defect is called "leaky" butter and results in a reduced score. This implies that availability of nutrients or inhibitor is limited by the fine dispersion of water droplets. Droplet size ideally is less than 10 μm.

3.3.7 Washing and Salting

Butter granules may be washed to remove excess buttermilk; however, this is not often done today. Salt added to butter inhibits microbial growth. However, salt must be distributed evenly in the moisture phase of butter effectively to inhibit microbial growth in water droplets. Insufficient working results in a nonhomogeneous distribution of salt in the water droplets. Salt creates an osmotic gradient between salt granules and buttermilk during working. This tends to cause aggregation of water droplets and can lead to free moisture ("leaky" butter) and a color defect called "mottling." Adequate working and use of finely ground salt or salt flour can minimize this defect.

The use of brine to salt butter is restricted to products with less than 1% salt, because the brine cannot contain more than 26% salt (w/w). Mostly, slurries of salt in saturated brine solutions containing up to 70% w/w sodium chloride are used. Salt granules used to produce a slurry should be less than 50 μm in diameter. Salt in the slurry should also be of high chemical purity, with insignificant levels of lead (<1 ppm), iron (<10 ppm), and copper (<2 ppm).

The microbiological quality of water used for washing or for brines is critical to production of a safe and stable product. Water with less than 100 cfu/mL total aerobic count when plates are incubated at 22°C and less than 10 cfu/mL total aerobic count when plates are incubated at 37°C has been deemed to be acceptable. Formerly, wash water was chilled and chlorinated at 10 ppm 2 h before use to control microflora. Little if any butter washing is done today.

Listeria survive in a saturated brine solution held at 4°C for 132 days. Thus, brines used to salt butter must be free of *Listeria*. Water is frequently contaminated with pseudomonads, and consequently care must be taken to insure water and brines used are free of these bacteria. The most common form of spoilage in butter occurs with species of *Pseudomonas*. Addition of salt to butter lowers the freezing point so that psychrotrophic microorganisms present may be able to grow at less than 0°C. Some psychrotrophic organisms multiply in salted butter stored as low as –6°C.

Distribution of salt in the moisture phase of butter has less impact on growth of yeasts and molds on the surface of butter as compared to bacteria. Humid conditions appear to have a greater

impact on mold growth than does the material on which they grow. Bacterial spoilage may occur in areas of butter with low salt in large droplets of moisture (poor working).

Varnam and Sutherland (1994), Kimenai (1986), and Munro (1986) have provided more detailed descriptions of continuous butter manufacturing processes.

3.3.8 Packaging

In batch operations, butter is loaded directly from the churn into hoppers and wheeled to packaging machines. Handling butter this way exposes it to air, workers, plant environment, and ambient temperatures that may accelerate spoilage. Control of the microbiological quality of air in the packaging room is therefore important. HEPA (High Efficiency Particulate Arrester) quality air with the filtration after temperature modification is desired. Practices that result in standing water on the floor or residual and spilled product facilitate growth of environmental contaminants. Practices that aerosolize contaminants often produce unacceptable levels of microbiological contamination in the air. Thus, maintaining dry conditions in the plant is preferred. Numerous approaches can be taken to monitor microbiological air quality, which include sedimentation, impaction on solid surfaces, impingement in liquids, centrifugation, and filtration. Air quality is particularly important in butter produced from continuous-type churns that may incorporate up to 5% air into the product (if a vacuum deaerator is not used). Most whipped butter does not have processing room air incorporated but instead uses purified compressed nitrogen gas. Gases used must be of acceptable microbiological quality.

Personnel hygiene is critical at this point of butter manufacture, because contaminants from hands, mouth, nasal passages, and clothing may be transmitted to butter during packaging. Few continuous churns are arranged to discharge product directly into the receiving hopper of packaging machinery. However, to ensure uninterrupted operation, it is common to transfer butter to a butter boat (open) or covered silo. Covered silos minimize the risk of further contamination from the plant environment. Screw augers in the bottom of the boat or silo move butter to the suction side of a rotary positive displacement pump which moves butter from the boat or silo to packaging equipment. Direct packaging into consumer-size containers is preferable over bulk packaging, because such butter must be reworked and repackaged before sale. Such

reworking increases the risk of contamination and subsequent spoilage of butter.

Cardboard boxes lined with vegetable parchment, parchment aluminum foil laminate, or a variety of plastic films are typically used for bulk packaging of butter. Polyethylene is the preferred material based on its physical properties (low density, high impact, cost effectiveness, absence of copper, and near sterile condition). Parchment, which supports mold growth under humid conditions, is still frequently used. Retail butter packs are typically wrapped in parchment, waxed parchment, or foil/parchment laminate and overwrapped with a cardboard container. Odors in storage refrigerators will permeate and ultraviolet rays from light will penetrate parchment wraps more rapidly than other wrappers and result in oxidized flavor. Individual butter packs, for example, continentals, cups, and chips, used in restaurants and food service are made at the time of packaging by appropriate high-speed equipment.

3.3.9 Pathogen Survival and Growth in Butter

Research conducted using the following pathogenic microorganisms has shown their growth in butter products: *L. monocytogenes* in butter at 4 and 13°C (made from inoculated cream), *S. aureus* in lightly salted (1% w/ w) whey cream butter at 25 and 30°C, and inoculated whipped butter at 25°C. *L. innocua* (not a pathogen but frequently associated with *L. monocytogenes* in environmental samples) was found in butter by Massa et al. (1990).

3.3.10 Food Poisoning Outbreaks

The incidence of documented food poisoning associated with butter is low. This is partially attributed to widespread use of pasteurization at elevated temperatures. Postpasteurization environmental contamination of cream or butter represents the greatest risk to butter contamination and spoilage. Several outbreaks of staphylococcal intoxication related to butter have been reported in the United States. In one instance, gastrointestinal illness developed in 24 customers and employees of a department store restaurant and was traced to whipped butter manufactured from whey cream. The same butter used to manufacture the implicated whipped product also resulted in one case of gastroenteritis. This butter contained 10 ng of staphylococcal enterotoxin A/g. In 1977, more than 100 customers of pancake houses in the Midwest became ill after consumption of whipped butter.

3.3.11 Spoilage

The two principal types of microbial spoilage of butter are surface taint and hydrolytic rancidity. Both conditions can be caused by growth of *Pseudomonas* spp. Some *Pseudomonas* spp. are psychrotrophic and produce proteases and lipases which may survive pasteurization and which hydrolyse protein and fat, respectively. *P. putrifaciens* can grow on butter surfaces at 4 to 7°C and produce a putrid odor within 7–10 days. This odor may result from liberation of certain organic acids, especially isovaleric acid.

Rancidity, the second most common spoilage defect, is caused by both microbial and nonmicrobial lipases, which degrade milkfat to free fatty acids. *P. fragi* and sometimes *P. fluorescens* are associated with this defect. Mold growth on butter also can cause hydrolytic rancidity for the same reasons. Molds that can cause this defect in butter include some in the genera *Rhizopus*, *Geotrichum*, *Penicillium*, and *Cladosporium*. Less common spoilage defects include malty flavor, skunk-like odor, and black discoloration. These defects are caused by *Lc. lactis* var. *maltigenes*, *P. mephitica*, and *P. nigrifaciens*, respectively. Other microbially induced color changes may result from surface growth of various fungi that produce colored spores. Heat-resistant proteases and lipases produced by pseudomonads that may grow during storage of raw milk or cream may result in spoilage of butter after manufacture even though spoilage organisms may have been destroyed by pasteurization.

3.3.12 Sources of Environmental Contamination

The necessity for milk, cream, and wash water to be of high microbial quality and the importance of pasteurization to public health have been described. Yeasts and molds are particularly resistant to dry conditions when compared to bacteria. Unlike bacteria, many of these fungi can grow at water activities (a_w) below 0.84. A few can grow below an a_w of 0.65. A study was reported in which molds would not grow on butter held at or below 70% humidity. Therefore, to prevent growth of osmotolerant yeasts and molds, a humidity of 60% or less should be maintained in the processing environment.

Ineffective sanitation of processing equipment could result in product contamination from equipment such as piping, pumps, silos, or other equipment. In our experience, the backplate of older positive displacement pumps (e.g., from pasteurized cream storage tanks) may be neglected during sanitation and become a microbial growth

niche, which in turn provides an inoculum to the product stream. Stress cracks in double-walled, insulated tanks can also provide a source of product contamination when the insulating material between walls becomes wet. Further, published data validating effective cleaning and sanitation on continuous churns through use of microbiological swabs are lacking.

Personal hygiene of employees working with butter is also important. Cross contamination from hands, mouths, nasal passages, and clothing must be precluded. Handling butter in restaurants may also result in cross contamination of a product; for example, when 1-lb prints are divided with knives used for cutting meat or when whipped butter is scooped with improperly sanitized equipment.

3.4 MICROBIOLOGICAL CONTROL OF BUTTER

3.4.1 Factors Limiting Microbial Growth in Butter

A variety of extrinsic (e.g., temperature) and intrinsic (e.g., salt in the moisture phase) factors combine to control the microflora of butter. Most important among these are (a) fine and uniform dispersion of moisture phase, (b) addition and uniform dispersion of salt, (c) low-temperature storage, and (d) use of lactic cultures (in ripened cream butter). Microbial growth is proportional to availability of nutrients and related to size of water droplets in butter. Thus, the smaller and more uniform the droplets, the lower the potential for microbial growth. Salt must also be distributed evenly in the moisture phase of the product effectively to inhibit microbial growth in contaminated water droplets. The approximate salinity of moisture in butter with 1.5% salt is 9%; this will inhibit growth of many bacteria. However, working may not result in a homogeneous distribution of salt in the water droplets. Data suggest that dispersion of water droplets, salt, and bacteria in butter made by continuous churns may be more uniform than in butter made with batch churns. Aerobic plate counts revealed a steady decrease in microbial contaminants in butter made in continuous churns compared with counts obtained on butter made from batch churns. Salt-free droplets were found in freshly worked salted butter made with a batch churn. Technological developments that allow for uniform dispersion of moisture, salt, and bacteria enhance both safety and shelf-life of butter.

Storage of salted butter at freezing temperatures is not adequate to guarantee complete cessation of microbial growth because of the depressed freezing point in the moisture phase of the product

resulting from elevated salt content and presence of other dissolved solutes. However, freezing is an effective means of storage for unsalted butter. O'Toole (1978) provided data that suggested that the lowest temperature limit for microbial metabolic activity in salted butter was –9°C. As a result of sensory evaluation, the flavor of butter held at –6°C was marginally less after 12 weeks; however, butter stored 8 weeks at 4 or 10°C dropped about one point in flavor score.

Some countries allow the use of potassium sorbate and sodium benzoate as preservatives in butter. However, countries such as the United States, United Kingdom, France, and Luxembourg prohibit preservatives in butter. Addition of 0.1% potassium sorbate inhibited growth of coliforms and molds in naturally contaminated butter. The inhibitory effect was enhanced when 2% salt was added along with 0.1% potassium sorbate. This inhibition occurred in all samples stored 4 weeks at –18 and 5°C.

Caution should be exercised in selection of any additives blended into butter products for flavor (e.g., honey, garlic, chopped herbs, and fruits), because they may contribute additional enzymes and microflora to the product. For example, unpasteurized honey added to butter will cause hydrolytic rancidity within 2 weeks because of lipase in the honey. Butter colorants that have not been mishandled have rarely contributed to the microflora of cream or butter.

3.4.2 Quality Assurance

Any quality assurance program should incorporate maintenance and documentation of good manufacturing practices (GMPs) and hazard analysis critical control points (HACCP).

3.4.3 Hazard Analysis Critical Control Points (HACCP)

An obvious critical control point for butter manufacturers is pasteurization or repasteurization of cream received at the manufacturing site. Control of the microflora in the manufacturing environment is also critical. Each plant must evaluate its individual process and develop its own risk assessment and HACCP plan. An environment sampling protocol should be aimed at monitoring for *L. monocytogenes*, *S. aureus*, and *Salmonella*. Recalls of butter because of *L. monocytogenes* contamination were reported as recently as 1994. Faust and Gabis (1988) have recommended areas of food plant environments that can be targeted for sampling for pathogens. Discovery of *Salmonella* or *Listeria* in the environment requires

immediate corrective action with documentation of the success of that action. Irbe (1993) has recommended that manufacturers of whipped butter develop in-plant guidelines for aerobic plate count and *S. aureus* at critical control points of manufacture. Finished products must be free of *Salmonella*, and *L. monocytogenes* and should be free of *Escherichia coli*.

Testing for these organisms can be done to validate success of the manufacturer's HACCP program. All testing of pathogens must be done away from the manufacturing site. Most in-plant laboratories are not equipped with the needed accessories to prevent spread of pathogens to the plant environment. Manufacturers should also test for lipolytic and psychrotrophic spoilage organisms in the finished product and develop a three-class attribute sampling plan. These data can be used to establish goals and measure success based on principles of continuous quality improvement. Sanitation of equipment used to manufacture product should be assessed regularly by testing environmental swabs for selected microbes.

The authors of this chapter recommend that pasteurized cream for butter manufacture has ≤5000 cfu/g (APC) with <2 coliforms/g. Finished butter should contain ≤5000 cfu/g (APC), <2 coliforms/g, no staphylococcal enterotoxins, no *Salmonella* in 375 g, no *L. monocytogenes* in 25 g, and <10 yeasts and molds/g.

3.5 MICROBIOLOGY OF RELATED PRODUCTS

Margarine, like butter, contains approximately 80–81% fat, 15% moisture, 0.6% protein, 0.4% carbohydrate, and 2.5% ash. In margarine, edible fats, oils, or mixtures of these with partially hydrogenated vegetable oils or rendered animal carcass fats are substituted for milkfat. Eighty percent fat in butter and margarine is considered too high by many individuals concerned about their diets. Consequently, numerous spreads have been manufactured with lower fat contents. In many countries, there are no legal standards or definitions for these low-fat spreads. However, a working categorization has been made based on fat content. *Full-fat spreads* are described as those with fat contents of 72–80%; *reduced-fat spreads* have 50–60% fat; *low-fat spreads* have 39–41% fat, and *very low-fat spreads* have less than 30% fat. Vegetable fats, mixtures of vegetable fat and milkfat, and milkfat alone have been used to develop these spreads. Another trend has been production of spreads in which fat has been replaced in part or completely by a variety of substances such as Neutrifat, Simplesse, and Stellar. Olestra a sucrose

polyester with fatty acids, was recently (1996) approved by the U.S. Food and Drug Administration (FDA) as a substitute for conventional fats and may appear in products in the future.

3.5.1 Dairy Spreads: Manufacture and Microbiological Considerations

Low-fat spreads are also water in oil emulsions but contain more moisture than butter. Consequently, there is increased likelihood of microbial growth in these products unless preservatives are added. The use of preservatives is allowed in some countries but not in others. Because of combining ingredients at 45°C, in an emulsifying unit, growth of thermoduric organisms (e.g., *Enterococcus faecium*, *E. faecalis*) and thermophils may occur. Higher fat dairy spreads are typically made using a swept-surface heat exchanger and texturizer where the aqueous blend of ingredients is mixed in the correct ratio with oil-soluble ingredients.

Crystallization of fat during working is critical to obtain desired consistency and spreadability in the finished product. Rapid supercooling to −10° to −20°C under high sheer conditions in the scraped surface heat exchanger initiates and maintains crystallization and disperses moisture within the fat matrix. Control of cross contamination during packaging is more critical than in butter manufacture because of the higher potential for microbial growth in spreads.

Microorganisms that cause spoilage in butter have been implicated in margarine spoilage. However, vegetable fats are typically more resistant to lipolytic breakdown than is milkfat. *Yarrowia lipolytica*, *Bacillus polymyxa*, and *E. faecium* are spoilage organisms of concern in low-fat spreads. Lanciotti et al. (1992) showed that *L. monocytogenes* and *Yersinia enterocolitica* can grow in "light" butter at 4 and 20°C. A class I recall of 60% butter, 40% margarine product occurred in 1992. More detailed descriptions of margarines, spreads, and industrial milkfat products can be found in the report by Varnam and Sutherland (1994).

The safety record of butter has improved considerably since the advent of cream pasteurization and improvements in churn design, sanitation, and water quality. However, rigorous adherence to GMPs with appropriate environmental sampling and HACCP are necessary to ensure the safety and prolong the shelf-life of butter and spreads.

4

Cheese Products

The origin of cheese is lost in antiquity. But, most assuredly, milk was contaminated with lactic acid bacteria, which through acidification of the milk, created conditions unfavorable for growth of other bacteria. As the story goes, milk held in storage vessels (animal stomachs) clotted, making cream cheese, the "mother of all cheeses." The acid environment caused milk proteins to clot. It was a great leap forward when centuries later humans discovered the use of coagulating enzymes. This led to production of less sour cheeses. Natural contamination of milk or cheese by bacteria, yeasts, and molds led to development of a multitude of flavor sensations in cheese as it aged. Imagine, a long time ago, when humans first tasted that odorous morsel covered with colorful molds, yeasts, and bacteria. But now consider a world without Roquefort, Stilton, Limburger, or Gruyere. Boring! Unthinkable!

Modern cheese making is controlled and has been refined through strict adherence to manufacturing guidelines and careful selection of specific lactic acid bacteria and ripening microorganisms. Even so, sometimes there are problems. No cheese is produced in a sterile environment, so contamination is inevitable. One of the chief causes of poor flavor quality in cheese is the undesirable metabolism of contaminating microorganisms. A preventable cause of poor-quality flavor is that many retailers sell products long after they have reached the end of their expected shelf life. The ability of a cheese to age well with regard to undesirable microbial growth depends on cheese composition, manufacturing protocol, level of contamination, and ability of the contaminants to grow in cheese.

Therefore, cheese maker, retailer, and consumer must be aware of limitations of the product with regard to growth of contaminants and defects that they cause. It must be kept in mind that not all undesirable attributes of a cheese result from contaminating microorganisms. Some cheese defects may be caused by poor milk quality (late lactation milk, milk from mastitic animals high in enzymes of animal origin, i.e. lipase and protease), inappropriate rate of acid development by the starter, or poor manufacturing and storage regimens.

Although there are more than 1000 named varieties of cheeses worldwide, this chapter discusses only the major types.

4.1 DAIRY CHEMISTRY AND THE CHEESE-MAKING PROCESS

For many cheese makers, there is an art to making cheese. To cheese manufacturers, it is commonly a routine, strictly controlled process. No matter how it is made, cheese is a complex entity in a constant state of change, which has been likened to an ecological community of living organisms in which microbiological activities affect and are influenced by chemical changes.

Production of cheese involves two interconnected phases: The first is to develop the desired composition and pH, and the second is to develop desired physical and flavor characteristics. The first phase is controlled through milk composition and manufacturing protocol, particularly rate and extent of acid development by the starter during the manufacturing process. The second phase is influenced by the first but is dictated by metabolism of a variety of microorganisms and by enzymatic and chemical reactions. This process is called ripening, curing, or maturation, and depending on the cheese variety, may take many months to complete.

In concept, manufacture of cheese is simple. In reality, it is a complex process governed by a series of interrelated chemical and physical phenomena. During cheese making, a coagulum is formed in which milk proteins (caseins) are clotted, entrapping the milk fat, water, and water-soluble components. Further manipulations of the coagulum (cutting, heating, stirring) and development of acid result in controlled moisture expulsion and desired physical and chemical changes of caseins. The resulting curd and whey mixture is separated, with curd being formed into blocks, wheels, or other shapes.

Development of desired flavor, body, and texture is brought about through a combination of the activity of specific introduced

microflora and enzymes as well as naturally occurring or contaminating bacteria and enzymes. Part of the initial maturation process involves physical changes to the protein brought about through a decrease in pH, loss of calcium, and hydration of casein. Without the ripening process, it would be impossible to distinguish one variety of cheese from another except to note that different cheeses may have different physical characteristics.

Milk solids are composed of protein (casein and whey protein), milkfat, lactose, citric acid, and mineral salts (usually associated with the casein) collectively called ash. The composition of milk varies considerably between species and individual animals. It is affected by breed and genetics of the animal, feed, environmental conditions, lactation number, stage of lactation, and animal health. All of these factors can also influence cheese making and cheese characteristics. An average composition of cow milk is as follows: 87.6% water, 3.9% milk fat, 3.1% true protein (82% caseins, 18% whey proteins), 4.6% lactose, 0.7% ash.

There are three basic ways to make cheese, but a given variety is made with only one method. All methods involve development of acid by a select group of lactic acid bacteria called the starter. All methods involve some means of concentrating the milk solids (mostly milkfat and protein) by expelling a portion of the aqueous phase of milk (serum or whey).

Rennet curd cheeses (most varieties) are made by clotting milk with a coagulating enzyme (all are proteolytic enzymes) such as chymosin (the most active ingredient in rennet). Acid curd cheeses (cottage, cream) are made with acidification of milk sufficient to cause casein to form a clot. Heat-precipitated curd cheeses (ricotta, queso blanco) are made with a combination of low pH and high heat to precipitate proteins (both casein and some whey proteins).

Fresh or nonripened cheeses such as cottage and mozzarella can be made by direct addition of acid (acetic, lactic, or citric). Cheeses made by this method are called direct acid cheeses (e.g., direct acid mozzarella).

4.1.1 Rennet Curd Cheese Manufacture

Rennet curd cheeses are those in which the coagulum is formed by activity of a coagulant, an enzyme mixture with particular proteolytic activity. Coagulants are commonly called rennets. Calf rennet is derived from an extract of calf stomachs, but there are

other rennets derived from different sources: fungi, other animals, and some plants, especially thistles. All contain proteolytic enzymes, which, through their activity, help to destabilize casein micelles in milk, an event that subsequently transforms milk from a liquid to a semisolid (coagulum). Chymosin is the desired coagulating enzyme in calf rennet, but because of cost, demand, and the lack of calf stomachs, most chymosin used in the United States is produced by genetically engineered bacteria, yeasts, or molds. Fermentation-derived chymosin is highly purified (100% purity) and is used in liquid or tablet form. Chymosin is the preferred coagulant, because it has specificity toward one peptide bond in κ-casein. Although chymosin hydrolyzes bonds in casein molecules at other sites when they are accessible, the specific site of hydrolysis that occurs during coagulation is Phe_{105}-Met_{106}. The nonspecific proteolytic activity of some other coagulants causes concern over excessive proteolysis, leading to a soft-bodied cheese, bitter flavor defects, and reduced cheese yield.

Caseins exist in complexes of discretely arranged molecules called micelles. There are four types of casein molecules, α_{s1}, α_{s2}, β, and κ-caseins. The exact molecular arrangement of molecules is not known, but it is hypothesized that micelles are composed of groups of casein molecules linked together through various types of bonding, including calcium phosphate bridges, and most importantly electrostatic and hydrophobic interactions. A hydrophilic (and negatively charged) portion of κ-casein molecules protrudes from the micelle surface, giving the micelle stability from spontaneous aggregation.

At the normal pH of milk (6.6–6.7), micelles carry a net negative charge because of the nonprotonated amino, carboxyl, and phosphate groups on caseins. Through electrostatic repulsion and stearic hindrance via the "hairs" of κ-casein, micelles are stable (show no tendency to flocculate or gel) and remain as individual entities. Activity of the coagulant removes the protruding, hydrophilic region on the κ-casein molecule. This eliminates stearic hindrance and reduces the negative charge at the micelle surface. With loss of these barriers, micelles begin to come together (clot formation). Ionic calcium (added as $CaCl_2$ or released from micelles through acidification of milk) allows adjacent micelles to aggregate through hydrophobic and electrostatic interactions. Eventually (20–30 min), casein micelles form a continuous network of aggregates called the clot or coagulum.

Milkfat, water, and water-soluble components (serum) are entrapped within the casein network. Undenatured whey proteins are water soluble and do not participate in forming the network but are trapped in spaces (pores) that form between aggregates of micelles.

Once the desired firmness of the coagulum has been reached, it is cut into small cubes or pieces (curd). The firmer the coagulum when cut and the larger the curd particles, the higher the moisture content of cheese. After the coagulum is cut, casein molecules continue to interact and squeeze out serum trapped between them, and with exogenous pressure, curds shrink and become firmer. This process is called syneresis and is enhanced by lowering the pH, increasing the temperature of curd (cooking process), and stirring the curd. Therefore, the rate of acid development by the starter has a great influence over moisture content of cheese and control over the rate of acid development is key to successful cheese manufacture. Body (soft to firm) texture (grainy to smooth), melt, stretch, chewiness, oil release during baking, casein hydration, and color of cheese are directly controlled by pH. In addition, growth and metabolism of microorganisms and flavor development are strongly influenced by pH.

Each variety of cheese has a desired rate and extent of acid development, which if not met or compensated for, may result in too much or too little moisture or too high or too low pH, creating undesirable physical and flavor characteristics in cheese. At the proper time, curd is separated from whey and treated appropriately as dictated by the variety of cheese. Curd may be continuously stirred as whey is being removed or it may be allowed to mat. Curd may be salted first and then formed into the desired shape or formed first and then salted by placing the cheese into brine. Pressing of blocks, cylinders, or wheels of cheese removes trapped whey from the cheese and helps individuals curds to fuse, forming a solid mass of cheese. Not all cheeses require pressing. The unripened cheese is then ready for maturation. Camembert and surface-ripened cheeses (Limburger) will be inoculated with specific microorganisms at this time.

4.1.2 Acid Curd Cheeses

Acid curd cheeses do not rely on activity of a coagulating enzyme to clot milk. Instead, milk is acidified by direct addition of acid or through lactic acid developed by starter bacteria. At a pH of approximately 5.2, caseins in milk begin to flocculate and eventually

gel as the pH decreases. Gelation is the consequence of acidification-induced physicochemical changes to caseins. At neutral pH, casein micelles remain as individual entities and are unable to interact or form aggregates. This is, in part, caused by charge repulsion (micelles are negatively charged). In addition, hydrophilic regions of κ-casein molecules protrude from the micelle core and prevent hydrophobic cores of adjacent micelles from interacting (stearic repulsion).

As the pH is lowered, the calcium-phosphate complex disintegrates and some casein molecules dissociate from micelles. There is also a reduction of the net negative charge on casein molecules, an increase in hydrophobic interactions, and it is thought that the protruding portion of casein molecules falls back onto the casein micelle core. The net result is that micelles and solubilized casein molecules begin to form aggregates, eventually leading to formation of a continuous network of aggregates and visible gel (pH ~4.95). In cottage cheese, the gel is cut into small cubes at a pH of 4.65–4.75. Serum (whey) is immediately expelled from the curd.

In cream cheese manufacture, the gel is stirred at pH 4.4–4.8 rather than cut as in cottage cheese, and whey is removed by centrifugation. Traditionally, clotted milk was put into bags of cheesecloth and hung to filter out serum. A low pH of cheese tends to produce a grainy or gritty product. Separated cheese is packaged (cold-pack cream cheese) or processed. Hot-pack cream cheese is made by blending cold-pack cream cheese with cream, whole milk, salt, stabilizers, and skim milk solids and heating the mixture to (72–74°C). The homogenized blend is packaged hot. Microbiologically induced defects are similar to those in cottage cheese but are less likely to occur, because the cheese is packaged hot.

4.1.3 Acid-Heat Coagulated Cheese

The premise for manufacture of acid-heat coagulated cheeses is to heat milk to 78–80°C and then acidify milk by direct addition of citric, acetic, or lactic acid to the desired pH (5.8–5.9 for ricotta, 5.2–5.3 for queso blanco). Milk for queso blanco can also be first acidified by lactic acid bacteria (*Lactococcus* spp.) and then heated. Heating of the milk (ricotta milk is usually a mixture of sweet whey, whey protein concentrate, and milk) causes coagulation and flocculation of caseins and whey proteins. In ricotta cheese manufacture, proteins and entrapped fat are removed or filtered from the remaining serum and drained until packaged. In ques

blanco cheese manufacture, curds are allowed to settle and whey is drained. Curds are then salted and pressed. Both cheeses are consumed fresh, and because denatured whey protein forms a network with the casein, the cheeses resist melting during frying or baking. Because of the high-heat treatment under acidic conditions, survival of bacteria other than spore formers is minimal, but contamination during packaging is of concern. Microbiologically induced defects are comparable to those of cottage cheese. Most defects are caused by growth of *Pseudomonas* sp., yeasts, and molds.

4.2 INFLUENCES OF MICROBIOLOGICAL QUALITY AND MILK COMPOSITION ON CHEESE QUALITY

The microbiological quality and composition of milk play an integral part in the quality of the cheese made from it. Cheese can be made from grade A or grade B milk, but cottage, cream, and mozzarella cheeses must be made from grade A milk only. The bacterial count of grade A milk, as determined by a standard bacterial count or loop count, cannot exceed 100,000/mL at the time of receipt or collection. The bacterial count of grade B milk cannot exceed 300,000/mL. Processors often pay premiums for low bacterial count milk as an enticement to farmers to produce high-quality milk. In practice, processors have recorded that milk from greater than 90% of producers has a bacterial count of less than 20,000/mL. The bacteria found in the milk arise from contamination (especially from air and biofilms on equipment) or from the animal itself.

The level of contamination is reflective of the cleanliness of the entire milking operation, including that of the animal before milking. Clostridia and lactic acid bacteria generally originate in silage and other feeds and are concentrated in feces. High levels of clostridia in silage indicate poor lactic acid fermentation. Feces can get on the udder, and if the udder is not cleaned, milk can become contaminated. Improper cooling rates or final holding temperatures of milk result in high numbers of bacteria reflective of an environment conducive to microbial growth. Most bacteria in milk are, not surprisingly, psychrotrophic bacteria and they are the contaminants likely to grow at the low temperature at which milk must be stored (not to exceed 7°C for grade A and 10°C for grade B within 2 h after milking). *Pseudomonas* spp. are usually the dominant psychrotrophic organisms found in milk. Although these bacteria are easily killed by pasteurization, they produce lipases and

proteases, which are not totally inactivated by this heat treatment. The enzymes are active in milk and can cause bitterness (protein hydrolysis) and rancidity (milk fat hydrolysis) in products made from milk if the level of activity is high enough. Milk may be held for 2 days (legally) after receipt at the factory and microbial counts will undoubtedly increase. It is growth of *Pseudomonas* sp. during refrigerated milk storage that concerns the cheese maker.

A more important cause of rancidity in milk and cheese is activity of endemic animal lipases (milk lipase). The level of activity of this enzyme is increased in milk obtained from animals with mastitis (udder infection). In this instance, lipase activators and somatic cells are secreted from blood into milk. Somatic cells are used as an indicator of cow health and limits have been set by individual states (not to exceed 750,000/mL). Milk from mastitic animals has decreased casein content, the major protein found in milk, although the total amount of all proteins (whey proteins increase) may decrease only slightly, if at all.

The composition, quality, and amount of cheese produced are greatly affected by the casein content of milk. The other proteins, collectively called whey proteins, are water soluble and contribute much less to cheese yield. The lower the casein content of milk, the lower the yield of cheese. Cheese makers do not routinely directly measure casein in milk, because the test is expensive and takes too long to complete. Instead, they use fast, inexpensive, automated tests to measure total protein. Casein content is calculated by multiplying the percentage of total protein by 0.82. In mastitic milk, however, the amount of casein as a percentage of total protein decreases. Cheese makers cannot predict this value. Rather a high somatic cell count indicates that the casein content of milk may be reduced. Consequently, the cheese maker commonly pays premiums for low somatic cell count milk.

4.3 MILK PRETREATMENT: CLARIFICATION, STANDARDIZATION, AND HEAT TREATMENT

All milk received by the cheese plant is first tested for the presence of antibiotics. Milk containing antibiotics must be dumped (liquid manure or landspread) even though, if diluted with other milk, a negative test could be obtained. Raw milk, as the cheese maker receives it, is almost universally filtered to remove extraneous matter (straw, hay, and large clumps of bacteria). The Code of Federal Regulations establishes fat (milk fat content by weight of

the cheese solids or fat in the dry matter [FDM]) and moisture limits for some cheeses. These values are called the standard of identity. The casein to milkfat ratio in milk determines the FDM of cheese, whereas moisture is controlled by the manufacturing process. The use of whole milk almost always results in cheese with an FDM of at least 50%. To manufacture cheeses with a lower FDM, such as part-skim mozzarella or Swiss cheese, milkfat is removed or skim milk is added to whole milk. The process of manipulating the composition of milk is called standardization and is becoming more popular for all cheese types because of economic considerations and a desire for uniformity of cheese composition and cheese yield.

4.3.1 Heat Treatment

Heat treatment given milk before cheese making varies from country to country, cheese maker to cheese maker, and cheese to cheese. Pasteurization of milk is a legal requirement in the United States for fresh cheeses such as cottage, mozzarella, and reduced-fat varieties. It is based on a 9-log destruction of *Coxiella burnetti*. Cheeses made from unpasteurized milk must be held for 60 days at a temperature not less than 1.7°C. It is thought that pathogens will die out during this time period because of acidic conditions in cheese and growth of nonstarter lactic acid bacteria. However, this may not be true, especially if the level of contamination is high. Manufacturers who do not pasteurize milk use another heat treatment (65–70°C for 16–20 s), but the trend is toward pasteurization. A main argument against pasteurization is that cheeses made from pasteurized milk tend to have a milder flavor (the flavor takes longer to develop or the flavor is atypical of raw-milk cheese). Research into development of flavor in cheese may provide means to overcome this perceived obstacle, but the question of safety of raw-milk cheeses remains. Pasteurization is not a guarantee of safety, because milk or cheese can be contaminated after the milk has been pasteurized. When cases of illness can be attributed to consumption of cheese containing pathogens (a rare event), often the cheese is manufactured under poor hygienic conditions, is a fresh cheese, is made from unpasteurized milk, or the rate and extent of acid development were curtailed. The rate of acid development is critical (as well as contamination in the first place), since some bacteria, especially coliforms, will not grow well at low pH and higher acid cheeses. It is not uncommon to find coliform bacteria in washed curd cheese varieties (lower in acid content—baby Swiss, reducedfat varieties)

or in cheeses where the acid development was slow (especially because of phagic infection).

The effectiveness of pasteurization in killing bacteria in milk depends on initial microbial numbers, composition (fat and sugar), and thermoresistance of individual microorganisms. The thermal death time of bacteria is logarithmic. This implies that within a given population of a single strain of microorganism, some individuals will survive pasteurization and other individuals will be killed. By definition, thermoduric microorganisms survive pasteurization, and by convention, thermoduric bacteria are classified as being thermoduric based on the potential for individual bacterial cells within a population to survive pasteurization. Genera containing thermoduric species include *Microbacterium, Micrococcus, Bacillus* spores, *Clostridium* spores, *Streptococcus, Coryneform, Enterococcus*, and *Lactobacillus*. Some of these bacteria are responsible for a variety of cheese defects, such as excessive softening of cheese, splits and cracks, off-flavors, and abnormal color. Thermoduric bacteria may colonize in the regenerative section of the pasteurizer. Indeed, a solution to keep numbers of thermoduric microorganisms low is to clean and sanitize the pasteurizer more often.

Although rarely used in the United States, a specially designed centrifuge called a Bactofuge (bactofugation) is used to remove most of the bacterial cells and spores (empirically 98%) from milk. Two streams of milk result from bactofugation, the "cleaned" milk and the bactofugate containing bacterial spores and cells. If used, the bactofugate is heated to 130°C for a few seconds, but the milk is pasteurized. The two fractions are then recombined. Bactofugation is used in Europe in lieu of sodium nitrate in controlling outgrowth of *Clostridium tyrobutyricum* spores, whose metabolism results in gassy, rancid cheese. The use of sodium nitrate in cheese is not permissible in the United States.

After heat treatment, milk is cooled to the temperature conducive for optimal starter activity and pumped into specially designed vessels called vats. Cheese vats vary in size, with the larger vats holding as much as 22,700 kg and the smaller commercial vats holding approximately 4500–6800 kg. Vats are generally double walled to permit controlled indirect heating of milk. If starter is used, it can be added while milk is being pumped into the cheese vat or after the vat is filled. The temperature of milk at the time starter is added is determined by the type of cheese to be made, type of

starter, and the desired temperature at the time of coagulant addition, but it is generally between 31 and 34°C.

4.3.2 Starters

The strains and balance of strains of bacteria used in starters is often dictated by tradition as much as it is by manufacturing protocol and desired cheese characteristics. The choice of starter depends on the desired rate and extent of acid development (pH) during manufacture, proteolytic activity of the strains, flavor (and gas formation if desired), and conditions encountered during manufacture and storage such as pH, acidity, salt, and temperature profiles. Mesophiles are sometimes used to manufacture mozzarella (non–pasta filata type) and Swiss varieties instead of the traditional thermophilic starters. In these instances, a lower cook temperature is used and the resultant cheese is generally higher in moisture and may have a slightly different flavor profile (more acid, less buttery). The amount of starter used is based on the rate of acid development desired by the manufacturer and is dictated by cheese variety, but it is influenced by strain and how the culture was propagated (conditions of growth such as media, pH control, and age). This is an important concept, because amounts of starter listed in literature for cheese manufacture can be misleading (e.g., use of 1% w/w starter grown with no pH control may be equivalent to using 0.2% w/w starter grown with pH control). The use of artisinal cultures is not common in the United States. These cultures are mixtures of several genera, species, and strains of lactic acid bacteria. They may contain lactococci, lactobacilli, leuconostocs, streptococci, and enterococci and probably give the cheese special flavor characteristics.

4.4 CHEESE MICROBIOLOGY

The diversity of cheese-manufacturing protocols, ripening regimens, and composition makes cheese a complex subject microbiologically. It is a misconception to think of cheese microflora in terms of the type of cheese; for example, all Cheddars, blue cheeses, and so on. Each individual cheese (not type) has its own unique microflora regardless of the starter or any deliberately added secondary ripening microorganisms (e.g., molds or yeasts). There is an extensive list of adventitious microorganisms that can grow in or on cheese, but their presence in any cheese is governed by chance. These nonstarter, nondeliberately added microbes are contaminants to milk or cheese. Thus, the contaminants that are found in any

cheese result because the specific microbes happen to be in milk or on equipment, in air, or on humans that have had direct contact with the milk or cheese. It is extremely difficult to interpret data on microbial content of cheese because of chance contamination. In addition, the cheese environment plays a critical role in growth of microorganisms.

Microorganisms that grow in cheese or at least maintain viability follow the same set of criteria (pH, moisture, salt, acidity/type of acid, redox potential, nutrient availability, competition, temperature, anaerobic/aerobic conditions) as in any food product. Two factors determine the microflora of cheese: presence and survival of the microorganism and ability of the microorganism to grow.

During cheese maturation, environmental conditions can change sufficiently to allow growth of initially inhibited contaminants, or conditions may become even more inhospitable. The cheese environment is dynamic. Thus, the microflora in cheese can be considered to be a dynamic ecological system. Few studies on bacterial viability in cheese have been completed in which changes in cheese chemistry during maturation are correlated with its effect on the microflora.

A complicating factor in the study of cheese microflora is methodology used to isolate microorganisms. Selective media may provide too harsh an environment for recovery and growth of injured or stressed cells. Microorganisms may be viable and metabolically active but not culturable with current methods. Nonselective media may not be appropriate to detect low numbers in a competitive environment.

Why is it important to study the microorganisms in cheese? Pathogens in cheese are of utmost importance. However, flavor quality (both desirable and undesirable) of cheese is also a consequence of the metabolism of microorganisms. Additionally, some textural defects can be directly attributed to growth and metabolism of microorganisms.

Molecular techniques are being applied selectively to determine the presence of individual species and strains of bacteria in cheese. The polymerase chain reaction (PCR) is a rapid procedure for in vitro enzymatic amplification of a defined segment of DNA. It is particularly useful in identifying the proverbial needle in the haystack and individual strains of bacteria. A unique oligonucleotide sequence (probe) can be used specifically to identify (through amplification)

the presence of DNA from particular bacteria in cheese. Enumeration of the bacteria is not necessary, but the bacteria may no longer be alive. DNA extracted from individual bacteria isolated using traditional techniques can also be tested to determine the exact species or strains of bacteria. Of particular interest is rapid detection of low levels of pathogens in milk and cheese. The technique has also been applied to identify species of *Clostridium* in cheese, new strains of *Lactococcus lactis* subsp. *cremoris*, individual strains of *Lactobacillus helveticus* and nonstarter lactobacilli in Cheddar cheese.

Many of the adjuncts used to enhance flavor of cheese are *Lactobacillus* spp. and are often not easily differentiated from other strains of lactobacilli by biochemical tests. Complicating the situation is that lactobacilli are the dominant nonstarter lactic acid bacteria found in cheese. Selective media for lactobacilli cannot differentiate between adjunct and contaminant lactobacilli. This makes it difficult to determine numbers of individual strains of lactobacilli in mixed populations of lactobacilli. It is important to follow numbers of individual strains of lactobacilli (or other bacteria) to study the cause and effect of *Lactobacillus* spp. (or other bacteria) on flavor development in cheese. In addition, the ability unequivocally to determine the presence of patented or licensed strains of adjuncts can be useful for legal purposes.

4.4.1 Cottage Cheese

Cottage cheese curd is made from grade A pasteurized skim milk. Fortification of milk low in casein (<2.4% casein or <9% total milk solids) with very low heat–treated nonfat dry milk can improve cheese yield and quality.

Milk is inoculated with *Lc. lactis* subsp. *lactis* and *cremoris*, with the latter being generally preferred. Commercially, cottage cheese is usually made with a "short set"; that is, 4–5 h elapse between time of starter addition (milk pH 6.60, 31–32°C) and time of cutting (coagulated milk pH 4.70–4.8). However, a "long-set" method is also used. To promote efficiency, the long-set method is sometimes used. Vats are filled with milk (20–22°C) and starter is added so that over-night (9–12 h) the pH of milk decreases to 4.90. Thus, when the cheese maker returns at the start of the workday, the milk coagulum is almost ready to cut. In the short-set method, the inoculation rate of the starter is 3–5% w/w of milk; whereas in the long-set method, much less (0.5–2% w/w) starter is used.

The pH at which curd is cut and the final pH of the curd after processing are critical for yield and cheese quality but vary among processing plants. This variability results from a variety of factors, including casein content of milk, heat treatment of milk, and rate of acid development by the starter. Overacidification or underacidification leads to brittle curd that shatters when stirred. Tiny pieces of curd may be lost in subsequent manufacturing steps, causing a loss in yield; or if retained, they may cause graininess (many hard bits of curd) and lack of uniform curd size (a visual defect that downgrades the product). Most manufacturers use a very small amount of coagulant. This enables curd to be cut at slightly higher pH. Curd is less fragile and yield is higher.

Once the coagulum is cut, the curd and whey mixture is heated to 54–57°C (in approximately 2 h) and held (15–20 min) until proper firmness is reached. Rate of heating and final temperature can prevent overacidification and firms curd (removes whey). Although strain dependent, most lactococci do not produce significant amounts of acid at temperatures above 40°C and are reduced in number by the cooking procedure. *Pseudomonas* spp. and Enterobacteriaceae, common spoilage bacteria of cottage cheese, are also sensitive to the cooking procedure, which greatly reduces their number. The lethality of the cooking procedure is time and temperature dependent and is determined by the initial bacterial load. Therefore, the lower the bacterial population at the time curd reaches the final cooking temperature, the more effective a given heat treatment is.

After correct curd firmness is reached, most whey is removed and curd is washed two or three times with cold water. The wash step removes lactic acid and lactose and helps to control the level of acidity (acidic taste) in the finished cheese. Water is acidified (pH 4.5–6.0) and chlorinated (5–10 ppm) or pasteurized to kill bacteria. Washing cools curd rapidly to less than 5°C, which is essential to keep growth of contaminating bacteria to a minimum. After the last wash water is removed, pasteurized cold cream dressing is added and the product is packaged. The amount of fat in the cream dressing determines the fat content of the final cheese, so reduced-fat cottage cheese is made by adjusting the solids and fat content of the cream dressing.

Contaminated equipment and air are the most likely sources of spoilage bacteria in creamed cottage cheese. Although cottage cheese curd is acid (pH approximately 4.5–4.7), the pH of the final

commercial product, creamed cottage cheese, is higher (5.0–5.3). The pH of the creamed cheese can be manipulated by the acidity of the cream dressing. Low product pH (5) may lead to free whey accumulation (clotting and syneresis of cream dressing) during storage, whereas a higher pH allows for increased growth of contaminating bacteria. The final product should be stored at less than 5°C. Although salt is added in the dressing, the salt in moisture ratio (S/M) of creamed cottage cheese (1–2%) is not high enough to hinder growth of contaminating bacteria. The dressing also contains lactose, which can be fermented by undesirable microorganisms and starter if they survive the heating step.

In properly manufactured creamed cottage cheese, the environmental conditions within the cheese (low acid, relatively high pH, low S/M) are not harsh enough strongly to inhibit growth of most psychrotrophic contaminants. Thus, similar to conditions in raw or pasteurized milk, microorganisms able to grow fastest at low storage temperatures are the dominant ones found in cottage cheese. Gram-negative psychrotrophic bacteria such as pseudomonas (particularly *P. fluorescens*, *P. fragi*, and *P. putida*), Enterobacteriaceae (coli-forms, especially *Enterobacter aerogenes*, *E. agglomerans*, and *Escherichia coli*), *Alcaligenes*, *Achromobacter*, and *Flavobacterium* are the contaminants most likely to be found in cottage cheese. All these bacteria are destroyed by pasteurization. *Pseudomonas* spp. are obligately aerobic and predominate at the surface, whereas coliforms are aerobic and facultatively anaerobic and sometimes can be found throughout the cheese. Their growth and metabolism, as well as that of yeasts and molds, result in undesirable flavors (called unclean, putrid, rancid, fruity, and yeasty), surface film, and discoloration.

As with other cheeses, consumer acceptance of cottage cheese flavor varies considerably. Cottage cheese is consumed as a fresh product (a few days to 4 weeks old) and the ingredients (milk, nonfat dry milk, cream) can all influence the flavor. However, three main concerns can be controlled microbiologically: level of acidity, diacetyl (aroma), and level of undesirable flavors. The wash treatment and cream dressing can be used to adjust pH and acidity of cheese. Diacetyl can be added directly as a starter distillate or can be formed in the cream dressing through metabolism of citric acid by *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* and *Leuconostoc* spp. However, in addition to development of undesirable flavors,

Pseudomonas spp., *Alcaligenes*, and *E. aerogenes* can oxidize diacetyl to acetoin, a flavorless compound. This results in cheese that is bland or flat in flavor.

Growth of microorganisms in cottage cheese is inhibited most effectively by low storage temperature (<5°C), but it is also affected by pH and antimicrobials. Potassium sorbate may be added to control yeasts, molds, and certain bacteria, although it may impart bitterness in creamed cottage cheese at levels greater than 0.075%. As with other acids, the effectiveness of sorbate depends on the sensitivity of spoilage organisms and is a function of antimicrobial concentration of the undissociated form of the acid in the aqueous phase (pKa). It is enhanced by lower pH, the symbiotic effect of other antimicrobials, lower initial microbial load, and lower storage temperature. Thus, the degree of shelf life extension resulting from the use of sorbate is directly related to the quality of the initial product and subsequent handling.

Microgard is grade A skim milk that has been fermented by *Propionibacterium freudenreichii* and then pasteurized. It is widely used by the cottage cheese industry to inhibit growth of gram-negative bacteria, some yeasts, and some molds. The actual inhibitory compound is a bacteriocin (700 D, heat stable, and proteinaceous in nature).

Direct injection of CO_2 into cream dressing has been shown to inhibit growth of *Pseudomonas*, *Listeria monocytogenes*, and *Clostridium sporogenes*. The technique has been used commercially without the side effect of "carbonation" flavor in the cheese. It is claimed substantially to improve the shelf life. It is believed that the CO_2 enters cells and inhibits growth or kills cells by lowering the pH within the cell. The technique is more effective at 4°C than 7°C.

The relatively short storage time (2–4 weeks) and rapid attainment and maintenance of low temperature during storage (5°C) probably preclude growth of contaminating lactobacilli. However, if the temperature of storage is high enough (7°C), as may occur in retail outlets, metabolism of lactobacilli may be a potential problem. Of particular concern is acid development through metabolism of lactose by either the nonstarter lactobacilli or surviving starter bacteria or lactococci used in fermentation of the cream dressing. Poor acidification results in free whey or watery cheese and an acid-tasting product. Growth and metabolism of psychrotrophic microorganisms are also increased.

In the past, mixed-strain cultures, which included high levels of *Lc. lactis* subsp. *lactis* biovar *diacetylactis* were inadvertently used. These bacteria produce gas (CO_2) from cometabolism of lactose and citric acid. The gas causes the curd to float, but the curd structure is also disrupted and weakened, leading to curd that is more easily shattered as the curd is stirred.

4.4.2 Internally Ripened Blue Mold Cheeses

Roquefort, Stilton, blue, and Gorgonzola are examples of cheeses in which development of flavor is dominated by metabolism of *Penicillium roqueforti* or *P. glaucum*. These molds grow throughout cheese (internally ripened) and are able to grow in the low-oxygen, high-salt conditions that are typical of these cheeses. To facilitate exchange of air with CO_2 produced in cheese (via mold metabolism), cheese is manufactured to produce an open texture and is pierced or punched with large-bore needles. If the texture is too tight, mold only grows near the puncture. In addition, internally mold-ripened cheeses may also be surface ripened with yeasts and bacteria (e.g., Stilton); a process that provides for distinctive taste sensations in a number of cheeses, including Limburger.

By international agreement, Roquefort cheese must be made from sheep milk, in the Roquefort Valley of France, and ripened in naturally air-conditioned, high-humidity caves near the town of Roquefort. Similarly, manufactured cheese produced from cow milk in the United States and other countries is called blue (bleu) cheese. Morris (1981) provides an excellent technical description of the manufacture of blue-veined cheeses.

Blue cheese is usually made from a blend of heat-treated (raw) or pasteurized skim milk and homogenized cream, whereas Roquefort is made from nonhomogenized raw, whole sheep milk. The purpose of homogenization is to break up large fat globules. Sheep milk naturally contains more small globules. Homogenization results in a whiter curd (and increased contrast with the blue mold), increased flavor development through enhanced lipase activity, and a more porous, crumbly texture. Pasteurization destroys most of the milk lipase, which is believed to aid in ripening of cheese and kills most nonstarter bacteria, especially lactobacilli, which might play an important role in overall development of characteristic flavor. Milk is inoculated with spores (10^{3-4}/mL milk) of *P. roqueforti*. Some manufacturers prefer to inoculate curd instead. Either method ensures that spores and thus flavor development will occur evenly throughout

cheese. During manufacture, steps are taken to produce a porous or open texture. The starter is *Lc. lactis*, and citrate-metabolizing strains (*Lc. lactis* subsp. *lactis* biovar *diacetylactis* and *Leuconostoc* sp.) are sometimes added. Carbon dioxide produced through metabolism of citric acid expands mechanical openings in cheese, which in turn allows for more intrusive growth of mold. The coagulum is cut when very firm into large cubes (0.95 cm diameter). The whey and curd mixture is heated to 35–37°C, held for 30 min with agitation, and then whey is removed. Curds may be salted. Dry curds are put into hoops (drained vessels) and allowed to sit for several days at 21–27°C. This encourages complete fermentation of lactose, results in a cheese with a pH of 4.8–4.9, and permits full drainage of whey. The body is desirably brittle and crumbly. Improper whey drainage may result in soft, mushy surface areas during storage. These areas are ideal for growth of yeasts and putrefactive bacteria such as *Pseudonomas* sp. and *Acinetobacter*.

Cheeses are brine salted or rubbed with salt for several days. Roquefort cheese must be dry salted by regulation. After salting, cheeses are pierced with 0.24-cm diameter needles and placed in a curing room (10°C at 90–95% humidity) for 2–4 weeks or until mold growth begins to appear at openings of holes. Piercing allows for transfer of oxygen and CO_2 to stimulate growth and metabolism of *P. roqueforti*. *P. roqueforti* is more tolerant of low oxygen, high CO_2, and high salt than most other species of molds. After sufficient mold growth, cheeses are wrapped and stored (matured) for 2–4 months at (10°C). In France, later curing of cheese occurs in the famous caves of Roquefort. After proper curing, cheeses are cleaned of surface growth (molds, yeasts) and repackaged for sale.

Metabolism of molds (lipolysis and proteolysis) during maturation is essential for development of the distinctive blue cheese flavor. A distinctive yeast flora also develops on Roquefort, including *Debaromyces hansenii, Candida* sp., and *Kluyveromyces lactis*. The intensity of mold-derived flavors is so strong that, although other microorganisms are present in such high numbers (yeasts, micrococci, and lactobacilli), their contribution to flavor of blue cheese cannot be ignored, nor neither can it be ascertained.

The salt in moisture in the interior of blue cheeses can be as high as 6–8%. This inhibits growth of lactococci and *Leuconostoc* sp. Free fatty acids released through lipolysis and via oxidative decarboxylation are converted to methyl ketones. Of particular

importance in blue-veined cheeses are 2-heptanone and 2-nonanone, without which there is no distinctive blue cheese flavor. Secondary alcohols, methyl and ethyl esters derived from fatty acid metabolism and proteolysis, are essential for the well-balanced and distinctive flavor of blue-veined cheeses. Molds require oxygen to grow, albeit at low levels, for *P. roqueforti*. With *P. roqueforti*, too little oxygen can result in a change in color from blue-green to greenish yellow. This situation can occur if cheese is vacuum packaged. Proper color returns when the cheese is exposed to air.

During initial salting and ripening of blue cheese, there is a conspicuous lack of visible growth of mold and yeasts on the surface. The low pH and high salt content keep the level of these microorganisms in check. Some manufacturers also use a hot brine treatment (72°C for 20) to kill microorganisms at the cheese surface. However, as cheese matures, salt diffuses in and the pH rapidly increases (5.8 up to 6.5). Yeasts and molds metabolize lactic acid and hydrolyze protein, releasing ammonia and amino acids. Both metabolic activities result in pH increase. During maturation, microorganisms once held in check by adverse conditions (low pH) can begin to grow. Salt-tolerant bacteria such as *L. monocytogenes* and *Staphylococcus aureus* are of particular concern. Since blue cheese is often added as an ingredient to salad dressings, the presence of undesirable bacteria such as heterofermentative lactobacilli can be a potential problem. Although the cheese does not contain any sugar, the dressing may. Metabolism of sugar by heterofermentative lactobacilli produces gas and an unattractive salad dressing.

4.4.3 Externally Mold-Ripened Cheeses: Camembert and Brie

Camembert and Brie are essentially the same cheeses, but in France are made in different regions. Brie cheese wheels are also larger in diameter and may be produced with *S. thermophilus* starter. Whole milk, sometimes with cream added (double-cream Brie), is inoculated with *Lactococcus* sp. After considerable acid development by the starter, coagulant is added. The coagulum is very firm when cut. This results in a higher moisture cheese. The coagulum is cut into large pieces, 1.6-cm diameter, stirred, and dipped into forms. Alternatively, uncut coagulum may be dipped directly into forms. The height of finished cheese is important, because the degree of ripening of cheese depends on its thickness. Curd settles in forms, which are turned approximately 6–8 h after being filled. There is no cooking or heating step. No pressure is applied. As in blue

cheese, the starter continues to produce acid until it becomes self-inhibited at pH 4.7–4.8.

Cheese is removed from forms and brined or dry-salted (salt rubbed or sprinkled on the surface). After salt is absorbed (1 day), spores of *P. camemberti* are sprayed onto the surface. Spores may also be added directly to the milk. Cheese is not pierced as in blue cheese, so mold does not grow in the interior of the cheese unless there is an area of unfused curd (mechanical openings or holes). Cheese is transferred to shelves in rooms of high relative humidity (90–95%) at 10°C. It is placed on mats or perforated sheets to allow air contact with as much surface area as possible. This permits growth of the mold evenly over the entire surface area of the cheese. Cheese is also turned regularly to expose bottom areas and keep soft cheese from being imprinted with the perforated mats or sticking to them. After approximately 2 weeks in ripening rooms, mold has developed sufficiently, and cheese is wrapped for sale. It is then stored at a low temperature (4–7°C) for further ripening (2–4 weeks).

Slow growth of mold may indicate a too-high salt content or too-low pH. To prevent the latter, water may be added to milk or whey to remove some of the lactose before curd is transferred to forms. The practice has also been applied in the manufacture of blue cheese.

Before growth of *P. camemberti*, cheese is firm, crumbly, and acid. As mold grows, it metabolizes lactic acid and hydrolyzes protein. Just beneath the surface growth, cheese is very soft, creamy, and appears slightly translucent and more yellowish than the interior portion of the cheese. As ripening continues, the interior becomes progressively softer and creamier just as at the surface. This progression is referred to as ripening from the outside to the inside. The change in the body of cheese results, in part, from migration of ammonia from the inside to the outside of the cheese. Migration of ammonia from the outside to the inside of the cheese raises the pH of cheese from 4.8 to >6.5. This alters hydration of casein with the net result of an increase in fluidity of cheese. With an increase in pH, naturally occurring milk proteinase, plasmin (not active at low pH), hydrolyzes protein, further softening cheese. Eventually, the entire cheese becomes soft and creamy. Overripening either by poor stock maintenance or by design results in cheese that is very fluid and that "runs" when cut open.

Metabolism of *P. camemberti* results in hydrolysis of milkfat (lipolysis) and subsequent oxidative decarboxylation of free fatty acids to methyl ketones. Of particular importance to the flavor of Camembert are 1-octen-3-ol, 1, 5-octadien-3-ol, and 2-methyl-isoborneol. In the United States, Camembert and Brie are generally ripened with mold only. However, Karahadian and Lindsay (1985) postulated that growth of *Brevibacterium linens* on cheeses imported from France resulted in development of sulfur compounds: dimethyl disulfide, dimethyl trisulfide, and methional. Other regional differences in flavor may arise from metabolism of particular microflora contaminating the surface of cheese. Nooitgedagt and Hartog (1988) found yeasts (predominantly *D. hansenii, Yarrowia lipolytica, K. marxianus,* and *Candida* spp.), and *Geotrichum candidum* and a few cheeses with greater than 10^4 staphylococci, greater than 10^5 *E. coli,* and greater than 10^7 Enterobacteriaceae.

4.4.4 Cheeses with Eyes

Swiss, baby Swiss, Gouda, and Edam are among cheeses characterized by development of circular openings called eyes. Eyes develop through formation of CO_2 by metabolism of specific secondary bacteria. In Swiss-type cheeses, gas (CO_2) is formed by *P. fruedenreichii* subsp. *shermanii* through metabolism of lactic acid. In Gouda and Edam cheeses, CO_2 is formed from metabolism of citric acid by *Leuconostoc* spp. and *Lc. lactis* subsp. *lactis* biovar *diacetylactis*.

These are the most difficult of cheeses to manufacture because of the strict grading regimen they must pass. Eye development is key, and this is sometimes the only criterion by which these cheeses are graded. Reinbold (1972) and Olson (1969) have provided detailed descriptions on manufacture of these cheeses. The method of manufacture is similar for all cheeses with eyes.

Starters for Swiss and baby Swiss cheese are predominantly *S. thermophilus* with small amounts of *Lb. delbrueckii* subsp. *bulgaricus, Lb. helveticus,* and *Lb. lactis*. Depending on the manufacturer, *Lc. lactis* may also be used to ensure fermentation of all sugar, including residual galactose. The propionibacteria are added with the lactic starter.

Lc. lactis is used as starter for Gouda and Edam cheeses. Gouda and Edam are manufactured similarly, but Edam is lower in moisture and fat content.

The main objective in making cheeses with eyes is to produce a pliable curd mass. This is necessary for development of round eyes rather than slits or cracks. Pliability or elasticity of cheese results from both protein density and physicochemistry (strongly influenced by pH and bound calcium). As CO_2 is formed, it accumulates at locations where air has been entrapped during processing or at sites where the curd is not tightly fused. Gas exerts pressure on the protein network. If the protein network is elastic, it bends or gives but does not break, forming an eye. If the protein network cannot withstand the pressure, it breaks and a slit is formed. Elasticity is a phenomenon related to hydration of casein, calcium-phosphate bonding, and electrostatic and hydrophobic interactions between casein molecules. Thus, rate and extent of acid development (pH) at whey drainage and pressing must be carefully controlled. To accomplish this, slow acid development is necessary and separation of curd and whey generally occurs at a relatively high pH.

In Swiss cheese, after cutting, curd is heated to 48–53°C and held for 30–60 min depending on the desired moisture content and pH. In Gouda and baby Swiss varieties, a portion of whey (25% of milk weight) is drained and replaced with hot water to raise the temperature of curds and whey to 38–39°C. Addition of water not only heats curd but also dilutes lactose, thereby controlling the pH of cheese. Alternatively, Swiss cheese manufacturers can control the pH by adding warm water to milk and cold water to cool curd before whey drainage (combined water addition is approximately 7–10% of weight of milk). The high heat used in Swiss cheese manufacture inhibits acid development and partially inactivates the coagulant (depends on type of coagulant). The starter is not killed and resumes acid development as curd cools.

In larger commercial manufacturing plants, regardless of cheese type, curd and whey are pumped together into a smaller vessel or rectangular tower that concentrates curd into a single large mass. Pressure is applied and serum is squeezed from curd.

In the traditional method of Swiss cheese manufacture, all curd from the cheese vat (a round kettle) is enclosed in a cheese cloth, lifted into the cheese form, whey is manually pushed out of the curd mass, and cheese is pressed. Because serum is at a high pH during pressing, less calcium phosphate is dissolved in whey as compared to cheeses of a more acidic nature at drain (Cheddar or mozzarella). Phosphate acts as a buffer and helps keep pH of curd from getting too low. Low pH (5.1) inhibits growth of *Propioni-*

bacterium sp. and is involved in development of a short, inelastic body in cheese. After pressing, the curd mass is brine salted.

To ensure curd fusion and complete sugar metabolism, cheese is held at 7°C (prewarm room) for several days. Cheese is then placed in a warm room (10–13°C for Gouda and 20–26°C for Swiss).

The temperature affects both growth of the eye former and elasticity of the protein network. The warmer the cheese, the more elastic the protein. Rate of gas development is critical. If gas develops too rapidly and the casein network cannot handle the gas pressure, the cheese splits. If gas forms too slowly, the cheese maker may leave cheese in the warm room for too long, resulting in too much proteolysis. When gas does develop, curd is no longer elastic, resulting in splits. After the eyes form, Swiss cheese is cooled and stored (5°C) to prevent further gas development.

Gouda cheese may be ripened for extended periods at the warm room temperature. Because citric acid is limiting in cheese, there is no fear of excessive gas being formed by the added lactococci or *Leuconostoc*. However, in Swiss cheese, there is excess substrate (L-lactic acid) and potential for continued gas formation unless the cheese is cooled. The search is underway to find a *Propionibacterium* sp. that does not form gas during storage. Cold cheese is not elastic, so if gas is formed, it expands existing eyes and they split. As cheese ages, casein is hydrolyzed (proteolysis) by residual coagulant, nonstarter bacteria, and plasmin (native milk proteinase). Proteolysis eventually destroys elastic properties of the casein network. Thus, if gas is formed in cheese after much proteolysis has occurred, slits are formed.

In all cheeses with eyes, undesirable gas formation can occur if large numbers of *C. tyrobutyricum* are active. Their growth usually occurs months after cheese is made and after much proteolysis has occurred. The result is split eyes or newly formed large slits called cracks. Metabolism by *C. tyrobutyricum* also results in rancidity and H_2S formation. The latter gives rise to the term *stinker cheese* or *stinkers* for short. Metabolism at the surface of cheese by *Pseudomonas* spp., yeasts, and enterococci also produce stinkers.

Certain varieties of cheese with eyes, for example, Gruyere and Danbo, are also surface ripened.

4.4.5 Surface-Ripened Cheeses

Limburger and traditional brick cheese are known for their highly malodorous character. For certain individuals, they literally stink; to

the connoisseur, they smell in a pleasant sort of way. The strong odors arise from putrefaction of protein, which releases ammonia and sulfide compounds (H_2S and methyl mercaptan).

These cheeses are made from whole milk with *Lactococcus* spp. starters. After cutting the coagulum, a portion of whey is removed (25–50% of milk weight) and replaced with hot water. This raises the temperature and removes lactose from curd, which prevents cheese from becoming too acidic, a development that could delay ripening. There are several variations to this procedure, but the net result is the same. After 30–60 min, whey is drained while curd is stirred. If an open-bodied cheese is desired, all or most whey is removed. Curd is put into forms and may or may not be pressed. Once pressed, cheese is brined, "smeared," and placed in a high-humidity room (90–95% relative humidity) at 13–15°C. Cheese is slightly acid after brining (pH 5.2–5.4). The pH depends on amount of whey removed and water added during manufacture. Once brined, cheese is inoculated with the smear. The smear is a mixture of several microorganisms, most importantly yeasts, micrococci, *Arthrobacter* and *B. linens*, which develop as a layer on a cheese surface as it ripens. Smearing is done by scraping the smear layer from an already ripened cheese into a brine solution and then rubbing fresh cheeses with the smear-containing brine. This procedure is repeated every few days until luxurious growth occurs. Microorganisms in the smear may be purchased separately, mixed, and the cheeses inoculated. *Arthrobacter* and *B. linens* give the smear a red-orange color. Yeasts (*D. hansenii, Candida* spp., *G. candidum*, and *Y. lipolytica*) metabolize lactic acid and the pH of the cheese increases. *Micrococcus* spp. (*M. varians, M. caseolyticus*, and *M. freudenreichii*) begin to grow, followed by *B. linens* and *Arthrobacter*. The pH must be greater than 5.5 for *B. linens* to grow. Yeasts also synthesize vitamins (pantothenic acid, niacin, and riboflavin), which may be essential for *B. linens* to grow. A symbiotic relationship thus exists between growth of yeasts and *B. linens*. The length of time the smear is left on cheese and size of cheese influence its flavor intensity.

Limburger cheese is cut into small loaves (6.4 × 6.4 × 13 cm) before smearing, and the smear is not removed. Traditional brick cheeses are larger pieces and, again, the smear is not removed. In less pungent brick cheese, the smear is washed off after 4–10 days. If the smear is left on cheese, ripening continues. Ripening of

cheese involves extensive proteolysis, with release of ammonia, H_2S, and methyl mercaptan. These flavor compounds diffuse into cheese. Metabolism of lactic acid at the surface of cheese, ammonia migration into the cheese, and proteolysis on the inside, caused by coagulant and plasmin, eventually lead to a fluid interior. The cheese is runny when cut. Because of pH increase, microorganisms once held in check by low pH can then begin to multiply. *L. monocytogenes*, staphylococci, and coliforms are of major concern.

4.4.6 Colby, Sweet Brick, Muenster, Havarti

Colby, sweet brick, Muenster, and Havarti are consumed with minimum ripening, generally between 1 and 3 months. Inferior products are often sold if the cheese is aged for a longer time. These cheeses are low in acid, because lactose is rinsed from the curd, and they have a pH of approximately 5.2–5.4. Lactococci are used as starter in Colby and brick cheese but *S. thermophilus* is preferred for Muenster. Some manufacturers also use *Lb. delbrueckii* subsp. *bulgaricus* if *S. thermophilus* is used. Havarti cheese is manufactured with lactococci with added *Leuconostoc* sp. and citrate-metabolizing *Lc. lactis* subsp. *lactis* biovar *diacetylactis*. Once the coagulum is cut, curds are heated to 36– to 37°C if lactococci are used as the starter and 39–41°C if thermophilic cultures are used. In Muenster and brick cheeses, there is little or no acid development before putting curd into forms.

In Colby manufacture, the pH of curd at whey drainage is approximately 6.1. Whey is drained and curds are continually stirred as water (30–32°C) is sprinkled on them. This not only cools the curd but also removes lactic acid and lactose. All whey is then removed and curd is salted, put into forms, pressed, and stored. The result is a cheese with a pH of 5.2–5.3 and many mechanical openings. Cool, firm curd does not fuse completely even when pressed. Vacuum packaging closes the openings and cheese forms a tight knit texture, but this is not allowed in authentic Wisconsin Colby.

In Muenster cheese, curd and whey are pumped into rectangular open-ended forms, lightly pressed, brined, and stored. In manufacture of brick and Havarti, once the coagulum is cut, a portion of whey is drained and replaced with hot water to heat curds to 36–37°C. Curds are then handled as with Muenster. The more whey removed before putting curd into forms, the more mechanical openings appear in cheese. Cheeses are brined and stored. Havarti is ripened at 13–

16°C for 2–6 weeks; Muenster and sweet brick are stored at 7°C and are ready for consumption within a month.

4.4.7 Cheddar Cheese

Cheddar cheese is consumed when it is anywhere from 1 month to several years of age. Pasteurized or heat-treated (67–70°C for 20 s) whole milk is used. *Lactococcus* spp. is the starter, with *Lc. lactis* subsp. *cremoris* being preferred for longhold cheese. Once the coagulum is cut, curd is heated to 38–39°C. After proper stir-out, whey is completely drained and curd is either continuously stirred (stirred curd Cheddar) or allowed to mat (Cheddared curd, also called milled curd Cheddar). Stirred curd is preferred if the cheese will be used for process cheese. Cheddared curd is preferable for table cheese. Cheddared curd cheese is thought to develop a better flavor and has a smoother body than stirred curd cheese. When the curd reaches the desired pH, curd is salted. In Cheddared curd, the matted curd is cut into large pieces, which are periodically turned (Cheddared) in the vat until the proper pH is reached (pH 5.4-5.5). The slabs of curd are then cut into finger-sized pieces (milled) salted, put into forms, and pressed. Cheeses are generally stored at 7–9°C. There is considerable variation in details of Cheddar cheese manufacture resulting from mechanization of the process, size of vats, rate of acid development by starter, and whims of the manufacturer. The pH of cheese at 1 week is generally 4.95–5.1.

4.4.8 Pasta Filata Cheeses: Mozzarella and Provolone

Manufacture of pasta filata cheeses is almost identical to milled curd Cheddar cheese. *S. thermophilus* (cocci) and *Lb. delbrueckii* subsp. *bulgaricus* or *Lb. helveticus* (rods) are used as starter. The ratio of cocci to rods used varies from manufacturer to manufacturer, but ratios of 1:1, 3:1, 5:1, or 1:0 are commonly used. The cocci are the main acid producers. Lactococci are sometimes added. As with Cheddar cheese, considerable variation exists in manufacture of mozzarella and provolone cheeses. *Mozzarella* is a common name applied to various cheeses made similarly. There is some debate over the appropriateness of allowing non–pasta filata cheese to be called mozzarella; these cheeses are being made and sold as mozzarella especially as a kosher product.

Provolone is lower in moisture but higher in fat than mozzarella cheese. Lipases are added to milk and lipolysis results in a light piquant or rancid flavor in provolone. After the coagulum is cut, the curd and whey mixture is heated to 42–43°C. At pH 6.1, whey

is drained and curd may be cut into slabs and stacked. At a pH of 5.15–5.35, curd is milled and placed in a hot-water bath (70–88°C) and kneaded. The pulling or stretching of the molten curd mass gives the pasta filata cheeses their name, but it also imparts a fibrous body to the cheese. The temperature of the cheese (57–63°C) varies with time of exposure to mixing and water temperature. Coagulating enzymes vary in their heat sensitivity and the residual coagulant can play a major role in determining the physical characteristics of cheese (melt, stretch, oiling off, burning, chewiness). Starter may or may not survive the heat treatment of the curd. This has a major impact on metabolism of residual sugar and consequently Maillard-browning reactions when cheese is subsequently heated on pizza. After curd is stretched, it is shaped (usually into cylinders), placed in cold water to cool, and eventually brined.

High-moisture fresh mozzarella can be eaten immediately, but the more familiar pizza-type mozzarella (low-moisture, part skim) is aged for a few days. This short ripening period (4–7 days) allows for equilibrium between hydrogen ions (H^+) and colloidal calcium phosphate and for any free moisture within the cheese to be absorbed by the casein network. If water is not absorbed, it (also referred to as expressible serum) will come out during shredding of the cheese. As cheese ages, proteolysis results in an increase in mel. and a decrease in stretchability when used in cooking. This occurs in all cheeses but is most noticeable in mozzarella because of demands that are placed on the physical characteristics of this cheese when baked or fried.

The physical properties of any cheese are determined by pH, composition, and proteolysis. Thermophilic starter strains do not use the galactose portion of the lactose molecule, and it accumulates in cheese. The use of galactose-fermenting starter strains may reduce the level of galactose. Residual galactose and lactose are responsible in part, for browning of cheese when baked. Dehydration and scorching of protein during baking results in browning with a darker color in the presence of sugar. Residual sugar is also a prime substrate for heterofermentative lactobacilli and coliforms. Gas formation by these bacteria leads to "blown" cheeses and puffy packages. Yeast contamination via brine is also a potential problem.

4.4.9 Parmesan and Romano

S. thermophilus (cocci) and *Lb. helveticus* or *Lb. delbrueckii* subsp. *bulgaricus* (rods) are used to manufacture grana cheeses,

Parmesan and Romano. The ratio of cocci to rods varies according to the manufacturer but ratios of 1:1, 3:1, or 8:1 are common. Some manufacturers also add a small amount of *Lc. lactis* to ensure complete sugar metabolism. Reduced-fat milk is used for both. Moisture content is low (32% maximum for Parmesan, 34% maximum for Romano). Parmesan must be aged 10 months and has a minimum FDM of 32%, whereas Romano must be aged 5 months and has a minimum FDM of 36%.

There is an effort to reduce aging requirements for Parmesan to 6 months. Indeed, some companies have received a temporary variance allowing for the shorter ripening period as long as the cheese has the same flavor as the more aged cheese. Parmesan and Romano are manufactured similarly. The coagulum is cut softer and finer than for other cheeses to ensure a drier finished cheese. Fast acid development by the starter is desired. After cutting, the curd and whey mixture is heated to 45–47°C and stirred until the pH of whey is approximately 5.8–6.0. Whey is then drained. Curds are continuously stirred until all whey is removed. The low pH and high heat during stir-out enhances syneresis. Curds are put into forms (usually 9- to 18-kg wheels), pressed overnight, and brine salted for several days to 2 weeks. Some manufacturers brine only a few days and apply salt to the cheese after it is removed from the brine. This method of salting is called dry salting and may require several days of application to achieve the desired salt level. Alternatively, curds first may be salted, put into forms, and then pressed. This process produces what is referred to as barrel cheese. The cheese is not brine salted, and the process usually requires a longer stir-out and application of less salt. If the salt content is too high, it may inhibit fermentation of all the sugar. Residual sugar may participate in Maillard-browning reactions, especially if the cheese is further dried (with heat) to produce grated cheese. After brining, the cheese is stored at 7–10°C. Traditionally, wheels of cheese are coated with an oil to prevent mold growth and coated with wax at a later date. Some modern manufacturers coat the cheese with a polymer containing a mold inhibitor (natamycin).

Although Parmesan and Romano cheeses are made similarly, they taste distinctively different. Pregastric esterase or lipase is added to the milk for manufacture of Romano but not to milk for manufacture of Parmesan. Thus, the flavor of Romano is rancid or picante, whereas that of Parmesan is described as sweet and nutty.

4.4.10 Reduced-Fat Cheeses

Demand by consumers has led to development of reduced-fat versions of popular cheese varieties. Early attempts did not meet with tremendous success because of poor physical and flavor characteristics. Adjustments to the manufacturing protocol, including the use of selected starter strains and particular attention to dairy plant hygiene, have greatly improved the quality of these cheeses. Young, mild-flavored cheese with a reduction in fat content of 25–33% as compared to the full-fat cheeses have almost, if not actually, duplicated the quality (flavor and body) of the full-fat counterparts. Cheeses with a fat reduction of greater than 50% have yet to achieve similar results. It is more likely that these cheeses have taken on their own unique flavor and are being accepted on their own merits rather than in comparison to other cheeses.

Reduced-fat versions of cheeses are similar to their full-fat counterparts in that they are subject to the same microbiologically induced defects and for the same reasons. However, the ecology (variety of bacteria and changes over time) of the cheeses may or may not be the same; this has not yet been studied. Cheese with less fat is firmer than cheese with higher fat content. To overcome this problem, reduced-fat cheeses are manufactured to contain much higher moisture contents. But higher moisture means higher lactose in the cheese, which, in turn, means that the cheese is high in acid after the starter ferments the sugar. To avoid producing an acidic cheese, many manufacturers of reduced-fat cheeses (regardless of type) use whey dilution or curd rinsing to remove some lactose. However, reduced-fat Cheddar is also being made commercially without a rinse treatment by using a specific manufacturing protocol to retain the buffering capacity of the cheese. Cheese contains more acid (up to 2% lactic acid compared with less than 1.6% lactic acid in full-fat Cheddar) but both may have the same pH. Compared with full-fat cheeses, most reduced-fat versions are higher in moisture and pH and generally lower in salt (lower S/M) and lactic acid. Thus, the cheese environment and chemistry is not the same between full-fat and reduced-fat cheeses, and the reduced-fat cheeses may be more susceptible to growth of undesirable bacteria, especially coliforms. Of course other contaminants such as lactobacilli also grow more rapidly in lower salt, lower acid, reduced-fat cheeses. Reduced-fat cheeses may have a tendency to increase in pH more rapidly than full-fat counterparts because of lower acid levels and

increased proteolysis. Conditions are thereby created that are more favorable to growth of bacteria in general.

The consequences of reduction of fat on flavor of aged Cheddar cheese are well recognized (i.e., lack of similar flavor intensity at similar age), but the difference in flavor is less evident in reduced-fat versions of cheeses in which the full-fat version is mild-flavored. Reduced-fat Cheddar cheese made with a curd rinse tastes similar to Colby. Reduced-fat Cheddar made without a curd rinse has more Cheddar flavor than one made with a rinse, but development of flavor still lags behind that of full-fat Cheddar.

Although reduced-fat cheeses have met with some consumer acceptance, there appears to be a universal concern that cheeses not ripened deliberately by yeasts or molds lack flavor. As a result, adjunct bacteria, particularly *Lactobacillus* spp., are being used commercially to enhance cheese flavor.

4.4.11 Process Cheese and Cold-Pack Cheeses

Process cheese, cheese spreads, and cheese foods are produced from other cheeses. A mixture of cheeses (may be several varieties, ages, and flavor) is blended with milkfat (butter oil), water, "melting salts" (such as sodium phosphates, citrates) and, in the manufacture of spreads and foods, added whey powders. Depending on type of product and shelf life requirements, the mixture is stirred and heated to 70–140°C for 2–15 min. It is then packaged (filled) or made into slices. The rate of cooling depends on size and shape of cheese but may take several hours to reach temperatures below 38°C. This is in excess of pasteurization, so most microorganisms are killed with the exception of spore formers. Of particular concern are *C. sporogenes, C. tyrobutyricum, C. butyricum, C. botulinum*, and *B. polymyxa*. The presence of coliforms or yeasts is indicative of low processing temperature, especially at filling or negligent sanitation. In addition to composition, pH and aw, the presence of melting salts may be inhibitory to the growth of clostridia. Nisin will also inhibit growth of clostridia.

Cold-pack cheese is prepared by mixing, without the aid of heat, a blend of cheese, acid, salt, flavoring, stabilizers, and water. Cold-pack cheese food may also include whey powder, buttermilk, and nonfat dry milk. Cold-pack cheeses must be made from cheeses made with pasteurized milk or held for at least 60 days at a temperature above 1.67°C. Because such cheese is not heated, microbial quality is subject to microbial content of ingredients. In

addition, cold-pack cheese food contains lactose, a readily available food source for many potential contaminants as well as starter bacteria. Starter fermentation of residual lactose can cause the pH to drop and free moisture to appear at the surface of the cheese. The major microbiological problem with these products is growth of yeasts and molds, especially if free moisture is available at the surface. Antimycotic agents such as potassium sorbate are permitted (not to exceed 0.3%).

4.5 CHEESE RIPENING—INFLUENCE OF MICROORGANISMS

Microorganisms found in cheese can be classified into two groups: those that are deliberately added, such as starters and adjuncts, and those that are adventitious contaminants. The primary role of starter bacteria is to produce acid at a consistent rate, but it would be wrong to assume that their role is limited to this. The starter has a major impact on flavor in cheese consumed fresh. As cheese matures, direct contribution to flavor by the starter diminishes as nonstarter flora grow. Although, in most instances, the exact means by which starter bacteria or adjunct microorganisms contribute to development of flavor is controversial, they can both influence cheese maturation. Development of flavor in blue, Camembert, Limburger, Romano, and provolone cheeses is clearly dominated by microorganisms or enzymes deliberately applied to them. With other varieties of cheese, however, development of flavor is not clearly understood. Scores of compounds with the potential to affect flavor have been isolated from a variety of cheeses. But the full duplication of cheese flavor chemically has eluded us.

Olson (1990) described the possible role of starter bacteria in cheese flavor development as follows: fermentation and depletion of fermentable carbohydrates create an environment that controls growth and composition of adventitious flora. This is accomplished through development of acids, creation of low oxidationreduction potential during early stages of cheese maturation, and competition for nutrients. In addition, starters can develop flavor compounds directly and indirectly through their metabolic activities.

Autolysis of starters (and adjuncts) releases nutrients that serve as metabolites for other microorganisms in cheese. Also, activity of released intracellular peptidases can contribute to the increase in the free amino acid pool within cheese. Amino acids can, in turn, be metabolized by other bacteria directly to flavor compounds or can

react chemically with other constituents in cheese to produce flavor compounds. Any bacteria thriving in cheese can potentially influence flavor of cheese through synthesis of flavorful metabolites. Characterization of flavor in most cheeses is lacking; thus the direct connection between microbial metabolism and cheese flavor is also limited. Another problem hampering the study of influence of starter and nonadded microflora on flavor in cheese is a lack of consensus on what constitutes cheese flavor, especially in varieties of cheese not ripened by yeasts or molds. There is an element of distrust in that what one person perceives to be true cheese flavor may not be the same as what another might consider to be cheese flavor. Consequently, results of experiments on flavor enhancement or acceleration of flavor development are often met with skepticism.

Starters are the dominant bacteria found in cheese initially. Numbers range from 10^6 to 10^9/g cheese. As cheese ages, their numbers decrease and numbers of nonstarter bacteria increase. The rate at which this happens depends on strain of starter and initial numbers and type of nonstarter bacteria. Lactobacilli constitute most nonstarter lactic acid bacteria in Cheddar cheese (and probably most cheeses), with the dominant species of quality cheese being *Lb. casei* and *Lb. plantarum*. Heterofermentative lactobacilli may be present with no visible sign of gas production. *Lactobacillus* numbers in raw milk are greatly reduced by pasteurization. The presence of lactobacilli in pasteurized milk generally indicates high (10,000/mL) numbers in raw milk or postpasteurization contamination. Type and strains of nonstarter bacteria found in cheese are dependent on initial numbers in milk (especially if raw milk is used), biofilm formation on equipment and subsequent contamination, and ability of individual strains to survive and compete in the cheese environment (pH, salt, a_w, acidity, temperature, availability of nutrients).

Addition of *Lactobacillus* adjuncts has been suggested as a means of controlling numbers of adventitious lactobacilli by, at least initially, outcompeting other microflora in cheese. However, depending on strain, the adjunct culture may die or may not compete well against nonstarter microflora; thus the ecology of cheese can change as it matures. Dominance of cheese microflora by lactobacilli has led to numerous studies advocating addition of defined strains of lactobacilli to milk or cheese to reduce bitterness, enhance flavor, or develop particular textural or physical attributes in the cheese. Other bacteria, particularly *B. linens*, have also been used

commercially to enhance flavor of Cheddar and reduced-fat cheeses. The use of adjunct bacteria to accelerate flavor development has met with some resistance by manufacturers, because the flavor developed in cheese is not the same as flavor of cheese without the adjunct. Consistency of flavor quality is a major goal of the cheese maker. Not surprisingly, reduced-fat varieties of cheese more closely mimic the full-fat counterpart if the same adjunct is used in both cheeses.

4.6 ASSESMENT OF MICROBIOLOGICALLY INDUCED DEFECTS IN CHEESE

It is difficult at times to assess quality problems occurring with cheese. A thorough knowledge of all aspects of cheese making is required for detective work necessary to determine cause and effect relationships that may lead to a cheese quality problem. Foremost is identification of the problem. Is the problem really microbially related or is it the result of mechanistic shortcomings of manufacture? For example, openings in cheese can be a result of either pressing cold curd (mechanical) or gas formation by heterofermentative bacteria.

Second, if the problem is microbially induced, how did the organism gain access to the product and is the problem exacerbated by the manufacturing protocol, handling, storage, pH, or composition of cheese? For example, residual sugar in cheese because of incomplete fermentation by the starter can be fermented by contaminating heterofermentative bacteria leading to gassy cheese. Incomplete fermentation, in turn, can result from a change in composition of starter because of improper starter preparation. Perhaps cheese was cooled prematurely, too much salt was added, or bacteriophage killed the starter. Regardless of circumstances, a contaminating organism must be present and must grow. If the microorganism is present but does not grow, there is no problem. Many legal questions have arisen because of this simple concept.

The problem results from *growth* of a microorganism. Perhaps, had cheese not been temperature abused, the microorganism would not have grown! Cheese is not made in a sterile environment. It is inevitable that contaminating microorganisms will be present in cheese. It is not inevitable that they will cause a problem in cheese.

Prevention of undesirable growth of microorganisms in cheese involves four steps: (1) Keep the microorganism out of milk or prevent its growth in milk (hygiene on the farm, quickly cooling

the milk, short time between milking and cheese making). (2) Kill the bacteria (pasteurization). (3) Manufacture the cheese to prevent contamination (dairy plant hygiene). (4) Create an environment within the cheese so that if the microorganism is present, its growth will be limited (proper pH, salt, fermentation of all sugar, low storage temperature). However, the most universally accepted (but not always properly practiced) method of preventing defects caused by microorganisms is sanitation. In this regard, development of biofilms is important.

Many bacteria can form biofilms or can become associated with them. Biofilms consist of microorganisms immobilized at a surface, typically embedded in an organic polymer matrix of bacterial origin. Biofilms can develop on almost any wet surface (equipment). Microorganisms attach to the surface or to other organic material already attached to the surface, excrete copious amounts of extracellular polymers, and grow vigorously, creating a biofilm. Bacteria can form biofilms within a few hours of initial attachment to a surface. As the biofilm becomes thicker, the outer layer is sloughed off as the result of turbulence (e.g., milk stirring in a vat). Microorganisms within sloughed-off pieces contaminate milk. Other organisms can also attach to the biofilm. Sanitizers are less effective against biofilms, because the sanitizer reacts only with the outer layer and extracellular polymers protect microorganisms. Therefore biofilms must be removed before sanitizers are applied. The cleaning regimen becomes paramount in controlling bacterial contamination.

4.6.1 Molds

Airborne mold spores are ubiquitous, but, upon germination, they require oxygen to grow and sporulate. Therefore, mold growth on the surface of cheeses exposed to air is to be expected. Molds are not supposed to grow on cheeses that are vacuum packaged, but they sometimes do. Molds tend to grow on cheese where pockets of air exist between the packaging material and cheese surface. Growth is limited by the amount of residual oxygen. Low oxygen levels may dictate species of molds found. The most common molds found on vacuum-packaged Cheddar cheeses are *Penicillium* spp. (especially *P. commune*, a blue mold), and *Cladosporium* spp. (especially *C. cladosporioides*, a black mold). Other molds found on different cheeses include *Aspergillus*, *Fusarium*, *Mucor*, *Scopulariopsis*, and *Verticillium*. *Penicillium* spp. appear to be the

dominant type of molds that grow on cheeses. *P. commune* is the most widespread and frequently occurring species found on all cheese types and in smear of surface-ripened cheeses. Although *Aspergillus* spp. and *Penicillium* spp. are the dominant fungi isolated from air in cheese plants, *Penicillium* spp. are the dominant fungi isolated from cheese with very low levels of *Aspergillus* also being present.

Potassium sorbate and natamycin are used to control mold growth. Sorbateresistant strains of *Penicillium* metabolize sorbic acid to yield 1,3-pentadiene, which has a kerosene-like odor. If sorbic acid is added to the cheese and the cheese is made into processed cheese, the sorbate is diluted. Sensitive strains are then able to grow and may produce 1,3-pentadiene. The maximum amount of sorbic acid permitted for use in cheese is 0.3% by weight, which is not enough to inhibit all strains of *Penicillium* but adequate to inhibit *Aspergillus* spp. In cheeses with a rind (e.g., Gouda, Parmesan), a polymer coating is applied to prevent mold contamination and for appearance. Sorbates or natamycin are incorporated into the coating. Sorbates diffuse into cheese and may cause off-flavors, but natamycin diffuses very little and does not give cheese an objectionable flavor.

4.6.2 Yeasts

Although growth of yeasts is desirable in surface-ripened and some mold-ripened cheeses, it is not desirable in most other varieties. The heterofermentative metabolic activity (alcohol and CO_2) of yeasts sometimes makes them particularly easy to identify as spoilage organisms even though visible colonies are not observed. The cheese tastes yeasty, a taste reminiscent of raw fermented bread dough. But not all contaminating yeasts produce the typical yeasty smell. Some very proteolytic yeasts produce stinker cheeses. The smell resembles that of rotten eggs and is often associated with white spots on the cheese surface. Lipolytic activity can lead to rancid flavors (free fatty acids), and the combination of alcohols and free fatty acids can lead to fruity flavors. Although yeasts are commonly associated with slimy surface defects, other putrefactive organisms such as *Pseudomonas* spp. and *Enterococcus* spp. contribute greatly to the defect. A major factor contributing to growth of yeast, or any contaminating organism, is a wet cheese surface. This situation can occur for several reasons. As cheese matures, proteolysis results in release of moisture held by the protein network. If cheese is warmed or if it is ripened at greater than 7–8°C, moisture collects at the surface of cheese; that is, the cheese "sweats." Moisture

(serum) laden with potential nutrients (lactic acid, dissolved peptides, amino acids) accumulates between the packaging material and cheese, setting up an ideal situation for rapid microbial growth. Cheese must first be contaminated. Excellent plant hygiene is necessary, because yeasts are common contaminants in the dairy plant environment (wet surfaces, spilled milk, whey). A major source of yeasts is brines, and thus brined cheeses tend to be more prone to yeast contamination. In addition, the high salt at the surface of the cheese draws moisture, creating an environment that favors yeasts. The most frequently isolated yeasts are *Candida* spp., *Y. lipolytica, K. marxianus, G. candidum, D. hansenii,* and *Pichia* spp.

Yeasts and molds are common on the surface of rind cheeses, a large group of traditional European cheeses. These are cheeses that are not covered or packaged but rather allowed to mature "in air." The humid conditions of storage and high-salt environment at the surface (most are brined cheeses) create conditions selective for yeasts and molds. However, with these cheeses, growth of mold and yeasts is expected if not demanded.

4.6.3 Gassy Defects in Cheese

In Swiss, Gouda, Havarti, Roquefort, and similar varieties of cheese, the controlled development of gas by bacteria during maturation is desired. The result of gas formation in these cheeses is development of eyes (Swiss, Gouda) and expansion of preexisting mechanical openings deliberately formed during manufacture. In any cheese, however, gas formation can lead to undesirable development of slits, small round eyes (sweet holes), or blown, "puffy" packages. Whether a slit or a sweet hole develops is determined by physical properties of cheese. Eyes are formed if cheese can be deformed without fracturing. This property is determined by cheese composition, temperature of cheese, rate of gas formation, and, most importantly, pH and degree of proteolysis.

In general, a minimum population on the order of 10^6 colony-forming units per gram is necessary before openness from gas production occurs. Nonstarter flora most often associated with slit formation in cheese are obligate heterofermentative lactobacilli, *C. tyrobutyricum,* and facultative lactobacilli. Others encountered but far less often are coliforms, yeasts, "wild" propionibacteria, and *Leuconostoc.* Incidence of slits or blown cheese and causative organism is reflective of microbial quality of milk, overall dairy plant hygiene, heat treatment given milk, post–heat-treatment

contamination, rate and extent of acid development, residual sugar, cheese environment, pH, and redox potential. Pasteurization is very effective at killing all coliforms, leuconostocs, and most strains of lactobacilli and greatly reducing the level of all microorganisms except clostridial spores.

Fermentation of residual sugar (lactose or galactose) is a common source of carbon dioxide in cheese. The level of sugar and speed at which it is eliminated by homofermentative starter is critical. Slow starter activity and incomplete fermentation by thermophilic starters are chief causes of residual sugar. *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* do not ferment the galactose moiety of the lactose molecule and release it into cheese. Addition of mesophiles or *Lb. helveticus* to the starter can eliminate galactose from cheese. However, in pasta filata cheeses, the heat treatment given cheese can greatly reduce the level of starter. The starter must be able to ferment sugar at the low temperature (7°C) at which the cheese is stored, an unlikely possibility with thermophilic starter.

Cometabolism of citric and lactic acids by facultative lactobacilli, *Lb. casei*, and *Lb. plantarum*, is another source of carbon dioxide. Because facultative lactobacilli are ubiquitous in cheese, their metabolism is regarded as the cause of tiny slits in cheese when no other potential gas-forming bacteria are found.

Lactic acid fermentation by propionibacteria and clostridia is also a major source of gas in cheese. These organisms are regarded as the culprits in late blowing of cheese. As cheese ages, extensive proteolysis results in an increase in pH and release of amino acids, which stimulate their growth. Although many strains of clostridia can ferment lactic acid, *C. tyrobutyricum* is probably the only one that is significant in cheese.

Other minor contributors to gas formation in cheese are amino acid catabolism (nonstarter lactobacilli, propionibacteria, *Lc. lactis* subsp. *lactis*) and the use of urea by streptococci. However, decarboxylation of glutamic acid into carbon dioxide and 4-aminobutyric acid is the main source of eye and split formation in cheese made with a particular thermophilic starter composed of *S. thermophilus* and *Lb. helveticus*.

4.6.4 Discoloration in Cheese

Color is an important sensory attribute of cheese, and consumers avoid cheese that is discolored. Annatto-colored cheeses (Cheddar

Colby) are susceptible to light-induced, oxidation, which turns affected areas pink. Govindarajan and Morris (1973) reported that hydrogen sulfide produced from amino acid metabolism by nonstarter bacteria in cheese is responsible for formation of a pink precipitate of norbixin, a component of annatto. Cheese color can also be bleached under acid conditions but the color returns as the pH of the cheese increases during maturation. This defect is common when whey is entrapped between curd particles (mechanical openings). Lactose in whey is fermented, forming localized areas of low pH (<5) and consequently bleached color. Color of non–annatto-colored cheeses is influenced by what the animal ate (more grass a more yellow color), fat content (more yellow cheese), homogenization (whiter cheese), and especially pH. At low pH (<5), casein molecules aggregate and diffract light (makes the cheese white). As the pH increases, casein aggregates become more separated and the color becomes more yellow or gray. In skim milk cheeses, cheese will become translucent.

Parmesan, Romano, and Swiss cheeses are susceptible to a defect known as pink ring. As the name implies, a pink ring develops around the outside of the cheese and can progressively develop throughout the cheese from the outside to the inside. The pink becomes brown with age. It is most common in air-ripened cheeses (non–vacuum-sealed cheese). Shannon et al. (1977) implicated metabolism of tyrosine by certain strains of *Lb. helveticus* and *Lb. delbruekii* subsp. *bulgaricus* as the cause for the pink ring defect. The presence of oxygen appears to be necessary for development of the defect. It is more common in stirred-curd direct-salted Parmesan cheese (nonbrined) in which air is incorporated during the lengthy stir-out and is not subsequently removed by fermentation or vacuum packaging. Mallaird browning has also been implicated in pinking in which residual galactose is present in cheese because of metabolism of thermophilic starters. Nonstarter lactobacilli may ferment residual sugar, creating compounds that eventually form the pink to brown color.

Brown or red spots in Swiss cheese have been traced to growth of certain strains of "wild props," *Pr. thoenii* or *Pr. jensenii*. White spots, which are also soft, have been observed on brinesalted cheeses and have been traced to growth of enterococci or yeasts. Enterococci may be in cheese rather than cheese being contaminated via brine. The cheese environment (higher pH, lower acid, lower salt content) may determine the potential for growth of enterococci.

4.6.5 Calcium Lactate Crystals

White crystalline material on the surface of Cheddar and Colby cheese is often confused with mold growth. It is, however, calcium lactate, a racemic mixture of L(+) and D(–)-lactic acid. Lactose fermentation by *Lactococcus* spp. produces L(+)-lactic acid. Growth of nonstarter lactic acid bacteria, particularly lactobacilli and pediococci, racemize L(+)-lactic acid to D(–)-lactic acid. Crystals can also form in the interior of cheese but generally form where moisture (serum) can collect. Not all crystalline material is calcium lactate but may be composed of tyrosine (from proteolysis) or calcium phosphate. Recently, crystals of only L(+)-lactic acid have been isolated from Cheddar cheese, and the cheese does not contain high levels of lactobacilli or pediococci. Manufacturing practices allowing for high calcium and lactic acid in cheese exacerbate calcium lactate crystallization. Loose packaging which allows moisture to collect at the surface of the cheese also leads to higher incidence of calcium lactate crystals.

5

Nutrition and Maintenance

The aim of maintenance metabolism is keeping the body in a good stage. A great number of processes are involved: (1) muscles contractions for obtaining and eating food, blood, circulation, respiration, and maintenance of muscle tone; (2) active absorption and transport of chemical compounds; (3) production of digestive and other enzymes and of hormones; (4) maintenance of ionic equilibria; (5) repair of damaged of worn tissues, including turnover; (6) regulatory activities, and (7) defense activities against adverse circumstances of physical, microbial, or other origin.

It will be clear that during production, e.g., in a man at work or a cow during lactation, it is difficult to discern precisely which part of the muscle contractions or of the synthetic processes belong to maintenance and which to production metabolism. The same holds true for nearly all other processes mentioned. A very difficult process in this respect is that of obtaining food: Should maintenance include only the act of eating, or in man's case, also shopping for food, and in the case of wild animals, catching the prey?

To solve these problems, data on maintenance usually are given for well described, rather ideal circumstances. Standardization has often gone so far that hey no longer apply to the true maintenance situation. For example, in man, basal metabolism data give information on the metabolism of the body lying at complete, but wakeful, rest in the postabsorptive state, with no adverse circumstances (no mental, climatic, disease, or other stress). The condition that the body be kept in a good state is no longer fulfilled completely, since the body in this postabsorptive state loses some of its reserves; otherwise most

of the variable maintenance processes are reduced to a low, nearly constant level, so measurement of basal metabolism has good repeatability. From a nutritional viewpoint, it can be said that the body uses reserve fat and some reserve protein for its metabolism under these basal circumstances.

With homeotherm animals, which are less cooperative than man and easily stressed, a true state of basal metabolism usually can not be reached. The term *fasting metabolism* is preferred in this case; it applies to the metabolism measured after a few days' fasting with no stress, except perhaps for some due to incomplete adaptation to the surroundings at a low level of activity (the animal is allowed to lie down or stand up). In ruminants a true postabsorptive state is not reached even after a fast of 4 days, so the length of the proceeding fast has to be known for a correct interpretation of the fasting measurement. Even so, estimates of maintenance metabolism for such measurements often differ considerably from those of estimates obtained from animals kept at energy equilibrium.

The results of experiments in which animals were both fasted and fed up to maintenance requirement have been used to convert the fasting metabolism, i.e., the heat production during fating, into an estimate of the maintenance requirement of metabolizable energy ($M_{E,m}$, kJ/day), which is equal to energy in food minus energies in methane, feces, and urine. In monogastric homeotherms, $M_{E,m}$ was found to be about 1/0.80 times fasting metabolism, while in ruminating homeotherms it was about 1/0.70 times.

The term *resting metabolism* is sometimes used in reference to ruminants to describe the metabolism of the animal at complete rest (lying down) just before the morning feeding. In this case a state of postabsorption after only 10-hr fast is not reached. The measurement is usually very short, and is useful only for obtaining data under comparable circumstances.

It is clear that resting metabolism is higher than fasting metabolism, and that fasting metabolism is slightly higher than basal metabolism. In both cases more energy is utilized than in a state of true basal metabolism.

The term *maintenance metabolism* is usually defined as the metabolism of the non-stressed animal when the body is in energy equilibrium, i.e., when energy balance is zero because energy input equals output. The only difference in regard to fasting metabolism is that the animal is allowed to eat, but only just enough to cover

its energetic expenses. Some research workers add the condition of zero nitrogen balance, as young animals may show a positive balance despite being in energy equilibrium; they mobilize fat and at the same time deposit protein. Nutritionally, in this case the animal utilizes for its maintenance nutrients absorbed from the gastrointestinal tract (monosaccharides, amino acids, fat, glycerol, and fatty acids); at fasting it uses mainly reserve fat.

There is not special name denoting maintenance metabolism during production of work, milk, eggs, body tissues, etc. Again we consider only the situation at low stress levels. Usually, for this measurement data are collected for the same ration type at various levels of intake and production. By means of regression from these data an estimate of maintenance metabolism is made, e.g., for the energy required for maintenance by extrapolating intake to zero energy production (sum of energy produced in work, milk, eggs, body tissues, etc.). By expressing intake and production per metabolic body weight (live weight in kilograms raised to the 3/4 power), one may account for difference in body weight of the animal leading to differences in maintenance requirements. It is clear that in this way all increments of metabolism that correlate with increments of production (like increased blood circulation and respiration, gastrointestinal and metabolic activity) are excluded from the maintenance estimate. Not excluded are changes of maintenance metabolism that are correlated not with the level of production but with the fact of production per se, e.g., due to the change in hormonal status from nonlactating to lactating. For the dairy cow such a lactation effect seems to be present, its size being +10 to+ 20%.

The regression method fails to give precise information on the maintenance of growing animals. Their maintenance metabolism theoretically should increase with increasing weight (for mature animals at a rate correlated with body weight to the 3/4 power), but the increase is smaller. With advancing age, growing animals show a decline in physical activity, and at he same time their nitrogen turnover decreases; both factors reduce the increase of maintenance metabolism at higher body weight. For this reason the usual regression method does not give precise estimates of the maintenance requirements of a growing animal; some suggestions for improved regression models were made. Also, such estimates cannot be obtained by feeding the young animal just enough to maintain itself,

for the animal then changes its behavior due to the imposed nutritional stress. Thus, estimates of maintenance metabolism of growing animals obtained by regression or by feeding maintenance rations are inevitably biased.

Measuring maintenance requirements for protein of animals is even more complicated. Because protein can be used very well for energy purposes the supply of energy from sources other than protein during this measurement should be sufficient. Furthermore, the amount of ingested protein should not exceed the requirement. Protein surpluses are only in small part converted into body protein; most of the excess nitrogen is excreted with the urine, whereas energy surpluses are readily converted into body fat. If one of these conditions is not fulfilled, a too-high estimate of the protein required for maintenance will be obtained. Protein supply below protein needs tends to lower these needs somewhat.

There is no common name for metabolism that includes both maintenance as defined above the increases of metabolism needed for maintaining the body under unfavorable circumstances. In such cases, estimates of these increases are mentioned separately and added to maintenance metabolism under nonadverse circumstances.

5.1 ENERGY REQUIREMENTS FOR MAINTENANCE

5.1.1 Maintenance Requirements and Diet Composition

Most of the maintenance activities mentioned in the first section required free energy, which can be obtained by hydrolysis of ATP to ADP. For the repair and turnover of tissues and for the production of enzymes and hormones the requirement for energy in the form of building blocks, i.e., in forms other than ATP, is fairly low; moreover, recycling takes place. Also for this part of maintenance metabolism the main need is for ATPase a source of free energy to link the building blocks in the proper way. Thus, at the cellular level, maintenance requirements for energy can be considered to be mainly requirements for ATP. The same holds true for additional energy requirements due to adverse conditions like low environmental temperatures, stress, etc.

The capacity of the various absorbed nutrients to form ATP during their oxidation in the intermediary metabolism differs: 3J of glucose gives the highest yield; 3J of fat, acetic acid, propionic acid, and butyric acid some 5, 18, 15 and 14% less, respectively; and 3J of protein (with urea, CO_2, and H_2O) as the end products of the

oxidation) some 25% less. In monogastric homeotherms the metabolizable energy (M_E) of the ingested diet, equal to energy in diet minus that in feces and urine, nearly equals the energy of the absorbed nutrients minus their residues due to incomplete oxidation (urea, uric acid, etc.). Clearly, such animals require slightly more M_E for maintenance when their diets contain more protein and less starch and sugars.

Due to the anaerobic microbial breakdown of food in the forestomachs of ruminants, a large part of the carbohydrates is converted into volatile fatty acids, but at a loss of methane and fermentation heat. The M_E of their food accounts for only the methane loss because M_E in ruminants equals energy in diet minus energy in feces, urine, and methane. Fermentation heat approximates 10% of the M_E in cattle and sheep, and only has value for the animal at very low environmental temperatures, so a reasonable estimate of the energy in the absorbed chemical nutrients is 0.9 times the M_E of the ingested ration. Due to fermentation, the absorbed nutrients contain very few monosaccharides. Ruminant rations seldom contain more than 5% fat. In view of the yield of ATP from the absorbed nutrients (i.e., acetic, propionic, and butyric acids, amino acids, little fat or long chain fatty acids, and hardly any glucose), this would mean that in ruminants the M_E of all rations would be utilized for maintenance purposes with nearly equal efficiency. Generally, this has been found to increase slightly as the ration became less digestible. This may be due to the greater amount of work involved in eating and digesting such diets, and to the relatively higher fermentation losses caused by them.

It will be apparent from this that omnivore monogastric homeotherms consuming diets with high levels of digestible carbohydrates will be most efficient in utilizing their M_E for maintenance. Carnivore homeotherms, absorbing mainly fat and protein, will be 10 to 20% less efficient. Ruminants, due top their loss of fermentation energy and to the conversion of carbohydrates into volatile fatty acids, will be 15 to 25% less efficient in utilizing their M_E than the above-mentioned omnivores. All M_E used for maintenance finally becomes heat, so if all animal species required the same quantities of ATP for maintenance due to variation in the composition of their M_E they would require different quantities, of M_E and would produce different amounts of maintenance heat: lowest for the omnivores, highest for the ruminants.

5.1.2 Maintenance Requirements and Body Weight

Originally, it as thought that maintenance requirement of homeotherms under nonadverse circumstances were related to body surface. If one considers the body as a sphere, in this concept the requirement should be related to body weight to the 2/3 power, but better insight into cellular metabolism and thermoregulation makes this concept untenable. In this respect, many factors play a role, and no one or two of them is much more important than the others. Thus, the main approach recently has been to collect a great number of data on maintenance metabolism to find the best fit between these data and body weight raised to a ceratin power by varying the value of this power. For many homeotherms the 3/4 power, a value that was recommended for use by an international group working on energy metabolism, was found suitable. The figure resulting form raising the body weight (in kilograms) to the 3/4 power is called the *metabolic weight*.

5.1.3 Variation in Maintenance Requirements Between and Within Species

Information on variation of maintenance requirements under nonadverse circumstances for different animals has come form various sources: maintenance estimates from data on basal and fasting metabolism, from balance and feeding trials in which animals were kept in energy equilibrium, and form extrapolation results of similar trials with producing animals.

Most mature monogastric homeotherm species were found to require 340 to 420 kJ M_E per unit of metabolic weight: about 380 for monogastrics, about 420 for cattle, and about 340 for sheep. The estimate for lactating cattle was slightly higher: between 460 and 500. Due to their higher activity and high rate of N turnover, young growing pigs required about 600, which decreased to 340 at a near-mature age.

In cattle and sheep, variation of maintenance requirements between animals was found to be 5 to 10%. Accurate information of this type for other animal species is scarce.

Data on man have usually been derived from measurements of basal metabolism, results of a few balance trials, and food intake studies. The recommended data on maintenance requirements for man include increases due to eating and very light physical activity. Variation among different populations and individual persons is considerable. Part of this may be attributed to differences in physical

activity functioning of the thyroid, and lean body mass. Imprecise measurements also may have increased this variation. Even an accurate measurement of the intake of M_E for a normal diet requires a lot of work because many of its ingredients (meat, legumes) are difficult to sample. The estimates of the energy retention of the experimental subject often were not very reliable as they were based on body weight changes or on measurements of heat production during only part of a day.

Recently, new attempts to measure man's energy requirements have started which include at least one measurement of heat production over close to 24 hr/day. In these studies as well as in long term studies in which M_E, intake and body weight change were determined, standardization of intake and of behaviour pattern considerably reduced the variation of estimates of maintenance requirements per metabolic weight between persons.

5.2 PROTEIN REQUIREMENTS FOR MAINTENANCE

First the classical factorial way of deriving protein requirements will be presented. Important recent developments which discredit the classical approach will be surveyed next.

At maintenance the healthy, nonstressed animal body loses endogenous nitrogen with the feces and the urine and slight amounts with shed hair, skin, scurf, and sweat. The *metabolic fecal nitrogen*, MFN consists of the nonreabsorbed part of the nitrogen of digestive enzymes and other digestive secretions and the nitrogen of lost epithelial cells of the gastrointestinal tract. MFN is estimated either by feeding N-free diets or by feeding two or more diets with different N content and extrapolation of faecal N to zero N intake. The total amount of MFN is related to the dry-matter intake of the animal, the microbial activity in the gastrointestinal tract, and, to a smaller extent, the composition of the diet (e.g, monogastrics lose 1 to 2 g MFN/kg dry-matter intake, cattle lose 4 to 6 g^1). *Endogenous urinary nitrogen*, EUN, is usually defined as at he loss of nitrogen in the urine during intake of a nitrogen-free diet of such size that the energy demands are met; estimates are 0.08 to 0.2 g/day per metabolic weight. The correct measurement of both MFN and EUN is far from simple. The amount of nitrogen given off in lost hair, scurf, and sweat depends on the animal species (e.g., in cattle, 0.02 g nitrogen per metabolic weight per day^1). Hair growth usually continues at only a slightly reduced rate under maintenance conditions, in animals like sheep nitrogen deposition in

the fleece (some 0.05 g nitrogen per metabolic weight) also has to be included in the protein requirements for maintenance.

More nitrogen than the sum of these quantities is needed to keep the animal in nitrogen equilibrium during maintenance because the pattern of the amino acids absorbed form the intestinal tract often differs from the pattern of the amino acids needed for maintenance; moreover, some of the amino acids may be oxidized instead of used for maintenance metabolism of nitrogen. The total amount of absorbed amino acid nitrogen needed for maintenance is usually estimated by dividing the sum of MFN, EUN, and other maintenance losses of nitrogen by the biological value of the protein of the food from which the absorbed amino acids resulted. This biological value is determined with young animals not growing too rapidly; in this way a biological value is determined with young animals not growing too rapidly; in this way a biological value applying to maintenance and moderate growth is obtained. Maintenance requirements for essential amino acids differ somewhat form those for moderate growth, especially in animals with a thick coat, but these differences, are ordinarily neglected. Biological values of the protein of highly digestible human diets are high on average (around 90), while those of monogastric farm animals are often lower. The final step in the estimation of nitrogen requirements for maintenance is the prediction of which part of the ingested amino acids will be absorbed into the blood. Data on apparently digestible protein can provide the required information if they are first converted into data on truly digestible protein by adding 6.25 times the MFN to be expected under the experimental circumstances involved. According to this factorial approach, the complete computation results in the following equation:

$$ADP_m = (EUN + MFN_m + HN_m) \times 6.25 \times \frac{100}{BV} - MFN_m \times 6.25$$

where ADP_m = apparently digestible protein (g/day) required for maintenance, EUN = endogenous urinary nitrogen (g/day), MFN_m = metabolic fecal nitrogen of the maintenance part of the ration (g/day), HN_m =nitrogen in hair, etc. produced or lost during maintenance (g/day), and BV = biological value of protein in the diet.

Research of the last decade has shown that the classical approach treated above can be criticized severely, especially in the case of ruminants. In the monogastric, results of work ^{15}N suggest that he

endogenous secretions into the gatroinestinal tract., and therefore MFN increase with the N content of the diet. If this proves to be true, it would make both methods to estimate the size of MFN unreliable. Work on turnover rate of the proteins of animals tissue has thrown doubt on the constancy of the size of EUN. This rate depends on the metabolic state of the animal; at low N supply, therefore also during estimating EUN, it probably is lower than at a sufficient supply. Finally, experiments with pigs equipped with ileocaecal cannulae have shown that due to microbial fermentation part of the endogenous and other protein in the large intestine is decomposed into ammonia which is absorbed into the blood. Again as a result of this fermentation the extent of which is variable, the estimates of EUN with the conventional techniques might be biased.

In the ruminant, work with sheep and cattle equipped with rumen and/or duodenal cannulae has led to a new, physiologically better based theory on amino-acid absorption form the gut. The main sources for absorption of amino acids form the small intestine into the blood are microbial, feed, and endogenous protein. The size of the first source depends on the extent of the fermentation a.o. limited by the low supply of ATP for microbial growth under anaerobiosis, on a sufficient NH_3 supply, and probably also on the presence of some peptides or amino acids and S. Microbial protein has a high biological value, but some 15% of it is hardly digestible. The size of the feed protein entering the duodenum depends on the degree of degradation by the microbes of the various feed proteins in the forestomachs. Feeds differ considerably as to this aspect but also the retention time of the feed as well as the conditions for microbial degradation in the forestomachs determine the degree of degradation. The BV of this feed protein mainly depends on the composing feedstuffs. Little is known with certainty as to the size of the endogenous protein which has a fairly high BV. Finally estimates are made of the degree of absorption, of the BV of the absorbed amino acids and of the protein needed for maintenance nearly similar to the classical method. Various models of N metabolism in ruminants have been proposed recently. All follow in general the above theory but mainly due to the absence of precise information of several of the factors involved they differ considerably as to the size of the constants used for each of the separate aspects.

It will be clear that both the classical and the new factorial approach of assessing maintenance needs for protein suffer still from

lack of detailed information. They probably underestimate maintenance needs for protein. This means that for the time being, great value should still be given to results of long-term feeding and N-balance experiments despite the difficult interpretation of the results of feeding trials and the high amount of work needed to obtain unbiased N-balances.

Few estimates from experiments with nonagricultural animals are available. Experiments with these other species that fulfilled the conditions necessary for correct measurement of the protein required for maintenance and that provided all data needed for its derivation have been performed only seldom. This also applies to estimates for man. The factorial method gave values for man that were some 30% lower than the protein supply necessary to maintain nitrogen equilibrium in a number of intake and nitrogen balance studies. The expert committee of the FAO/WHO recommended the higher figures in 1973. To ensure a sufficient supply of protein to the major part of the individuals of a population, another increase of 30% is proposed. This should take account of the variation in protein requirement between individuals, estimated to be 15%. Since then, attempts were made to prove the correctness of these recommendations. Very careful and pretty lengthy N-balance trials were performed and long-term intake and metabolic studies were made. It is remarkable that in some of these studies the highest FAO/WHO recommendation proved only just enough. Little is known with certainty on possible increases of protein requirements under adverse circumstances like low energy supply, vitamin or mineral deficiencies, intestinal parasite infection, recovery from disease or operation, etc.

6

Nutrition and Muscles

Muscles is the most abundant tissues in the body; somewhat over 50% of the body bulk in muscle. Hence anyone interested in the effects of nutrition on the whole body can not afford to overlook the effects on the musculature. This is particularly true because the musculature is very responsive to under nutrition. Indeed, striated muscle may be regarded as a food storage tissue.

"Muscle is meat" and for this reason a considerable amount of research has been directed towards understanding how muscle grows. Particular emphasis has been placed on the nutritional requirement for optimum muscle growth. A major proportion of this chapter, therefore, deals with the effects of various nutritional factors on muscle in agricultural animals. Perhaps one of the most fascinating aspects of muscle is its ability to convert chemical energy into mechanical work. In recent years it has become apparent that there are different types of striated muscle. These have different contractile characteristics and discrete physiological roles. The contractile characteristics of a particular muscle are determined by the number and size of the different types of constituent fiber. Recent work has shown that the different types of fibers respond very differently to undernutrition. It is now realized that a correct level of nutrition is not only necessary for maintaining total force production of the muscle but also for maintaining other contractile characteristics, such as rate of contraction and rate of fatigue.

6.1 UNDERNUTRITION IN LOWER VERTEBRATES

As the lower vertebrates (reptiles amphibian, and fish) are "cold-blooded" (ectothermic) they have no firm obligation to maintain

their body temperature. For this reason they are usually able to withstand food deprivation for much longer periods than "warm-blooded" (endothermic) animals. Only fish will be dealt with in this section as very little information is available for the other groups.

Periods of severely reduced food intake appear to be a regular seasonal occurrence in the lives of many species of fish. The extraordinary ability of fish to with stand prolonged inanition is illustrated by laboratory studies involving enforced starvation. In such experiments, the cod, *Gadus morhua,* was found to survive for 78 days at 14°C, the herring *Clupea harengus* to survive for 129 days at 6 to 12°C, and the eel *Anguilla anguilla* has been reported to live for as long as 3 years without food. In most cases, the initial responses of the fish to starvation included the mobilization of lipid and glycogen reserves, followed by the breakdown of protein. As may be anticipated, the precise nature and magnitude of these events is dependent on the water temperature and the level of activity and the species of fish.

One of the main adaptation to starvation exhibited by fish is the differential rate at which various organ sand tissues are depleted. Organs absolutely vital to the animals' continued existence, such as the heart and brain, appear to be maintained at the expense of other less essential tissues. The myotomal musculature provides the largest store of protein in the body of the fish. This is particularly well endowed with catheptic (autolytic) enzymes that are considered to be there in readiness for the mobilization of amino acids during times of reduced protein intake. During such times, the musculature has been the subject of several investigations, but these have been mainly concerned with the white component of the myotome. However, recent studies by Walker on muscle fiber diameters, and by Johnson and Goldspink on fat, glycogen, and protein levels, indicate that during starvation the white muscle is more severely depleted than the red.

Studies also have been carried out to determine the effect of starvation n the ultrastructure, DNA and RNA levels, and fiber diameter of red and white myotomal muscle. Changes in swimming performance also were monitored. The ultrastructural study involving the crucian carp revealed starvation to result in a considerable degree of erosion of the myofibrillar proteins from the white fibers, while only a slight breakdown of these proteins could be observed in the red muscle. The reduction in fiber diameter was found to be more

marked in the white than in the red fibers. However, there was a marked reduction in the concentration of mitochondria in the red fibers. The fall in the "sprint" swimming speed of this species was greater than the fall in the maximum sustained swimming speed. This reflects the selective action of starvation on the white fibers as these fibers are used for sprint swimming, the red fibers being used only for cruising. Most fish under "normal" conditions appear to spend much of their life swimming at relatively low speeds. It seems reasonable, therefore that during starvation the fish should preferentially degrade its white muscle protein especially as white muscle is used only very occasionally yet it forms the bulk of the myotome.

6.2 EFFECT OF VARIOUS NUTRITIONAL FACTORS ON MUSCLE OF AGRICULTURAL ANIMALS

The muscle of agricultural animals, i.e., principally the ox, pig, and sheep, is important to man as a source of food, namely meat. This muscle is produced in the animal by converting, albeit inefficiently, plant protein into muscle protein. It is not surprising, therefore, that much of the research on the muscle of agricultural animals has been directed towards the various nutritional factors associated with the efficiency of meat production and with the proportion of lean meat to other tissues. These aspects of research are reviewed as well as the effects of nutritional factors on the cellular structure and chemical composition of the muscle of agricultural animals. Some of the work on nutritional muscular dystrophy "myopathies", particularly those associated with vitamin E and selenium deficiencies, also is discussed.

6.2.1 Carcass Composition

In 1932, Hammond showed that the level of nutrition can have a marked effect on the amount of fat in lamb carcasses. McMeekan, working on pigs, concluded that the proportions of developing body tissues (muscle, bone, and fat) could be greatly influenced by the plane of nutrition. He also concluded that different areas of muscle were affected to different extents. Pomeroy employed more severe levels of undernutrition on pigs and deduced that tissues are affected in the reverse order of development, i.e., fat first, followed by muscle, then bone. Different joints also were affected in the reverse order of development. Robinson fed mature ewes at two nutritional levels so that one group reduced weight an the other

increased weight. Muscle weight was shown to follow a linear change in both cases although muscle proportions in various joints remained constant.

Palsson and Verges found that various body regions in the lamb also develop at different rates with growth gradients from the head caudally, and from distal limbs proximally towards the loin. Butterfield and Berg showed that different muscle groups in cattle also grew at different rates. In a nutritional experiment similar to McMeekan's Palsson and Verges concluded that low nutritional levels affected the later developing regions the most. Johnson also found that in bulls the muscles with a high postnatal-growth impetus were retarded the most (upto 50% reduction in weight) when dietary restrictions were employed. Richmond and Berg, however, found that two different energy rations had not effect on muscle distribution except for the distal thoracic limb group, whereas the total percentage of muscle was higher and fat lower in carcasses fed the lower energy ration.

In an interesting reappraisal of the data of McMeekan's Palsson and Verges, Elsley and McDonald found that when variation in carcass fat content were excluded, there is not evidence for any effect of plane of nutrition on the proportion of muscle and bone. There also was no significant relationship (except for the head and neck region) between the order of maturity of various joints and their relative retardation of development during periods of restricted nutrition; these later calculations also excluded variations in carcass fat content. Elsley and McDonald did state, however, that more extreme undernutrition, such as that used by Pomeroy, may have an effect on tissue proportions.

The time in the life of the animal when it is subjected to undernutrition is very important. McCance found that by restricting the level of nutrition very early in life he could produce 1-year old pigs weighing only 5 kg in comparison with their 1-year-old well-nourished littermates weighing about 150 kg. The weight of the gastrocnemius muscle of these severely undernourished pigs was, in fact, only 2% of the well-nourished controls. It is worth mentioning here that 10 of these 1-year old undernourished pigs were rehabilitated on an excellent diet but stopped growing at the same chronological age as the controls, so that they did not attain the same adult size as the controls. Less severe nutritional conditions produced similar results. It is interesting to note, that whereas in severely under nourished pigs the proportions of muscle and bone

were altered, the rehabilitated pigs of Lister and McCance had muscle to bone ratios within the expected normal range.

Many more studies with a more economic bias have been carried out to establish the optimal levels of protein and calorie in the diet that produce the best growth rates and efficiency, as well as the best carcass parameters, such as high percentage lean meant and low back fat thickness. Fluctuations in feed intake have been found to have only trivial effects on carcass performance but the physical form of the diet may have some effect.

Some of these "economic" studies have shown that there is a genetic influence of breed on the efficiency of an animal to convert food into lean meat or on the proportion of muscle and fat laid down for a given daily food intake. Also, sex has been shown to have an effect on these parameters at several protein and energy levels. Pay and Davies concluded that boars had a better food conversion efficiency and higher lean meat content than castrates or gilts at given protein intake levels. Strains of the same breed of pig also differ in their requirements. Lean pigs need less protein per pound gain than fat-strain pigs and, whereas higher protein levels can improve carcass leanness in lean-type pigs, they have far less effect on fat-type pigs. Some explanation of this has been provided by the work of Martin et al. who showed that obese-type pigs had higher lipogenic and gluconeogenic enzyme levels than-lean-type pigs. The response of these enzymes to fasting-refeeding regimes was found to be more dynamic in the lean pigs.

Diet in later pregnancy also has been shown to affect rate, percentage carcass muscle, and other carcass parameters in the offspring of agricultural animals. Wallace showed that a ewe's diet in later pregnancy affects birth weight and muscle weight of the offspring. Later work on pigs has shown that maternal dietary energy levels and maternal protein levels also affect various muscle parameters in the offspring.

Mention should be made of certain specific substances that have been shown to have beneficial effects on the rate and amount of lean meat produced. Becker et al. have shown that antibiotics in many feeds will increase rate of gain. Jacobson et al. showed that supplementary cholesterol also improved weight gain in calves. Hormones have been shown to improve food conversion efficiency and leanness in pigs and steers. The interrelationships between hormonal and nutritional effects on skeletal muscle have been

reviewed by Trenkle, although there is little reference to agricultural animals. Certain minerals, including low levels of Cu in pigs have been shown to have beneficial effects on muscle weight gain. However, different levels of Mn in heifers and subclinical levels of Pb were shown to have no appreciable effect on muscle weight gain, and increasing Cd levels have been shown to have a detrimental effect on weight gain in pigs as have high levels (above 3%) of Na and K in lambs. It is known that adequate vitamin levels are required for normal muscle growth including vitamin B in pigs and vitamin A in calves. The importance of selenium and vitamin E in the diet on muscle is discussed below.

It may be concluded that although nutritional level can affect the total amount of muscle in animal, its relative proportion in the body, expressed as a percentage of fat-free carcass weight, is fairly constant except under severe conditions. Fat is by far the most variable component; breed differences can explain a 50% variation and plane of nutrition up to 45% or sometimes more in swine. Experiments on the effects of nutrition should, therefore, concentrate on obtaining carcasses with the desired fat content as this is the only component whose proportion can be altered to any extent. Nevertheless, meat production is a very inefficient system of food production. Pryor and Butterfield found that the gross energetic efficiency (edible carcass energy as a percentage of food energy) of calves falls from 27% at 4 weeks of age to 2% at 22 weeks. Nitrogen retention also is inefficient. As was expressed by Trenkle and Willham, it is, therefore, important to make agricultural animals less competitive with humans for feed grains, possibly by making use of genetic variations still available in these animals. Another interesting line of research put forward by Bergen is to elucidate the mechanism whereby calories are diverted to protein synthesis rather than fat synthesis.

6.2.2 Muscle Structure

it has been discussed in the above section how nutritional factors can influences muscle mass in an animal. Several studies have been carried out to investigate how these nutritional factors affect the structure of muscle at the cellular level.

6.2.2.1 Ox

The early investigations of Robertson and Baker showed that full-fed steers had larger muscle fibers in their muscles than those

steers that were either half-fed or rough-fed (i.e., fed roughage only). They also found that glycogen was present in all muscle connective tissue of full-fed steers only. Yeates showed that starvation of mature beef animals caused a reduction in muscle fiber diameter with no loss in the number of muscle fibers. Rehabilitation of these animals brought about a recovery in the muscle fiber diameter. Yeates also showed, in the same investigation, that, whereas percentage of muscle connective tissue increased in the starved animal the absolute amount did not. Later studies, including Johnson, have confirmed that dietary restrictions can considerably affect muscle fiber diameter in the ox.

6.2.2.2 Sheep

Joubert, in an extensive study, showed that the diameter of muscle fibers also was reduced in lambs maintained on a low level of nutrition. These studies were carried out at different ages and in all cases there was a strong correlation between muscle weight and muscle fiber diameter. Hight and Barton, however, found no effects of plane of nutrition on muscle fiber diameter in the longissimus dorsi of Romney ewes, although the nutritional states were enough to affect various compositional factors in the muscle.

6.2.2.3 Pig

As far as the big is concerned, McMeekan showed that muscle fiber diameter also was very much affected by nutritional status with different muscles being affected to differing extends. Moody et al. subjected pigs to a 42-day fasting period with the result that muscle fiber diameter was reduced as was the number of sudanophilic fibers and the amount of intramuscular fat. A 16-day rehabilitation period restored body weight but was not of sufficient length to completely restore the other parameters to control values. The extensive work of Staun showed that feeding pigs either a diet of insufficient protein or intensively feeding them a high energy diet of low biological value caused a decrease in muscle fiber diameter. Conversely, a high protein diet brought about an increase in muscle fiber diameter. Based upon estimations of the total number of muscle fibers in whole muscle cross sections. Staun concluded that there was no effect on muscle fiber number. The effect of different foods on muscle fiber diameter also was shown by Berry and Kroening who found that fiber diameter also was shown by Berry and Kroening who found that fiber diameters were bigger in hogs fed wheat and

barley grain than those fed on corn. The effects of very severe levels of low protein and low calorie nutrition on porcine muscle development was investigated by Stickland et. al. using the pigs developed by McCance. They found that there was a marked effect on muscle fiber diameter but no significant effect on total muscle fiber number. This latter parameter appeared to be genetically determined and fixed at or near birth in the pig. Swatland, however, found that there was an increase in the "apparent" fiber number (number of muscle fibers passing through a mid-section) of 300/ day in the sartorius muscle of the growing pig. This "apparent" fiber number was reduced in pigs fed a diet that prevented growth (over 28-day periods). This was probably due to the effect of nutrition on the length of the intrafascicularly terminating fibers found frequently in large muscles. It is probable that Stickland et al. found no effect on muscle fiber number (seen in a mid-section) because of the very small muscle used (flexor digiti V brevis), which must have very few intrafascicularly terminating muscle fibers.

6.2.2.4 Fowl

Relatively little work has been carried out on the adult fowl. However, mention should be made of the study by Montgomey et al., which showed that undernutrition in adult fowl did not reduce the number of fibers in the sartorius muscle.

It may be concluded, therefore, that nutritional levels in agricultural animals may have a significant effect on the diameter of muscle fibers in a given muscle but not on the total "real" number of muscle fibers.

6.2.3 Chemical Composition of Muscle

6.2.3.1 Food restriction

In lambs Palsson and Verges showed that a low level of nutrition reduced marbling (intramuscular fat) but increased the water content of meat. This was confirmed by Hight and Baron who also showed that the muscle of poorly nourished ewes had a lower protein content. This latter point was investigated by Lindsay et al. who analyzed the amino acid content of both arterial and venous blood supply of muscles in sheep. They found that for most amino acids the arterial concentration (i.e., input) was lower in starved than fed sheep. The venous blood (i.e. output) contained a higher concentration of amino acids (except for phenylalanine) in the fasted sheep. The review of Swick and Benevenga explains that the rate of protein synthesis

exceeds the rate of protein degradation in muscle during a good dietary supply so that protein is accumulated. When the animal is fed on a poor diet, however, the rate of protein synthesis in muscle decreases below the degradation rate; muscle breaks down its protein store so that amino acids are available for energy supplies and, during lactation for example, for milk protein synthesis.

Most other work in agricultural animals on the effects of restricted nutrition on muscle composition seems to have been carried out on the pig. McMeekan showed that low nutritional levels in the pig reduces intramuscular fat protein, and increases water content of muscle, in agreement with the work on sheep already mentioned. Lee et al. also showed that fasted pigs had more moisture and less fat in their muscles than both control and fasted-refed pigs, with the latter having the highest intramuscular fat content. In the same study, glycogen content was fund to increase in the muscles of the treated pigs with a subsequent lower postmortem lactic acid production and a slower rates of postmortem ATP depletion.

The effect of food restriction on the chemistry of muscle development has been reviewed elsewhere in this handbook. It is pertinent, however, to mention some of the work here where it relates to agricultural animals. Dickerson and Widdowson showed that normal porcine muscle development is associated with an increase in the concentration of cellular constituents, N, K, P and Mg and a decrease in concentration of the extracellular ions, Na and Cl, as well as a decrease in Ca. Severe undernutrition during the first year of life caused a reversal of these trends in the muscle of developing pigs. Widdowson found that an abnormally large proportion of total muscle N was present as collagen. This was due to the continued slow growth of extracellular components with a cessation of cellular growth during the year of undernutrition. Gilbreath et al. found that in both mature sows and piglets, the collagen concentration in muscle was inversely proportional to the dietary protein level. Protein restriction during pig development also affects the normal increase in the total content of DNA and RNA in muscle with rehabilitation capable of bringing the values ultimately to normal if the period of rehabilitation is long enough. In the severely energy and protein deficient pigs used by Stickland et al., it was found that the total number of nuclei (also confirmed by DNA estimations) was directly related to body weight and size of the muscle studied, whereas nuclei concentration as highest in pigs of lower body weight. This is

in accordance with the findings of Montgomery et al. in the sartorius muscle of the fowl. Restrictions in dietary tryptophan alone, however, show no apparent effect on RNA and DNA content of porcine muscle. There is evidence that DNA synthesis and RNA synthesis in muscle also is reduced in the progeny of undernourished gilts. Atinmo et al., however, found that there was no effect in undernourished gilts on muscle RNA or DNA synthesis although there was a difference in the growth rate of the progeny.

6.2.3.2 Quality of food

The type of food given to an animal may have an effect on the chemical composition of muscle. Intensive rearing of steers accelerates the rate of gain in these animals, but there seems to be no effect of this treatment on the concentration of protein, iron, thiamine, riboflavin, or niacin in muscle, yet there is an increase in the fat content. Fatty acid composition of muscle is, in fact, quite sensitive to changes in diet. Sumida et al. showed a difference in fatty acid composition of the longissimus muscle between steers raised on feedlots and those raised on pasture, whereas there was no breed difference. Supplementary cholesterol was shown by Jacobson et al. to have no effect on muscle cholesterol levels in calves.

The relationship of muscle fatty acid content to the source of dietary lipids in illustrated by the following three studies on pigs. Mason and Sewell found that a diet deficient in esterified fatty acids reduced diene and tetraene fatty acids and increased monoene and triene fatty acids in muscle. This situation was reversed when beef tallow or, more markedly, when corn oil was added to the diet. Brooks found that the linoleic content of intramuscular fat was almost tripled in pigs fed soybean oil over a basal diet. Filer et. al. showed that the muscle of sow-reared pigs had increased myristic, palmitoleic, and linoleic acids and decreased stearic and oleic acids than pigs fed a semisynthetic diet. The difference was related to the fatty acid content of sow milk and lard (the fat source in the semisynthetic diet).

6.2.3.3 Minerals

An adequate concentration of ions is important for the functioning of many enzymes and the correct balance of certain ions is necessary for muscle exitability. Although a potassium-deficient diet in dogs has been shown to affect muscle ion composition and transmembrane potential, there appears to be no such significant

effect in stress. K and Na content of muscle in 6-week-old calves was shown by Hall et al. to be unaffected by vitamin A intake.

Magnesium-deficiency in cattle may result in hypomagnesaemic tetany which can be classified as either milk tetany or grass tetany depending on where the deficiency lies. Todd and Horvarth produced hypomagnesaemic tetany in calves by feeding a low Mg milk diet up to 20 weeks. They stimulated muscles either directly or indirectly (via nerves) and found that the current required to cause contraction fell to one third of the normal value for indirect stimulation during tetany only. Normal values were required for indirect stimulation in control and hypomagnesaemic calves and for direct stimulation in control, hypomagnesaemic, and tetany. They concluded that the lesion is due to facilitation of transmission during tetany stages at the neuromuscular junction. The studies of Harrington showed that a magnesium-deficient diet had no detectable effect on muscle concentration of Ca, P, or Mg in foals.

The level of dietary iron in the diet fed to steers was found by Standish et al. to have no effect on Fe, Cu, Zn, Ca, Mg or Mn in the longissimus dorsi muscle. The dietary iron concentration does, however, have an effect on the pigment content, hence color, of veal.

Low levels of zinc in the diet of calves increases the level of zinc retained in the muscle due to a homeostatic conservation of zinc. Miller et al. showed that zinc content in muscle is not affected by cadmium levels in the diet of calves and goats.

A higher concentration of dietary manganese is reflected by a higher muscle manganese content in heifers. Subclinical lead intake, however, has no effect on the muscle lead content in calves; about 95% of the lead is, in fact, excreted in all cases.

6.2.4 Nutritional Muscular Dystrophy and other Myopathies

This section deals mainly with the condition of nutritional muscular dystrophy associated with low dietary levels of selenium or vitamin E or both. The main symptoms of the disorder are associated with muscular weakness producing a staggering gait usually caused by stiffness of the hind legs (hence the old term "stiff-lamb disease"). Muscles of the back also are affected. On examination, the muscles of affected animals are seen to contain small, grey-yellow lesions that can become large, white lesions in severe cases (hence the common term "white-muscle disease"). The condition can lead to

death, which could usually be due to lesions in the cardiac muscle producing cardiac failure. Young calves and lambs, especially suckling animals, are primarily affected although the condition also occurs in suckling and older pigs and foals.

Willman et al., were among the fist to artificially produce still-lamb disease by feeding lambs a diet of cull beans and alfalfa hay. William et al. and Whiting et al. found that ewes with a low level of vitamin E in their milk often produced progeny with stiff-lamb disease, but that a vitamin E dietary supplementation prevented this disease when fed to the ewes, or cured it when given to the affected lambs. Culik et al., and Bacigalupo et al. produced the disease in lambs by feeding them a purified vitamin E-deficient diet. The symptoms were alleviated in 3 to 5 days by administration of α-tocopherol (prototype of vitamin E). Vawter and Records studied the symptoms of white-muscle disease in young calves and suggested that the condition in calves also was probably associated with a lack of vitamin E in the diet. Although this is confirmed by the work of Blaxter et al., MacDonald et. al., Safford et al., Schofield, and Marr et al., all the agricultural animals, other authors found that vitamin E therapy was ineffective against white-muscle disease. In abut 1958, however, the importance of selenium as an adjunct to vitamin E therapy was being realized. Effects of both selenium and vitamin E in preventing white-muscle disease was shown experimentally in lambs by Muth et al. and subsequently confirmed by others, including Sharman et al., Kuttler and Marble, and Lagace. For further discussion of this earlier work on agricultural animals see the review of Hartley and Grant, Blaxter, Muth, and Oksanen.

From the investigations already mentioned, it seems quite clear that deficiencies in either vitamin E or selenium or both may lead to nutritional muscular dystrophy. It should be noted that these deficiencies also produce other diseases such as, in swine, mulberry heart disease and hepatosis dietetica, often in association with muscle lesions.

It has been suggested by several workers, including Zayed and van Gils, working on calves, and Nafstad and Tollersrud working on pigs that vitamin E deficiencies may sometimes be due to a high content of unsaturated fatty acids in the diet. Such a diet given to pigs produced muscle dystrophy symptoms that were relieved by α-tocopherol but not by selenium. The interrelationships between vitamin E and polyunsaturated fatty acids are discussed in a review

by Dam. Preservation of cereal by propionic acid may also produce vitamin E deficiency syndromes, including myopathy. Tri-o-cresyl phosphate also has been shown to be a vitamin E antagonist, inducing stiff-lamb disease in lambs. The results of Dvorak et al.. suggest that pigs raised under stressful conditions require an increased level of vitamin E than those pigs raised under optimal conditions.

Certain factors also may affect the availability or utilization of selenium by an animal. Sulfate added to the soil can interfere with selenium uptake as well as copper uptake and so induce white-muscle disease. Acid or neutral soils also deplete the available selenium. Although the action of selenium and its relationship with vitamin E are not fully understood, its action in the animal does seem to be antagonized by sulfur and arsenic. There also appears to be a heat labile antagonist to selenium in beans fed to lambs. Unaccustomed exercise is another factor that may enhance the effects on muscle of selenium-deficiency in cattle and lambs. The latter work on lambs showed that immobilization of one limb in selenium-deficient lambs reduces the severity of muscle lesions with respect to the contralateral limb.

Muscle selenium levels increase with increasing dietary selenium levels although over certain limits selenium is increasingly excreted by the kidney. Ku et al. also showed that natural selenium (associated with amino acids) is more significant in influencing tissue selenium levels than supplemental sodium selenite. For further information on selenium deficiencies see the reviews of Allaway and Ammerman and Miller.

Vitamin E and selenium deficiencies lead to several biochemical changes in the affected muscle. Increasing severity of myopathy is associated, in the affected muscle, with high lysosomal enzyme levels, decreased cytoplasmic enzyme levels, and increased plasma enzyme levels. These abnormal enzyme levels return gradually to normal with age, or more rapidly, by treatment with vitamin E and selenium supplementation. Broderius et al. showed that there was a decrease in total and protein sulfhydryl groups and an increase in nonprotein sulfhydryl groups (and reduced glutathione) in affected muscle of lambs with white-muscle disease. They concluded that selenium is involved in the metabolism of sulfhydryl groups. Evarts and Oksanen have shown that affected muscle exhibits a decrease in cardiolipin (mitochondrial phospholipid) in muscle and an increase in sphingomyelin (extramitochondrial).

This last report confirms electron microscopic examinations of the structure of myopathic muscle, associated with vitamin E deficiency, made by Iksanen and Poukka. These authors observed electron-dense particles between the myofibrils, which they concluded were probably changed mitochondria. A complete account of the gross and microscopic lesions seen in affected muscle of cattle and sheep was given by Cordy. More recently, other ultrastructural studies have shown that myopathic conditions associated with selenium/vitamin E deficiency exhibit, firstly, lesions in small blood vessels, connective tissue, and neuromuscular elements. This is followed by myofibrillar lysis and disruption of mitochondria, sarcoplasmic reticulum and plasma membranes. The areas of degeneration are then invaded by macrophages and myoblasts, the latter forming new muscle fibers within the remaining basal lamina of the old fibers. Ruth and Van Vleet showed that affected muscle exhibited a selective destruction of type I fibers (red, slow contracting fibers) and a lack of phosphorylase in Type II fibres (fast contracting fibers). Despite these many advances in research into nutritional muscular dystrophy, naturally occurring cases of the condition are still being reported, for example, in sheep, a pregnant heifer, and yearling bulls.

6.3 EFFECTS OF UNDERNUTRITION ON HUMAN MUSCLE

Body proteins are mainly synthesized form the amino acids that are derived from dietary proteins. Although most of the amino acids can be synthesized in the body given a nitrogen source (again dietary proteins from the important nitrogen source), the essential amino acids must be obtained directly from the dietary proteins. An adequate supply of dietary protein is, therefore, very essential for the building up of muscle mass during growth. Many of the nutritional problems encountered in the underdeveloped country are in fact those of protein deficiency rather than overall calorie intake.

The prevalence of kwashiorkor (a protein-calorie malnutrition disease), in human infants in many tropical and subtropical countries, has aroused considerable interest. Therefore, there have been many studies of the effects of protein malnutrition on both humans and laboratory animals. However, only in recent years have studies been carried out at the tissue and cellular levels. Vincent and Radermecker and Montgomery have reported that the mean fiber diameters of muscles of children and infants suffering from kwashiorkor were reduced. It also is known that DNA and RNA levels are decreased.

The reduction in the DNA levels almost certainly means that the number of muscle cells (fibers) is reduced and this will result in muscles with a decreased ultimate size, irrespective of whether the person receives an adequate diet later in life.

The effects of protein or calorie malnutrition have not, to the authors' knowledge, been studied on muscle in mature humans. Extrapolating from the studies on laboratory animals, we would expect there is to be considerable changes in muscle fiber diameter but no change in the number of fibres.

6.4 STUDIES ON LABORATORY ANIMALS

The cellular mechanisms that operate during period of undernutrition cannot conveniently be studied in human beings. Therefore, many investigators have had to resort to working on laboratory animals. These studies have the advantage that very pure genetic lines of rodents are available and the environmental conditions as well as food intake can be controlled very carefully. In this review the cellular mechanism involved in depletion will be dealt with under several heading.

6.4.1 Muscle Fiber Number

As stated above, protein restriction during prenatal or early postnatal life may be very critical as this is the period of rapid cell division. Indeed, Aziz-Ullah showed that the biceps brachii muscles of the progeny of female mice, which were given protein diet during the later half of their pregnancies, had a reduced a total number nuclei and a reduced total number of fibers.

However, the situation with mature muscle is certainly very different. Ward and Ward and Goldspink working with both hamsters and mice, showed that there was no change in the total number fibres following a period of undernutrition.

6.4.2 Muscle Fiber Size

The decreased in muscle bulk resulting form a period of undernutrition is known to be associated with a decrease in muscle fiber diameter. The way in which this happens has been studied in some detail by Goldspink and Rowe. In mice the mean fiber diameter changes considerably over a certain range of food intakes; however, less change occurs at very high or very low intakes. Some muscles in rodents are composed of large and small fibers. The effect of starvation was to reduce the number of large phase fibers so that the fiber size distribution plots tended to become unimodal instead

of bimodal. It is now known that the large ones are fast contracting fibers of the glycolytic type while the small ones are fast contracting fibers of the oxidative type. As will be mentioned below, the fast glycolytic fibers are much more susceptible to reduced food intake than other type of fibers.

The combined effects of exercise and food intake also have been investigated and it was found that in spite of being maintained on a low level of nutrition the muscle fibers in exercised mice still underwent hypertrophy. Indeed they almost hypertrophied to the same extent as muscle fibers in exercised mice fed ad libitum. The reason for this may be increased nitrogen retention and anabolic efficiency or it may be the result of an increased blood supply to the muscles.

The decreased in muscle size following a decrease in the nutrition level in invariably associated with loss of myofibrils from the fibers as well as sarcoplasmic proteins. Goldspink showed that the reduction of fiber size in mice skeletal muscle resulted from a decrease both in the number and in the size of its myofibrils. Receka et al. reported a loss of myofilaments in varying degrees in protein deficient Rhesus monkeys. Roy et al. reported that individual myofibrils were much thinner in the protein-deficient monkeys than in control animals. Aziz-Ullah showed that the mitochondria in malnourished muscles underwent varying degrees of degeneration from swelling to complete disappearance of their cristae. Using lineal analysis he also showed that there was a significant reduction in the areas occupied by myofibrils.

The way in which the myofibrils are lost from the fibers is not known, but Bird et al. found increased levels of cathepsins after a period of 5 days of reduced food intake, Wechsler claimed that the myofibrils at the periphery of the fiber are the first to be removed. Patterson and Goldspink in a study of starvation of carp noted the breakdown of myofibrils especially in the peripheral regions of fibers in the white myotome. The biochemical control of protein metabolism is not fully understood especially in relation to starvation. It appears that not only is the rate of protein degradation increased during starvation, but also the rate of synthesis of new protein is decreased. The decreased rate of protein synthesis may be due to a reduction in the protein synthesizing capacity of the polyribosomes, or may be due to a shortage of one or more essential amino acids.

The force production of a muscle is dependent on the cross sectional area of the myofibrils. Therefore, muscle strength would

be expected to decrease during a period of undernutrition. Goldspink showed that this was certainly the case; however, it was found that in relative terms the muscle strength actually increased. The reason seems to lie in the fact that the sarcoplasmic proteins are depleted more rapidly than the myofibrillar proteins. It also is interesting to note that the immediate energy supply for contraction, ATP, and phosphoryl creatine also increase in relation to muscle mass during protein malnutrition. This indicates, therefore, that the essential functions of the muscle tend to be preserved as much as possible during starvation. The loss of muscle strength during undernutrition in mature animals was found to be completely reversible.

6.4.3 Effect on Different Types of Muscle Fibers

In recent years it has been recognized that not all striated muscle fibers are the same. Indeed most muscles in mammals are made up of populations of different types of muscle fibers.

The fiber types in mature muscle can be described as slow-twitch oxidative fibers (STO), fast-twitch glycolytic fibers (FTG) and fast-twitch oxidative-glycolytic fibers (FTOG). Studies on the recruitment of muscle fibers during different types of activity, and on the chemical energy utilization during different kinds of contraction suggest that these types of fibers have different physiological roles. As the physiological properties of a muscle are dependent upon the size and type of its constituent fibers, it is important that we understand the effects of undernutrition on these different types. It is known that the response of skeletal muscle tissue to starvation is not uniform. Those muscles that lose weight most rapidly in inanition are those that respond most to growth-hormone. Rowe found that fibers of the fast-twitch muscles of the laboratory mouse became smaller, while the size of those of slow-twitch (soleus) muscle remained virtually unchanged. Only recently has the response of the different types of fibers within the same muscle, been investigated. Using histochemical staining methods for myosin ATPase, oxidative, and glycolytic enzymes, Ward, Goldspink and Ward, identified the three major muscle fiber types in hamsters and mice and measured their response to a period of undernutrition. Starvation did, however, have a profound effect on the size of the different fibers. Muscle fiber size was considerably reduced but the extent of atrophy varied between the different muscle fiber categories in the different muscles studied. Generally speaking, the fast contracting fibers were mor severely affected than the slow contracting

fibers. In the mouse biceps brachii muscle, which is without any slow fibers, of the two categories of ATPase-high fibers, the fast glycolytic fibers seemed to be more susceptible than the fast oxidative fibers. It is not known why the different types of fibers are affected to different extents. It may be that the slow fibers are spared more because they have a rich blood supply. However, it is more likely that the mechanism producing the differences in response is related to an intrinsic property of the muscle fibers, which could operate through the protein turnover mechanisms. The ATPase-low fibers are believed to be used for both postural activities and for slow isotonic movements and hence they are in much more frequent use than the ATPase high (fast contracting) fibers. Certainly, there would seem to be a selective advantage in sparing the muscle fibers whose function is primarily to maintain body posture.

The preferential atrophy of the fast contracting fibers during undernutrition means, of course, that the muscle will contract more slowly when maximally stimulated in the laboratory. However, this is not necessarily the case in vivo as the different fiber populations are recruit sequentially. Although the fast contracting fibers are recruited last, they are nevertheless required for activities involving a high power output (work per unit time). Therefore, the capability of the muscle to perform very rapid and very powerful movement. Therefore, the capacity of the muscle to perform very rapid and very powerful movement will be much diminished. The last contracting fibers are recruited very infrequently; most of the muscular activities being carried out by the slow contracting fiber and, therefore, general muscle function tends to be relatively unimpaired, unless the undernutrition is very severe.

7

Nutrition and Bone Formation

7.1 BONE FORMATION

Osteogenesis (or ossification) is the process by which bone is formed. It refers to the formation of all components of bone. Calcification denotes mineralization of bone, i.e., deposition of calcium salts in the interstitial substance and collagen. The bone cells participating in bone formation are osteoblasts, which secrete the specific intercellular substance and collagen, which later may calcify. Two types of bone formation are know: intramembranous and endochondral. Osteogenesis can be studied either from developing embryonic bone or through the process of fracture repair.

7.1.1 Intramembranous Ossification

This process of bone formation is found in the vault of the skull, face, clavicle, and partially in the compacta of diaphyses. In its initial process (7^{th} to 8^{th} weeks of embryonic life in humans), the areas in which these bones develop are occupied by mesenchyme. Clusters of mesenchymal cells differentiate in to osteoblasts to form centers of ossification. The osteoblasts start to secrete the characteristic organic intercellular substance of bone. When those cells are completely surrounded by organic matrix (osteoid), they are called osteocytes. However, many of the mesenchymal osteogenic cells do not differentiate immediately to osteoblasts but continue to proliferate to supply as many osteogenic cells as necessary. This phenomenon occurs not only in the centers of ossification but also in periosteum of bones.

As ossification continues, bone trabeculae form. Bone that consists of scaffolding of trabeculae joined together is called *cancellous* bone.

Osteoblasts cover the surfaces of bone trabeculae while osteocytes are embedded inside the trabeculae. As the covering osteogenic cells continue to proliferate, more bone is added of the periphery of the trabeculae also. In humans, new bone is now added in the form of lamellae.

Young forming bone is immature and spongy. However, subsequent bone that forms is more mature and compact. When new lamellae are added the spaces between trabeculae become narrowed so that the bone becomes more compact with only little, narrow bone marrow spaces.

7.1.2 Endochondral Ossification

This process of ossification results in the formation of most bones of the skeleton. The mesenchyme of the future bone condenses and differentiates into cartilage and, thus, cartilage models of the future bones are formed. The cartilage model is surrounded by the perichondrium in which the inner layer of mesenchyme is the chondrogenic layer. The formed cartilage model grows both in length and width. There are no blood vessels in these primary cartilaginous models.

Chondrocytes in the middle of the cartilage model start to mature and hypertrophy and the intercellular substance calcifies. During that time, blood vessels from the perichondrium start to invade the cartilage model. With the invasion of capillaries, cells from the inner layer of the perichondrium differentiate into bone-forming cells (osteoblasts and osteocytes) and a collar of bone, usually in the midshaft region, starts to form. Thus, the perichondrium now covers bone, and is therefore termed periosteum. It is probably the penetration of capillaries that causes perichondrial cells to transform into osteoblasts. The same cells may transform to cartilage cells to transform into osteoblasts. The same cells may transform to cartilage cells at the appropriate stimulus, i.e., fracture and repair.

As the calcified cartilage in the middle of bone model beings to disintegrate, capillaries and osteogenic cells penetrate and invade the model and form the primary ossification center. Although it is commonly believed that osteoblasts are brought in by the capillaries, there is some evidence that cartilage cells can transform directly into osteoblasts.

Osteoblasts arrange themselves around the remnants of calcified intercartilaginous interstitial substance, secrete bone matrix (osteoid) and form bone trabeculae. As these longitudinally arranged

intercartilaginous septa are to stay in the center of the forming endochondral trabeculae which are spongy in appearance, this enchondral bone is cancellous in type.

Whereas the center of ossification enlarges, the whole of the midshaft is gradually transformed into bone. This ossified part is the diaphysis, leaving the cartilaginous epiphyses at the ends. The cartilage cells in the epiphysis close to the diaphysis (metaphyseal area) arrange themselves into three typical zones of proliferating (columnar), maturing and calcifying (hypertrophic) cartilage. The hypertrophic cartilage columns in the vicinity of the diaphysis are destroyed, and more bone is deposited. Around the calcified interstitial substance by the osteoblasts brought in with blood vessels penetrating from the metaphysis.

7.1.3 Epiphyseal Ossification

In many small animals such as mice and rats, cartilaginous epiphyses are devoid of blood vessels until ossification starts. In human fetuses and larger animals, however, epiphyses are invaded by blood vessels (arterioles, venules, and capillaries) which form cartilage canals. This process might precede ossification by a long time .The formation of cartilage canals is probably related to the nutritional needs of cartilage in larger animals. At the start of epiphyseal ossification, cartilage cells in the region of the future ossification center enlarge and become hypertrophied. Interstitial substance calcifies, and vascular buds from cartilage canals or from the perichondrium invade the area bringing in osteoblasts. Endochondral bone trabeculae radiate from the ossification center in all directions. During epiphyseal remodelling, many of the inner trabeculae are reabsorbed and the newly formed trabeculae are arranged parallel to the mechanical forces operating on them. In some animals, including man, epiphyseal ossification in certain bones starts postnatally (i.e., most long bones in human). Once an epiphyseal center is well established, bone formation continues from the center to the periphery, leaving the epiphyseal plate (of cartilage) between epiphysis and diaphysis. The latter serves as the center for longitudinal growth of endochondral bone, persisting as long as growth continues. When longitudinal growth ceases, it is replaced by bone.

7.1.4 Bone Remodelling

The process of remodelling usually involves the removal of bone trabeculae and formation of new trabeculae to maintain bone shape

during growth. This process begins shortly after bone formation begins (early in fetal life and in the human fetus at the age of 4 to 5 months). Since the metaphysis is wider than the remainder of the shaft, metaphyseal bone trabeculae are removed from outside (periosteal) and are formed from inside (endosteal surface). In the midshaft, however, bone is removed from inside, a process during which most endochondral trabeculae are removed, and is laid down from the outside (by periosteal, intramembranous ossification).

Because the removal of newly formed trabeculae is more prominent in the metaphysis just below the level of the growth zone, numerous osteoclasts are found there. During bone remodelling, the external shape of bone is formed and this involves local formation or resorption of trabeculae. This process of remodeling is called external or anatomical and normally ends when growth is completed. Internal remodelling is the turnover of bone tissue generally occurring inside bone by which trabeculae are destroyed and rapidly replaced without inducing change of external shape. This process continues until death. The metabolic unit of bone is commonly known as "Haversian system" or "osteons." Those units of lamellar bone that are usually formed near term or postnatally are separated from neighboring units by "cement lines, and the osteocytes of one unit do not communicate directly with those of adjoining units. Not only do osteoclasts participate in bone remodelling but osteocytes do also ("peri-osteocytic resorption"), enlarging the osteocytic lacunae. The osteocytes might also secrete into their lacunae a mineralizing "fibrous osteoid" which is metabolically more active than the lamellar bone in the osteones.

7.2 CALCIFICATION

What causes cartilage and bone to calcify while other tissues do not? During calcification, chondrocytes and osteoblasts are probably involved intracellular uptake of mineral and its extracellular transfer in a specific form that causes bone to mineralize. In endochondral bone formation, calcification occurs in cartilage, while during intramembranous bone formation, calcification occurs directly in bone.

7.2.1 Calcification in Cartilage

Transmission electron microscopic (TEM) studies as well as scanning electron microscopic (SEM) studies in the interstitial substance of epiphyseal cartilage showed calcium-containing "globules" or "matrix vesicles" which serve as loci of initial calcification. These globules covered the longitudinal intercartilaginous septa and varied

in size (ranging from 300 to 10,000 Å) and in their mineral content. They were especially abundant in the zones of maturing and calcifying cartilage.

Recent SEM studies demonstrated that these globules are composed of numerous smaller "calcifying globules" about 0.2μ in diameter. Progressive treatment with NaOCl gradually removes the membrane, exposing mineral crystals. On the basis of ultra structural findings, it is suggested that he calcifying globules originate as buds from the plasma membrane of chondrocytes. During endochondral bone formation, calcification starts in the calcifying globules (which are located in the interstitial substance) in the form of needle-like calcium slats. Crystals nucleated in those structures might give rise to others by secondary nucleation. As calcification continues, calcifying globules coalesce, fill interfibrillary spaces, and then proceed to the collagen fibers to cover the surface and to be deposited within fibrils. Thus, mineralization is completed.

7.2.2 Calcification in Bone

There are different opinions as to the earliest mineral deposits in bone. They were thought to be deposited within the collagen fibrils, on their surface, on the interfibrillar ground substance. Electron microscopic studies, however, have located mineral not only within osteoblasts but also within extrusions from osteoblasts into the osteoid. These extrusions, termed "osteoblastic extrusions" by Bonnucci and "bone nodules" by Bernard and Pease, are found in the interstitial substance and on collagen fibers as well as on the surface of osteoblasts and serve as loci for initial calcification of bone.

Osteonic bone calcifies in two stages. During the primary phase, mineralization occurs as soon as bone matrix is laid down. Later, during the secondary phase, the mineral content rises slowly and progressively. Although calcification of collagen occurs during the primary phase, the earliest mineral deposition in the loci of initial calcification takes place in the matrix as scattered areas of relatively high electron density. The loci of initial calcification probably bear no relation to collagen. In the next phase, mineralization continues within collagen fibers in close relation to fibril banding.

7.3 EFFECTS OF NUTRIENTS ON BONE FORMATION

7.3.1 Energy and Protein

The effects of dietary energy and protein on bone formation will be considered together since their separate discussion is often

difficult or even impossible. Diets limited in energy sources are generally deficient in protein, and even when, under experimental conditions, the protein level is "adequate," protein will preferentially be used by the organism as fuel rather than for growth and cell replacement. On the other hand, dietary lack of protein leads to diminished food intake, thus creating energy deficient. Not all investigators studying the effect of protein deficiency in experimental animals have used pair-fed, pair-weight, or age-matched animals fed a diet adequate in protein as controls.

7.3.1.1 Experimental studies

7.3.1.1.1 Starvation

The most severe deprivation of energy and protein is starvation. In immature rats, it induces slowing of cartilaginous growth with more extensive calcification than normal, followed by a reduction in chondroblastic activity. In adult rats deprived of food for 72 hr, femur dry weight and bone calcium and phosphorus decreased, whereas contents of lactic, pyruvic, and citric acids increased. Intermittent starvation had a profound retarding effect upon increase in bone length and weight and on skeletal maturity.

7.3.1.1.2 Undernutrition

The effect of limited food intake (undernutrition) on bones have been studied in pigs and cockerels. In pigs undernourished form an early age, growth of long bones was retarded. Their cortex was thin and brittle, the marrow cavity enlarged by medullary (endosteal) erosion, remodelling was poor, and there was a paucity of trabeculae. The growth cartilage and adjacent part of cartilaginous epiphysis became narrow, and after some weeks, they ceased to function effectively. The zone of maturing cartilage cells disappeared, endochondral ossification became retarded, and the matrix of the zone of calcifying cartilage calcified. Thus, the calcium/collagen ratio was higher than in normal pigs of the same age or the same size. Humerus length and thickness of undernourished pigs were greater than in controls of equal weight (but not in comparison to normal age-matched pigs). This finding demonstrated that limiting the supply of nutrients and growth materials accentuates the development of those parts of the body that have a greater structural stability and perhaps mitotic activity than the rest, thus altering the whole form and shape of the animal. Since bone has relatively high nutrients priorities, growth in weight. Since undernutrition delays bone

maturity, bone age becomes dissociated from chronological age. On rehabilitation, the growth cartilage began at once to recover its normal appearance and function, but measurable length increase began later, growth assuming a normal rate.

In underfed cockerels, cartilaginous growth was severely reduced. Endochondral ossification was disturbed, the undifferentiated cells in the subperiosteal zone ceased to divide and differentiate, and osteoblasts disappeared. Breaking strength was higher than in control animals of similar weight, and there was no evidence of deformity. Modulus of elasticity, an index of bone stiffness, was also higher than in weight or age-matched controls fed a nutritionally adequate diet. Nutritional rehabilitation by unlimited food supply following under feeding from 15 days to 6 months of age resulted in the formation of a wide zone of hypertrophic cartilage and associated deposition of endochondral bone. Bone length increased, but final dimensions were not attained. This failure is probably due to disturbances in cartilage growth during the period of undernutrition.

7.3.1.1.3 Protein deficiency

The effects of diets varying in protein content on bone have been studied in pigs, rats, dogs, and monkeys. Growth rate of pigs maintained on a low protein diet was reduced, and this effect was intensified by feeding extra carbohydrate. Bone radiographs revealed rarefaction due to a reduction in the number and density of longitudinal trabeculae. However, the number of transverse trabeculae increased. The intensity of transverse trabeculation varied inversely with the protein value of the diet. Histologically the epiphyseal cartilages were thin, the columns of proliferating and maturating cartilage cells were short, cell maturation retarded, and the individual cells. There were few normally placed longitudinal trabeculae but many transverse trabeculae and the amount of calcified cartilage within the trabeculae was excessive. There was also defective remodelling and disproportionate growth of skull and vertebral bones. The total mineral content of bones of protein-energy-deficient pigs was low when compared with that of controls receiving isoenergetic quantities of an adequate diet, but the ratio of ash to bone matrix was not affected by the protein value of the diet. Additional dietary calcium did not improve either the growth or the radiographic appearance (rarefaction, transverse trabeculation) of the bone, but partial replacement of the dietary carbohydrate by protein largely prevented the disorder.

In a well-controlled experiment to test the effect of protein deficiency on bone development, young rats were maintained for 6 weeks on diets providing 0%, 3% 6% or 24% casein as their source of protein with controls being fed restricted amounts of the 24% casein diet. All the animals receiving insufficient protein, whether because of a low-dietary protein concentration or because of restricted intake of the adequate protein diet, sowed similar bone changes, but they were more severe in the animals fed the low-protein diet than in their pair-fed controls. Growth was retarded but less than weight gain. Histological examination revealed a decreased width of the epiphyseal cartilage plate due to diminished number and size of cells, increase in cartilaginous ground substance, slowing down and final arrest of cartilage erosion and bone formation, resulting in fewer and coarser bone trabeculae. In rats fed the protein-free diet, the changes were most severe; ossification ceased and the cartilage was "sealed off" on the epiphyseal side by a thin layer of bone. Adult rats maintained on a diet of low-protein value containing suboptimal amounts of calcium lost minerals from their bones. This loss could neither be prevented not reversed by increasing the calcium content of the diet, whereas additional protein induced bone mineralization. In another study of adult rats, protein deficiency failed to induce epiphyseal changes, but there was a progressive loss of both organic and inorganic bone constituents. Thus, the ash: matrix ratio did not vary much. The effects of maternal protein deprivation and of postnatal food supply on the initiation of ossification and of bone length and width have also been reported. Appearance of ossification centers was delayed in the fetuses and offspring of dams fed a low-protein diet. Postnatal augmentation of food supply (by reduction of litter size suckling from normal foster mothers) resulted in compensatory changes in body weight but not in the time of appearance of ossification sites. Their formation was influenced by maternal diet and chronological age rather than by postnatal nutrition.

Studies on bone metabolism in protein-deficient rats revealed a lower bone formation rate as measured by calcium accretion. The exchangeable calcium pool was smaller, and ^{14}C-proline incorporation into hydroxyproline decreased. However, bone resorption was barley changed, and a disproportionally high fraction of the calcium pool was extracted. Intestinal calcium absorption decreased resulting from decreased formation of calcium-binding protein. The authors believe that he disturbance in intestinal calcium transport and the depressed

collagen synthesis affect growth of bones that are shorter and lighter than in age-matched controls, "the epiphyseal region bearing the burnt of growth retardation."

Dogs reared from weaning on a low-protein diet also had shorter long bones and the metaphyses showed slight transverse trabeculation. In protein-deficient young monkeys, endochondral and periosteal appositional bone formation were reduced. The number of osteoblasts was decreased more than that of osteoclasts, suggesting a low bone-remodelling rate.

7.3.1.1.4 Amino acid deficiency

Diets devoid of single essential amino acids produced nonspecific effects on bone (lysine, phenylalanine, tryptophan, threonine, leucine, histidine, and arginine). Generally, each of these deficiencies led to retarded bone growth, thin epiphyseal cartilage, and low total amounts but normal concentrations of bone ash and calcium.

It appears that the various forms of energy and protein deficiency produced in different species lead to a decease in the proliferative activity of the epiphyseal cartilage cells. Depending on the severity of the deficiency state, varying degrees of retardation of epiphyseal cartilage cell proliferation have been reported. Thus, "cartilage is a critical index of the nutritive status of the organism. Most effects produced are non-specific, interfering with osteoblastic activity so that periosteal and endosteal bone formation is retarded and, hence, osteoporotic changes result.

7.3.1.2 Epidemiological and clinical observations

Radiographs of bones of children suffering form protein-energy malnutrition (kwashiorkor, marasmus) showed the following abnormalities: trabecular bone loss, thinning of cortex of long bones, delayed or poor ossification, smaller ossification centers, and irregularly calcified epiphyses without a clearly distinguishable zone of provisional calcification. These findings are indicative of retarded maturation and could largely be reversed by supplementary feeding. Higginson, studying autopsy specimens, reported thinning and immaturity of the epiphyses of long bones. Composition of bone was, however, not affected.

Careful radiological studies were performed by Garn and associates. They measured the total subperiosteal, cortical, and medullary cavity areas of tubular bones in children and adults of six Central American countries. The total subperiosteal area was smaller

in population where protein-energy malnutrition is common, lack of dietary energy being more important than availability of protein. Despite bone mass, growth at the subperiosteal surface was not necessarily diminished. Endosteal surface resorption was found to be excessive, thus enlarging the marrow cavities. Since the total subperiosteal area was only moderately restricted (or not restricted at all), cortical thickness was grossly reduced. Since subperiosteal appositional growth was close to normal and bone was lost even during the period of recovery, the investigators concluded that the reduction of cortical bone area is due to actual bone loss rather than failure to form bone.

Garn delineated some generalization concerning the effect of protein-energy malnutrition in early childhood bone. As the energy intake decreases relative to requirement for growth, both longitudinal and transverse bone growth are slowed down, calcification of the postnatally appearing calcification centers becomes delayed, and the amount of compact bone per unit length may decrease. As energy intake is reduced further so that weight remains constant, there may still be some longitudinal bone growth and appearance of new ossification centers, but the increased periosteal bone volume may not be paralleled by a normal increase in total or absolute skeletal volume. As protein malnutrition enters the picture superimposed on energy deficiency, size reduction is especially pronounced. "As the child slips over the edge into negative nitrogen balance and wasting of soft tissues, bone growth becomes most economical, not only failing to add bone volume in proportion to periosteal volume but even borrowing bone material to build new bone to the point where a given periosteal volume may have but half of normal or expected volume of compact bone." Thus "bone apparently tends to grow at he expense of bone" and protein-energy malnutrition can result in "a thin shell of bone of reduced mineral mass and even loss of bone."

7.3.2 Vitamins

At least three vitamins are know to be involved in bone formation: retinol (Vitamin A), ascorbic acid (vitamin C), and the calciferols (vitamin D).

7.3.2.1 Retinol (vitamin A)

The effect of retinol on bone formation has been studied both under conditions of deficiency of the vitamin and its excess.

7.3.2.1.1 Hypovitaminosis

Mellanby reported in 1931 that dietary lack of vitamin A in dogs resulted in a disturbance of movement coordinated and related this dysfunction to central nervous system lesions. This observation was the starting point of numerous studies by many investigators on the role of vitamin A on formation, remodeling, and destruction of bone. Mellanby, who conducted his studies mainly on dogs, later confirmed these observations with rats. They are summarized in his monograph of 1950.

Hypovitaminosis A in dogs resulted in bone overgrowth leading to extensive nervous lesions, particularly of the eighth nerve (but also of other cranial nerves). Overgrowth of vertebrae (narrowing of the spinal canal) and the femur (reducing the marrow cavity) were also reported. There were no changes in the epiphyses or in growing cartilage. Microscopic examinations revealed a decrease in the number of osteoclasts on the bone adjacent to the brain, but they were abundant on the marrow surface. Thus, their position seemed to have reversed from that normally found. Similar changes were found in the vertebrae. The increased bulk, the coarseness, and the poor remodeling of bone was related by Mellanby, therefore, to excessive osteoblastic activity and periosteal bone deposition accompanied by reduced osteoclastic resorption without significant changes in endochondral growth. Whereas in normal animals the growing central nervous system in accommodated by removal of bone from the inner surface of the bony case and deposition of bone on the outer surface consequently enlarging the capacity of the skull or the spinal canal, this mechanism breaks down in vitamin A deficiency because of the change in localization and activity of osteoclasts. Thus, retinol acts as a specific chemical controller of the activity of osteoblasts and osteoclasts.

Wolbach and associates attributed the bone dysplasia in vitamin A-deficient mammals and birds to a cessation of endochondral growth without any alteration in appositional growth. This would explain the shortness and bulk of the bones while retaining however, their normal shape. Microscopically, there was irregular and less-extensive-than-normal tunneling of epiphyseal cartilage. The zone of proliferating cartilage cells was less clearly demarcated the intracellular matrix was increased, and there was a broad zone of enlarged cells in noncalcified matrix. There were fewer osteoblasts than normal, less cancellous bone in the metaphyseal region and the adjacent

cortical bone was thinner and less dense than normal. Bone resorption ceased where re-modelling should occur, and the number of osteoclasts was scarce. Wolbach also believed a disproportionate growth of the central nervous system in relation to the surrounding bone case, and he explained it on the basis mainly of retardation of endochondral growth, a different interpretation than that of Mellanby.

Irving working with vitamin A deficient rats, found neither altered endochondral growth nor decreased osteoclastic activity. He did, however, report a marked and uncontrolled increase in osteoblastic activity. Similar findings were reported by Howell and Thompson in young and adult cocks and hens. The endochondral changes observed by Wolbach were attributed by these investigators to inanition and the compression of the central nervous system to excessive subperiosteal apposition of bone, as has been done already by Mellanby.

The altered bone growth found in vitamin A-deficient calves was also assumed to result form increased osteoblastic activity, whereas osteoclasis was apparently not affected. Others reported an "altered drift pattern" in calves maintained on a diet low in vitamin A, because subperiosteal resorption failed and was often replaced by growth. In particular, the mandible accumulated twice as much lamellar bone on its medial border while medial endosteal resorption of fiber bone increased. Other bones of the skull and vertebrae were likewise thickened. The authors assumed that retinol may be required for the differentiation of osteoclasts and suggested that the vitamin "affects the ability of the periosteal progenitor cell to differentiate as an osteoclast." This pattern "supports the hypothesis that the basic function of retinol may be related to cell differentiation."

In an attempt to explain the alteration in bone brought about by vitamin A deficiency, Havivi and Wolf studied certain metabolic alterations in bones of vitamin A-deficient chicks. Their main findings were an increase in organic deposition in bone, in concentration of chondoitin sulfate in the epiphyses, and in certain parameters of metabolic activity (as indicated by glucose and acetate conversion to CO_2 and incorporation of leucine, sulfate, and proline into the matrix). These results "tend to suggest that the increased metabolic activity of deficient bone may accompany...overgrowth of the deficient bone, possibly by the intercellular matrix." In a subsequent study, incorporation of radioactive calcium was found to be increased in bone of deficient chicks. Firschein injected ^{85}Sr and ^{14}C-labeled

proline into vitamin A-deficient rats and found a decreased content of hydroxyproline in bones (the hydroxyproline:proline:collagen ratio being unchanged). Rates of collage synthesis and mineral accretion were reduced.

7.3.2.1.2 Hypervitaminosis

Further information on the role of retinol in bone formation and destruction was obtained in studies in which excess vitamin was given to intact animals or added to bone rudiments in tissue culture. Wolbach and associates showed that excess retinol leads to early destruction of epiphyseal cartilage accompanied by loss of chondromucoprotein, thinning of cortical bone, and decreased bone, and decreased bone mass. These alterations were attributed to an increased rate of cartilage destruction without corresponding cartilage proliferation so that the thickness of epiphyseal cartilage became reduced. The zone of proliferating cells became narrowed, and that of growing cells widened without a clear demarcation line between the. Further, the diameter of the shafts and narrow cavities were reduced. This effect was not related to cessation of periosteal and endosteal bone cell activity but resulted form a partial reversal of the sites of osteoclastic and osteoblastic activities. Osteoid and poorly calcified matrix were formed on the endosteal surface, and osteoclasis dominated on the periosteal bone.

More recent investigations with chicks, pigs, kittens, and rats confirmed and enlarged these observations. All investigators reported that excess retinol damages the epiphyseal cartilage and retards growth of the epiphyseal plate by inhibiting the activity of osteoblasts. Thus, alkaline phosphatase activity of bone was found to be lowered. The epiphyseal plate affected by retinol toxicity was invaded by blood vessels inducing its fragmentation and dissolution. Osteoclasis was reported to be increased in kittens but unchanged in rats and pigs.

Excess retinol exerts not only a systematic action on the skeleton but also has a local effect on bone. Barnicot attached small crystals of retinol acetate to parietal bones of mice and embedded them in the cerebral hemisphere of litter mates; 7 to 14 days later, there was a strong osteoclastic reaction in areas adjacent to crystals leading in some instances to perforation. Thus, retinol can exert a direct action on bone tissue enhancing osteoclasis and increasing bone resorption.

The deleterious effect of toxic amounts of retinol on bone can be prevented by calcitonin, both in vivo and in vitro. It is suggested

that calcitonin acts directly on the osteoblasts to inhibit release of calcium from bone and breakdown of mature collage. Treatment with anabolic steroids also prevents the effect of excessive doses of retinol on the skeleton.

Fell and associates studied the effect of retinol on bone by adding the vitamin to limb rudiments of mouse fetuses and chick embryos maintained in tissue cultures. Limb bone cultured in plasma, to which large doses of retinol had been added, grew less and exhibited a rapid loss of cartilage matrix. Matrix depletion as determined histochemically was most evident in the epiphyseal plates and articular surfaces. Reynolds, studying fetal rat calvaria in vitro, reported excess retinol to increase formation of osteoclasts, to decrease osteocyte population, and to transform osteoblasts into fibroblast forms. Calcitonin, as mentioned above, prevented osteoclast formation and bone resorption. In a study in which costochondral junctions from young rat were grown in vitro in the presence of excess retinol, loss of zone of hypertrophic cells was observed. The cartilage became reduced in size and detached from the rest of the explant. Excess retinol not only affected cultured epiphyseal cartilage but was also found to be effective in promoting lysis of extracellular matrix of ear cartilage.

7.3.2.1.3 Biochemical systems

The effect of retinol on bone may be, at least in part, attributed alterations of specific biochemical processes. These were often and conveniently studied in tissue culture to which large amounts of retinol had been added. Although it may be questioned whether the effects of retinol at relative high concentrations are significant to understanding the normal function of the vitamin, these studies have thrown light on certain effects of retinol on biochemical systems.

Cartilage rudiments of chick embryos cultured in the presence of excess retinol released a proteolytic papain-like enzyme acting on normal cartilage. The protease attacks the protein moiety of the mucoprotein complex and leads to a loss of chondroitin sulfate and eventual destruction of the cartilage. Fell and Dingle suggest that he cause of the release of the protease by retinol is an alteration of the composition or the stability of the membranes of the cells and of subcellular particles (lysosomes). In other words, retinol excess may influence membrane permeability. The increased membrane permeability and release of protease result in loss of amino sugars, RNA and DNA, form cartilage cells. The loss of glycosaminoglycans

(GAG; mucopolysaccharides) from cartilage matrix of costochondral junctions cultured in the presence of retinol could be prevented by a protease inhibitor and a lysosomal stabilizer (chloroquine). The assumption that excess retinol affects lysosomal membranes of cartilage cells is supported by studies with erythrocytes and liver mitochondria. Rabbit erythrocytes, hemolyzed readily in vitro by incubation in the presence of rational, and retinol, added to a suspension of liver mitochondria, caused a rapid swelling of these particles. Thus, one site of action of retinol appears to be within or on membranes. Optimal concentrations of the vitamin are apparently required for the maintenance of the normal structure and functioning of the membranes of cells and of subcellular particles.

This effect of retinol in facilitating the passage of hydrolytic enzymes through lysosomal membranes may explain its inhibitory effect on hydrocortisone. This hormone, known to have a stabilizing action on membranes, retards the effect of excess retinol in cartilage tissue cultures. On the other hand, hydrocortisone was found to be capable of inhibiting retinol-induced enzyme release from cultured rat calvaria without, however, altering bone resorption.

Retinol is also involved in the metabolism of glycosaminoglycans of bone and cartilage. In endochondral ossification, the proteoglycans and their GAG compounds are considered to be of importance in the sequence of biochemical processes that characterize the transformation of epiphyseal cartilage into bone. The main GAG in epiphyseal cartilage is a galactosaminoglycan consisting of the two isomers: chondroitin-4-sulfate and chondroitin-6-sulfate. As mentioned above, bone rudiments cultured in the presence of retinol lose amino sugars. Loss of previously administered $^{35}SO_4$ from cultured cartilage and removal of chondroitin sulfate from cartilage have also been reported. On the other hand, vitamin A deficiency is reported to increase concentration of chondroitin sulfate in chick epiphyseal cartilage.

Retinol also influences the synthesis of epiphyseal GAG. Deficiency of vitamin A decreased the incorporation of radioactive sulfate into chondroitin sulfate of rat cartilage matrix but increased radioactivity in pig cartilage.

The reports on the effects of hypervitaminosis A on incorporation of radiosulfate into cartilage or GAG are also conflicting. Radioactive sulfate was injected into hypervitaminotic mice, the total sulfate pool of these mice was increased and uptake of coastal cartilage decreased.

Thus, synthesis of chondroitin sulfate appears to be diminished. In rats and rabbits, hypervitaminosis A inhibited incorporation of radiosulfate into cartilage. According to McElligott, the primary effect is an inhibition of GAG synthesis, since this occurs in cartilage in absence of matrix depletion. The depletion may be due, according to this view, to degradation of protein-chondroitin sulfate complex in the absence of synthesis, but the accelerating effect of a proteolytic enzyme can not be excluded. Mukherji and Bachhawat reported a reduction in formation of 3'-phosphoadenine-5' phospho-sulfate by epiphyseal cartilage and, hence, in synthesis of sulfated GAG. Other investigators, working with hypervitaminotic chicks, studied both uptake of injected radiosulfate by cartilage and its incorporation into epiphyseal GAG. They found that uptake was enhanced and breakdown retarded. Thus, the turnover was slower, and since activity of enzymes degrading GAG was not increased, it was concluded that the activity of the synthesizing enzymes was apparently enhanced.

Presence of excess retinol in cultures of chondrocytes has been reported to depress synthesis of GAG. According to Solursh and Meier, inhibition of GAG synthesis is not the result of cytotoxicity of retinol, since incorporation of leucine into acid insoluble material or collagen synthesis was unaltered. Since the incorporation of glucose into polysaccharides was inhibited faster than that of glucosamine or sulfate into GAG, they suggest that retinol acts to inhibit an early step in the pathway of GAG synthesis.

The effect of excess retinol on GAG synthesis counteracts that of vitamin D. Rats were treated with large doses of one of these vitamins or of both. Toxic amounts of vitamin D increased GAG content, whereas retinol decreased it. Administration of both vitamins partially prevented the skeletal pathology associated with large doses of vitamin D. It is suggested that the beneficial effect of retinol in preventing the bone changes produced by excess vitamin D results from increased turnover of GAG and of collagen. Excess retinol is believed to remove the excess GAG and proteins resulting in a more normal bone composition and structure.

7.3.2.2 Ascorbic acid (vitamin C)

7.3.2.2.1 Histological changes in bones of scorbutic animals

The role of ascorbic acid in bone growth is related to the function of the organic matrix. This fact became clear from studies on animals requiring ascorbic acid as a vitamin. Wolbach, summarizing his extended studies, pointed out that scurvy is characterized by the

inability of the supporting tissues to produce and maintain intercellular substances. The cartilage cells fail to produce matrix, develop an abnormal appearance, become shrunken and irregular in shape, and their nuclei stain very deeply with basic dyes. The cells are separated from each another by material that fails to give the normal matrix staining reaction. More recently, these observations have been extended. The bone matrix of deficient guinea pigs was described to be "scanty and easily torn." The inhibition of matrix production caused cessation of osteoblastic activity. In the metaphyses, blood flow was impaired and bone resorption increased. Fibroblast-like cells were found in the metaphyses and beneath the periost. Cortical bone was thin and fragile.

Administration of ascorbic acid resulted in an increase in the number of proliferative and hypertrophic cartilage cells, and calcification became normalized. Follis summarized the effect of ascorbic acid deficiency: "it would seem best to designate scurvy as that part of the overall picture of ascorbic acid deficiency which is characterized by a failure of certain specialized cells, i.e., fibroblasts, osteoblasts, and odontoblasts, to promoter the deposition of their respective fibrous proteins: collagen, osteoid, and dentin."

More recent evidence supports this conception. Skeletal deposition of radiocalcium and phosphorus was found to be reduced in scurvy and their release was increased. This increased liability of deposited bone minerals led Thornton to the suggestion that the type and amount of matrix formed is altered in ascorbic acid deficiency.

Therefore, much effort has been directed to elucidating the effect of ascorbic acid on the biosynthesis of those components which are characteristic of bone matrix and intercellular substances, viz. Collagen and glycosaminoglycans.

7.3.2.2.2 Ascorbic acid and bone metabolism

Collagen formation. Collagen is the predominant protein of bone and connective tissue. Its biological role and the effect of ascorbic acid deficiency on its formation have been reviewed.

Ascorbic acid stimulates collagen synthesis in vitro, as has been shown in tissue culture of granulous tissue of scorbutic guinea pigs, of human fibroblasts derived from skin and fetal lung, and also of bone cells. Embryonic chick tibias that grew and synthesized collage, for several days displayed a marked dependence upon the presence of ascorbic acid.

A unique feature of collagen is its content of hydroxyproline and hydroxylysine and ascorbic acid has been shown to be an essential cofactor for hydroxylation of proline and lysine. Hydroxyproline and hydroxylysine in collagen are derived not from free hydroxyproline and hydroxylysine but from free proline and lysine, respectively. This implies that hydroxylation occurs at a level other than that of the free amino acid, after the incorporation of specific prolyl (and lysyl) residues into peptide linkage. After it was made possible to separate proline incorporation from proline hydroxylation, it was shown in vitro that in the absence of hydroxylation, a hydroxyproline-deficient polypeptide precursor of collagen, protocollagen, is formed. Protocollagen has been shown to a accumulate in granulation tissue from ascorbic acid-deficient guinea pigs, and the participation of the vitamin in hydroxylation of peptide-bound-proline during collagen synthesis has been demonstrated. In cultured fibroblasts, a prototype of collagen-synthesizing cells, it has been found that ascorbic acid activates an enzymatically inactive protein and converts it to active enzyme. It has also been found that peptidyl-^{14}C-proline, formed by incubation of isolated connective tissue, is converted in the presence of a soluble enzyme system containing appropriate cofactors including ascorbic acid to peptidyl-^{14}C-hydroxyproline. Hydroxylysine formation is closely analogous to the synthesis of collage hydroxyproline. In under hydroxylated collagen, abnormal physical properties were found, such as reduced stability of the collagen triple helix, dissociation into single chains, and increased susceptibility to degradation by pepsin.

However, attempts to detect protocollagen in vivo have failed. Barnes et al studied dorsal skin of ascorbic acid-deprived guinea pigs after administration of labeled proline. The extent of incorporation of proline indicated that collagen synthesis was severely impaired, but no protocollagen (after treatment with collagenase) was detected. It was suggested that the apparent absence of the hydroxyproline-deficient precursor of collagen does not necessarily imply that none is formed in vivo during scurvy. It may be that its formation is short lived and that in continued absence of ascorbic acid its formation, like that of collagen, ceases in vivo.

Glycosaminoglycans. In endochondral ossification the proteoglycans and their glycosaminoglycans components are considered to be of importance in the sequence of biochemical processes that characterize the transformation of epiphyseal cartilage

into bone. The main GAG in epiphyseal cartilage is a galactosaminoglycan consisting of the two isomers: chondroitin-4 sulfate and chondroitin-6-sulfate.

Histochemical studies showed that granulation tissue of scorbutic animals contains decreased amounts of GAG. This defect is probably due to a reduced formation of GAG when ascorbic acid is lacking. Thus, incorporation of ^{35}S-sulfate into-scorbutic granulation tissue and into cartilage is reported to be decreased. Greatly reduced uptake of radiosulfate into cartilage of osteochondral junction of scorbutic guinea pigs has also been demonstrated autoradiographically. Changes occurring in the biosynthesis and chemical composition of GAG of granulation tissue and cartilage of scorbutic guinea pigs have been investigation by Kodicek and associates. Studies with U-^{14}C-glucose, a precursor of tissue hexosamine, showed a decreased incorporation of the label into galactosamine in both cartilage and would granulation tissue. Since galactosamine is an important constituent of chondroitin-sulfate, the decrease of this material in ascorbic acid deficiency can be explained by the block of the biosynthesis of galactosamine. According to Kodicek, the destruction of already formed galactosaminoglycans is increased in scurvy and can account for the low ^{14}C-count in galactosamine. The block is formation of galactosamine results form impairment of the UDP-epimerase reaction which catalyzes the conversion of UDP-acetylglucosamine to UDP-acetygalactosamine by an oxido-reduction mechanism. The impaired formation of galactosamine explains the decrease in biosynthesis of chondroitin-sulfate and in uptake of radiosulfate in scorbutic tissues. Kodicek, and Bourne, believe that the studied of Staudinger et al., an microsomal electron transport and hydroxylation reactions in the presence of ascorbic acid, are the most consistent approach to elucidate the mode of action of the vitamin. These investigators have isolated a NADH-monodehydroascorbate oxidoreductase a flavoprotein which catalyses proton transfer from NADH to ascorbic acid radical to form reduced ascorbic acid. In this manner, ascorbic acid would be involved indirectly in the electron transport chain.

Thus, there appear to be two major metabolic defects that occur in scorbutic cartilage; a decreased formation of hydroxyproline and hydroxylysine essential constituents of collagen, and a defect in formation of chondroitin-sulfate, resulting in part form the failure of galactosamine synthesis. These alterations account largely for the changes observed in scorbutic cartilage and bone.

7.3.2.2.3 Effects of large doses of ascorbic acid

Sine the body is capable of readily excreting water-soluble vitamins, administration of large doses of these vitamins is generally not believed to induce untoward effects. However, administration of unphysiologically large doses of ascorbic acid may affect bone metabolism. Thornton incorporated ^{45}Ca into the skeletal system of chicks. Injection of ascorbic acid resulted in a markedly increased blood level of ^{45}Ca and of acid phosphatase followed, after 24 hours by a decreased blood ^{45}Ca level. These findings were indicative of mobilization of minerals from the bone. In subsequent experiments, movement of ^{45}Ca and ^{32}P from previously labeled chick bone was monitored following ascorbic acid administration. Mobilization and excretion of both isotopes were stimulated during the initial 24 hr of treatment, and the specific activities of each isotope decreased in compact and cancellous bone. These results suggest that ascorbic acid affects not only mobilization of bone minerals and their excretion but also subsequent repletion with is table isotopes, resulting in changes of specific activity. Ascorbic acid added to chick embryonic tibia cultures (50 μg/ml) increased calcium turnover and oxygen consumption and decreased lactic acid production. When injected into vitamin D-deficient chicks, bone ash decreased more than by dietary lack of vitamin D alone. Supplementation of a vitamin D-deficient chick diet with ascorbic acid intensified the rachitogenic properties of the diet; the epiphyseal plate width increased further and the effect of vitamin D deficiency appeared sooner. This impairment of ossification of the epiphyseal plate and mineralization of new bone was enhanced. In pigs receiving 1000 mg ascorbic acid per day for 32 days, excretion of hydroxyproline was increased, indicating increased catabolism of collagen.

In view of these findings, brown commented on the suggestion that large doses of ascorbic acid may be effective in reducing the incidence of severity of common cold in man. Without discussing the pros and cons of such a treatment, he cautioned that those recommending it should take in account a possible untoward effect on bone.

7.3.2.3 Vitamin D (calciferols)

Vitamin D (cholecalciferol or vitamin D_3 and ergoalciferol or vitamin D_2) is involved in bone formation as well as in bone resorption. There is much evidence to show that although the vitamin promotes bone resorption, it apparently exerts no direct action on

the bone matrix but rather on calcium and phosphorus homeostasis, thus ensuring physiological concentrations of these minerals in the fluids bathing the bone-forming tissue and creating optimal concentrations for mineralization. Irving stated recently that "it is still impossible to decide if vitamin D has a direct calcifying action on bone, but the balance of the evidence is against this view." However, vitamin D (or its metabolites) affects certain parameters of cartilage and bone function and metabolism that may be relevatant to bone formation and calcification. On the other hand, the effect of vitamin D on bone resorption seems to be accepted by most investigators.

7.3.2.3.1 Vitamin D deficiency

The effect of vitamin D deficiency on bone formation has been extensively studied and recently described. The primary disturbance caused by vitamin D deficiency is a failure to mineralize newly formed osteoid tissue and cartilage matrix, hence, the unusual softness of the bone which under stress of weight and locomotion gives rise to the characteristic deformities of rickets or osteomalacia. Normally, the epiphyseal cartilage cells proliferate in columns and calcium salts are laid down in intercartilaginous longitudinal septa. In rickets, the proliferating cartilage does not differ much from the normal in extent or in arrangement of its cells, but the calcification along the cartilage columns id defective, the blood vessels invade in a disorderly fashion, and the zone provisional calcification is not reabsorbed. Since the cartilage cells continue to proliferate, the zone of provisional calcification becomes wider than normal. As a result, large amounts of osteoid tissue appear at the base of the widened epiphyseal plate. A thick osteoid layer may also be deposited under the metaphyseal periosteum increasing the bone diameter.

Changes have also been described in the arrangement of collagen fibers. Engfeldt and Zetterstrom studied osteoid form rachitic puppies in polarized light. The collagen bundles, instead of being arranged in a plane parallel to the Haversian canals, were found in short bundles running perpendicularly to the Haversian canal. The Haversian system itself was poorly developed and the osteoid was deposited under the periosteum as thick, poorly calcified concentric layers with little reorganization into osteons. Crystallographic studies suggested that the bone apatite of rachitic rats is chemically less mature than that of normal controls. The mitochondrial granule distribution in the growth plates of rachitic rats was also found to

be modified, as revealed by electron microscopic observation. Mineral granules were noted only in the mitochondria of a few cells adjacent to the zone of provisional calcification. The authors suggested that one specific effect induced in chondrocyte mitochondria by a low phosphate, vitamin D-deficient diet in their reduced ability to form granules and an apatite binding material, and that sufficient supply of phosphate is needed for this purpose.

Disturbances of intramembranous bone formation have also been described in rachitic rats. The bone studied, a part of the maxilla, was a narrow palate where bone was deposited on the upper surface and removed from the lower. The earliest change was an increase in bone width resulting from a failure of uncalcified osteoid to be reabsorbed. The osteoblasts continued to form bone matrix but gradually lost their ability to calcify. Since osteoclasts on the lower resorption site disappeared, osteoid tissue accumulated. Healing began with calcification of the osteoid. Osteoclasts then reappeared, and resorption of the newly formed calcified osteoid took place.

The failure of osteoid to be absorbed unless it is calcified means that the normal continuous remodeling of bone cannot take place; deformities which are easily produced in the weakened bone persist.

Recently, Baylink et al., using tetracycline labeling and quantitative histological methods, studies the effect of vitamin D deprivation in rats. The main changes associated with vitamin D deficiency were a decrease in matrix formation by osteoclasts, an inhibition of osteoid mineralization, and an increase in osteoclastic bone resorption. These findings resulted from changes in the rate of matrix formation and of bone resorption per unit area of forming or resorbing surface rather than from changes in the area of the surfaces. Hypocalcemia occurred since bone resorption, although greater than normal, failed to compensate for the reduced intestinal absorption. The authors concluded that "the primary effect of vitamin D deficiency on bone is a reduction in the capacity of osteoclastic and probably osteolytic resorption. This, along with decreased intestinal absorption of calcium, promotes hypocalcemia and secondary hyperparathyroidism. Bone cells presumably can respond only suboptimally to the increased concentration of parathyroid hormone in blood and the response becomes less effective... hypocalcemia becomes more severe eve though the concentration of parathyroid hormone in blood presumably increases". These investigators believe that hypocalcemia and hyperparathyroidism rather than lack of vitamin D per se, cause the reduced rate of bone

matrix formation and mineralization. In osteomalacia, as in rickets, the characteristic findings is an excess of osteoid tissue. It differs from rickets inasmuch as little new bone growth occurs.

7.3.2.3.2 Hypervitaminosis D

In hypervitaminosis D, calcification of the epiphyseal cartilage is normal. However, wide osteoid borders appear, an indication that osteoblasts activity is continuing. In the metaphyses and the cortex, new bone matrix is found in excess though devoid of inorganic material. In guinea pigs, excessive doses of vitamin D increased osteoclastic resorption and fibrous replacement in bones, mimicking hyperparathyroidism. In hypervitaminotic rats, numerous resorption cavities with hyperplastic osteoblast were observed in the widened epiphyseal plates. Widened epiphyseal plates, abnormal chondrocytes, and congenital bone defects were also found in rat offspring whose mothers had been treated with large doses of vitamin D.

Kinetic studies also pointed to an increased bone resorption which was accompanied by an increased turnover and augmented exchangeable calcium poll which may explain the increased amount of osteoid tissue. Bone of chicks overdosed with vitamin D failed to take up tetracycline, indicating no new bone deposition and a high degree of resorbing activity in these bones. Bone slices from animals poisoned with vitamin D mineralized normally when incubated with normal rat serum, however.

7.3.2.3.3 Effect of vitamin D on bone formation

Functional Metabolism of Cholecalciferol. The elucidation of the metabolism and mechanism of action of vitamin D is one of the greatest achievements of nutrition research in recent years. The metabolites most important for the present discussion are 25-hydroxycholecalciferol (25-OH-D_3) and 1,25-dihydroxycholecalciferol (1,25-$(OH)_2$-D_3). They act on three target organs; the intestinal mucosa, the kidney tubules, and bone. The newer knowledge of cholecalciferol metabolism, and the mode of action of cholecalciferol metabolites has recently been reviewed.

Calcium and phosphorus homeostasis. Cholecalciferol, in form of its metabolites, acts on different organs creating conditions that are optimal for bone formation and mineralization.

1. 25-OH-D_3 and 1,25-$(OH)_2$-D_3 are involved in the induction of active intestinal calcium transport, thus increasing availability of calcium for mineralization.

2. 1,25-$(OH)_2$-D_3 increases, the concentration of phosphorus in the serum of rats on a low phosphorus diet. This increase is believed to be a specific effect of the metabolite in stimulating phosphate reabsorption in the kidney tubules. This effect helps to explain the antirachitic effect of 1,25-$(OH)_2$-D_3. Classical rachitic lesions in rats can be produced only with a low phosphorus, vitamin D deficient diet. If the diet is normal in phosphorus and low in calcium, vitamin D deficiency will result in hypocalcemia, retarded bone growth, and osteoporosis; but typical rachitic lesions will not develop. Thus, rickets in rats is a low phosphorus disease, and in man also, rickets is characterized by a low serum phosphorus level. Thus, elevation of serum phosphorus level, which is necessary for normal mineralization and reversal of the bony lesions in rachitic rats must be recognized as an important component of vitamin D action.
3. The action of vitamin D metabolites on bone is more difficult to understand organ culture studies with fetal bones and calvaria from newborn mice showed that 25-OH-D_3 or 1,25-$(OH)_2$-D_3 stimulated the release of previously incorporated ^{45}Ca. 1,25-$(OH)_2$-D_3 was about 100 times more active than 25-OH-D_3. Calcitonin was found to block the induction of bone resorption by 1,25-$(OH)_2$-D_3. This action of the vitamin metabolites in vitro is believed to be basic to our understanding of their effect in vivo. Their effect on both intestinal mucosa and on bone are of great importance in maintaining a plasma calcium × phosphorus product that is essential for proper mineralization of bone. According to this school of thought, vitamin D has not direct effect on bone formation. Increased calcification in response to vitamin D is thought to be secondary to an elevated calcium and phosphorus product in plasma. Reynolds recently stated that "there is probably no direct action of vitamin D and its metabolites on the formation of bone" and this its role in increasing serum calcium level "mainly by controlling the intestinal transport of calcium may be sufficient to ensure that mineralization proceeds in a normal animal."

A direct effect of cholecalciferol on bone formation was, however, reported by van Nguyen and Jowsey. These investigators perfused one fore limb of a dog with a cholecalciferol-containing solution and drained out the venous return so that an effect of the vitamin independent of the reaction of the body could be studied. After 4

hr, the dog was killed and metatarsal bones were examined. The rate of bone formation in the perfused limb was found to be greater than in the nonperfused, while resorption remained essentially unchanged. Criticism has been leveled against this experiment on the ground that the high, unphysiological dose used (0.5 mg/kg body weight) "precluded any hypothesis regarding the possible role of unmodified vitamin D in bone formation.

The notion of a direct and immediate effect of vitamin D in promotion of mineral accretion by bone has also been advanced by clinicians. It has been pointed out that the osteomalacia that occurs in chronic renal failure can often be reversed by judicious use of vitamin D in doses that apparently do not affect the concentration of blood calcium or phosphate or their solubility product. However, as far as we are aware, none of the mono- and dihydroxylated active metabolites of cholecalciferol have been shown so fare to exert any direct effect on bone formation per se in this condition.

The view that bone may be a target organ of $1,25\text{-}(OH)_2\text{-}D_3$, in calcium homeostasis was challenged by Edelstein et al. on the basis of the distribution of cholecalciferol metabolites in bones of chicks fed diets that were adequate or inadequate in calcium or phosphorus. Even under conditions of severe restriction of these minerals, the amounts of $1,25\text{-}(OH)_2\text{-}D_3$, recovered from the bones were only very small. Thus, the authors believe that $1,25\text{-}(OH)_2\text{-}D_3$, does not play a role in the maintenance of plasma calcium homeostasis via bone resorption.

Indirect effects possibly related to bone mineralization. It is, however, possible that vitamin D (or its metabolites) exerts effects on cartilage and bone that may promote mineralization and/or correct metabolic defects in the organic bone matrix that permit calcification.

Autoradiographic studies of epiphyseal plates of rachitic rats pointed to profound involutional changes of the cells of the maturation zone. Incorporation of labeled cytidine (RNA synthesis), glycine (protein synthesis), and sulfate (glycosaminoglycan synthesis) was much decreased indicated a "virtual suspension of synthetic activities." Vitamin-D therapy was followed by a rapid increase of isotope labels in the epiphyseal region. The authors believe that the principal defect in the maturation zone of the epiphyseal plate of rachitic rats may be a local unavailability of high-energy phosphate required for metabolic activity. Polysaccharide synthesis was found to be markedly diminished, glucose oxidation reduced, and anaerobic

glycolysis sharply increased with production of large amounts of lactic acid and a decrease of glycogen deposition. It may be assumed by thermodynamic principles that the ATP level was reduced and that the cells in this area converted over to a low-efficiency energy system, Dixit suggested that normal glucose and glycogen metabolism stimulates the formation of calcifiable matrix by the cartilage cells. Kodicek and Thompson reached similar conclusions on the basis of autoradiographic localization of labeled cholecalciferol in bones of rachitic rats. The labeled vitamin was taken up by the chondroblasts in contrast to its absence in osteoblasts, suggesting that "the vitamin is concerned with biochemical processes leading to the elaboration of a component of organic matrix so that it may become calcifiable." The finding that vitamin D administered to rats on a low-calcium diet increased calcium turnover but did not have a calcifying effect is in line with this conclusion.

Belanger and Migicovsky attempted to separate the effect of vitamin D on maturation of the cartilage matrix and of the subepiphyseal bone cells from that of homeostasis of blood calcium level. They concluded that calcium can only be considered as a partial substitute of vitamin D and that vitamin D stimulates both replacement and maturation of bone-forming cells.

There is conflicting evidence on the role of vitamin D in collagen synthesis. Studies employing labeled proline in rachitic rats and chicks pointed to an increased rate of collagen synthesis. On the other hand, it was found that an early effect of vitamin D in rachitic chicks was to stimulate bone collagen synthesis, thus providing new organic matrix for calcification. It is noteworthy that this effect of vitamin D was specific to bone collagen and was not observed in skin collagen.

More recently, the structure of bone collagen synthesized in rachitic chicks and rats was investigated. Bone collagen of rachitic animals had the same chain composition but differed in that in both $\alpha 1$- and $\alpha 2$-chains, more lysine was converted to hydoxylysine. No such change took place in collagen from skin or cartilage from rachitic chicks. It has been suggested that the state of hydroxylation of bone collagen lysine may be important in mineralization and that the increased hydroxylation in rickets may be of a contributory factor in the lack of mineralization in this condition. In a more recent paper, Barnes and associates attributed this effect in rickets to a reduced blood calcium level, since feeding a low calcium diet

produced the same effect, as well as in parathyroidectomized animals. This claim was not confirmed by Mechanic and associates, who showed that he increased lysine hydroxylation of diaphyseal bone collagen of rachitic chicks was not correlated with blood calcium level. They concluded that "in absence of vitamin D metabolites, more immature collagen is synthesized and the organization, packing, and structure of the matrix is insufficient to ensure normal bone mineralization and turnover." In a subsequent paper, the same authors reported to have measured the activity of lysyl oxidase (the enzyme responsible for the production of aldehyde precursors of lysyl-derived collagen cross-links) in tibial metaphyses from chicks receiving different levels of vitamin D and calcium. In vitamin D deficient chicks, enzyme activity was higher than in controls. Addition of calcium to the vitamin D-deficient diet had no effect on the oxidase activity. This is apparently the first demonstration of an effect of vitamin D on an enzyme system involved in maturation of bone matrix.

These abnormalities in collagen structure in rickets may explain the abnormal arrangement of the collagen bundles and perhaps also the immaturity of apatite crystals and the failure to form an apatite-binding matrix mentioned above.

Evidence has been presented suggesting that lipids may participate in bone mineralization. Lipids were found at sites of calcification, and their content increased during proliferation and calcification of epiphyseal cartilage. Biochemical studies showed that during transformation of osteoid to bone, neutral fats disappeared and phospholipids appeared. During mineralization calcium may be bound to phosphatidyl serine or to a phospholipid-protein complex.

Reports on the effect of hypovitaminosis D on amounts and composition of cartilage and bone lipids are conflicting. In an histochemical study, sudanophil material disappeared from the epiphyseal cartilage of rachitic rats and it appeared again in cartilage after dosage with vitamin D. However, in a chemical study, a total lipids extractable from epiphysis, metaphysis, and diaphysis of rachitic rat bones increased as well as diaphyseal phospholipids. In another investigation, not only were increased concentrations of lipids, (including phospholipids), found in rachitic rat cartilage (per DNA content and dry weight), but also an increased incorporation of labeled palmitic acid into cartilage lipids, indicating increased lipid synthesis were found. Recalculation of the data on in vitro

incorporation of ^{14}C-labeled palmitic acid into phospholipids yielded, however, a lower incorporation rate in rachitic cartilage than in cartilage of normal controls. Since the concentration of phospholipids increased, it appears that the rate of phospholipid degradation was decreased. Howell et al., studying costochondral plates of rachitic calves, found a decreased phospholipid content with an altered pattern of different components.

Hypervitaminosis D is reported to stimulate incorporation of radiosulfate in the phospholipids of intestine, kidneys, and bone and to increase all lipid components with the major increase being in the phospholipid fraction. The authors suggested that vitamin D is involved in bone formation through its role in phospholipid metabolism. Since in hypervitaminoses D the rate of matrix formation is greater than normal, it may be that the rate of calcification is inhibited by the presence of excess lipids (and in particular, phospholipids) and that calcification cannot occur unless the phospholipids are removed. In view of the conflicting results with hypovitaminotic animals, it is difficult to apply this explanation to the effect of vitamin D deficiency on cartilage and bone lipids.

Summarizing the mode of action of vitamin D (or its metabolites) on bone formation it may be stated.

1. It regulates, together with parathyroid hormone and calcitonin, calcium and phosphorus homeostasis ensuring a plasma × phosphorus product that is optimal for matrix calcification.
2. It appears to make bone matrix classifiable. In its absence, normal matrix calcification fails, even when the plasma calcium × phosphorus product is normal.

7.3.3 Minerals

In this section there minerals will be considered: calcium phosphorus, and magnesium. The calcium content of the body is estimated as 1200 g in the young adult male and 1120 g in the young adult female. The corresponding estimates of phosphorus are 670 g in men and 630 g in women. Of the total, 99% of the calcium and almost 90% of the phosphorus are in the skeleton. Salts of these minerals form the main fraction of the inorganic components of the bones, conferring to them the strength to bear the weight of the body and other loads and the capacity to resist the pull of the muscles and to withstand and stresses and strains of active life. In some species, dietary lack of calcium or phosphorus or excess phosphorus interferes with the homeostatic regulation of

the composition of the fluids bathing the bone-forming tissues, ultimately leading to faulty bone formation. Moreover, in rapidly growing animals, such as the young rat, and optimal proportion of dietary calcium and phosphorus is necessary in order to avoid deleterious effects on bone formation.

Magnesium plays only a minor role compared to calcium or phosphorus. The skeleton contains about 60% of the 20 to 28 g of magnesium present in the adult body. However, both deficiency and excess of magnesium may interfere with normal bone formation.

7.3.3.1 Calcium

7.3.3.1.1 Experimental studies in animals

Much information on the effect of calcium on bone formation in various animal species has been obtained by investigating the effects of dietary calcium deprivation. Sherman an McLeod, in their classical paper on the effect of dietary calcium insufficiency on the growing rat skeleton, initiated a large number of such studies. The main findings were the stunting of growth and bone rarefaction. Subsequent studies of bones of growing rats, mice young and adult cats, dogs, and pigs revealed a consistent picture of brittle and easily fractured bones. Cortical thickness of tubular bone was reduced, and often only thin periosteal and endosteal shells were observed. In the endosteal region, large resorption cavities were found and the marrow cavity was increased. Bone resorption occurred apparently under both the periosteum and endosteum, and resorption cavities also appeared in the compacta. A calcium-free zone of organic matrix intervened between the endosteal and calcified endosteal bone. Numerous hypertrophic osteocytes were found in wide osteocytic lacunae indicating osteocytic resorption. A large number of osteoclasts appeared in the areas with extensive resorption. Between the narrow periosteal and endosteal zones, there were few trabeculae of apparently poorly developed or fragmented tissue. Microradiographic examination, however, revealed that these trabeculae, although thinner than normal, appeared to be normally mineralized. If care was taken to supply adequate amounts of vitamin D, there was no increase in the width of the osteoid border nor any excess of uncalcified osteoid matrix as seen in rickets. These changes were accompanied by an increase in the periosteal bone mineralizing surface. The number of osteoblasts has been reported unchanged or increased. By means of light and electron microscope, it has been found that bone resorption in calcium-deficient rats takes place in two stages: the removal of

calcium from the osteocytic perilacunar bone matrix (osteocytic resorption) and from the endosteal area followed by dissolution of the organic matrix. Further findings of these investigators were an increase in rough-faced endoplasmic reticulum in the hypertrophic osteocytes and endosteal cells suggesting an increase of protein synthesis and possibly of production of proteolytic enzymes.

In calcium-deficient rats, the bone matrix was fibrous and arranged in a disorderly manner suggesting that the normal trabecular arrangement had been disrupted. Engorged blood vessels and myeloid tissue penetrated between the trabeculae.

Stauffer and associates, using quantitative histological methods, attempted to obtain a comprehensive conception of the changes occurring in the skeleton of calcium-deficient rats. They found that matrix formation was decreased largely because of diminished apposition suggesting impaired osteoblastic synthetic activity. The resulting inhibition of mineralization was associated with increased bone resorption and parathyroid gland volume. Thus, hypocalcemic calcium-deficient rats developed a mineralization defect that was similar to that seen in vitamin D deficiency. The principal difference was the much increased bone resorption in calcium deficiency.

Pathophysiologically, the first stage in the development of osteoporosis resulting from dietary lack of calcium is hypocalcemia which stimulates the parathyroid glands. This is apparently the main cause of the excessive bone resorption. This assumption was supported by the finding that hypoparathyroidism protected the skeleton from the osteoporosis resulting from calcium deficiency but at the expense of somatic and skeletal growth. Osteoporotic changes in the skeleton of rats maintained on a low-calcium diet were, however, observed even in the absence of the parathyroid glands. On the other hand, parathyroidectomized cats fed a low-calcium diet did not develop osteoporosis. From indirect evidence based on urinary excretion of hydroxyproline, it has been suggested that hypocalcemia per se stimulated bone resorption.

The histologically demonstrated increase in bone formation was confirmed using radioactive strontium. Retention was greater in osteoporotic rats and dogs than in normal controls.

Calcium deficiency may also effect the structure of bone collagen. Bone collagen of deficient chicks had the same chain composition as that normally synthesized but differed in that the hydroxylation of lysine was increased in both $\alpha 1$- and $\alpha 2$-chains. The significance

of the increased hydroxylation of bone collagen is unknown. Although it seems unlikely that collagen involved in the initial induction of bone mineralization, it is still arguable that a critical collagen structure is required for controlled formation and growth of the mineral phase of bone.

Demineralization of bone has been demonstrated biochemically and radiographically in animals fed calcium-deficient diets, e.g., rats, mice, cats, and dogs. Mice appear to be less sensitive to dietary calcium deprivation than rats, their capacity to adapt to a low-calcium diet is greater than that of rats, probably by improving calcium absorption. Their slower growth rate as compared to that of rats enables them to better withstand dietary lack of calcium.

Animals are able to adapt to different levels of dietary calcium and to form normal bone with varying calcium, intake. Bone formation and resorption may proceed at equal rates under conditions of widely different metabolic activity. In each instance, calcium equilibrium is attained at many different levels of intake. Thus, when weanling rats fed a low-calcium (0.2%) diet were switched over to a high-calcium (0.9%) diet they retained a larger percentage of dietary calcium than rats accustomed to a higher amount of calcium. During the first 3 weeks on the high calcium diet, their bones contained (per unit bone weight) less calcium than bones of rats fed the high-calcium diet (or switched over form the high to the low-calcium diet). Later this difference disappeared, indicating that the skeletal calcium stores may control calcium retention.

Long-term experiments on rats showed that during early life, bone calcification depends on the calcium content of the diet, but after the age of 1 year, calcification was rather independent of dietary calcium. Thus, before calcification was complete, calcium retention from a diet low in calcium (0.13%) was influenced by skeletal calcium stores. Most investigations on the effect of dietary calcium deprivation on bone were performed on young, growing animals. Adult rats were reported to be more resistant than young animals to diets low in calcium. There were no histologically and microradiographically demonstrable changes in long bones and only a slight reduction of bone mass and weight of ash. On the other hand, aging mice developed bone porosity irrespective of dietary calcium intake during their adult life.

Adequate supply of calcium during early growth period apparently confers the ability to better resist subsequent calcium deficiency.

Middle-aged (15 months old) rats fed a calcium-deficient diet of meat survived for a long time, sometimes over a year, without developing gross skeletal abnormalities and with little or no demineralization. The provision of calcium supplement to weanlings for graded period, before restriction to meat without calcium, increased their ability to grow and to thrive. With only 3 weeks of supplementation, for example, the rats grew normally during 4 months and they had normally shaped bones. To a marked degree, the urge for growth had taken precedence over the demand for normal mineralization of bones. Thus the ash content of their femurs was only one half of that rats receiving calcium continuously. "These observations emphasize the crucial influence of age, in rats, previously adequately supplied with calcium on their resistance to subsequent dietary calcium deficiency.

Several investigators have reported that alterations in bone tissue induced by dietary calcium deficiency can be reversed by subsequent feeding of an adequate or high-calcium diet. Feeding a high-calcium (1.2%) diet reduced the medullary cavity of tubular bones of rats. This was due to decreased endosteal bone resorption and, particularly, to increased endosteal bone formation. Bone formation at the periosteum and at the epiphyses was unchanged, indicating that the high-calcium diet did not cause a generalized increase in bone formation. The increase in endosteal bone formation was limited to those sites along the endosteum where greatest bone loss had occurred during calcium depletion, indicating that a local factor was at least partially responsible for the stimulation of endosteal bone formation during calcium repletion. In dogs, the numerous soft osseons present in microradiograms of long bones at the end of depletion became fewer with repletion. Densitometry showed that remineralization was a linear function of time on the repletion diet. The biomechanical qualities of bones improved with time and calcium content of the repletion diet.

7.3.3.1.2 Human primary osteoporosis

Osteoporosis is an atrophic process of bone associated with a negative calcium balance or a "calcium deficiency in widest sense. Balance and radiocalcium kinetic studies and refined morphologic techniques have shown that:

1. New bone formation is commonly normal.
2. Osteoblastic activity is not diminished.
3. Osteoblastic activity is increased.

4. The ability to absorb calcium is often decreased.
5. Intravenously administered calcium is subnormally retained.

It is believed that osteoporosis "is a multifactorial disease, in its homeostatic varieties produced whenever bone is forced to provide calcium which the organism fails to obtain form its environment," such as dietary deficiency, intestinal malabsorption, or imbalance between internal (bone) and external (intestine, kidney) responses to homeostatic stimuli. Bone mass is reduced because of "the calcium needs of the organism." Thus, dietary calcium deficiency may contribute to human osteoporosis, although it is doubtful whether it is the primary cause.

Riggs and associates reported that the estimated calcium consumption of 166 osteoporotic patients was lower than that of 83 control subjects matched for age and sex. In another, study, it was found that the vertebral mineralization measured by quantitative radiographic densitometry of 53 patients with osteoporosis was lower by 60% and their mean estimated habitual calcium consumption lower by 21% than those of 53 control subjects of the same age and sex. Some correlation between the crude dietary intake of calcium, protein, and phosphorus and the bone status was reported in 250 Glasgow women. However, this was accounted for by the fall in body weight and dietary intake that accompanies aging and was eliminated when the diets were corrected for age and body weight. Other investigators failed to observe any association between degree of osteoporosis and habitual calcium consumption. Garn and associates concluded that calcium intake does not relate to bone loss. High intakes (above 1500 mg/day) did not seem to be protective, and low intake (below 300 mg/day) were not demonstrably associated with bone loss. The later loss of cortical bone," from the fifth decade on, is a physiological phenomenon as much as "the earlier gain" in youth and adolescence. Primarily osteoporosis "is a common feature of aging."

7.3.3.2 Phosphorus

7.3.3.2.1 Deficiency

Two early studies described the effects of phosphorus deficiency in animals. The administration of low phosphorus rations to weaned pigs was followed by decreased growth (weight, height, and length), lowered blood phosphorus, and poorly developed bone. The long bones and the ribs of these animals were less dense, more fragile to

breaking by an external load, smaller (length and diameter), and contained significantly less ash than those of animals on a control diet containing adequate phosphorus. In young rats, administration of a diet extremely deficient in phosphorus (0.017%) caused a decrease in growth and the rapid development of extreme skeletal rarefaction. When these animals were subsequently fed diets containing a higher level of phosphorus, marked recovery was noted. These early observations of phosphorus-deficiency-induced bone resorption have since been confirmed in rats, pigs, and dogs. Baylink and associates found that a low-phosphorus diet fed to rats resulted in a hypophosphatemia and hypercalcemia. There were a number of changes observed in the tibia, namely, the resorbing surface was increased and the medullary cavity was enlarged due to an increase in the rate of endosteal bone resorption. The magnitude of the increase in bone resorption was similar in thyroparathyroidectomized and intact rats, indicating that neither parathyroid hormone nor calcitonin was involved in this cellular response. Alterations were also noted in matrix formation and mineralization. The bone matrix formation rate and the osteoid maturation rate were decreased, indicating a delay in the onset of mineralization; the initial rate of mineralization was decreased. The number and size of osteoclasts in the tibial diaphysis increased in response to phosphorus deficiency. In further studies by this group of investigators, radioactive calcium was injected at the beginning of the treatment with a low-phosphorus (0.04%) diet. In the experimental animals, specific activity in bones was lower and in serum it was higher than in controls indicating an inhibition of calcium deposition in bone. Cuisinier-Gleizes and associates reported widening of the epiphyseal plate with thinning out and loss of metaphyseal trabeculae and narrowing of diaphyseal cortex width in rats. The percent of osteoblasts and osteoclasts increased and femur ash decreased. Parathyroidectomized animals responded to phosphorus deprivation qualitatively like the sham-operated controls, i.e., by an increase in bone-mineral resorption, indicting that these responses were parathyroid-independent. The investigators concluded that the increased bone resorption is the cause of the hypercalcemia and hypercalciuria observed in this condition.

In phosphorus deficient rats, there was also an increased excretion of injected bone-seeking isotopes and an elevation of their urine: tibia ratio. These effects also pointed to an increased bone

resorption. Examination of bone resorption rate with unusual markers (lutetium, neptunium) showed that cortical bone resorption slightly increased as son as after 1 week on the phosphorus-deficient diet. Composition of bones of phosphorus-deficient rats changed with preferential removal of phosphorus; this tended to increase the Ca:P ratio in bone ash.

In weaning pigs, "deleterious results" were reported when the calcium intake was high without increasing dietary phosphorus. Depending on the absolute amounts of dietary calcium, the following changes occurred in bone: incomplete calcification of new bone (as seen by osteoid borders of the trabeculae), reduction or complete disappearance of the zone of provisional calcification in the epiphyseal cartilage and retardation of increase and removal of hypertropic epiphyseal cartilage resulting in the formation of a wide epiphyseal plate and atypical rachitic metaphysis. From this study, it is apparent that not only the absolute amount of dietary phosphorus is important but the proportion of calcium and phosphorus is also.

In bone tissue culture studies, it was found that a low-phosphorus concentration in the incubation medium enhanced bone resorption. Hence, from animal and tissue culture studies cited here it may e assumed that it is the hypophosphatemia induced by restriction of phosphorus that mediates, directly or indirectly, an enhanced resorption of bone.

7.3.3.2.2 Excess

Phosphate supplements given to gods resulted in increase bone resorption accompanied by an elevated level of serum immunoreactive parathyroid hormone. The relatively free influx of excess dietary phosphorus into the blood stream probably causes a mild depression of plasma calcium level, stimulating parathyroid hormone secretion. Similarly, bones of rabbits fed for 4 to 8 weeks with a commercial stock diet with phosphate added (Ca:P ratio of 0.42 or 0.55 as against 1.1 or 1.7 in the supplemented diet) exhibited porosis. Tibial defects made at the beginning of the experimental period were replaced with bone tissue containing many areas of resorption, probably resulting from increased secretion of parathyroid hormone.

In a series of reports, Draper and associates demonstrated that excess dietary phosphate accelerates the rate of bone resorption in rats and mice. Long-term studies on adult mice also clarified a relationship between dietary phosphorous intake expressed either in absolute terms or in terms of a Ca:P ration and the rate of bone

loss in aging. When the dietary Ca:P ratio was high (2.0), increases in amounts of dietary calcium were associated with increments in ash content, cortical thickness, and breaking load of femur. At a lower Ca:P ratio (1.0), these parameters were depressed and unresponsive to increase in dietary calcium. The highest values for bone mineral were observed by feeding a diet with 1.2% calcium and 0.6% phosphorus. These findings indicate that although calcium can modify the deleterious effect of excess phosphorus, a high dietary phosphorus enhances the resorption of bone, even in the presence of a high-calcium concentration. The accelerated bone resorption, as demonstrated by mobilization of ^{45}Ca from the skeleton and decreases in bone ash, dry fat-free weight, dry fat-free organic matter, and breaking strength, caused by dietary phosphorus excess, was not the result of decreased calcium absorption but was abolished by parathyroidectomy and arrested by reducing the intake of phosphorus. Further investigation showed that feeding the high-phosphorus diet for several days lowered the plasma calcium level slightly but significantly. This stimulated parathyroid hormone synthesis as evidenced by an increased uptake of the nonmetabolizable amino acid, α-aminoisobutyric acid, by the parathyroid glands followed by increased release of ^{45}Ca from "deep labeled" stable bone. Thus, as in the studies with dogs and rabbits mentioned above, "the parathyroid gland is under constant stress in order to maintain blood calcium homeostasis and consequently enhanced bone resorption occurs continuously. ... Enhancement of parathyroid activity is the key to the action of dietary phosphorus on bone resorption." The significance and implications of the effect of high-phosphorus diets on the homeostasis of adult bone to human nutrition is discussed in a review article by Draper and Bell.

7.3.3.2.3 Calcium:phosphorus ratio

AS already referred to, not only the absolute amount of dietary calcium and phosphorus is important for normal skeletal growth, but so are their proper proportions, particularly in rapidly growing animals, such as chicks, pigs, and rats. Steenbock and Hertig conducted a series of experiments on rats, measuring growth and bone ash when dietary calcium was fixed and phosphorus varied or with fixed phosphorus and varying calcium. When the diet contained 0.45% calcium, optimal growth was achieved with 0.32% phosphorus (Ca:P – 1.4) and highest bone ash concentration was achieved with 02 to 0.6% phosphorus (Ca:P–0.8 to 2.2). With a phosphorus level

of 0.315% optimal growth and highest bone ash were found when the diet provided 0.43 to 0.83% calcium (Ca:P—1.4 to 2.7). In another study on rats, highest values of femur weight and concentrations of calcium in fat-free bone were obtained with a diet containing 1.6% calcium and 1.6% phosphorus (Ca:P—1.0), and lowest values were obtained when the diet contained 0.2% calcium and 0.1% phosphorus (Ca:P–0.5). In young rats, biomechanical properties of bones, such as breaking strength and limit and modulus of elasticity, were less affected by feeding a diet limiting in phosphorus (0.06%) than by a calcium-deficient (0.11%) diet when the corresponding mineral was present in adequate concentration (0.56% calcium and 0.34% phosphorus, respectively). Deficiency of both calcium and phosphorus (Ca:P—1.73) had less of a deleterious effect on bone strength than deficiency of calcium with Ca:P ratio of 0.34. The levels of calcium and phosphorus in the ratio of young pigs are apparently less important than their mutual proportions. They could vary widely with little effect on growth as long as the Ca:P ratio remained about 1.3.

7.3.3.3 Magnesium

Both dietary lack and excess of magnesium are reported to affect bone formation.

7.3.3.3.1 Deficiency

Growth retardation resulting form dietary magnesium deprivation has been described in young mice rats and dogs. Histological studies of long bones showed a reduced thickness of the epiphyseal plate. The epiphyseal cartilage had a widened hypertrophied zone and a narrower than normal proliferating zone containing few rows of chondrocytes. Practically to new trabeculae were seen at the subepiphyseal region. The capillaries surrounding the thin and poorly ossified spicules were dilated. The diaphysis was thin and contained immature fibrillar matrix with chemical studies of epiphyseal cartilage showed that the polymerization of protein-polysaccharide complexes was reduced, curtailing the calcification of the ground substances. Thus, magnesium deficiency may reduce enzyme activities that are involved in protein synthesis and thereby interfere with the sequence of events leading to normal calcification. Moreover, the amounts of chondroitin sulfate present in the cartilage as well as the rate of sulfation of glucosaminoglycans and collagen formation in bone matrix of magnesium-deficient rats were decreased. On the other hand, no changes were found in the nonlipid organic content of

marrow-free humeri from rats fed a magnesium-deficient diet. Thus, "the skeletal effects of magnesium deficiency at the epiphyseal plate... are the most important since they influence general bone growth and eventual size of the body.

Other abnormalities were observed in femurs of magnesium-deficient rats by the use of absorption densitometry and Grenz rays. Osteoclastic activity was reduced and bone density was increased, finding that apparently confirms the presence of dense immature matrix reported above.

Glaster and Gibbs reported on the localization of radiomagnesium in mongrel puppies. When Mg was injected, radio autographic femur scans showed highest densities in the epiphyseal lines, the growing, activity metabolizing portion of bone in immature animals.

The effect of magnesium was also studied in bone tissue culture. Reduction of magnesium concentration in the medium from 1.0 to 1.5 m*M* enhanced calcification in the shaft of the long bones of fetal rats, whereas cartilage dry weight and collagen content were significantly decreased. Nielsen monitored the uptake of calcium by calvaria from newborn mice from the surrounding medium and the release of previously incorporated ^{45}Ca from the osseous tissue. Incubation in a low-magnesium (.08 m*M*) medium resulted in a net release of calcium from the bone when the initial calcium concentration in the medium was low (0.16 m*M*). However, at a physiological calcium concentration (1.60 m*M*) the low-magnesium medium increased uptake of ^{45}Ca into bone. Nielsen concluded, on the basis of these and of other experiments, that magnesium in physiological concentrations exerts an inhibition action on the calcification of bones from young mice and suggested that magnesium may have inhibited the conversion of amorphous calcium phosphate to hydroxyapatite. Previously, Bachra and associates had demonstrated this phenomenon using synthetic amorphous calcium phosphate.

The reports on the mineral composition of bones of magnesium-deficient animals are conflicting. Orent et al., who were probably the first to observe the effects of dietary magnesium restriction on bone composition, reported that the bones of deficient rats became unusually heavy and abundant in absolute content of minerals due to a large measure to excessive deposition of calcium. These findings have not been consistently confirmed by others. Thus, increased bone ash was reported in magnesium-deficient rats but no great

change in rats and guinea pigs, and even a decrease in young and adult rats, dogs, and chicks was reported. As regards calcium in bone ash, some investigators have reported increases, no change, or decreases in various species. Similar contradictions abound when considering bone phosphorus content. This variability of bone ash quality and composition in magnesium deficiency from different laboratories remains unexplained.

Dietary magnesium deficiency generally resulted in a decreased bone magnesium concentration. In rats, Hunt reported that bone magnesium concentration decreased in proportion to dietary intake, irrespective of age, but that the degree of response was greatest in young rats. Thus, the decrease of magnesium concentration may be attributed, at least in part, to dilution of the ion present initially in bone as a result of continued bone growth in the absence of magnesium, i.e., the deposition of magnesium-poor bone.

7.3.3.3.2 Excess

The effect of feeding a diet high in magnesium (0.5 to 1.0%) on bones of adult rats has been described. The lesion observed in tibias appeared histologically similar to those found in rats fed diets very low in magnesium. The epiphyseal plate contained fewer than the normal amount of chondrocytes, and several of these cells appeared degenerative. Staining of the diaphysis was irregular and patchy in appearance. Alpha-radiography revealed a marked decrease in number and size of lacunae in the matrix, similar to findings in rats fed a low-magnesium diet (less than 0.25%).

7.3.4 Trace Elements

Five trace elements appear to be involved in bone formation: copper, manganese, zinc, silicon, and fluorine. The exact nature and mechanism of this involvement is being intensively studied, yielding still unanswered questions. Reviews on the role of these elements in bone physiology and mineralization are available.

7.3.4.1 Copper

7.3.4.1.1 Deficiency

Bone abnormalities resulting from copper deficiency have been reported in rabbits, dogs, pigs, chicks, lambs, and cattle. In young dogs, the disorder is characterized by bowing of the front legs, thin cortices, few trabeculae, wide and dislocated epiphyses, and spontaneous fractures. Histological studies of bones of these dogs and of copper-deficient pigs disclosed essentially similar abnormalities.

Cartilage growth was normal, but osteoblastic activity was reduced, resulting in a failure of bone deposition in the cartilage matrix. Bone resorption was excessive. Concentrations of ash, calcium, and phosphorus were normal. In copper-deprived chicks, impaired osteogenesis as well as increased brittleness and fragility of bones with normal concentrations of ash, phosphorus and magnesium were reported. The bone lesion found in copper-deficient lambs differed form that found in pigs, dogs, and chicks in that the epiphyseal cartilage was not thickened and there were no gross deformities. Osteoblastic activity was one of the first functions to be impaired in lambs born to copper-deficient ewes. The osteoporosis that developed in copper deficient cattle was associated with a decrease in size and number of osteoblasts. Epiphyseal ossification was delayed. Cortical thinning and deficiency of trabecular bone were accompanied by zones of osteitis fibrosa particularly in vertebrae and radial metaphyses where increased osteoclastic activity and fractures associated with hemorrhages were observed.

7.3.4.1.2 Effect on collage on mineralization

The skeletal abnormalities in copper-deficient animals are probably related to defective collagen formation. The collagen extracted form copper-deficient bone contained less aldehyde and was more easily solubilized than collagen form control bones. Since solubility is inversely related to the degree of intramolecular cross-linking, it appear that copper deficiency blocks cross-links in collagen. The mechanism of collage cross-linking is presumed to involve the condensation of aldehyde groups that are derived from the e-amino group of lysine. The conversion of lysine to such an aldehyde can be mediated by an amine oxidase. Copper containing amine oxidases, apparently necessary for the cross-link formation, have been shown to be present in connective tissue and bone and to be reduced in bone of copper-deficient chicks and in plasma of deficient cattle. It can be assumed that the defect in cross-link formation of bone collagen results in faulty mineralization. Thus, the primary biochemical lesion in the bones of copper enzyme, amine oxidase.

Studies with tissue cultures of embryonic chick cartilage and bone showed that addition of excessive amounts of copper (5 to 20 μg/ml medium) produced deleterious results. The chondrocytes became swollen, rounded and basophilic. They were detached form their lacunae; the quantity of matrix was reduced; osteogenesis ceased; and the cells failed to divide and mature, lost their enzymes and diet.

Defective formation of bone collagen may possibly be involved in the skeletal abnormalities found in rats and mice fed a meat diet. Bone ash of mice kept for 6 weeks on a diet composed of muscle meat was markedly diminished, and bone rarefaction could be demonstrated radiologically and histologically. This disorder was partially prevented by replacing one quarter of the muscle meat by beef liver. Although this dietary manipulation did not increase the calcium content of the diet, which remained very low, it considerably increased the amount of copper. In subsequent studies, the effect of copper added to meat on the mineralization of bones from mice was investigated. Addition of small amounts (2.5 to 10.0 ppm) of copper significantly increased mineralization and decreased the enhanced incorporation of radioactive calcium indicating a lowered exchangeability of bone calcium which accompanied the improved calcification.

Bone rarefaction and increased skeletal uptake of radioactive calcium could also be produced in mice by treatment with parathyroid extract. Thus this treatment bears some resemblance to that of feeding a meat diet. Furthermore, as with feeding a meat diet, the effect of treatment with parathyroid extract could be largely prevented by supplementation of the diet with small amounts (10 to 40 ppm) of copper. Although the mechanism of action of copper on bone mineralization in meat-fed animals is not known, it may be assumed that feeding this diet, being deficient in calcium, stimulates the parathyroid glands, thus maintaining a normal blood calcium level. Stimulation of the parathyroid glands results in breakdown of osseous collagen and removal of bone minerals. The reported increase in stability of collagen structure in normal rats induced by treatment with copper would explain the effect of this element on bones of animals treated with parathyroid extract.

7.3.4.2 Manganese

7.3.4.2.1 Deficiency

Investigators working with rats, rabbits, pigs, and chicks noted the following abnormalities: reduction of length, density, and breaking strength of bone and, particularly, shortening and bowing of legs. Ash content is variously reported to be normal or slightly or significantly decreased, whereas bone volume and composition remain unaffected. Manganese deficiency may lead to severe clinical disorders, such as lameness, enlarged hook joints, and crooked and shortened legs in pigs, or perosis (slipped tendon) in chicks.

Histological studies of pig bones showed selective retardation of endochondral ossification in the radius, whereas the ulna continued to grow, thus resulting in bowed front legs. Chondrogenesis in chicks was found to be impaired by retardation or suppression of proliferation and maturation of epiphyseal cartilage cells and of matrix formation. Endochondral bone growth was likewise retarded or suppressed. Leach reported reduced extracellular matrix and disorganized cartilage cell arrangement.

These results were largely confirmed and extended by others who produced manganese deficiency in newborn rats by feeding females a manganese-deficient diet form weaning on. The offspring of these females showed characteristic signs of manganese deficiency: retarded growth, shortened and bowed legs, and ataxia resulting from dissimilar growth rates of cranial bones. Histological examinations of the tibiae of the offsprings failed to exhibit any abnormalities in the ossification centers during the first 10 to 12 postnatal days. In order animals (21 to 48 days), however, the epiphyses were smaller than normal recessed in a pocket-like depression in the metaphyses, and became partially fused with them. Even when the "pocketing" was incomplete, the epiphysis was small, misshapen, poorly vascularized, fragmented, and rarefied. Bony spicules frequently perforated the narrow epiphyseal plate. These findings likewise point to a defect in chondrogenesis.

7.3.4.2.2 Role in mineralization

Although blood and bone phosphatase activity were found to be reduced, kinetic studies using radioactive calcium showed calcification not being impaired. Variations in dietary manganese did not alter much the amount and location of deposition of radioactive calcium or phosphorus in chick tibiae. Deficient bones appeared to be normal when examined by X-rays or with silver nitrate staining. Thus if calcification per se is not rendered defective by manganese deficiency, manganese must be implicated in some other process of bone formation. Attention was then focused upon a possible involvement of manganese in the biosynthesis of glycosaminoglycans. It was found that uptake of radiosulfate was lowered in cartilage of manganese-deficient chicks and that the amounts of glycosaminoglycans, and especially of chondroitin sulfates, was reduced in this tissue. The relative amounts of glucosamine and galactosamine in epiphyseal cartilage were changed, with most of the decrease in total hexosamine content occurring in the galactosamine fraction. Further studies

showed that two enzyme systems involved in the synthesis of chondroitin sulfate are affected by manganese deficiency: (a) polymerase enzyme, which is responsible for the polymerization of UDP-N-acetyl-galactosamine and UDP-glucuronic acid to form the polysaccharide and (b) galacto-transferase, an enzyme that incorporates galactose from UDP-galactose into galactose-galactose-xylose trisaccharide which serves as the linkage between the polysaccharide and the protein associated with it. The evidence is based on the requirement of manganese for the in vitro activity of these enzyme systems as well as the observation that preparations from manganese-deficient chicks incorporated more substrate than do comparable preparations form normal chicks. This observation is interpreted to mean that the number of available acceptor sites is increased in the deficient tissue, reflecting suboptimal in vivo enzyme activity. These studies suggest that manganese deficiency may affect bone formation by causing biosynthesis of glycosaminoglycans.

7.3.4.3 Zinc

7.3.4.3.1 Deficiency

Skeletal abnormalities are a conspicuous result of zinc deficiency in many species. In chicks and turkeys, growth is stunted and long bones are shortened and thickened. The length of the tibiotarsus is particularly affected, and disproportionate growth of this bone and of the tarsometatarsus results in perosis often found in zinc-deficient birds. The boned disorder appears to arise form a failure of cartilage cell maturation in the epiphyseal plate region and decreased osteoblastic activity in the thin bone collar. The cells in the hypertrophic and calcifying region the epiphyseal plate were found to degenerate abnormally unless they were close to blood vessels. More drastic abnormalities in the bones of the head, limbs, and vertebrae were found in chick embryos from eggs of zinc-deficient hens.

A great variety of congenital skeletal malformations were observed in offspring of female zinc-deficient rats. Fetal endochondral bone formation was reduced, resulting from a decreased cell number rather than cell size.

Radiographically, bone density was reduced in zinc-deficient chicks. Bone ash was diminished in chicks and rats or in mice, chicks, and turkey poults, without marked change. Alkaline phosphatase activity in bones of deficient rats was normal or reduced, and it was lowered in tibias of deficient turkeys. In zinc-deficient

chicks, activity was normal only in epiphyseal plate cells in the proximity of blood vessels or in calcifying areas.

7.3.4.3.2 Excess

High levels of dietary zinc (0.75% or more) have a deleterious effect on bone. Rat femurs were reduced in weight and ash content. With a low calcium diet, the resulting osteoporosis was more severe than that expected from calcium deficiency alone. The decrease of bone mineral could be avoided by dietary supplements of calcium and phosphorus. Calcium alone was less effective.

Zinc, added to cultures of embryonic chick cartilage and bone, stimulated chondrocyte division and vacuolation of cytoplasm. With high concentrations (over 7.5 μg/ml medium), toxic changes of granular basophilia, lacunar detachment, and necrosis were observed.

7.3.4.3.3 Effect on mineralization

The mechanism of action of zinc on calcification is not well understood. Zinc may act on different processes associated with mineralization. Thus, zinc-deficient rats differed form controls in various parameters of calcium metabolism, such as the rates of calcium entering and leaving bone, pool size, the rate of slow exchange, and the slowly exchangeable calcium in bone. These findings suggest that zinc is required for normal calcium metabolism. Since zinc was found to be progressively incorporated within preosseous tissue as evinced from autoradiographic and histochemical studies, Haumont and McLean assumed that zinc "helps to catalyze the calcification process." It has been hypothesized that it may take part as a metallic ion in crystal seeding or nucleation. Thus, Brudevold and associates reported that zinc is readily acquired by synthetic apatite competing with calcium on the apatite crystal. Similarly, studies with one powder showed that bone mineral takes up (and presumably binds) zinc more rapidly than matrix.

Evidence is also available that zinc deficiency affects the organic bone matrix. Thus, less radiosulfur was incorporated into epiphyseal cartilage and primary spongiosa of zinc-deficient chicks than of controls. These findings suggest that the synthesis of glycosaminoglycans is reduced in zinc deficiency. It is of importance that this metabolic alteration occurs in the area of bone elongation which is impaired in zinc-deficient birds.

Collagen metabolism might also be altered in zinc deficiency. Although we are not aware of any study on the effect of zinc

deficiency on cartilage or bone collagen, the effect on collagen of connective tissue has been investigated. A reduced incorporation of thymidine-methyl-^{3}H into skin DNA and lower polyribosomal yields associated with defective skin collagen synthesis has been reported in zinc-deficient rats. Thus, zinc may play a fundamental role in collagen formation. Furthermore, since the congenital skeletal malformations of zinc-deficient rat fetuses were associated with a diminished uptake of tritiated thymidine when compared with controls, Swenerton et al. expressed the opinion that the skeletal defects in zinc deficiency are caused by impaired DNA synthesis.

7.3.4.4 Silicon

Evidence that silicon is associated with calcium in an early stage of bone calcification has recently been presented. With an electron microprobe technique, silicon was shown to be localized in active growth areas in bones of young rats and mice. The amount was related to the "maturity" of the bone mineral. In the early stages of calcification both silicon and calcium content of osteoid tissue was low; however, as mineralization progressed, silicon and calcium contents rose. In more advanced stages of calcification, the amount of silicon fell, and the more "mature" the bone mineral became, the smaller was the amount of measurable silicon, until it reached or fell below detection limit as the calcium concentration approached the proportions present in bone apatite. A similar relationship between silicon and calcium was shown to exist in the periosteal region of young bone. These findings suggest that silicon takes part in the sequence of events leading to calcification.

Further evidence that silicon is involved in calcification at an early stage is provided by the calcium: phosphorus ratio. In mature bone, the ratio is generally 1.67. At silicon-rich sites, the ratio is in the range of 0.6 to 0.8, resulting form the presence of organic phosphate that is not yet combined with calcium in an inorganic calcium phosphate precursor to bone apatite or as apatite itself. Thus, silicon appears to be involved with phosphorus at an early stage of bone formation.

Under certain conditions, silicon hastens the rate of bone mineralization. In rats fed a low-calcium and low-silicon diet, mineralization proceeded at a lower rate than when the diet was low in calcium and sufficient in silicon. Silicon deficiency was without effect on rate of mineralization when the dietary calcium was sufficient. Thus, silicon appears to affect bone maturity.

Silicon appears to be essential for normal growth of rats and chicks. Silicon-deficient chicks had shorter leg bones with smaller circumferences and thinner cortices; they fractured more easily under pressure. The tibial-metatarsal and tibial-femoral joints were smaller; the epiphyseal ends had less articular cartilage and were narrower. Concentration (but not content) of minerals was higher and concentration of water was lower than in controls.

Further studies showed that silicon is involved in the synthesis of glycosaminoglycans of articular cartilage and connective tissue. It occurred in small amounts in chondroitin-6-sulfate from cartilage, apparently as an ether derivative of silicic acid. Silicon deficiency lowered he glycosaminoglycan content of articular cartilage of cockerels. Thus, "silicon may function as a biological cross-linking agent and contribute to architecture and resilience of connective tissue. Its site of action may be in the glycosaminoglycan-protein complexes of the ground substance.

7.3.4.5 Fluoride

Although the apparent essentiality of fluoride for optimal growth, (and possibly for reproduction) in animals has only recently been indicated, its beneficial effects on bone under certain conditions have long been appreciated. Increased skeletal density following fluoride ingestion is presumed to be a result of new bone formation and/or fluoride incorporation into bone hydroxyapatite structure, the latter being more stable and more resistant to agents causing mineral dissolution and bone loss. Hence, it has been suggested that fluoride might be used profitably in the prevention and treatment of bone conditions involving bone loss and demineralization, such as primary osteoporosis in man. Under certain conditions, however, treatment with fluoride may exert deleterious effects on the skeleton.

7.3.4.5.1 Effect of fluoride on composition of bone

Fluoride is an avid bone seeker. More than 90% of total fluoride in the body may be found in the skeletal tissue, and the fluoride content of bone ash may reach a theoretical 35,000 ppm. The levels of fluoride deposition in bone increase in proportion to the amount, duration of exposure, and continuity of fluoride uptake. In addition, bone fluoride uptake depends (most importantly among others) on the following factors: age of the animal type, region and metabolic activity of the bone, and calcium content of the diet. Fluoride is not irreversibly deposited in bone, and there is a slow mobilization through several mechanisms.

Because of the readiness of fluoride to deposit in bone, it is pertinent to describe the effect of this ion on the chemical structure of bone. A rise of bone fluoride concentration alters the concentration of various components both in the mineral and in the organic phase of bone from several pieces, including man, both in vivo and in vitro. The concentration of carbonate in fluorotic (800 to 8000 ppm fluoride) bone isolated form deceased individuals, after prolonged ingestion of a water supply containing up to 4.0 ppm fluoride, decreased about 10%. This was confirmed in bone samples from fluorotic rabbits and chickens. At least part of the carbonate in bone mineral seems to be surface limited, and there is evidence to suggest, at least in part, that fluoride can be deposited on the surface of bone mineral at the expense of carbonate.

There is a decrease of citrate in fluoride-rich bone. This decrease is not due to inhibition of its formation nor an acceleration of its utilization. Although the citrate content of bone from fluoride-treated rats was about 30% lower than control bone, neither citrate formation from exogenous acetate precursor nor citrate utilization (measured as isocitrate decarboxylation) was affected. Instead, the evidence supports the hypothesis that citrate is released form the bone crystal surface via an exchange with fluoride.

Magnesium increases in bone after fluoride ingestion. Weidmnn an associates an Griffith and associates using whole bone, suggested that fluoride increases the affinity of bone mineral for magnesium possible interaction with magnesium to form a stable compound that could precipitate on the crystal surface. Others have supported this hypothesis form their observations at different periods of animal growth and with more than one level of fluoride. Using microdissected fluorotic bone from three morphological different areas of the tibia mid-diaphysis of growing rats, Rosenquist reported differential concentrations of fluoride and increased magnesium concentrations. However, there was no direct relationship between the magnesium and fluoride concentrations. Thus, the possibility exists that the increased magnesium concentration in fluorotic bone reflected an increased metabolic activity rather than formation of a magnesium-fluoride compound.

To test the effect of fluoride on collagen synthesis, the incorporation of labeled proline into newly formed collagen hydroxyproline was investigated. A decrease in collagen synthesis was observed in fluorotic bone from osteoporotic human subjects

and growing rats receiving prior does of fluoride and in mouse calvaria maintained in organ culture in the presence of fluoride. While these investigations are suggestive of a fluoride effect on bone matrix collagen synthesis, no clear evidence for the presence of fluorosis was presented.

A 200-ppm dose of fluoride administered in the drinking water of growing rats for a 2-week period caused a sharp decrease in total lipid content of metaphyseal and diaphyseal bone from femur and tibia, accompanied by a significant decrease in lipid formation from two radioactive precursors, acetate-^{14}C and citrate-^{14}C. This effect of fluoride inhibition of bone lipid formation was subsequently confirmed in newborn rat calvaria in an in vitro system.

Fluoride uptake by bone is also accompanied by well-documented crystallographic change. In addition to ionic exchange of fluoride with carbonate and citrate on the mineral surface, it is generally thought that the major reaction by which fluoride is incorporated into calcified tissue is that of isomorphous substitution with hydroxyl ions involving the exchange of hydroxyl ion in the hydroxyapatite lattice to form a more stable fluorapatite. High resolution X-ray diffraction patterns of normal and osteoporotic human and animal bone apatites reveal that characteristic reflection peaks are sharpened or better resolved as fluoride content increases; presumably, this is indicative of enhanced bone mineral crystallinity and a larger crystal size. As a result, there is an attendant reduction in the specific surface of the apatite crystal with fewer and larger crystals and, hence, less total surface per unit mass of bone. This more stable apatite seems to be more resistant to agents causing mineral dissolution and bone loss.

7.3.4.5.2 Histopathological changes in fluorosis

It has been established that exposure to high doses of fluoride in the diet or drinking water results in new bone formation and increased bone density in animals and man. However, these changes are associated with qualitative bone abnormalities, and it has been suggested that the quality of the bone formed by fluoride stimulation is poorly organized and poorly mineralized.

In man, at high fluorine intakes (over 8 mg per day) taken over a number of years, skeletal fluorosis may arise characterized by an increased density of bone as demonstrated by radiographical thickness and coarsening of the trabeculae and similar changes in the cortex. Biopsy specimens showed disordered lamellar orientation

and an enlarged, poorly formed Haversian system. In the spongy bone, wide areas of increased osteoid tissue were found among well-formed trabeculae. Most of the wide osteoid seams were undergoing mineralization but probably at a reduced rate. Bone trabeculae were very dense and contained considerable amounts of calcium.

The effects of fluoride administration on human bone were confirmed by Schenk and associates. They observed an increase in the total volume of bone and described the main effects of fluoride administration: (a) much of the newly formed bone was of an irregular, coarsely bundled, woven type and was unmineralized or partially mineralized; (b) there was an increase in osteoclast count and total resorption surface; (c) there was an increase in total osteoid surface but with a retardation of osteoid mineralization.

Some of the more characteristic changes associated with excessive fluoride intake in cattle were abnormal bone formation on periosteal surface, thickened cortex, irregular bone pattern and mineralization, coarse and haphazardly arranged collagen fibers, and excessive osteoid tissue.

From the studies of skeletal fluorosis in sheep and rabbits, it was found that the newly formed bone is histologically similar to normal fetal bone, that the exostoses of fluorotic bone are less calcified than nonfluorotic bone and contain wide areas of osteoid, and that bone resorption may also, but not invariably, occur. In rats and rabbits, the major effect were increased periosteal bone formation, decreased mineralization rate in young bone, and increased endosteal bone resorption resulting from increased osteoclastic activity. Thus, cross-section area, cortical thickness, and diameter of femurs were increased as well as the medullary area.

The results from these studies clearly point to fluoride-induced defects in the mechanism of bone remodeling. The presence of broad osteoid seams or borders in fluorotic bone has suggested osteomalacia, and the way in which bone formation is often accompanied by bone resorption is akin to Paget's disease. Increased bone resorption was a frequent but not invariable consequence of fluoride ingestion. Faccini has suggested that this may attributed to a hyperparathyroidism secondary to fluorosis; the bulk of evidence supports this hypothesis.

7.3.4.5.3 Effects of fluoride on fetal bone

Most studies have indicated that there is at least partial transplancental transfer of fluoride in both animals and man.

However, there is only limited and somewhat equivocal information as to whether exposure of the maternal organism to fluoride before pregnancy affects the total amount of fluoride that is transferred via the placenta to the offspring. If fluoride is incorporated into fetal bone, effects on the growth, physicochemical, or histological characteristics of the tissue might be expected. In two rodent species and man, high parental maternal fluoride intake resulted increases in bone fluoride contents of the fetus or newborn. A significant increase in the calcium concentration (but not phosphorus) of the bone ash of human fetuses was associated with an increase in the fluoride content of the mother's drinking water. In a study on neonate rats, the magnesium content of osseous tissues paralleled increases in bone fluoride content as a result of increased maternal intake of fluoride. Forsyth and associates reported that the amount of fluoride contained in the bones of newborn pigs increased in direct proportion to the fluoride intake of the dams. The mineral composition of the bone ash of the pigs at birth was not altered by the fluoride incorporated. Length, width, volume, and weight of the newborn pig humeri decreased with increased fluoride intake of the sows; this depression of skeletal growth was not accompanied by simultaneous decreases in birth weight. Histological analysis seemed to indicate that this depression of fetal skeletal growth persisted at least up to the fourth week of life. Although in this study and in another cited here a high prenatal maternal fluoride intake was capable of causing increases in fetal and neonatal bone fluoride content, no benefits to bone structure or composition could be ascribed to fluoride in these experiments.

8

Nutrition and Reproduction

Plane of nutrition is intimately involved with maintenance of the functional integrity of the vertebrate reproductive system. Inadequate nutriton of nutreint deficiency as well as overnutriton and excess food have deleterious effect on reproductive performance. The mechanisms of action under these conditions may be at least wtwofold, either through a direct effect on the reproductive organs themselves or through altrations in the synthesis and/or release of the reproductive hormones. In the latter indirect mechanism, hormone levels might in turn regulate or influence nutritional requirements so a type of feedback is established.

Being more specifric with respect to conditions effectign the endocrine system and hence reproductive persormance, other parameters examinmed are the effect of actue and chornic starvation, caloric restriction,and quantity andquality of protein and vitamin and mineral deficiency. Regarding vitamin levels, not much is known concerning their influence on hormone activity. Vitamin are, however, essential to the functioning of the main metabolic pathways and they could possibly after hormone activity through these means.

A great number of nutritional experiments have been conducted onfarm animals, so this review will deal primarily with them. Experiments using rodent species have also been cited.

8.1 CALORIC INTAKE

When exaaminint the influences of nutrition on reproduction performace, the two caloric extremes, starvation and excess feed, must be ijncluded. Since thse arethe two ends of the nutritional scale, results cannot be explained as being dependent on the

elimination of a single factor. Some of theproposed mechanisms of action for these two experimental conditions are noted.

8.1.1 Starvation

Drakers starved for 2 weeks during the spring, show a reduced testicular weight increase, interrpution of spermatogenesis beyond secondary spermatocytes, and Leydig cells which remain quiescent. Thi is associated with regression of the FSH cells and a failure of neurosecretory material mobilization form themedian eminence.

In the fall, similar starvationfollowed by exposure to continuoous light resuls in a considerable incdrease intesticular eight, completed spermatogeneis, and active leydig cells; but FSH, LH and prolactin cells in the pituitary are less active than those of full-fed controls on continouous light. The results may be explained by assuing that stimulation of thehypothalamus may overcome the nutritional inhibition of gonadotropic funciton of the pituitary. The ffect of differetn light conditions have not been correlated.

Leathem concludes that the effects of starvation on gonadotropins cannot be generalized due to experimental variation in different laboratories. The conclusions are important when restriction of energy intake is imposed and when the effects are measured.

Starvation of mice seems to deplete energy ore than either proteins, vitamins, or minerals. This is evidenced by the fact that a "fasted" group given a 75% glucose solution does not differ form normally fed controls with respect to the incidence of mated females that have litters.

Starvation has marked effect if it occurs at or close to the time of implantation in rats and mice. The signs of starvation consists of:

1. Intercellular hemorrhage close tot he periphery of the unattached parts of the deciduomata.
2. The deciduomata and embryos becoming necrotic.
3. The tissue being invaded by leukocytes.
4. The deciduomata liquefyir ; and debris being absorbed. Sometimes blood and debris are discharged through the cervix.

Short duration fasting of male mice had no effect on mating behavior and fertility when limited to a 30-hr period. When the starvation period is extended to 36 or 48 hr the proportion of mated females decreases; the effect lasts for bout 48 hr after starvation is ended. Fasting for as long as 48 hr does not decrease fertility,

which can be explained by the long duration of spermatogenesis and the time that it takes for sperm to be transported form the testes to the ductus deferens.

The concept that starvation effects the gonads via an effect on the pituitary has been supported by experiments on laying chickens. Lack of feed results in atresia of the large follicles; however, injection of FSH, PMSG, or avian pituitary extracts prevents such atresia and the follicles can be ovulated.

8.1.2 Excess Feed

In hereditarily obese mice, problems of excessive feed intake have been examined. Sperm are present in the ductus deferens but the males are sterile if fed ad libitum, Restriction of fed intake reveals that some males with this genotype can be fertile. The endocrine function of mice with this syndrome is also obstructed as evidenced by the small seminal vesicles and the histology of the testes. Feed restriction does not improve the hormonal production of the testis, indicating that overeating is not the cause of the impairment. Obesity induced in animals by overfeeding and restricted exercise causes some atrophy of the testes. However, effect on tubules and interstitial cells have not been clarified.

Obese female mice are sterile. However, ovulation can be induced by gonadotropin treatment. Either unfertilized or fertilized eggs can then be transferred to normal foster mothers, and either fertilization and gestation or gestation alone will occur in the foster mother. A probable reason for the sterility of obese mice is a lack of gonadotropin secretion.

Electrolytic lesions of the ventromedial nucleus induce hyperphagia and obesity in rats plus an initial nonspecific pseudopregnancy. These rats later show normal estrous cycles; mating behavior does not occur, however, even in animals which show vaginal estrus.

8.2 PROTEIN

Protein is of considerable importance in maintaining the functional integrity of both the male and female reproductive systems. The major potion of gonadal dry weight in the male is protein, and it is estimated that the minimum need of dietary protein to support reproduction is 14 to 17% in rodents. In the female protein deprivation effects several reproductive parameters including onset of estrus and pregnancy (i.e., implantation), gestational length, and morality.

8.2.1 Male Reproduction

8.2.1.1 Spermatogenesis and sperm motility

Immature rats, when on a protein-deficient diet for a length of time allowing maturation in controls, show no permanent testicular damage. Refeeding adequate amounts of protein (18% casein) results in reappearance of spermatozoa in 70% of the animals. Removal of protein form the diet of adult male rats for 1 month has little effect on spermatogenesis, testicular weight, or protein concentration. Prolonged protein depletion is required before the rest is exhibits a loss in size and protein, but the effect is not uniform.

In addition to examining the effects of general protein deprivation, studies depriving male rats of specific amino acids have been reported. Shettles noted an influence of arginine deficiency of spermatogenesis in the rat after 3 weeks. Deficiencies of lysine and tryptophan impair spermatogenesis, whereas leucine, methionine, and valine are without effect. However, adverse influences on testes have been reported for valine and for methionine. Proper levels of amino acid supplementation are necessary, as excess amino acids are toxic. The feeding of 5% L-tyrosine in an adequate diet causes testis degeneration, with tubules lacking sperm and spermatids and Leydig cells being subnormal.

Not only is quantity of protein in the diet important for the integrity of the male reproductive system, but quality or nutritional value must be considered. A poor protein such as maize or gelatin will, when fed at high levels, decrease sperm motility and increase the number of abnormal sperm. Thus feeding adult rats gelatin for 20 days exhibits no influence on the testes, as evidenced by analysis of testis protein and histology, even when caloric intake is reduced to 65% of normal.

Although most of the effects of protein deprivation have been evaluated in rodents, various avian species have also been studied. Roosters fed rations containing various percentages of protein (16.0, 9.0, 6.75, and 4.5) were examined to determine effect on spermatogenesis. Sperm production before 24 weeks of age was obtained only in the cocks fed the 16 and 9% protein diets. Birds on the 9% protein diet showed sperm production considerably below that found on the 16% protein diet. Following the feeding of a 17% protein diet, a sharp increase in sperm production was observed, and by 30 weeks of age, the groups produced similar amounts of sperm. It

is apparent that a certain, minimal level of protein is required for early and optimal sperm production and that damage done as a result of restricted feeding is reversible.

8.2.1.2 Maturation and fertility

Protein requirements of mature, male chickens are much less than those for females, and rearing young males on low-protein rations does not appear to affect their fertility subsequent to their delayed maturity.

Marked protein deprivation in pubertal bulls causes a reduction of semen volume and sperm number as compared with those on normal protein levels, but the conception rate of the former bulls is not impaired. A low-protein diet will also reduce seminiferous tubular diameter. But calves fed high levels of protein exhibit no delay in sexual maturation.

Protein-free diets fed to make rats and mice prevent sexual maturation of the testes and secondary sex organs and cause an increase in lipid content of the testes. The testis weight is reduced but the ratio of testis weight to body weight increase, attesting to the greater liability of body protein other than that in the testis. Refeeding of 20% casein results in a major restoration of testis protein, demonstrating that the damage is reversible. Hypophysectomy prior to refeeding prevents the restoration of testis weight as prounced atrophy occurs. Proteins of lower nutritive value than casein, i.e., wheat gluten and peanut flour, slow the rate of testis weight increase, and 20% gelatin prevents a testis weight increase.

8.2.1.3 Androgen production

Protein depletion reduces androgen production by the adult rat testis, as reflected by a decrease in accessory organ weight. This reduction in androgen production is probably explained by a significant loss in Leydig cells in male rats fed a nonprotein diet. Immature rats lacking protein recover androgen production slowly following re-initiation of feeding. This is evidenced by the changes in seminal vesicle weight.

8.2.1.4 Gonadotropins

Deletion of protein from the diet of immature, male rats results in a reduction in total, hypophyseal gonadotropin content after 2 weeks. Removal of protein from the diet decreases hypophyseal gonadotropin content in adult, male rats, in comparison with pair- and ad libitum-fed controls. After 1 month, a significant lowering of

hypophyseal gonadotropin levels occurs. Both FSH and LH are reduced, with the impression that LH is reduced initially. Resulting hypophyseal gonadotropin levels are sufficient to maintain testis involution in rats fed a diet devoid of protein. Testosterone does not maintain either spermatogenesis or testicular weight in these animals. Thus testis function will be modified by diet via the hypophysis and possibly the hypothalamus.

The immature mouse testis responds to exogenous gonadotropin despite 10 days of dietary protein deprivation by stimulation of the tubules and Leydig cells and androgen secretion. However, the response to pregnant mares serum is reduced, if the period of protein depletion is extended to 20 days. The gonadotropin does not quicken the recovery of spermatogenesis in mice being refed protein. The degree of response of reproduction tissue to gonadotropin may be related to the nutritive value of the protein.

8.2.2 Female Reproduction

Female rats on a protein-free or protein-deficient diet exhibit the same general characteristics as the male following such treatments, i.e., delayed, sexual maturity, and atrophy of the ovaries and abnormal estrous cycles in the adult. Removal of protein form the diet of the rat at the time of mating does not prevent implantation, an effect different from that observed after total starvation at about the same time. Upon initiation of an adequate diet, rapid changes in the ovary occur, vesicular follicles can be seen, and estrogen is released. The elimination of one or more of the essential amino acids gives similar results. However, such omission is confounded by insufficient intake of other nutrients.

8.2.2.1 Estrus

Heifers raised on protein-deficient diets show no symptoms of estrus, with the ovaries and uterus remaining infantile. The impairment of reproductive performance by both low caloric and low protein intake is reversible, since adequate feeding usually restores normal function.

8.2.2.2 Egg production

The significance of specific amino acids in reproduction has been studied in chickens with emphasis on hens' egg production. Leaving out one of the essential amino acids (arginine, lysine, methionine, tryptophan, glycine, histidine, leucine, isoleucine, phenylalanine or phenylalanine and tyrosine, threonine, and valine)

decreases the food intake of hens immediately. When birds are force-fed such a ration, normal egg production does not result, indicating that the interruption of egg production is not a result of the decreased energy intake.

8.2.2.3 Pregnancy

8.2.2.3.1 Implantation

Further studies regarding specific amino acids and protein showed that their content in the uterine fluid changes markedly at the time of implantation. These changes can be specifically noted only in the rabbit. The concentration of most amino acids is much higher in rabbit uterine fluid than in blood serum at implantation. Glycine, alanine, taurine, and glutamic acid are particularly abundant and their concentration is progesterone-dependent.

8.2.2.3.2 Maintenance of pregnancy

The critical level of dietary protein to sustain pregnancy is the rate appears to be 6%, with levels less than 6% causing fetal resorption in over 85% of the animals by day 20 of gestation. Henricks and Bailey, studying the effects of protein deprivation, provided support for the claim that there is a threshold level of dietary protein needed to support pregnancy, at least until its midpoint. This threshold level might be needed to support the development of the corpus luteum that is necessary during the first half of pregnancy if adequate placental development is to be achieved for pregnancy to continue.

8.2.2.3.3 Litter size and survival

Studies with pigs have shown that as little as 5% protein (either provided by sesame meal, gelatin, or corn plus soybean meal) is sufficient to obtain litter size, number of live pigs farrowed, weight of live pigs, and survival of piglets similar to figures obtained with a 16% protein diet. Nitrogen balance studies revealed that an intake of 3% protein at a food intake of 1.8 kg/day was slightly in excess of maintenance requirement for pregnant gilts.

8.2.2.3.4 Reproductive efficiency subsequent to pregnancy

Severe protein restriction during gestation and lactation in swine impairs subsequent reproductive efficiency as indicated by an increased number of days from weaning to estrus, reduced ovulation rate, and lower uterine weight. These detrimental effects from previous protein restriction are more severe in younger animals, but estrus

and ovulation can be induced in such noncyclic animals by injecting PMSG and HCG.

8.2.2.3.5 Hormones and embryonic survival

A diet containing less than 6% protein results in resorption of the fetuses in the majority of female rats (85%). The injection of progesterone during the period of days 3 to 10 of pregnancy counteracts the effect of dietary protein deprivation. The injection of prolactin from days 3 to 12 of pregnancy has the same beneficial effect as progesterone on embryo survival. Corticosterone injections on days 5 to 9 of pregnancy could also replace progesterone as a means of maintaining pregnancy. This ability of corticosterone may be due as much to its inducement of prolactin secretion as to a mobilization of tissue stores of nutrients for use by the fetus. Further support has been provided by Morishige and Leathem who showed that plasma prolactin level increases in the protein-deprived rat when treated with corticosterone may be due as much to its inducement of prolactin secretion as to a mobilization of tissue stores of nutrients for use by the fetus. Rats fed protein-deficient diets for 5 weeks show reduced plasma and pituitary levels of prolactin compared to fully red rats.

Henricks and Bailey studied the effects of moderate and extreme protein deprivation and plasma prolactin and progesterone and luteal progesterone concentration in rats. They confirmed the concept established by the earlier investigations, i.e, dietary protein deprivation can cause the hypophysis to become sufficiently hypofunctional with respect to prolactin secretion so that by the time the gland is no longer necessary, the status of pregnancy is in jeopardy.

8.2.2.4 Gonadal response to exogenous gonadotropins

The gonads of rats fed protein-deficient diets respond to exogenous gonadotropic hormones, although the response is qualitatively different, (i.e., the follicles are stimulated but do not become luteinized after PMSG in protein-deficient rats, but they luteinize in rats on an 18% casein diet) and quantitatively diminished. They are, however, more sensitive to PMSG when they are on protein-deficient diets. In hypophysectomized rats, protein-deficient diets cause an increased sensitivity to LH and HCG but not to FSH.

8.3 LIPIDS

The physiological role of essential fatty acids, linolein, and linolenic and archidonic acids has yet to be completely elucidated,

although it is known that the male as well as the female reproductive organs are influenced by the dietary levels of these acids.

8.3.1 Male Reproduction

8.3.1.1 Essential fatty acids

Leathem notes that the mating potential of male rats is reduced when fats are excluded from the diet. Thse rats exhibit degeneration of the seminiferous tubules with reduction in spermatids and spermatozoa, which after 5 months results in testes devoid of sperm. Furthermore, testis degeneration occurs despite dietary supplements of vitamins A and E and in animals whose health appears quite normal. The damage done by essential fatty acid deficiency can be accentuated by adding cholesterol to the diet of rats and rabbits.

Adult rats fed 10% hydrogenated coconut oil for 25 weeks were then fed 10% corn oil to examine the recovery of the testis from essential fatty acid deficiency. Arachidonic acid was more readily incorporated into testis lipids than any other acid. Docosapentaenoic acid the major W6 acid of the control testis, was slow to recover on refeeding, and this fatty acid has been related to fertility in the rat. The linoleic acid content of testis will vary only slightly with changes in dietary linoleate unless the diet is increased several times.

Linoleic acid-deficient diets fed to birds form 4 weeks of age cause a 90% reduction despite a normal sperm volume sperm motility, and sperm count. Rations high in linoleic acid that are deficient in either vitamin E or an antioxidant results in impaired fertilizing capacity of sperm but in no reduction of rooster testis size. Such infertility is reversible when vitamin E is restored to the ration, which is interesting in light of earlier research indicating vitamin E damage to the avian testis to be permanent.

Essential fatty acid deficiency may invoke some of the testicular changes by influencing the pituitary. Morphological changes in the anterior pituitary have been observed. Furthermore, hypophysectomy not only reduces total testis lipids but invokes a greater decrease in phospholipids than in nonphospholipid components. Hypophysectomy produced similar testis changes in the rat previously fed a fatty acid-deficient diet for 26 weeks. Feeding linoleate following hypophysectomy resulted in the characteristic interconversion patterns anticipated in the normal testis. Conversion of linoleic to archidonic was less efficient, however. It is possible that the biosynthesis of phospholipids in the testis is a reflection of membrane turnover.

8.3.1.2 Nonessential fatty acids

The addition of certain nonessential fatty acids to the diet cause marked impairment of reproduction. The inclusion of 10% erucic acid (cis, 13-docosenoic acid, 22:1) in the diet of male rats causes a decrease in testicular weight, degeneration of the tubules, and complete infertility. Decreasing the percentage of erucic acid to 5% causes infertility in three out of six rats with no testicular weight changes. Testicular recovery after undergoing marked atrophy is poor and almost nonexistent. Erucic acid induced in a semisynthetic diet has no effect; no explanation for the occurrence is known.

Weanling rats fed 14% arachis (peanut) oil for 15 weeks exhibit a marked impairment of spermatogenesis, and after 28 weeks testicular damage is of such an order that prolonged feeding of ethyl linoleate does not restore fertility.

8.3.2 Female Reproduction

In chickens, linoleic acid is required for optimal egg production, egg weight, fertility, and hatchability. Linoleic acid deficiency causes an increase in the concentration of eicosatrionic acid in the tissues, yolk, and plasma. The concentration of this fatty aid is negatively correlated with the expression of the above-mentioned reproductive characteristics. Some of the polyunsaturated fatty acids like menhaden oil have a depressant action on the formation of eicosatrionic acid and thus seem to improve reproductive performance.

Linoleic acid deficiency in the diet of female rats causes irregular ovulation and atrophic changes of the uterine mucosa. The addition of hydrogenated arachis oil (14%) to the diet decreases the conception rate in females and increases embryonic mortality to 100%.

Uric acid acts as an antagonist to essential fatty acids and causes sterility in the rat in 3 months. Females have regular estrus cycles and mate normally but do not conceive. Mated rats might become pseudopregnant early in experimentation, but later no pseudo-pregnancy is observed.

Infants rats from females that had been on the erucic acid diet for 4 months and had conceived died because of involution of the mammary glands after parturition. Recovery from the effect of the erucic of the acid diet is slow and generally considered poor. The effect of erucic acid on lactation could be prevented by adding vitamin E to the diet, however, this vitamin does not prevent the damage to the testes induced by erucic acid.

The feeding to laying hens of as little as 25 mg of *Sterculia foetida* oil, which is high in sterculic acid and malvelonic acid (both cycloprenoid fatty acids), cause 80% embryonic mortality; the reasons for the effect have not been clarified.

Feeding of *Sterculia foetida* oil to female mammals causes a high incidence of atresia, the alteration of atretic vesicular follicles into lobules of interstitial tissue with central cavities formed by the zona pellucida, luteinization of the granulosa of medium-sized follicles, and underdevelopment of the uteri myometria and endometria. When fed to pregnant mammals, a diet containing only 3% foetida oil results in 100% embryonic mortality. In males the feeding of sterculia oil has no effect on reproductives organs or performance.

8.4 CARBOHYDRATE

Carbohydrates are found in testicular tissue, but quantities vary with species. Rat testis carbohydrate increases after 56 days of age and may relate to androgen secretion. When testicular atrophy is induced by estrogen, however, testis carbohydrate concentration does not decrease.

More information on species variations is needed, as evidenced by the fact that 72 hr of starvation does not alter testis glycogen in the mouse but reduces testis glycogen 50% in hamsters

8.4.1 Germinal Epithelium

In adult rats, tolbutamide or insulin will produce lesions of the germinal epithelium that are inhibited by simultaneous administration of glucose. Furthermore, inositol synthesis from glucose using rat testis homogenates exceeds that of other tissue.

8.4.2 Leydig Cells

A hyperglycemic state occurs in A-O mice with hereditary obesity. The testes of these mice are small, and atrophic Leydig cells are observed. The interstitial cells contain some 3α hydroxysteroid dehydrogenase, but the seminal vesicles are hypoplastic. When obesity is induced by gold thioglucose, no effect is observed on testis function, suggesting that overeating alone is not the factor in depressing the reproductive system in A-O mice. Severe diabetes in young rats prevents testes tubular maturation and Leydig cell function.

8.5 RESTRICTED FEED INTAKE

The effects of general restricted feed intake are quite numerous and are dependent on the stage of the life cycle the animal is on

when the experiment is carried out. Effects can be direct via acting on the reproductive organs themselves or indirect by acting on the transport of hypothalamus-reducing, gonad-releasing factor sto the anterior pituitary. Both of these mechanisms of action will be examined.

8.5.1 Male Reproduction

8.5.1.1 Testes development and onset of puberty

Male rats weaned prematurely show a high incidence of impaired spermatogenesis and a decrease in the diameter of the seminiferous tubules. These rats consume more calories than normal once they start to eat, and this greater food intake continues to about 40 days of age. It has been suggested by van Tienhoven that body size and food intake are determinants to a large extent of the onset of puberty in the rat, and that these factors become more important the earlier undernutrition begins.

After weaning, underfeeding of male rats causes a regression of interstitial cells in the testes along with an increase in testicular weight. Development of the seminiferous tubules is not affected, and all effects are reversible on refeeding.

During inanition the rat testis responds to gonadotropin by a stimulation of Leydig cells, in increase in gonad size, and a return of spermatozoa. Fasting of male mice at the time of mating adversely influences fertility. These effects can be eliminated with injections of gonadotropin or by feeding glucose.

When total digestible nutrient (TDN) intake is restricted in bulls, the rate of growth is decreased and age of puberty increases. Testis growth is also retarded while sperm output and semen volume are reduced. The number of motile sperm per ejaculation is reduced to 50% of that in normally fed animals, but this difference is erased with time.

The ages when Holstein bulls raised on high-, medium-, and low-TDN intakes come into semen production averages 39, 46, and 58 weeks, respectively. The delay in development on the low level of intake appears to be a direct effect because approximately, 1700 pounds TDN were required to develop bulls to semen-producing age on all three levels. The accessory sex organs in the underfed, young bulls exhibit a decreased responsiveness to testosterone, further indicating a direct nutritional effect on the reproductive organs. The restricted intake also delays by several

months the age at which young sires can be used in artificial breeding, although no difference in fertility has been found. Any effects from the restricted feeding are reversible, but irreparable damage does occur if undernutrition of the young bull is continued for too long. This damage is reflected in testis size, sperm production, and sperm replenishment.

Restricting feed to (85% of ad libitum intake) to growing turnkeys of age 12 to 24 and 24 to 40 weeks results in the later appearance of sperm when compared to full-fed toms.

Underfeeding of boars during the first year of life delays the onset of puberty and restricts testis weight and spermatogenic function (small tubules). Severe feed restriction causes regression of Leydig cells and spermatogonia, as well as an enlarged and edematous glans penis.

Marked reduction of calorie intake in rams causes a reduction in the diameter of the seminiferous tubules, the total semen volume, the sperm content of the epididymis, and the weight and fructose content of the seminal vesicles. Spermatogenesis was found in all of the rams. These effects are reversible upon initiation of proper feeding.

Upon restricted feeding, one observes a more sensitive response of the Leydig cells to initiation in comparison with the seminiferous tubules. Using monozygotic twin, bull calves, Mann et al. noted that restricting the food intake to ne half of normal to one of them had a marked delaying effect on the onset of seminal vesicle secretion but a lesser delaying effect upon spermatogeneis.

8.5.1.2 Androgen production

The testis of the above-mentioned but calf contained less testosterone. Samples of spermatic vein blood showed the testis secreted less testosterone. The testosterone/androstenedione ratio being lower than normal might suggest that the normal maturation process had not occurred. The full-fed animal exhibited the anticipated testis steroidogenic change with maturation; testosterone production increased while androstenedione decreased. Underfeeding may influence testis enzymes in steroid biosynthesis. Histologically, Δ^5-3B-ol-hydroxysteroid dehydrogenase was reduced in the Leydig cells.

Berliner and Ellis suggested that desmolase activity in the testis was reduced by underfeeding the rat for 20 days. Using pregnenolone and progesterone as precursors, in vitro conversion to progesterone

and 17α-OH progesterone was not altered, but androstenedione and total androgen production were decreased.

8.5.1.3 Effects on adult animals

The reproductive organs of the adult male are more resistant to changes imposed by diet than are those of the immature animal. Bulls have been maintained in good condition for years when fed a good quality hay plus limited amounts of a concentrate containing 14% total protein. Excessive caloric intake should be avoided as obesity favors reduced male reproductive performance. The same principles apply to rams, boars, and stallions.

Restricting either total feed or calorie intake of adult male chickens to the extent that 15 to 30% of their body weight is lost results in decreased volume and fertilizing capacity of semen. Testis size is also reduced, indicating a decrease in gonadotropic hormone output. Parker suggests that undernourishment reduces the passage of gonad-releasing factors (GRF) from the hypothalamus to the anterior pituitary.

8.5.2 Female Reproduction

8.5.2.1 Estrus and onset of puberty

In prepubertal animals, restricted calorie intake may cause hypoplasia of the reproductive organs and delayed puberty. The age at puberty may be influenced by many other factors (i.e., social environment, season of year, breed, and/or disease), however only nutrition's influence will be examined here.

Kennedy and Mitra showed that vaginal opening, first estrus, and mating occurred on the same day in the most optimally grown control rats. In fast growing and retarded rats, first estrus and first mating were noted at approximately the same body weight. If rats are retarded during the pre-weaning period (by having one rat nurse 3 pups and another rat 15 to 17 pups) vaginal opening precedes first estrus by about 3 days and first mating takes place during the 24-hr cycle. Feed restriction, when started after weaning and continued, completely blocks the onset of estrus. Estrous cycles are initiated within a week after refeeding is begun.

In horses, the length of the normal estrous cycle varies form 16 to 24 days, with an average of 22 days. This length is prolonged under poor nutritional condition.

In beef heifers, a lowered plane of nutriton delays the onset of puberty, whereas a high plane hastens puberty but results in a heavier

weight at the time. Rapid weight gains between birth and weaning and between weaning and 1 year of age considerably decrease the age of puberty in beef heifers. Anything that causes slow growth, inadequate rations, or other factors delays the onset of puberty.

If nutrition is maintained at a normal level, puberty occurs when body weight reaches 60% of the adult body weight in sheep and at 45% of the adult weight in cattle. A similar relation does not exist in pigs. In swine, van Tienhoven suggests, age may be more important in determining onset of puberty than the plane of nutrition.

The domestic pig receives an intake of dietary energy sufficient not only for body maintenance but also usually for an optimum growth rate. Limiting energy intake to half that of full-fed controls delays puberty by more than 40 days. Restriction energy intake to 60 to 70% that of ad libitum feeding for from a few days to several weeks results in hastening onset of puberty (e.g., -11 days) in some trials and delaying puberty (e.g., + 16 days) in other trials.

When swine are deprived of a diet adequate for growth and development for prolonged periods they are capable of reproduction if allowed to consume adequate diets later in life. Pigs limited to 4 to 14 kg body weight during their first 2 years and then allowed to grow on adequate diets eventually exhibit reproductive cycles.

Inadequate nutrition may affect the time of onset of the sexual season. Ewes that are unhealthy because of an extremely low energy level or lack of nutrition such as vitamin A or phosphorus in the ration do not show estrus as early in the breeding season as ewes which are properly fed and healthy. The length of the estrous cycle tends to be longer on a low as compared to a high plane of nutrition, but breed differences are not clear cut. Meat breeds have slightly shorter estrus cycles as compared with wool breeds.

The delay of the onset of puberty because of undernutrition may be due to a lower level of gonadotropins produced by the anterior pituitary gland, a lack of responsiveness on the part of the ovaries, or possibly a failure of the ovaries to produce adequate amounts of estrogens. It is not clear if any one or all are involved.

8.5.2.2 Ovulation rate

In sheep management it is known that increasing the plane of nutrition ("flushing") or increasing the gain in body weight of ewes prior to mating results in higher ovulation rates and thus more lambs per ewe. The flushing does not need to be started until about 3 to 5 weeks prior to mating. Allen and Lamming found that flushing

does not increase the ovulation rate above that of ewes kept in fat condition. They speculated that gonadotropins released for ewes on submaintenance rations may be incomplete. As a follow up they gave PMSG injections to ewes on submaintenance diets, resulting in increased ovulation rates and showing that the ovary can give a normal response to exogenous gonadotropins. Poor nutrition may quiet ovulation, especially near the onset of the breeding season.

Acceleration of the ovulation rate by flushing provides a practical means of increasing the lamb crop, although it must be noted that an increase in the number of fetuses and in the number of lambs born increases the nutritional needs both during late pregnancy and lactation and also in the care required at lambing time. The tendency for ovulation rates to be higher for sheep kept at latitudes closer to the poles and for sheep with faces free of wool covering, as has been shown in a number of breeds, may be related to seasonal or nutritional effects but are not well understood.

High-energy diets fed for a restricted duration induce a higher ovulation rate in swine. The number of ovulation is predominantly affected by genetic background, ovulation rate, however, is usually affected in a positive way with increasing levels of energy intake. The level of energy restriction before feeding the pigs a high-energy diet is an important factor influencing ovulation rate. A low level of energy intake is usually given before high-energy diets. The optimum duration of a high-energy diet seems to be 11 to 14 days before the expected estrus or mating. Protein levels in high- and low-energy diets are usually similar; there is little evidence that increased protein intake during brief periods increases ovulation rate.

Restriction of feed intake prior to mating reduces the ovulation rate of gilts. Similarly, energy restriction imposed during the first estrus cycle reduces the number of ovulations at the second heat.

The follicular development of undernourished rats is not affected by restricted feeding. Corpora lutea, however, are not detectable by the eleventh week, while controls show corpora lutea at 7 weeks. Follicular development is depressed by drenching with glucose twice daily during the estrous cycle. The blood concentrations of glucose are increased during this interval.

The physiologic basis for the relationship between plane of nutrition and ovulation rate is not clear, but the level of nutrients available immediately prior to the time of ovulation is thought to be of primary importance. This is suggested by the low correlations of

backfat thickness or body size with ovulation rate and the fact that a maximum response can be obtained by increasing the energy intake during a relatively short feeding period. The mechanism of action may be through a direct effect on the developing follicle or perhaps indirectly through an increase in the release of gonadotropins. A severe restriction in the energy intake does not influence the gonadotropin concentration but will cause a decrease in the weight of the anterior pituitary.

The level of nutrition also affects postpartum reproductive activities. Nutrient requirements are elevated substantially during the last trimester of pregnancy and during lactation. Reproductive functions have secondary priority in the cow and ovarian follicular development and ovulation are delayed in those on low-energy rations either near the end of pregnancy or during the postpartum interval.

8.5.2.3 Response to superovulation

Immature animals are able to respond to superovulatory conditions as soon as the follicle develops an antrum. The sensitivity to gonadotropins develops gradually and at different rates in different species. The ovaries of immature sheep can be stimulated at 16 weeks of age and those of rabbits at 10 to 20 weeks, depending upon the breed and plane of nutrition. The development of embryos into normal young indicates that eggs obtained from immature donors are functionally equivalent to those obtained from adults.

The superovulatory responses of mature animals vary with the species, breed, live weight, stage of estrous cycle, age, postpartum interval, season of the year, and plane of nutrition.

8.5.2.4 Gonadotropin Content

In malnourished, experimental animals, hypophyseal gonadotropin content has been reported as decreased, increased, or unchanged. Plane of nutrition may also effect pituitary size rather than pituitary concentrations of FSH and LH. If the effect of undernutrition is to influence synthesis and release, then gland content does not reflect circulating levels of hormone. Piacsek and Meites have shown that undernutrition reduces the concentration of luteinizing hormone-releasing factor in the hypothalamus. This brings a major regulatory mechanism under the control of nutritional level.

Watanabe et al. demonstrated that actinomycin D and paromycin can block the FSH-releasing action of hypothalamic extracts in vitro without interfering with the resting release of FSH. This, then,

suggests that protein synthesis is required for the action of FSH releasing factor in inducing release of FSH from the anterior pituitary. Undernutrition may then affect protein synthesis, thereby altering hypothalamic releasing factor action on the anterior pituitary.

A high level of nutrition is associated with higher pituitary and adrenal weights, higher ovulation rates, more numerous larger follicles, more follicular fluid, and probably higher LH concentrations in the pituitary. The higher LH concentrations in the pituitary are interpreted as a higher rate of production, whereas Allen and Lamming, finding a higher content of gonadotropins in the ewes on a low level of nutrition, considered this evidence of lack of release of gonadotropins. In this respect pituitary gonadotropin content is not a valid measurement for either production or release unless one also has values for the plasma concentrations of the hormone. The data may indicate that energy restriction prior to mating reduces the number of ovulations and that body weight of the ewe is of importance in affecting the rate of ovulation.

8.5.2.5 Fertility

The incidence of pregnancies in mice can be reduced by caloric restriction after weaning. When full feeding is started at 8 months of age, a high incidence of pregnancies results at up to 12 months of age. The incidence of pregnancy was low at this age in mice kept either on the restricted or on the full-feeding treatment after weaning. Effects of feed restriction on egg-laying age, egg production, egg size, and mortality in hens have been examined. The food intake during the growing period was 70% eaten by the birds fed ad libitum. Results showed:

1. A 2-week delay in onset of sexual maturity.
2. The rate of egg production was higher for the restrictively fed birds, but both full fed and restricted-fed laid the same number of eggs.
3. As to size of eggs, fewer small eggs were laid by the restrictively fed birds than by controls, and mortality for restrictively fed birds was 11.4% against 15% for full fed.

The effects of energy restriction in pigs have been found to depend on the stage of development at which the restriction is started. Reid suggested that one might expect optimal reproductive performance by a sequence of limited feeding prepubertally (to attain earlier puberty and save feed at the same time), a high level of

energy intake for a brief prior to breeding (flushing), and a low-energy (flushing), and a low-energy intake during pregnancy.

Cows on a low level of nutrition or improper quality ration during or just preceding the breeding season require more services per conception for successful pregnancy. This, together with longer intervals from parturition to first estrus, results in later successful conception dates.

8.5.2.6 Litter Size

In the rat, restriction of food intake to either 25 to 50 or 75% of that of controls during days 9 to 20 of pregnancy increased the incidence of resorption of fetuses. The resorption process was an all-or-none response. Transitory, unrestricted feeding reduced the incidence of resorption. Feeding starting on day 8 had the most effect and feeding starting on day 10 had no effect. The data indicate that the period between days 8 and 11 or pregnancy are crucial with respect to the onset of resorption. A maternal gain in energy is observed during pregnancy and is reflected by an increased fat storage and a positive nitrogen balance. These reserves have been suggested to be used during the later part of pregnancy when food intake declines and the conceptuses increase in size.

The nutritional status of the swine dam seems of little significance to the high proportion of embryos that die during gestation. During the first 25 days after mating, pregnancies are maintained on protein-free dieters, low-energy diets, or during brief periods of complete inanition. Restricting metabolizable energy intake throughout gestation has no effect on embryo survival rates and only a slight decrease results in piglet birth weight. There is an increase in litter size between the first and the third gestation, but this is unrelated to the nutritional status of the dam. The results indicate that dams given restricted energy levels deliver more pigs during the second and third gestation than do fully fed controls. Thus there is no beneficial effect on fetal survival. Long term effects of energy intake on reproductive performance through three consecutive gestation periods indicate that the total number of pigs farrowed declines, but birth weight of living pigs increases with increasing levels of metabolizable energy for the dam. Body weight change from breeding to 24 hr postpartum is markedly affected by level of dietary energy intake.

Maternal nutrition in the ewe affects the litter number and the degree of development. The preconception reduction in litter number is compensated for by a weight increase in the individual cotyledons.

Using rabbits with a high incidence of fetal atrophy, Hammond found that restriction of food intake (energy and other nutrients) so that animals showed no weight increased depressed conception rate but increased litter size. Ovulation rates in energy-restricted animals were comparable to rates in animals on a high plane of nutrition, however, the number of fetuses in the uteri was 3.8 for the animals on the high plane and 6.0 for those on the low plane.

8.5.2.7 Gestation Length

Mice that are fasted for 48 hr terminate pregnancy within a few hours after the fasting period. The fasting causes hemorrhage and necrosis of the deciduomata, leukocytic invasion, and liquefication and absorption of the debris. In sheep and mares, a low plane of nutrition tends to reduce gestation length, particularly in late pregnancy and for twins.

8.5.2.8 Mortality

Prenatal development is influenced by several factors: heredity, size, parity, duration of pregnancy, competition between littermates, placental size, and nutrition of the mother. The influence of the plane of nutrition of the sow on embryonic viability is not clearly understood, but a deficiency of specific nutrients doe not appear to be the cause of the high prenatal death rates commonly observed. However, the level of energy intake is established to be related to embryonic survival. High caloric intake increases ovulation and thereby increases the incidence of embryonic mortality prior to implantation. Following implantation, however, fetal death is decreased with unlimited feeding.

The effect of caloric intake on prenatal death in cattle is at present controversial, although hypoglycemia induced by insulin reduced conception rates in lactating cows. This is probably due to embryonic mortality.

In sheep, full feeding before breeding also increases ovulation as well as embryonic death. Most losses between implantation and weaning occur during the perinatal period (a period consisting of before, during or up to 24 hr after parturition at normal term) as a result of starvation of the neonate and of dystocia among lambs born to maiden ewes and ewes on poor pasture.

During the first two thirds of gestation in the ewe dam, growth of the fetus is apparently independent of both the caloric intake of the dam and its litter size. In the last trimester of pregnancy, there

are marked differential changes in fetal weights reflecting variations in genetic factors, litter size, nutritional status, and health of the dam. Undernutrition of the ewe in late pregnancy leads to the production of stunted lambs even though a normal energy level had been present earlier. Conversely, a reversed type of feeding program results in normal-sized lambs. Survival after parturition is best in breeds with longer periods of gestation, as these lambs tend to be heavier at birth and survival is best from birth weights slightly above average. Neonatal mortality may be a result of weakness of the mother or the young, poor maternal behavior, or delayed onset of location. Experiments with turnkeys wherein food intake was restricted between 20 and 34 weeks of age showed no effect on mortality, fertility, or hatchability of fertile eggs.

8.6 VITAMINS

Many of the major metabolic reactions depend upon the presence of a particular vitamin. Obviously, then, the proper functioning of every system including reproductive is vitally dependent on proper vitamin levels, and vitamin deficiencies cause several deleterious effects.

8.6.1 Vitamin A

An interesting discovery in the study of the function of vitamin A, especially with respect to reproduction, has been the interrelationship among three of its forms. These relationships are

Vitamin A alcohol = Vitamin A aldehyde $\rightarrow$ Vitamin A acid

Females rats fed a diet lacing vitamin A alcohol but containing vitamin A acid have normal estrous cycles, conceive normally, but resorb the fetuses during the last one third of gestation. On day 15 or 16 of gestation, necrosis can be observed at the edge of the placental labyrinth; when vitamin A alcohol is given in sufficient quantities pregnancy continues normally. These effects also have been noted in guinea pigs. Excessive intake of vitamin A by pregnant rats causes exencephaly, cleft palates, and inner ear abnormalities in the fetuses.

Intact, female, vitamin A deficient rats show metaplastic changes inteh uterine epithelium, but in ovariectomized rats such changes fail to occur. Castrated, vitamin A-deficient rats treated with estrogen show metaplasia, but if vitamin A is also given, no adverse effects can be noted. In preventing these morphological changes, vitamin A alcohol is effective, but vitamin A acid is not.

In the vagina, vitamin A deficiency causes cornification of the epithelium in intact and spayed rats. This vagina is histologically indistinguishable from that of a rat in estrus. Vitamin A prevents the vitamin A-deficiency-induced cornification and also prevents the effect of simultaneous local application of estrogen on the vagina of spayed rats. The relationship between vitamin A and β-estradiol proved to be a log does response curve. The results may indicate that vitamin A inhibits keratinization through an effect on protein anabolism. In vitro experiments have shown that vitamin A deficiency can cause keratinization of epithelia.

Female chickens fed a vitamin A alcohol-deficient diet but supplied with vitamin A acid lay normally; the eggs are fertile, but upon incubation the embryos die within 2 days. As in rats, injection of Vitamin A alcohol prevents this mortality, and healthy chicks hatch. The problem may be that vitamin A acid either does not reach the egg, or else it cannot be utilized by the embryo.

Cows deficient in vitamin A may produce calves with low viability and have fetal resorption or fetal abnormalities and keratinization of the vaginal epithelium predisposing them to infection. Vitamin A causes irregular estrus, anestrus, retardation in prenatal development, and lowered viability of the neonate in swine.

Hypervitaminosis A in immature, male rats causes complete degeneration of the germinal epithelium and a decrease in weight per 100 g body weight of seminal vesicles and prostate. In adult males these effects are either absent or reduced. An organ cultures of testis tissue also has proved sensitive to overdosage with vitamin A.

Short-term (5-week), vitamin A deficiency in the 30-day-old rat increases most testis lipids at a time when no changes in weight or morphology are evident. The lack of vitamin A deficiency results in a reduction in testis phospholipid that is somewhat less than that observed with a lack of vitamin E.

Adult, male rats and guinea pigs on diet that is vitamin A deficient but includes retinoic acid show degeneration of the germinal epithelium with some spermatocytes and spermatogonia remaining. Leydig cells also decrease in number. On refeeding, the epithelium can regenerate itself. Neither FSH nor testosterone injection could prevent the degeneration of the seminiferous epithelium. The castration cells in the pituitary seem to be secondary to the degeneration of the germinal epithelium.

Vitamin A deficiency induces metaplastic keratinization of the epithelium lining the male accessory sex glands. Retinoic acid prevents the pathology without correcting the organ atrophy. Young bulls deprived of vitamin A have al low intensity of sexual behavior at their normal breeding age. Gross deficiencies of vitamin A result in low sperm numbers, a high proportion of abnormal sperm, and degeneration of germinal epithelium of the seminiferous tubules. The same effects are observed when a vitamin A-deficient diet is fed to mature rams. Morphologic abnormalities attributable to vitamin A deficiency appear earlier in rams on an otherwise high plane of nutrition.

Vitamin A deficiency in roosters decreases the number of sperm produced and increases the percentages of nonmotile and abnormal sperm. Roosters fed a Vitamin A deficient diet but containing Vitamin acid, however, appear to be normal with respect to testicular histology and fertility. In direct contrast to other results, Nockels and Kienholz found that the bird testis is stimulated by Vitamin A deficiency with an increase in weight and the presence of testis sperm at an earlier age than in vitamin supplemented birds.

Palludan injected vitamin A into the testes of vitamin A-deficient boars and observed local effect on the histology of the germinal epithelium. From these experiments it has been suggested that vitamin A has a direct effect on the seminiferous tubules. Scott and Scott found that gonadotropin injections can reverse the effects of vitamin A deficiency in cats, which suggests that the deficiency affects pituitary gonadotropin secretion. In rats, however, testicular lesions caused by vitamin A deficiency cannot be prevented by either FSH o testosterone, although atrophic accessory sex organs are stimulated. The endocrine deficiency remains unclear, and perhaps consideration should be given to the fact that prolonged vitamin A deficiency causes a significant reduction in the thyroid secretion rate.

8.6.2 Vitamin E

Female rats on vitamin E-deficient diet have normal estrous cycles and mate normally; the blastocysts implant, but resorption starts at about day 3 of pregnancy. In sheep and cattle, simple dietary vitamin E deficiency does not affect reproduction.

Turkey hens on a diet deficient in vitamin E show normal egg production or fertility but decreased hatchability. Embryos are smaller than normal and lens defects are present. A diet high in linoleic acid and without antioxidants causes a decrease in egg production,

low fertility, and 100% embryonic mortality; either ethoxyquin or vitamin E can prevent these disturbances of reproduction. Vitamin E may act as a biological antioxidant rather than as a vitamin, for other antioxidants have the same effect.

Vitamin E deficiency in male rats causes irreversible damage to the germinal epithelium. Histologically, vitamin A and vitamin E deficiency damage to the testis is quite similar, but the effects of lack of vitamin A are reversible. Seminiferous tubular damage may progress to the point where only Sertoli cells remain, but the Leydig cells are not influenced. A regeneration of damaged testes in hamsters after vitamin E supplementation was demonstrated by Mason. The reversibility of vitamin E deficiency-induced testicular damage in guinea pigs and roosters has not been established. Little or no effect from vitamin E deficiency has been noted in the rabbit, mouse, or livestock such as bulls, rams, and goats.

Changes in the rat testis tissue during vitamin E deficiency are quite numerous and include a decrease in glycogen, aspartic transaminase, esterase, and β-galacuronidase. Testis lipid and phospholipid are reduced in the degenerate testis. Increasing the dietary level of saturated, medium-chain triglycerides exerts a protective effect against vitamin E deficiency in rats but not in chickens. At the pituitary level, vitamin E deficiency causes changes described as a "partial castration syndrome." Administration of FSH and LH increases testis weight in the vitamin-E deficient rat, but testosterone has an adverse effect. Furthermore, FSH in creases the testis phospholipids in an action counter to that vitamin E deficiency. Chorionic gonadotropin administration to vitamin E deficient rats restores accessory sex organ weights.

The effect of vitamin E deficiency in roosters is the presence of atrophic testes, but the incidence is not 100%. This effect is observed after prolonged (2-year) vitamin E deficiency. Arscott et al. fed rosters a diet containing 7% linoleic acid, which resulted in a decrease in fertility in about 5 weeks and near the end of the experiment fertility had dropped to zero. Sperm concentration markedly decreased duirng the experimental period. The effect of the high linoleic acid intake could be prevented by feeding of the antioxidant ethosyquin or of vitamin E.

8.6.3 Vitamin D

Excessive vitamin D_2 in rats has several effects; it impedes fertilization, changes the estrous cycle, alters the implantation process,

and causes resorption of implanted embryos if administered to rats before mating or during the first 5 days of gestation. Gestation is normal when administration of the vitamin is started after day 5 of pregnancy.

Vitamin D deficiency in laying hens results in reduced egg production, smaller eggs, decreased shell thickness, and increased incidence of blood spots in the eggs, although the ovaries are normal and the hens seem to be in good health. The effect of vitamin D deficiency in roosters are depression of testicular weights and of secondary sex characteristics. vitamin D-deficient roosters also weight less, which has been suggested to reflect a lower feed intake. This makes results from vitamin D deprivation in rooster difficult to distinguish form those caused by lowered energy and protein intake.

8.6.4 Ascorbic Acid

The effect of ascorbic acid deficiency in monkeys consists of atresia and degeneration of follicles, regression of the corpora lutea, and hyperplasia of the endometrium. If the deficiency occurs during early pregnancy, abortions and resorptions may occur; during the late pregnancy the effect consists mainly of premature delivery.

Ascorbic acid deficiency is of importance only in the relatively few species for which this act has to be supplied in the diet (i.e. guinea pigs, monkeys, man). Deficiency of this vitamin causes degeneration of the germinal epithelium starting near the center of the seminiferous tubule and progressing toward the periphery. The Leydig cells are not affected. Chronic lack of ascorbic acid in the laboratory rodent will cause a degeneration of both Leydig cells and seminiferous tubules. This acute deficiency is accompanied by changes in RNA, DNA, and total nitrogen per testis cell. Most of the effects, however, have been found to be caused by undernutrition.

8.6.5 Thiamine

One of the effects of thiamine deficiency in the female rat is an abnormal estrous cycle. If the female becomes pregnant, resorptions occur, gestation is prolonged, and many of the young born are not viable. Thiamine deficiency in rats causes smaller than normal testes, small diameters of the seminiferous tubules, abnormal spermatogenesis, underdeveloped Leydig cells, and small seminal vesicles, but there is still some androgen secretion, as indicated by the normal ventral lobes of the prostate and the ductus deferens. The addition of ascorbic acid to the diet largely prevents these effects.

Associated with these testicular effects, vitamin B_1-free diets in male rats decrease pituitary gonadotropins. Response to chorionic gonadotropin is obtained so that fructose and citric acid levels are restored to normal despite the need for thiamine in carbohydrate metabolism. The relationship between thiamine depletion and exogenous hormone response is still not clear.

Thiamine interactions in the testes have gained some importance since it was found that radioactive thiamine is largely incorporated in rat testis mitochondria and soluble fractions and that these fractions are responsive to B_1 depletion.

8.6.6 Vitamin B_{12}

Vitamin B_{12} insufficiency causes degeneration of the germinal epithelium, reduction in testicular size, and a decrease in the number of Leydig cells of rats and reduces testicular size in cockerels. Associated with this decrease in Leydig cell number is a subsequent delay in maturation of the seminal vesicles.

Vitamin B_{12}-deficient female rats have reduced ovulation rates and atrophic ovaries and uteri. In pregnant rats the deficiency results in hydroencephaly and malformation of the eye of the fetuses. In chicken embryos, B_{12} deficiency decreases the cephalin and sphingomyelin. Embryonic mortality occurs so early that it appears the eggs had not been fertilized.

8.6.7 Pyridoxine

Lack of pyridoxine in female rats and mice results in the presence of normal, mature follicles, but atretic follicles are found. While the interstitial cells are atrophic, the cycles are irregular. No pair-fed controls were used.

In the above mentioned experiments on mice, the same effects are found in the pair-fed controls. Pyridoxine might not be required for normal gonadal function except in the sense that the deficiency reduces food intake, Egg production in chickens is affected more severely by deficiency of pyridoxine than is hatchability. Injection of desoxypyridoxine or methoxypyridoxine to induce pyridoxine deficiency inhibits early embryonic development.

Prolonged feeding of pyridoxine-deficient diets to adult and immature rats causes lesions in the seminiferous tubules and Leydig cells and reduces testicular weight to 50% of that of pair-fed controls. The secondary sex organs are small, indicating low androgen secretion. Mild deprivation of pyridoxine for 3 weeks causes an

inhibition of testicular growth in immature rats that involves the seminiferous tubules and interstitial tissue. Severe vitamin deficiency to immature rats for 2 months invokes tubular degeneration involving all but he Sertoli cells and is not mimicked by inanition.

The influence of pyridoxine deficiency on the gonad is at least in part a reflection of changes in the hypophysis. The testis responds to testosterone and gonadotropin treatment. The pituitary involvement in the male may be anticipated form prior studies in female rats. In B_6-deficient female rats, a tenfold increase in hypophyseal FSH and a slight increase in LH content occurs. Nevertheless, the gonad may be directly involved, as suggested by the decreased sensitivity of the ovary to FSH.

8.6.8 Biotin

Biotin deficiency shows atresia of follicles in rats and mice but in the rat the estrous cycle seems to remain normal. Although the evidence is incomplete, it appears that biotin deficiency does not affect gestation adversely.

In chicken eggs, deficiency of biotin causes high embryonic mortality on the third day and toward the termination of the incubation period. The embryos have crooked tibia, twisted tarsometatarsi, and parrot beaks. Biotin-free dieters fed to adult, male rats cause cryptorchidism, retarded testicular development, and damage to the adult male organ. Seminal vesicle weight is subnormal. Testosterone hastens the development of the vitamin deficiency and enhances the severity of the biotin deficiency. Lack of biotin in the diet has affects similar to thiamine deficiency in the male reproductive system, but the effects on the germinal epithelium can be distinguished histologically. In thiamine deficiency, the primary spermatocytes do not undergo chromatin reduction, whereas in the case of biotin deficiency, there is condensation of multinucleated giants cells, a phenomenon which also occurs during either vitamin E, riboflavin, or pantothenic acid deficiency. Lesions of the paraprostatic ganglion are found in the case of biotin deficiency and also in the case of vitamin E deficiency.

8.6.9 Folic Acid

A period of folic acid deficiency as short as 48 hr in female rats between days 7 and 12 of pregnancy results in 70 to 100% abnormalities in pups. Some of these defects are edema, cleft palate, and abnormalities of the paw and lung. Chicks embryos exhibit bends

in the tibiotarsus, syndactylism, and beak defects. Independent of inanition, folic acid deficiency diminishes the response to estrogen, probably because the deficiency prevents adequate nucleic acid synthesis. Oviductal response to estrogen and progesterone in folic acid-deficient rats can be obtained after administration of DNA.

Lack of folic acid n the rat has no prominent effects, divorced form inanition, on the testis. Terroine and Lys observed a reduction in testicular 5-ribonucleotidase following folic acid deficiency in rats and guinea pigs, and they found seminal vesicles and prostates reduced in size. The response of the testis to PMSG is diminished but the response of the prostate and the seminal vesicles to androgen is normal. In cockerels folic acid deficiency causes a decrease in the tubule diameter of the testes.

8.6.10 Riboflavin

The ovaries of riboflavin-deficient rats are atrophied and lack corpora lutea, the rats are in anestrus, and the uterus is atrophied. Pregnancies have been obtained in rats after instituting a riboflavin-deficient diet a few days prior to mating so that the effect of the deficiency can be studied with respect to pregnancy. Resorptions are found in riboflavin-deficient rats.

Riboflavin deficiency in male rats causes a reduction in testicular size, damaged to the germinal epithelium (the damage being more severe than in the case of thiamine or biotin deficiencies), and atrophy of the Leydig cells, the seminal vesicle, and prostate.

Chicks embryos form eggs deficient in riboflavin show a high mortality rate. Embryos from such eggs show edema, degeneration of the Wolffian bodies, "clubbed" down and dwarfism.

8.6.11 Pantothenic Acid

Deficiency of pantothenic acid causes delayed puberty in rats. In sexually mature rats, the deficiency results in smaller ovaries that lack mature follicles, atrophy of the uteri, and permanent anestrus. During pregnancy it leads to abortion and malformations. The incidence of defects can be decreased by the addition of ascorbic acid to the diet.

The lack of pantothenic acid in avian embryos results in high embryonic mortality at 12 to 1 days of incubation. The embryos show subcutaneous hemorrhages and severe edema.

Pantothenic acid is required for normal testicular funciton, and deficiencies cause the appearance of multinuclear giant cells in the

seminiferous tubules. Deficiency also results in reduction in size of seminal vesicles in rats and mice. Supplements of pantothenic acid have resulted in a stimulation of spermatogenesis and of seminal vesicle weight. Furthermore, testicular coenzyme vitamin A content increased in response to supplements of the vitamin.

8.6.12 Niacin

In a diet deficient in niacin, mortality of chicks is 100%. The embryos show hypoplasia of the musculature, generalized edema and, sometimes shortened upper beaks.

8.7 MINERALS

Information on the effects of mineral deficiencies on reproductive performance is scarce compared with other areas reviewed, but experiments will nevertheless be cited dealing with effects of some of the more common mineral restrictions.

8.7.1 Zinc

Zinc deficiency has an effect on the testis that is more marked in the prepubertal state. A moderate zinc deficiency causes subnormal testis size and atrophy of the seminiferous tubule. Severe zinc deficiency in the rat causes marked atrophy of the germinal epithelium with a reduction in the testis zinc content. Seminal vesicles and prostates of the deficient rats are also small. Changes in the gonad are not due to inanition and are not reversible when zinc is added to the diet. The deficiency has been found not to alter protein synthesis in the testis but appear to increase protein and RNA catabolism.

Administration of gonadotropin to immature rats stimulated the testis despite a zinc-deficient diet and enhanced the uptake of Zn into the testis following hypophysectomy. In the adult, however, zinc is required for spermatogenesis as tubular atrophy, typical of zinc deficiency, develops in spite of gonadotropin treatment. These results would suggest that the reduction in growth rate and development of immature testes in zinc deficiency is primarily due to a pituitary hormone deficiency.

Despite severe zinc restriction, Holstein bulls exhibit only a temporary reduction in testis size and no effect on semen volume or sperm motility. However, sufficient Zn for incorporation of high concentrations into spermatozoa during final sperm maturation seems essential to spermatogenesis. An influence of zinc deficiency on the testis has been observed in mice, calves, lambs, and dogs.

8.7.2 Calcium and Phosphorus

Calcium and phosphorus deficiencies can cause infertility in the rat. Low phosphorus intake reportedly will arrest spermatogenesis in the adult rat, but the inanition effects have not been closely examined. A low -calcium diet for 10 weeks does not influence spermatogeneis but does reduce the number of Leydig cells as well as their esterase activity. The Leydig cells do respond to exogenous gonadotropin treatment.

Reproduction may depend upon the actual levels of calcium and phosphorus fed as well as the Ca:P ration. Reproduction is poor when levels of either element are high, regardless of the ratio.

Lack of calcium has not been observed to affect the gestation of either the rat or the pig. If such a ration is fed to rats for several generations, however, decrease in fertility and an increase en embryonic mortality are found.

Hens on a calcium-deficient diet produce eggs if given gonadotropin, but the birds die with severe skeletal defects. Hens that lay eggs without shells ovulate at a higher rate than normal hens and will do so even when on a severely calcium-deficient diet.

In range cattle, phosphorus deficiency causes ovarian dysfunction (which in turn results is delayed puberty and an increase in quiet ovulations), irregular estrus and eventually, complete cessation of estrus. A high calcium intake may bring about reduction in phosphate utilization. Phosphorus deficiency apparently has little effect in pigs.

8.7.3 Molybdenum

Although important in body processes (i.e., flavoprotein enzymes), no information is available to relate a molybdenum deficiency to testis function. An excess of dietary molybdenum will, however, result in a lack of libido in young male bovine. The testes of these animals exhibit tubular damage with little or no spermatogenesis and damage to the Leydig cells. Fertility in the female is not affected.

8.7.4 Cobalt

Cobalt is required for the synthesis of vitamin B12 and a deficiency in cobalt is widespread. Cobalt deficiency hinders reproduction, but the mechanism is unknown.

8.7.5 Copper

Low fertility in cattle has been associated with copper deficiency in several areas. However, the content of copper in animal tissues

form different parts of the world varies greatly. Thus interactions of copper with molybdenum and sulfur and those of iron utilization may all play a role. Copper and cobalt deficiencies result in reduced viability of the offspring in ruminants. Copper is apparently involved in the myelinization of the fetus for lambs, and calves born from copper-deficient dams show degeneration of the brain and motor ewes.

8.7.6 Potassium

Low-potassium diets (0.01% K) causes a prevalence of abnormal spermatozoa and a lowered sperm motility and result in sterility, although matings do occur. Lack of potassium in female rats results in resorptions and abortions, which can be prevented by estrogen and progesterone treatment.

8.7.7 Sodium

Sodium deficiency delays spermatogenesis in rats so that at 99 days only a few, primary spermatocytes are evident, whereas spermatogenesis is complete in pair-fed controls. The deficient rats also have smaller secondary sex organs than pair-fed controls. The reproductive system of the female on a sodium-deficient diet is characterized by normal ovarian weight, with a few corpora lutea and fewer large follicles, probably accounting for the weight difference. The vaginal epithelium is multilayered and the lumen is filled with keratinized cells and leukocytes; the uteri have columnar epithelial cells which show secretory activity. Cycles are irregular and fertility is reduced.

8.7.8 Manganese

Manganese deficiencies in rats and rabbits can reduce libido and invoke testicular damage and sterility. After 90 to 100 days of age, sperm motility is reduced and tubular degeneration occurs thereafter. At 9 months the tubules lack spermatids, and desquamated cells appear in the epididymis. The manganese requirement for these rodents is 0.05%. Gilts or cows fed a manganese-deficient diet experience ovarian disturbance ranging from weak signs of estrus to anestrus.

8.7.9 Iodine

The influence of hypothyroidism on the testis si more pronounced when induced before puberty, although the dog and possibly the guinea pig may not be markedly affected. Cretin offspring exhibit

marked retardation of testis development, the tubules containing only spermatogonia and a few spermatocytes. The Leydig cells are atrophic. In the adult, a decline in libido and a reduction in semen quantity have been noted in iodine-deficient bulls and stallions. A seasonal reduction in semen quality in rams has also been associated with a mild hypothyroid state.

8.8 FETAL NUTRITION AND METABOLISM

The supply of nutrients in prenatal life is achieved in four stages. In the first, the cleaved egg obtains its nutrition from its own duetoplasm, which is only temporary in farm animals. In the second, the blastocyst absorbs fluids and nutrients from the uterine luminal fluid. As the blastocyst increase in size, it can no longer absorb sufficient uterine fluids by diffusion. The third stage, the histotropic nutrition, is aided during implantation by the vitelline circulation in the yolk sac and the trophoblastic cells. Fluid, unaltered fats and tissue debris (endometrial fragments) may be engulfed at this time by phagocytosis. Lastly, after the formation of the placenta, absorption of nutrients from the maternal circulation occurs across the placental membrane.

The fetus may be regarded as a parasite living within the mother and is assumed to have priority in the event of insufficient maternal nutrition so that its development can proceed unimpaired. It is known, however, that the fetus is most likely to suffer from maternal undernutrition when it occurs toward the end of gestation.

Proteins as such are not transferred via the placenta. Amino acids cross readily against a concentration gradient; a high fetal-maternal amino acid ratio exists in sheep (2 to 3:1). A concentration of amino acids by the placenta occurs during transport. The accumulation occurs on the maternal side, and the transfer from trophoblast to the fetal circulation takes place by simple diffusion down a concentration gradient. Immunoglobulins are transmitted in man and some animals but not in farm animals. This can be explained by structural differences in the various placental types.

The lipid content of maternal blood is higher than in the fetus in sheep and pigs fed a high-fat diet. The placenta is not permeable to fat as such, but constituents, namely fatty acids and glycerol, pass freely.

In ruminants, the fetal blood sugar level is higher than in the mother. Peculiarly, fructose comprises about 70 to 80% of the sugar in fetal blood while glucose is predominant in maternal blood. In

sheep the rate of transfer of C^{14}-labeled glucose form mother to fetus is about 10 mg/min and varies with the level of glucose. The placenta converts a ceratin fraction into fructose, which is then transferred to the fetus independently. C^{14}-labeled fructose injected into the fetus crosses the placenta in very small amounts.

Throughout gestation, retention of calcium, phosphorus, and iron increases in relation to fetal body weight as well as absolutely. In addition, the fetus has the unique ability to deplete maternal, skeletal stores of calcium if feeds are very low in calcium. Iron is used for hemoglobin synthesis, but little is known about its distribution and metabolism. Copper readily traverses the bovine placenta. Manganese is found in the fetal calf liver but apparently does not accumulate.

After birth the continuous supply of glucose and other nutrients obtained from the mother is severed. To prepare for the new environment, the fetus accumulates large amounts of glycogen in the liver and both the skeletal and cardiac muscles during the later part of gestation. These carbohydrate reserves are utilized rapidly after birth, the rate of decline occurring much faster at lower environmental temperatures. Thus any undue stress puts the neonate under a severe nutritional requirement that must be overcome for it to survive.

8.9 CONCLUSION

From the material reviewed it is apparent that the development and functioning of the reproductive organ depends upon the animal's plane of nutrition. Nutrient deprivation has various affects, depending on the time the diet is initiated and duration of the deficiency. In many circumstances the duration of feeding determines the degree of reversibility of the effects from deprivation.

The involvement of the endocrine system in nutrition's affects on the reproductive organs has been shown in many of the experiments reviewed. Starvation as well as feed excess may cause a failure in the mobilization of neurosecretory material from the median eminence leading to interruption of reproductive functions. It is interesting that fasting for short durations does not cause as marked an effect as prolonged inanition. This may bed due to the long duration of spermatogenesis and sperm transport from the testes to the ductus deferens.

Inadequate supplies of protein or specific amino acids and poor-quality protein both have detrimental effects on both male and female

reproductive organs. These two factors, protein supply and quality, determine to some extent the onset of maturity and fertility. In the female a certain threshold level of protein is required for maintenance or pregnancy until its midpoint and has been suggested to be necessary for the development of the corpus luteum until placental development is achieved. Protein requirements for various animals differ, as evidenced by the fact that in swine as little as 5% protein is sufficient for maintenance of litter size, whereas in rats a diet containing less than 6% protein results in resorption of fetuses in 85% of females. Interestingly, rats fed protein-deficient diets show reduced plasma, and pituitary levels of prolactin and injections of progesterone, prolactin, or corticosterone counteract the effects of protein deficiency.

Essential fatty acids (EFA) are necessary for proper reproductive functioning, and deprivation causes serious reductions in fertility. In contrast, a high level of EFA (e.g., linoleic acid) without vitamin E activity as an antioxidant impairs fertilizing capacity. However, this effect is reversible.

The majority of the experiment cited in this review deal with the effects of restricted feed intake on reproductive performance. It is apparent form experimental results that body size and food intake are, to a large extent, determinants of the onset of puberty. In the male, caloric restriction has marked effects on the prepubertal animal, slowing down or diminishing androgen production. These changes may result from the influence of under feeding on testis enzymes involved in the biosynthesis of sex steroids or a reduction in the passage of gonad-releasing factors from the hypothalamus to the anterior pituitary.

In the female, feed restriction also delays onset of puberty. In both sheep and cattle, age at puberty is influenced by the animal's percentage of adult body weight. This relationship is nonexistent in swine, as age is probably more important here. Ovulation as flushing. The mechanism for this effect may be through a direct action on developing follicles or indirectly through increasing the release of gonadotropins.

Vitamins are involved in many of the major metabolic reactions taking place in cells and their importance to reproduction is well documented. It is interesting to note that vitamins effect on reproduction depends upon the time in life that they are withdrawn from the diet. Vitamins E, D, and C, when deficient in the diet

during pregnancy, all cause defects in the embryo or loss of the fetus.

The last section "Fetal Nutrition and Metabolism" is meant to be only a very short review of some of the current literature. Obviously, all the topics covered throughout the chapter, i.e., protein and lipids, could be examined in the final section. The lack of proper maternal nutrients toward the end of gestation causes adverse effects on final fetal development. Unquestionably, as the fetus is a parasite throughout gestation any severe nutritional inadequacies on the maternal side will to some extent effect fetal nutrition. Obviously, then, diet should be under strict control prior to and during gestation to ensure low fetal morbidity and high rates of fetal survival.

9

Nutrition, Regeneration, and Repair

Problems related to growth, and in particular, regeneration are among some of the most interesting in contemporary biology. During the development of an organism, cellular activity proceeds in a highly organized manner, the end result begin a form in which individual cells are able to survive.

Any drastic alteration in the organism such as loss of a part or essential structure stimulates the process of regeneration to restore the organisms. If the regenerative potential is lost, then the organism may perish.

Although the world is not ideal, "regeneration" is the best general term to cover all types of restoration of body form. Vital structures cannot regenerate because, by definition, their loss cannot be endured for a period long enough to permit their regeneration. The best known form of regeneration is redevelopment *in situ* of a lost appendages, as in Crustacea or Amphibia which is an example of epimorphic regeneration, a vertebrates have lost. This relatively slow form of regeneration is characterized by dedifferentiation of the formed tissues in the remaining stump, resulting in mesenchyme-like cells which migrate distally into a mound called the blastema. Grown and differentiation of the blastema then reforms the missing part. Epimorphic regeneration is a developmental feature confined to those creatures in which the loss of an extremity does not constitute an immediate threat to life. However, tissue regeneration such as occurs in wound healing and repair is of prime importance

to man and is a simpler, more rapid phenomenon which gives rise to a less perfect repair than epimorphic regeneration. Nevertheless, tissue regeneration involves the same process of cell dedifferentiation, proliferation, migration, and differentiation. According to Needham, wound healing is epimorphic regeneration on a small scale. Compensatory hypertrophy and hyperplasia of the liver, to which the term regeneration is generally applied, are really exaggerations of the normal processes of growth and cell renewal.

9.1 REGENERATION IN LOWER ANIMALS

The relationship between nutrition and regeneration was first studied by Zeleny in a series of brilliant experiments. He showed that the increase in size of the regenerating arms of brittle star and crayfish legs is partly determined by the number of appendages removed. The more parts removed the faster each regenerates. In discussing the possible relationship between this result and the food supply, Zeleny pointed out that the larger the number of appendages removed, the greater is the temporary surplus of food, since the amount necessary to nourish the entire leg may be greater than that used at first in the growth of the small new part. Morgan was careful when comparing regeneration in well-fed and starved organisms to distinguish between rapidly of development of the new part and its size. He found that the formation of new parts in the earthworm and salamander takes place at the same rate, whether the animal is fed or starved, provided there is still enough nutrient for the formation of new material. Nevertheless, the size of the new part is affected by the amount of food in the same way as the rest of the body. Additionally when starvation has gone beyond a certain point, regeneration is delayed or stopped before the animal perishes form hunger. Morgulis found an abundant supply of food was favorable for regenerating worm in that fed worms regenerate more segments and longer tails than unfed worms. Curiously enough, alcohol was found to influence the rate of regeneration, depending upon the strength of the solution. A weak solution exerted a stimulating effect and a stronger one an inhibiting effect.

Since regeneration of an organ or part of an organism is the function of the organism as a whole rather than a local one at the site of injury, it might be expected that nutrition would inevitably modify the regenerative response; however, this is not so, The ability of lower animals to regenerate limbs and vital parts despite restricted diets shows considerable variation, and there are many unquestionable

cases on record in which no definite relationship could be found between food supply and regeneration. The experiments of Morgan showed that planarians in the last stages of starvation still draw upon their emaciated bodies to make good an amputated part. Also, Ellis found that fasting tadpoles could regenerate as well as their fed counterparts. More recently, Hui and Smith reported that *salamanders* unfed for 10 weeks regenerated hind limbs fully and as rapidly as controls, despite a 50% loss in body weight. Obviously, much must depend upon the nutritional reserves available for the formation of new material. If the reserves are completely exhausted, as show in plants by Kupfer, no regeneration takes places.

Biological studies have confirmed the importance of nutrition for regeneration in a variety of creatures. Using populations of sponges of known age and initial size growing under standard conditions, Rasmont has shown that feeding speeds up the onset of gemmulation, as compared with starved sponges. Comparing diets of *Staphylococcus aureus* and *Escherichia coli*, it was noticed that although *S. aureus* accelerated the onset of gemmulation more than did *E. coli*, it is a diet of *E. coli* that allows the largest yield of gemmules.

Nutrition exerts a profound influence on regeneration of hydra. Certain body regions of hydra appear to possess a low regenerative capacity, but, according to Burnett, this may be a nutritional effect, since rich feeding with brine shrimp instead of *Daphnia*, the traditional food of the animal, increases the regenerative capacity. However, Kass-Simon and Potter have reported on arrested regeneration in the budding region of hydra as a result of abundant feeding. When such budding animals are transected in the vicinity of the bud head, regeneration is significantly delayed. The results suggested that large quantities of food cause the tissue of the budding region to assume a budding condition, which simultaneously renders it temporarily incapable of responding to a wound stimulus.

The insect *Rhodnius* is able to regenerate the distal parts of its legs. Maximal regeneration has been found when the insects were fed 5 days after amputation at a time when the mitotic rate is maximal. The effect of fasting on regeneration in the African lungfish, which is capable of prodigious growth and full limb and tail regeneration, has been studied by Conant. In the fasting lungfish, the regenerating pectoral limb shows healing and latent phases comparable to well-fed controls, but growth itself is considerably

slower and averages only 50 to 60% replacement after several hundred days. This slowing of regeneration is of particular interest because lungfish are capable of prolonged fasting and may survive 6 months without food in estivation.

9.2 REGENERATION IN MAN AND OTHER ANIMALS

Most studies dealing with the effect of nutrition on regeneration are concerned with metabolic adaptations to starvation or protection deprivation. The latter can be more accurately designated protein-calories malnutrition, since animals invariably respond to protein deprivation by reducing their overall dietary intake. It is clear that many of the metabolic processes essential for cell proliferation are adversely affected by food deprivation and protein-calorie efficiency. Observations on the liver indicate that the generation of chemical energy is impaired by a diminution of mitochondrial respiratory capacity, decreased hepatic gluconeogenesis, and a reduction in liver enzymes. In plasmic and nuclear constituents, including protein, RNA, polyribosomes, and DNA.

The protein content of the liver is dependent upon the dietary intake of protein. Rats transferred from a stock diet to a protein-free diet lose one third of their liver phospholipid, and nucleic acid. Total RNA is reduced, as in transfer RNA, and there is an increased breakdown of messenger RNA.

Abundant evidence shows that the number of hepatic ribosomes and their aggregation into polyribosomes is regulated by the dietary supply of amino acids. A deficient amino acid supply results in a breakdown of polyribosomes and a loss of cytoplasmic RNA, which can be correlated with a decreased rate of protein synthesis.

The most severe effects of food deprivation on DNA synthesis occur in early life in the period of maximum growth, when malnutrition results in a proportional reduction in DNA in all organs except the brain and lungs. A prompt and sustained decreases in DNA synthesis in the hepatocyte nuclei of growing rats follows a protein-free diet. This is in contrast to mitochondrial DNA, which at first remains stable prior to increasing to more than double the control value, an adaption which favors preservation of function at he expense of cell proliferation. The tissues most frequently employed in experimental studies of regeneration are the discontinuous replicators with their slowly expanding cell populations such as the liver, kidney, and salivary glands. Most tissues in this category are "conditional renewal" systems in which surgical depletion of tissue

mass induces proliferation of the remaining remnant of tissue. Par excellence, this type of system is provided by the liver and to a lesser extent by the kidney, in which cell proliferation following unilateral nephrectomy is much less marked.

9.2.1 Liver Regeneration

Hepatocytes are endowed with a remarkable latent capacity for proliferation. In the rat, resection of two main lobes, comprising about 67% of the liver, is followed by a burst of proliferative activity which reaches a peak after about 28 hr. This precisely regulated process in the residual hepatic lobes, generally referred to as regeneration, is one of compensatory hypertrophy and hyperplasia. Under controlled conditions, this proliferative response is reproducible but may be modified by a number of factors, including diet.

Early studies found liver restoration became impaired when food was restricted. However, Perez-Tamyo et. al reported that liver restoration was not affected by semistarvation, while Doljanski et al., considered liver restoration after partial hepatectomy in starved rats to be similar in extent and pattern to that occurring in normally fed animals. However, Doyle et al. found that he weights of regenerated livers in fed and starved rats, when expressed as a percentage of the liver weights of pair-fed controls, were significantly greater in fed rats. This observation was confirmed by Stirling et al. who found that the proliferative response after partial hepatectomy is inhibited in starved rats.

Recent studies on the effect of protein deprivation on liver regeneration have yielded conflicting results. Deo et al. found higher labeling and mitotic indices in protein-deficient rats after partial hepatectomy when compared with control rats. This was thought to indicate a prolongation of the DNA synthetic phase. Montecuccoli et al. reported that regenerating liver from rats fed a protein-free diet incorporated ^{3}H-thymidine into DNA at about the same rate as did control rats. However, Dallman and Manies and Siimes and Dallman showed that DNA synthesis decreased to less than one half of control values in protein-deficient rats and was less than normal in protein-deficient rats after partial hepatectomy. These observations were confirmed by Stirling et al., who found that in rats fed a protein-free, calorie-rich diet, DNA synthesis is reduced, the mitotic index is diminished, and the proliferative response is impaired.

Partial hepatectomy induces a series of metabolic changes preceding the onset of regeneration, including hypoglycemia, loss of

hepatic glycogen, and accumulation of lipids in the liver remnant. The possibility arises that inhibition of the proliferative response in starved rats after partial hepatectomy is an energy effect; however, such inhibition, according to Stirling et al., is not affected by the administration of glucose or glucogon. In fact, the administration of massive supplies of glucose and fructose sufficient to prevent the decrease in blood glucose and hepatic glycogen content also suppress liver-cell mitosis. This observation is consistent with the observation of Bengmark et al. that a large oral intake of glucose results in retardation of weight restoration of the regenerating rat liver. It is also consistent with the finding of Simek et al., that a continuous intravenous administration of glucose causes some suppression of DNA synthesis after partial hepatectomy.

The reason for this might be the change in energy metabolism of the liver following hepatectomy. According to Simek and Sedlacek, energy metabolism is shifted from a predominant utilization of glycides to an increased utilization of lipids, with a corresponding decrease in the content of glycogen and an increase in the content of lipids. Interference with this shift in energy metabolism by the injection of insulin and glucose results in a decrease in the regenerative capacity of the liver.

Liver cells can be stimulated to divide without removing part of the liver. Leduc found that in young weanling mice, mitotic activity in the liver ceases during starvation and reappears upon subsequent re-feeding. These results are in accord with those of Morpurgo, who conducted similar experiments with young rabbits. In adult mice, Leduc found that re-feeding after fasting is also followed by a wave of mitotic activity in the liver. Furthermore, after a short period on a low-protein diet, a change to a higher level of dietary protein is followed by a similar wave of mitotic activity. The time of appearance of the peak of mitotic activity was influenced not only by the amount of dietary protein but also by the duration of the period on a low-protein depletion.

Short and colleagues reported DNA replication and cell division in the livers of rats changed from a protein-free diet to one containing protein or amino acids. The extent of the stimulation was, up to a point, related to the level of protein. Protein or amino acids in the preparatory diet blocked the response of the liver to the second diet. A diet lacking tryptophan and lysine had no effect and induced only little DNA formation when it was supplemented

with only one of the amino acids. The addition of both amino acids evoked a good response. The formation of liver DNA after the nutritional shift began several hours earlier than after partial hepatectomy.

Present knowledge of adaptation to protein-calorie deficiency on the part of hepatocytes offers little in the way of explanation for this remarkable stimulation of DNA synthesis and mitosis. The factory common to both the protein-deficient rat fed a high-protein diet and the partially hepatectomized rat is that the capacity of the liver to metabolize amino acids is exceeded, and it appears that some how an excessive amino acid burden is translated into a stimulation of DNA synthesis by a direct or an indirect effect on the nucleus. The hypothesis offered by Short et al., is that an accumulation of products of amino acid metabolism induces the critical changes of the DNA pre-replicative period, perhaps by altering gene function.

9.2.2 Compensatory Renal Hypertrophy

Removal of one kidney from a laboratory animal or man is followed by compensatory growth of the other. Control of this growth has been studied for more than century, but there is still no clear explanation of the mechanism involved. Compared with the rapid and intense restorative activity of the liver after partial hepatectomy, compensatory growth following unilateral nephrectomy is slow. Although kidney visible. The process is one of compensatory hypertrophy and hyperplasia. No new nephrons are formed, even when nephrectomy is carried out in the neonatal period. Glomeruli enlarge, but not new ones are formed. Most of the tubule growth is from hypertrophy, but hyperplasia plays an appreciable role. Five days after unilateral nephrectomy in the rat, the 7% increase in renal cells accounts for about one quarter of the increased mass, with hypertrophy being responsible for the remaining three quarters. The nephron increases in length, diameter, and cell volume, but the distal segments contribute less than the proximal tubule to the added size of the nephron.

Unilateral nephrectomy produces two significant changes. First, the total number of kidney cells is halved, and secondly, the excretory load on the remaining tissue is doubled. Therefore, it is possible that compensatory renal hypertrophy and hyperplasia, if related to cell mass, might be due to a decrease in some growth inhibitor substances or the production of a growth stimulator, but no such chemical mediator has been identified. However, compensatory

growth might be a response to the increased work load placed on the remaining kidney, which has to cope with the excretory functions normally shared by the two organs. Attempts to confirm this hypothesis include supplying the animal, whose kidneys are intact, with an excess of some substance which must be excreted via the kidneys and measuring the effect on kidney weight. Osborne et al. found that diets supplemented with inorganic salts or urea had no significant effect on renal size. Similarly, Addis et al., reported that diets containing added acid or alkali did not affect kidney weight. A fundamental difficulty in these and other indicator of kidney growth, particularly since changes are small and slow to develop of DNA content as a measure of cell number and RNA content of the kidney as a measure of cytoplasmic mass, was able to demonstrate that metabolic acidosis following the ingestion of NH_4Cl causes true growth of the rat kidney. Cell number and size increase to the same order of magnitude as that seen in the kidney remaining after unilateral nephrectomy. Also, renal growth associated with the stimulatory effects of acidosis and unilateral nephrectomy are additive. Later work by Halliburton and Thompson showed that the growth changes produced by administering NH_4Cl, although similar, were not identical to those produced by unilateral nephrectomy.

It has been known for many years that feeding high-protein diets to normal animals produces renal hypertrophy, while low protein intake or starvation leads to atrophy. A high-protein diet was found by Konishi to significantly increase the incidence of mitosis in the kidneys of control rats and in the remaining kidneys after unilateral nephrectomy. Moreover, the rise in the RNA/DNA ratio that follows unilateral nephrectomy is greater in animals fed a high protein diet. A comparison by these authors of the effects of partial nephrectomy with the effects of a high-protein diet showed that the total contents of DNA, RNA, protein, and lipid phosphorus resulting from the feeding of a high-protein diet were of approximately the same magnitude as those in the remaining kidney 4 days after unilateral nephrectomy.

The type of protein given appears to have little influence in the degree of renal hypertrophy except in the case of gelation. A diet containing 30% gelatin produced nearly a 60% increase in kidney weight. The effect was due not only to kidney hypertrophy—as demonstrated by increases in mean cell mass, RNA, and protein content of cells—but also to an increase in cell number, as manifested by a 25% increase in DNA. The increase in cell number could not

be explained by an imbalance of amino acids or the abnormally high glycine content provided by the gelatin diet.

The problem of whether the kidney hypertrophy that follows a high protein intake is a manifestation of the known relationship that exists between protein intake and the protein content of the kidney or whether it is due to the increases work involved in excreting the extra urea produced by protein metabolism has been investigated. If the latter hypothesis is correct the addition of large amounts of urea to the diet of normal animals should lead to kidney hypertrophy. Early reports on this point are contradictory. Some authors, including Wilson and Allen and Mann, reported an increase in kidney size, whereas Osborne et al., MacKay et al., and Baxter and Cotzias did not find any such effect. However, later studies by Halliburton and Thomson and Halliburton revealed that the effect of giving a diet supplemented with a substantial amount of urea, equivalent to three times the normal dietary protein caused hypertrophy, but the effect on both kidney size and composition was relatively small as compared with the effect of unilateral nephrectomy. A weakness inherent in many work hypertrophy experiments is that excretion constitutes only a small amount of all the work performed by the kidney, so that even doubling its excretory work would be the work performed by the kidney, so that even doubling its excretory work would be unlikely to result in detectable hypertrophy. In particular, the concept of increasing urea feeding is incorrect, since urea is handled passively by the kidney.

The effect of starvation and water deprivation on compensatory renal hypertrophy has also been investigated. Early reports showed that compensatory growth of the kidney after unilateral nephrectomy was almost completely suppressed in starved animals. In agreement with this, Williams has shown that mitotic activity in the kidney remaining after unilateral nephrectomy in a starved animal is considerably diminished compared with that in fed animals. More recently, Royce showed that withdrawal of food and water during the entire 48-hr experimental period resulted in inhibition of P^{32} incorporation into kidney DNA and no increase in kidney weight following unilateral nephrectomy. Similarly, Reiter found that both starvation and water restriction markedly decreased the mitotic response and DNA synthesis of the surviving kidney following unilateral nephrectomy in the mouse. An investigation of the effect of diet and unilateral nephrectomy on the composition of the rat kidney showed that he fall in the DNA concentration and rise in the

RNA/DNA ratio in the remaining kidney take place regardless of whether the animal is fed or fasted postoperatively. The increase in protein/DNA ratio, however, occurs only if the animal is fed. The increase in RNA content per cell, the most dramatic early change in compensatory renal hypertrophy, is not significantly affected by starvation.

9.2.3 Epidermal Mitotic Activity

It has been shown that starvation has a powerful effect in depressing epidermal mitotic activity in the mouse, so that after 36 hr such activity is almost entirely eliminated. A similar effect is produced by restricted diets. Animals rationed to 50% of what they would eat if fed ad libitum have an epidermal mitosis rate of about 15% of that of controls.

When epidermis from a starved mouse in incubated in a simple saline medium, a great burst of mitotic activity occurs. The obvious explanation for the diminution of mitotic activity in starvation, i.e., that the cells lack sufficient nutrient, is therefore not the correct one. Bullough and Lawrence suggested that the epidermis of the starved mouse contains many cells that have already completed their preparation for division. The latter is prevented by some inhibitor which, being water soluble, is washed out and diluted to an ineffective concentration when the skin is separated and immersed in a saline medium. Speculating on the possible nature of such an inhibitor, Bullough and Lawrence suggested that it might be adrenaline. Adrenaline secretion is increased during stress, and it may be assumed that starvation causes stress. It is known that adrenaline can inhibit mitotic activity and that adrenaline in the form of a salt is highly soluble in water and would readily wash out of the skin.

9.2.4 Intestinal Epithelial Cell Renewal

The lining epithelium of the small intestine has the highest cell turnover of all body tissue. The concept of a continuous replacement of the intestinal epithelium by regeneration is now established. Cells lining the free surfaces of the villi do not divide; rather, new cells are produced in the crypts and move upwards to be finally shed at the tips of the villi.

Starvation and protein deprivation have a marked effect on intestinal cell renewal. In starved mice, Brown et al. found that the rate of cell renewal was reduced to about one half of the normal rate as measured by injection of thymidine-H^3, microautoradiograpy,

thymidine-C^{14}, and chemical extraction methods. This change was accompanied by morphological changes and impaired differentiation of the epithelial cells. Also, the rate of migration of the epithelial cells to the villous tips was reduced. This slowing of epithelial cell migration has also been observed in protein-deficient monkeys by Deo and Ramalingaswami, who suggested that the prolongation of transit time may represent an adaption to protein deficiency in that it would result in an increased survival of intestinal cells. Other changes included a decreased population of crypt glands and reduced mucosal thickness, resulting mainly from a reduction in the total number of epithelial cells.

9.2.5 Compensatory Hypertrophy and Hyperplasia of the Small Intestine

The treatment of obesity by intestinal surgery has resulted in a renewed interest in the mechanisms by which the gut adapts to the loss of absorptive surface following partial enterectomy and intestinal by-pass procedures. An adaptive response could, for example, account for the decrease in the rate of weight loss in some morbidly obese patients treated by intestinal by-pass procedures.

In 1896, Monari reported that after partial resection of the small intestine in dogs, the mucous membrane and the circular muscle layer of the remaining remnant were thickened, and the villi were larger and more numerous than normal. Flint resected more than 75% of the small intestine in dogs and observe postoperative hypertrophy of the villi, the mucosal epithelium, and the inner circular layer of muscle. He stressed the marked increase in mucosal surface area which resulted from villous hypertrophy. These pioneer studies and work by Clatworthy et al., Loran and Althansen, Bochkov, and Nygaard have established beyond any doubt that compensatory hypertrophy and hyperplasia of the small intestinal mucosa occur in the remnant remaining after partial resection of the gut. Loarn and Althansen have also shown that compensatory hypertrophy and hyperplasia are accompanied by an increased rate of epithelial cell migration in the ileum and jejunum, as shown by tritiated thymidine labeling. In addition, Porus has described hyperplasia of mucosal epithelial cells in the small intestinal remnant of patients who had previously undergone partial resection.

The question of whether or not nutrients in the lumen of the gut remnant stimulate compensatory hypertrophy and hyperplasia is still undecided, although the balance of evidence suggests that they

are at least partly responsible. Booth et al. found that intestinal hypertrophy following partial resection of the small bowel in the rat was more marked in the ileum than the jejunum. Dowling and Booth suggested that the increased load of nutrients derived from the lumen of the bowel could be the stimulus, since following ileo jejunal transposition, without resection, the transposed ileum exposed to jejunal chyme showed marked hypertrophy and enhanced glucose absorption. Confirmation comes from Altmann and Leblond, who found that in ideal segments inserted into the jejunum, villi enlarged to the size of local jejunal villi. In jejunal segments inserted into the ileum, villi decreased almost to the size of local ileal villi. Thus, villus size was influenced by the environment, most probably by the different types of chyme in jejunum and ileum. As shown by Friedman, increased dietary bulk alone does not produce villus hypertrophy, which suggests that the nutritional component of the diet exerts the stimulatory effect on the mucosa.

A feature common to many experiments found to evoke the adaptive response in the small intestine mucosa is an increase in the nutrient load presented to the intestine. For example, hyperphagia associated with hypothalamic lesions, lactation, or chronic alloxan diabetes results in villus hypertrophy.

However, there is evidence that a factor or factors other than intraluminal nutrients are concerned. Wilmore and Dudrick have observed villus hypertrophy in partially enterectomized beagles maintained on intravenous hyperalimentation. Additionally, Tilson and Wright have shown that a self-emptying segment of by-passed ileum, through which there was no flow of chyme, hypertrophied in parallel with a functioning segment of ileum after jejunectomy. This suggests the presence of a systemic stimulus for epithelial growth in response to a demand for increased absorptive surface. However, the degree of hypertrophy observed was greater in the functioning segment than in the by-passed segment, indicating that intraluminal contents have a role in the production of growth, although these contents are not the sole stimulus. There is also the possibility that work hypertrophy is concerned, for as pointed out by Goss, the size of an organ is largely determined by the work it must do, since the physiological mechanisms which regulate function also control growth.

9.2.6 Would Healing

Both clinical and laboratory studies have shown that the rate and efficiency of wound healing depend to a great extend upon the

presence of adequate proteins and vitamin C, and to a lesser extent upon certain minerals and hormones. The recently acquired ability of the surgeon to support nutrition by means of intravenous feeding and elemental diets (those which are nutritionally complete, predigested, and leaves minimal residue) in the catabolic phase following surgery, extensive burns, fractures, and other trauma has led to a revival of interest in the effects of malnutrition on wound healing.

During the past 5 decades, the importance of proteins in wound healing has been particularly emphasized.

Studies of the tensile strength of wounds in protein-deficient rats, by Kobak et al., showed that he wounds of these rats were only one third as strong as those of rats on a normal diet on the fifth day after wounding. Furthermore, this reduction is tensile strength is still apparent at 21 days. More recently, Caldwell et al. demonstrated that the rate of gain of tensile strength is directly related to the severity of the protein depletion and that even moderate depletion results in a decrement of gain of tensile strength. Wounds in the malnourished could be weaker because of a structurally inferior scar or because normal wound healing progresses at a slower rate. Temple et al., investigated this problem in rats rendered malnourished by shortening of the gut and found that malnutrition did not alter ultimate wound strength and that early wound weakness probably resulted from slow healing. They concluded that nutrition plays an important role in early strength and survival of wounds but not in ultimate wound healing. This finding confirms the clinical observation of higher rate of leaks rate of leaks from gut anastomoses and wound dehiscence in malnourished patients early in the course of healing. It is rare for wounds to disrupt once healing is complete, except in specific deficiency states such as scurvy.

Visceral collagen is removed to a lesser extent than skin collagen in malnourished subjects. Rats fed a protein-free diet showed a decreased colonic weight with a relative increase in colonic collagen as noncollagenous materials are removed from the colon. Nevertheless, there is general agreement that the tensile strength of colonic wounds in malnourished animals is inferior to that of control animals. Daly and co-workers found that as the duration of protein depletion and percent of weight loss in rats increased, bursting strength of a standard colonic anastomosis and total circulating serum albumin decreased linearly.

No agreement exists as to the degree of malnutrition at which wound weakness becomes manifest. Daly et al. reported a striking reduction in the tensile strength of colonic anastomoses in rats fed a protein-free diet, with changes apparent even in rats deprived of protein for only. 1 week prior to operation and with only a 2% weight loss. However, Irvine and Hunt found little evidence of breakdown and removal of colonic collagen until the animals had been started of protein for 7 weeks and loss of body weight amounted to 3.4%. Only then was the tensile strength and collagen content of colonic anastomoses significantly lowered.

The question of whether a single limiting factor is responsible for the retarded wound healing associated with protein deficiency has been investigated. The parenteral administration of methionine or methionine and cystine supplements to protein-depleted rats has been reported to accelerate wound healing in spite of continued protein depletion. However, Caldwell and associates were unable to detect any beneficial effect upon the rate of gain of tensile strength of wounds in protein depleted animals given supplements of L-methionine, DL-valine, L-cysteine, or choline chloride. They concluded that the labile methyl group is not a single limiting factor.

As may be expected, knowledge of the effect of protein deficiency on wound healing in man is less extensive than that available on experimental animals. Protein deficiency has been reported in one half of the patients with disrupted abdominal wounds. Koster and Shapiro found serum albumin values of less than 3.75 g/100 ml in 86% of patients with wound healing complications, but 20% of patients with normal wound healing had the same degree of protein deficiency. By testing skin wounds in volunteers 5 days after the wound was inflicted, Lindstedt and Sandblom demonstrated significantly weaker wounds in patients with low serum protein or serum albumin values than in patients with normal protein values. However, the patients presenting with metabolism; it is rare to encounter deficiency of single factor.

Of all the process of tissue synthesis that take place in wound healing, most attention has been concentrated on the biosynthesis of the fibrous protein collagen. Wound repair a substantial synthesis and accumulation of collagen, with subsequent cross-linking of the fibers to give tensile strength to healing, as shown by the close correlation between collagen content and tensile strength in the first weeks of wound healing.

There are a number of stages of collagen biosynthesis in which nutritional deficiencies might interfere with collagen formation and thereby retard wound healing.

1. During fibroblastic proliferation in the wound margins in the first few days after injury Kobak et al. found a decreased number of fibroblasts present in the wounds of protein-depleted rats on the fifth post operative day.
2. In the initiation of collagen biosynthesis in the fibroblast, which involves nuclear DNA, messenger RNA, transfer RNA, and ribosomes. Observations previously cited suggest a diminution of these substances and organelles in the protein-deficient animal.
3. During amino acid chain assembly in the cytoplasm of the fibroblast. For example, animals deficient in lysine (an amino acid which, with its derivative hydroxylysine, is required for collagen formation) show a decrease in collagen precursors and, accordingly, collagen. Fibroblast structure but not number is altered.
4. In the hydroxylation of proline and lysine, an essential step in the synthesis of collagen. This is effected by hydroxylases in the presence of a number of cofactors, including vitamin C. Lack of these substances impairs hydroxylation and therefore collagen synthesis.

The older medical writings on vitamin C and repair of injured tissues have been admirably summarized by Bourne. For centuries, it has been known that wounds do not heal normally in scurvy, and the role of ascorbic acid in wound repair has since been established. The demonstration that the impairment of collagen synthesis in scurvy is not accompanied by a comparable impairment of general protein synthesis suggested that ascorbic acid is involved in the synthesis of only certain protein synthesis suggested that ascorbic acid is involved in the synthesis of only certain proteins and, more specifically, collagen.

In vivo studies by Gould, which revealed the dependence of collagen formation upon the presence of ascorbic acid at the site of synthesis, were later confirmed by in vitro studies which emphasized the need for ascorbic acid by fibroblasts synthesizing collagen. The participation of ascorbic acid in the enzymic hydroxylation of prolyl and lysyl residues (previously incorporated into peptide linkage) to form collagen hydroxyproline and hydroxylysine has since been demonstrated. A small but significant amount of underhydroxylated-

collagen has been found in connective tissue from scorbutic guinea pigs. This is consistent with the suggestion that scurvy results in an intracellular accumulation of severely underhydroxylated collagen which may, after a time, inhibit further synthesis of protocollagen. The administration of ascorbic acid is wounded scorbutic guinea pigs exerts a marked effect on the fibroblasts of the wound tissue. Dilated endoplasmic reticulum reverts to normal, and there is a significant increase in the proportion of large membrane-bound polyribosomes, with a concomitant decrease in monoribosomes.

There have been few experimental studies on wound healing in vitamin C-depleted subjects. Crandon et al. found biopsy evidence of satisfactory healing on the tenth post operative day in a man completely deficient in vitamin C for 3 months. In the same individual after 6 months on the deficient diet, a similar wound showed no signs of healing and no study of tensile strength was carried out. In 1942, Bartlett et al. studied the ascorbic acid content of small, healing, human thigh wounds in six supposedly healthy individuals with normal serum proteins, and related this to tensile strength. One individual, markedly deficient diet, a similar wound showed no signs of healing and no study of tensile strength was carried out. In 1942, Bartlett et al. studied the ascrobic acid content of small, healing, human thigh wounds in six supposedly healthy individuals with normal serum proteins, and tissue ascorbic acid, had a tensile strength determination only one fifth the average of the other supposed normals. Administration of an adequate amount of vitamin C resulted in a healing scar in a second wound, the tensile strength , of which was slightly above the mean for the group. Confirmation of these observations comes from Wolfer et al., who showed that human subjects on a diet vitamin C-deficient diet for a period of 7 months showed a 50% diminution in tensile strength of healing wounds that were deficient in collagen and reticulin.

Few vitamins other than vitamin C have been shown to influence regeneration and repair, though it is reasonable to assume that they are as essential for these processes as they are for normal growth.

Mayer and Krehl found that young vitamin A-deficient rats developed a scurvy-like conditions accompanied by a lowering of tissue vitamin C. Conversely, increased fibroplasia and collagen formation in the wound tissue of rats given vitamin A was reported by Freiman et al. Herrmann and Woodward, and Seifter et al. Recent experiments by Hunt and Zederfeldt demonstrated that in

nonhealing wounds, larger doses of vitamin A significantly increase healing rates. Frassantio and Colonna studied the effects of nicotinic acid on the healing of wounds in the rabbit and found an apparent acceleration of healing when the vitamin was injected but not when it was applied to the wound. Finally, Hautvast and Barnes showed that folic acid deficiency causes a significant impairment in collagen synthesis due in part to involvement of folic acid in general protein synthesis.

Although its precise role in tissue repair has yet to be defined, there is evidence that zinc, an essential trace metal, is active locally at the site of injury. Pories et al. demonstrated accelerated healing of granulating wounds caused by excision of pilonidal sinus tracts in young healthy males given zinc sulfate by mouth during the period of repair. Healing rates were nearly three times greater in the treated group than in untreated controls. Savlov et al. demonstrated experimentally that radioactive zinc-associates, zinc-deficient patients fail to concentrate zinc in wound granulation tissue and have problems with wound healing. These investigations suggest that the explanation for accelerated wound healing observed by Pories and co-workers is probably related to increased zinc requirements following tissue trauma. Acceleration of wound healing need not involve increases in collagen production, and since wound tensile strengths were not determined, there is no evidence to suggest collagen production was increased. Waters et al. demonstrated that zinc does not have a direct accelerating effect on either cellular proliferation or collagen biosynthesis in human skin fibroblasts in vitro.

9.2.7 Fracture Repair

Early stages of bone repair involve cellular proliferation, with the formation of fibrous tissue and osteoid, both of which are largely collagen. It is to be expected, therefore, that a deficiency of vitamin C would delay bone repair. Bourne has shown that in vitamin C-deficient guinea pigs with fractures, the degree of healing was proportional to the amount of vitamin C given. In completely deficient animals, the osteoblasts of the periosteum failed to multiply, and fibroblasts die not migrate into the fracture site; collagen formation was reduced or absent. MacLean et al. found vitamin C deficiency caused delay in the differentiation of mesenchymal cells to osteoblasts.

These effects of partial and complete vitamin C deficiency in the early stages of bone repair have been supplemented by the

observations of Murray and Kodicek on prolonged partial vitamin C deficiency in guinea pigs. They reported slow and reduced callus formation, as compared with that found in normal animals. Whereas in normal animals the callus consolidated into compact bone by thickening of the trabeculae, in the partially vitamin C-deficient animals this did not occur. Fractures of the fibula are often accompanied by injury to muscle fibers. In dietetically normal animals, the muscle fibers quickly regenerated, whereas in vitamin C-deficient animals, they were replaced by fibrous tissue. These authors also found that vitamin D-deficient diets, in which the balance of salts was not altered, caused no delay in callus formation or any other effect, whether alone or combined with a partial deficiency of vitamin C.

These changes in scorbutic animals indicate that fracture healing ought to be delayed in scurvy, and this has proved to be the case. The many observations to this effect extending back to the 18th century have been summarized by Bourne.

The possibility that an amount of vitamin C in excess of that required to saturate an animal would accelerate bone repair has been investigated. Halasz and Marx have shown that a large excess of vitamin C in guinea pigs did not regenerate bone any faster, an observation confirmed by Bourne. Using 1-mm holes bored in the femurs of rats and guinea pigs, Bourne found that an excess of vitamin C had no accelerating effect on bone regeneration. Results in the rabbit are difficult to interpret, but, according to Bourne, the only explanation of the various observations is that a normal rabbit can synthesize its own vitamin C. However, the healing of a fractured bone calls for more vitamin C than the animal can provide.

The effects of vitamins A and D on skeletal metabolism, which are probably germane to fracture repair, have been studied by Clark and Smith. They found that the administration of large amounts of vitamin A to rats caused a loss of collagen and mucopolysaccharide from bone, with no demonstrable changes in ash content. However, large amounts of vitamin D increased the absolute amounts of collage and mucopolysaccharide and decreased the ash.

9.2.8 Bone Marrow

The bone marrow, an actively proliferating tissue, is known to respond to blood loss and chronic anoxia by increasing the rate of red cell replacement. The effect of protein malnutrition on this regenerative process was studied by Siimes and Dallman. They found

that protein-deficient rats were handicapped in their bone marrow response to blood loss in that increase in DNA synthesis was delayed.

9.2.9 Proliferative Activity of the Thyroid Gland

Loeb observed that the degree of compensatory hypertrophy in the thyroid glands of guinea pigs is proportional to the changes in weight that the animals undergo in the period following partial removal of the thyroid. Rabinovitch found that underfeeding guinea pigs led to a cessation of acinar epithelial proliferation, small acini, and solid colloid. He suggested that the overall reduction in general metabolism that occurs in undernutrition is intensified by the changes which take place in the thyroid gland.

10

Nutrition and Senescence

It is generally accepted that intakes moderately above the recommended allowances are optimal for the well-being of an organisms. However a number of studies performed on animal have demonstrated that longevity was increased when the intakes of certain nutrients were lower than the recommended allowances. Similarly, very high intakes of some nutrients have been reported to shorten life span. Furthermore, little information is available regarding the influence of nutritional status at one period in life on the remaining periods. In the past, emphasis has been placed primarily on the establishment of the nutritional requirements of young growing animals. Few efforts have been made to determine whether changes occur in nutritional requirements following growth cessation. Many age changes in physiological functions may result in increased nutritional needs in later life. For example, cellular loss and decrements in biochemical systems such as active transport may impair absorption in the elderly. In addition, the greater displacement from basal levels and the slow rate of return following various challenges such as glucose administration may increase metabolic demands and, therefore, nutritional requirements. Finally, age-associated decreases in hormonal secretions may result in reduced efficiency of the metabolism of specific nutrients. Therefore, an attempt will be made to review the pertinent literature on (1) the effect of age on nutritional requirements after the cessation of growth and (2) the effect of the nutrition on life span. This information may provide useful knowledge for the optimal nutrition of the aged and for an understanding of the basic mechanisms of biological aging. Studies concerned with the first problem area have been

carried out principally with human subjects, whereas those concerned with the second have been performed with animals.

10.1 EFFECT OF AGE ON NUTRITIONAL STATUS OF MAN

Unfortunately, there are many variables that make the assessment of nutritional requirements in human subjects difficult. This is especially so in the United States due to the marked differences in genetic background, social environment, and economic status of the population. These variables have a significant impact on the nutrition of the individual. Furthermore, the great selection of foods available complicates the problem even more. Other difficulties that arise because of the various ways of assessing the nutrient; urinary or fecal excretion under various intakes; and the measurement of studies of assessment of dietary intake and nutritional status. The first represent national surveys of large segments of the population, while the second includes studies on limited numbers of subjects as part of specific laboratory investigations. It is important to describe the characteristics of each of these populations in terms of socioeconomic and other factors.

10.1.1 National Surveys

In order to assess the nutritional status of the general population in the United States, the Department of Agriculture has obtained information on nutrient intakes of large numbers of individuals at intervals since 1936. The most recent, in 1965, covered 14,500 persons from 6174 households. The survey did not include those living in institutions and rooming houses, thus omitting many of the aged who were ill or disabled. The results showed that the average nutrient intakes per day for men aged 55 and over were adequate except for calcium, which declined with age and averaged 85% of the recommended dietary allowances (RDA's), whereas the intakes of thiamine, riboflavin, and calcium of women in this age group were evaluated separately, it was found that dietary adequacy (based on RDA's) declined with income. Of those with annual incomes of less than $3000 after taxes, 63% had inadequate diets.

The Ten-state Nutrition Survey conducted by the U.S. Department of Health, Education, and Welfare was designed to assess the nutritional status of certain groups considered to be at risk for undernutrition, such as poverty groups, migrant workers, Spanish-speaking people in the southwest U.S. inner-city residents, and individuals in industrial states who had migrated from the south in the previous 10 to 20 years. These industrial states who had migrated

from the south in the previous 10 to 20 years. These groups were selected from districts with average incomes in the lowest quartile according to the 1960 census. The assessment involved a series of clinical and biochemical measurements and a dietary evaluation. It was concluded that persons 60 years of age and older consumed far less food than needed to meet the nutrients standards for their age, sex, and weight. In addition to a low caloric intake, other limiting nutrients were protein, iron, and vitamin A. Although the differences were minor, the Ten-State Survey tended to report lower intakes than the USDA study. Obesity was more prevalent in higher than lower income groups. The percentages of obesity in females in the 45- to 55-year-old age groups were 50% for blacks and 40% for whites. These percentages declined markedly with age to about 20 to 25% in both races by age 75 to 85. Men of both races had lower incidences of obesity as compared to females, and there were no age-associated patterns. The clinical assessments did not indicate a high incidence of severe malnutrition in the older subjects. Similarly, the biochemical tests for nutritional status did not suggest marked age-associated nutritional deficiencies.

At present, the findings of the first Health and Nutrition Examination Survey (HANES) for the U.S. population in 1971-72 are only available in a preliminary report. The sample studied represented civilian, non institutionalized persons from 1 to 74 years of age. The design allows for detailed analysis of the data for the total population, as well as for those groups considered to go at high risk for malnutrition — the poor, preschool children, women of childbearing age, and the elderly. The preliminary results include data on 10,126 persons, representing a 72.8% response by the individuals selected for sampling. The results indicated that, among persons over 60 years of age with incomes above the poverty level, 16% of the white and 18% of the black population consumed less than 1000 cal/day. In those with incomes lower than the poverty level, these percentages rose to 27 and 36%, respectively. The intake of protein as well as of calories in this age group was also related of income in both races; however, protein intakes per 1000 cal showed no variation with race or income. Calcium intakes were less than the standards for 37% of all persons over 60. The intakes of vitamin A were below standards in 52 to 62%, and consumption of vitamin C was low in 39 to 59% in all adults in this age group. The only biochemical indications of nutritional problems among the elderly in this study were the high percentages of black aged 60 years and

over with low values for hemoglobin (29.6%) and hematocrit (41.7%). However, iron deficiency was not considered to be the cause, since most of this group did not have low levels of serum iron or percentage of transferrin saturation. The biochemical tests have not been completed, nor are the clinical assessments available from this study. The final, still unpublished report will include data on serum folate, vitamin C, magnesium, cholesterol and total iron-binding capacity, and urinary creatinine, thiamine, riboflavin, and iodine.

These surveys do not indicate consistent evidence of poor nutritional status or of marked deficiencies in nutrient intake among older members of the general population in the U.S. However, significant percentages of many of the groups studied consumed less than recommended amounts of certain nutrients, especially of protein, calcium, ascorbic acid, and vitamin A. One of the most consistent findings was that low intake were more likely to occur if income was low. Watkin reached the same conclusion, indicating that nutrient intake or nutritional status of the elderly was more related to health and poverty than to age *per se*.

10.1.2 Laboratory Investigations of Nutritional Requirement

There are some data from a small number of carefully conducted laboratory studies which provide information regarding the following questions:

1. Is aging accompanied by changes in nutritional requirements?
2. Is there evidence that such changes are correlated with changes in physiological functions?
3. Are these changes reversible?
4. What appears to be the cause of these changes?

10.1.2.1 Vitamins

The effect of age on nutritional status in relation to various water-soluble vitamins estimated by a variety of techniques. These data do not offer strong evidence to indicate that age influences the vitamin requirements in man. However, in certain old individuals, vitamin deficiency states may exist.

10.1.2.2 Proteins and amino acids

Data presently available on the effects of age on protein requirements were obtained by the nitrogen balance technique. Unfortunately, the results of these studied are not in complete

agreement. A similar lack of agreement of the effect of age is found when the requirements for amino acids are considered. For example, Tuttle et al. fed five men aged 52 to 68 a diet that contained amino acids in amounts that exceeded Rose's minimal recommended allowance for 25-year old male subjects. When fed either this diet or one that contained twice the amount of tryptophan, all subjects were in negative nitrogen balance. These data indicate an increased requirement for one or more of the essential amino acids with age. On the other hand, in a study carried out by Watts et al., six black men 65 to 85 years of age were fed semipurified diets containing essential amino acids in ratios corresponding to the FAO protein recommendations and the pattern of milk protein. Although the amounts of the essential amino acids fed to these subjects were actually lower than those fed to the subjects of Tuttle, both diets were adequate for maintaining nitrogen equilibria in all subjects. A comparison of the other variables in these two studies failed to explain the apparent age-associated differences in requirements. Interpretation of these nitrogen balance studies is made difficult by the quality of protein used, caloric intake, and the observation that subjects can be maintained at nitrogen equilibrium at various levels of nitrogen intake. Therefore, at present there is no strong evidence to indicate that age affects the requirements for proteins or amino acids.

10.1.2.3 Calcium

Most of the studies performed to establish calcium requirements as a function of age failed to include young subjects. These studies carried out primarily on old subjects indicate that the calcium intakes necessary to establish equilibrium exceed the RDA of 800 mg/day. This suggests that old individuals require higher calcium intakes. However, in the study of Ohlson et al., where the subjects ranged form 30 to 89 years of age, no correlation with age and calcium requirements was observed. It should be pointed out that the possibility of establishing calcium equilibrium at various levels of intake make these data difficult to interpret. Therefore, at present, age does not appear to markedly affect the requirement for calcium. However, in the study of Ohlson et al., where the subjects ranged from 30 to 89 years of age, no correlation with age and calcium requirements was observed. It should be pointed out that the possibility of establishing calcium equilibrium at various levels of intake make these data difficult to interpret. Therefore, at present, age does not appear to markedly affect the requirement for calcium.

10.1.3 Nutritional Deficiencies and Physiological Impairments

Unfortunately, there is little evidence presently available to correlate age-associated nutritional deficiency states with clinical findings, physiological functions, or biochemical changes. In one such study employing 106 subjects aged 16 to 99 years. Chieffi and Kirk failed to demonstrate significant correlations between serum vitamin A level and dark-adaption time, the number of epithelial cells excreted daily in the urine, and the percentage of keratinized cells in the urinary sediment. However, the frequency of dryness of the skin, conjunctivitis, and the percent of keratinized cells in conjunctival smears were higher in subjects with low vitamin A plasma levels (1 to 15 μg%) than in those with high levels (25 to 60 μg%). The differences between the groups were not marked and could not be considered clinically useful. Morgan et al. studied the frequency of gingivitis in subjects over 50 years old who were divided according to levels of serum ascorbic acid. They found at levels of 0 to 0.47 mg%, 0.5 to 1.09 mg%, and 1.1+ mg% that the frequencies of gingivitis were essentially the same, i.e., 22, 17, and 13%, respectively. In addition, although thickening of the bulbar conjunctiva was noted in 94% of the subjects, this condition was not marked in individuals whose serum vitamin A levels were low. In a more recent study, Davis et al., attempted to establish biochemical and hematological changes attributable to low serum levels of vitamin B_{12}. In 275 subjects between the ages of 49 and 89 year, no significant correlations were found between the serum vitamin B12 level and serum lactic acid concentration, serum lactic dehydrogenase activity, and hematocrit. These data indicate that the vitamin deficiency states that may exist in some old subjects are generally nor severe enough to be manifested by clinical or biochemical changes.

The importance of calcium nutrition in the elderly is best exemplified by its possible relationship to the increased incidence of osteoporosis with age. For example, recently, Gitman and Kamholtza performed routine X-ray examinations of the dorsal lumbar spine on all admissions to a large geriatric facility. They found that in a group of 933 females, approximately 50% between the ages of 65 to 70 had osteoporosis. The incidence increased essentially linearly to 90% in women over 90 years. The incidence in men, however, was lower than that found in women, and ranged from approximately 15% in the 65-70-year-old group to 30% in the individuals over 90. Unfortunately, the reports presently available are not in complete

agreement regarding the relationship between calcium intake and the incidence of osteoporosis. Lutwak and Nordin reported that the average intakes of calcium of subjects with osteoporosis were statistically lower than those of normal control subjects. On the other hand, Garn et al. found no correlation between the intake of calcium and the cortical thickness of the second metacarpal in 382 subjects ranging in age from 25 to 85 years old. Likewise, Smith and Frame, in a radiographic survey of 2000 women 45 years of age or older, found no significant differences in the calcium intake of the subjects with high or low vertebral densities or vertebral compressions.

10.1.4 Nutrient Supplementation

In addition, these studies provide evidence that, contrary to common belief, there is no impairment in absorption of vitamins in elderly subjects. The findings are in arrangement with other studies on the absorption of nitrogen in older individuals. For example, Chinn et al. reported no decrements in the rate of digestion and absorption of ^{131}I-labeled albumin in 12 subjects 72 to 88 years of age. Similarly, Watkin et al. and absorption with age. These data clearly indicate that if selected vitamin deficiencies occur in old individuals they can be readily corrected through the administration of the specific nutrients.

10.1.5 Causative Factors of Deficiency States

Since older individuals apparently can correct nutritional deficiencies by increasing their dietary intake of the nutrients in question, a logical explanation for the existence of age-wise deficiencies in various nutrients is a decreased intake among the older people. In order to explore this possibility, a study was carried out by McGandy et al. in which 7-day dietary histories were reported by 250 healthy men between the ages of 23 and 99 years. Most of the subjects were highly educated, financially successful men engaged in or retired from professional and managerial occupations who resided in the Baltimore-Washington area. The economic variable, so important in national surveys, was absent among these subjects. The data are presented in graphic form with median values and first and third quartiles; the National Research Council recommended allowances of the various nutrients are included for comparison.

Other studies have shown that the total caloric intake diminishes with age in man. However, it is not clear how much of this reduction is a reflection of age decrements in basal oxygen consumption as

described by Shock et al. and how much is due to a reduction in physical activity in the aged. The data on the effect of age on daily caloric intakes were analyzed the age decrement in total dietary calories per day while basal metabolism of the same subjects in calories per day. The decrease in basal metabolism amounts to 5.23 cal/day/year, while total caloric intake falls by 12.4 cal/day/year. Consequently, the difference, which amounts to 7.8 cal/day/year, must be related to the reduction to calories required for other purposes including physical activity. Another approach to the problem of assessing age decrements in nonbasal energy requirements involved interviews with 167 of the subjects with regard to their physical activities.

10.1.6 Frequently of Nutritional Deficiencies Among the Aged

Some estimates of the frequency of nutritional deficiencies among the aged are found in studies reported by Brewer et al. and by Chinn. In the latter study, the nutritional status of approximately 500 elderly patients admitted to a hospital for long-term illnesses over a period of 3 years was assessed. The data showed that only 35 (7%) had significant primary nutritional problems. Of these, 15 were undernourished, whereas the remaining 20 had a problem of obesity. Similar data were obtained by Brewer on 107 subjects who were admitted to county institutions for the aged in Michigan. Nutritional assessment was made on the basis of the concentration of hemoglobin and plasma levels of ascorbic acid, vitamin A, and carotene. Only 5 to 10% of the residents could be considered in a poor nutritional state with respect to vitamin A and ascorbic acid. Brin et al. examined 234 elderly subjects whose average age was 71.0 ± 8.9 years (mean ± standard deviation). They measured hematocrits and evaluated nutritional status with respect to ascorbic acid, vitamin A and carotene, riboflavin, and thiamine. The later was estimated on the basis of urinary excretion as well as three parameters of erythrocyte transketolase activity. They concluded that 5% of the men and 13% of the women had hematocrits in the deficient range according to ICNND criteria. Plasma ascorbic acid levels were low in only 8% of the whole population. Plasma vitamin A and carotene levels were in the acceptable to high range, and urinary riboflavin excretion values showed no deficiencies. Thiamine deficiency was indicated for 18% of the population if the standard of ICNND was used and for 21% on the basis of Pearson's criteria. However, on the basis of the erythrocyte transketolase data, a

biochemical defect was evident in only 6% of the group. The authors concluded that this ambulant, well, aged, surveyed population was fairly well nourished. Therefore, on the basis of these low frequencies of nutritional deficiencies and the complete lack of information on the effect of continued long-term vitamin therapy in older people, it seems unwise to propose mass vitamin and other nutrient supplements to the aged at the time, and therapy should be administered on the merits of individual cases.

10.2 RELATIONSHIP OF DIETARY RESTRICTION AND AGING

Dietary restriction has been shown to increase the life span of a variety of species. In general, dietary restriction has been brought about by reducing the daily intake of a nutritionally adequate diet (one which supports maximal growth); intermittently feeding a nutritionally adequate diet (Feeding every second, third, or fourth day); and feed *ad libitum* a diet containing insufficient amounts of proteins to support maximal growth. The results of these experiments have been categorized according to whether dietary restriction was imposed on young growing animals or on adult organisms.

10.2.1 Dietary Restriction of Adult Animals

It has been generally believed that nutritional manipulations that increase life span had to be imposed during early growth. This concept probably originated due to the early work of Minot postulating that senescence follows the cessation of growth. In addition, McCay et al. showed that increased life span of rats was associated with growth retardation. Furthermore, Lansing indicated that aging in the rotifer involves a cytoplasmic factor whose appearance coincides with the cessation of growth. However, more recent studies have indicated that dietary restriction imposed in adult life was effective in increasing life span.

It is apparent that the life expectancy of adult animals can be increased by dietary manipulations. However, experimental data are not in agreement regarding the effectiveness of various methods of imposing dietary restriction. In addition, Kopec and David et al. have shown that dietary restriction in adult *Drosophila* was ineffective. Similarly, Barrows and Roeder did not demonstrate an increase in life span in 13- or 19-month-old female adult rats whose dietary intake was reduced 50%. Thus, it is apparent that further studies must be carried out to define effective ways of consistently increasing the life span of adult organisms.

10.2.2 Biochemical and physiological Variables

In an effort to establish the biological mechanisms responsible for the increased life span associated with dietary restriction, comparisons have been made among various biochemical and physiological variables in animals whose life span was increased by dietary manipulations.

Unfortunately, little information is available on the effect of body temperature on the life span of homeothermic animals. Nevertheless, the life span of poikilothermic animals increases with decreased environmental temperature. It is generally assumed that this latter finding is a result of a decreased metabolic rate due to the lowering of the rate of biochemical reactions at the reduced temperatures. However, the low body temperatures of mice were associated with an increased oxygen consumption in which poikilotherms have been exposed to different temperatures at various times in the life cycle, suggest a more complicated mechanism which may be independent of oxygen consumption.

Complete agreement on the effects of oxygen uptake on the life span of animals is not found in data presently available. For many years an inverse correlation has been described among various species of mammals, i.e., the higher the oxygen uptake per unit of body weight, the shorter the life span. Indeed, Kibler and Johnson showed that rats exposed to cold temperatures throughout their lives experienced a marked decrease in longevity and 40% increase in oxygen consumption. However, Weiss reported that although the life span of the F-1 generation was longer and the basal metabolic rate (BMR) lower than either of the parental strains, the BMR of the parents was essentially the same in spite of marked differences in longevity. Finally, Storer reported a direct relationship between oxygen consumption and life span among 18 strains of mice. Should the longevity of individuals within a strain vary inversely with BMR, the increased oxygen consumption due to dietary restriction would shorten life span; should the converse relationship exist, the increased oxygen consumption of these mice would result in an increased life span. Therefore, these data indicating an increased oxygen consumption and reduced rectal temperature in dietary restricted animals cannot presently contribute to our knowledge of the biological mechanism responsible for the increased life span.

On the basis of these data it was assumed that enzymatic activities could be used as expressions of genetic programming and

that these biochemical indices are intimately associated with physiological ones (at least with egg production in the rotifer). The enzymatic activities were considered adequate expressions of program on the basis that under all conditions the following always occurred: (1) the pattern of age change in the enzymatic activities were identical, (2) The maximal levels of activity were the same, and (3) age-dependent decreases in the ratio of two enzymes, namely malate dehydrogenase (MDH) and lactate dehydrogenase (LDH), always occurred. Rotifers and *Daphnia* apparently are programmed to produce equal numbers of eggs. However, the duration of the different intervals during the life cycle varied as longevity was altered by nutritional conditions. The data seem to support the concept that there was a program for the total life span of the organism, and those nutritional conditions that altered the length of life did so merely by altering the rate of occurrence of specific events.

A delay in genetic informational transfer would be advantageous if aging was the result of deleterious genes in late life. However, there is not evidence to support this. Furthermore, later studies in which enzymatic activities had been based on DNA or numbers of hepatocytes do not support the concept of delayed genetic informational transfer; rather, they may suggest a reduced use of the genetic code throughout life span. Therefore, an additional proposal has been offered: Dietary restriction reduces the use of the genetic code and thereby minimizes genetic imperfections as they may occur in late life.

10.2.3 Diseases

Although the incidence of many diseases increases with age, the relationship between disease and aging remains unknown. At present no explanation has been offered for these relationship.

Nutritional surveys fail to consistently provide evidence that a poor nutritional state exists among members of the aging population in the United States. However, significant numbers of many of the groups studied consumed less than the RDA of certain nutrients, including protein, calcium, ascorbic acid, and vitamin A. One of the most consistent findings in the national surveys was that low dietary intakes were associated with poor health and low income. The frequency of serious nutritional problems among the aged is estimated to be approximately 5 to 10%. A number carefully conducted laboratory studies in which only small numbers of subjects participated substantiate the findings of the national surveys.

Furthermore, they fail to provide strong evidence to indicate that age influences the vitamin requirements in man. In general, little correlation has been observed between low plasma levels of various vitamins and physiological impairments associated with their deficiencies. Low plasma levels of vitamins in old individuals can be increased by the administration of the particular vitamin. Laboratory studies in which the economic variable was absent indicated that age was accompanied by a decrease in the intake of all nutrients. The decrement in total caloric intake was approximated by an age-associated decrease in basal metabolism as well as a decline in physical activity.

Dietary restriction has been shown to increase the life span of a variety of species. Recent studies have shown that the beneficial effects of dietary restriction can be observed when underfeeding is initiated in adults as well as young growing animals. Studies that have compared age-associated changes in biochemical and physiological variables in normal as well as underfed animals failed to establish the biological mechanism responsible for the increased life span associated with dietary restriction.

11

Nutritional Factors in Teratology

Some of the fundamental questions in biology concern the relationship of organisms to their internal and external environments with nutrition being an important aspect. The earliest forms of life probably utilized elements from the earth's crust, atmosphere, and oceans as well as radiation from the sun in their early evolution. In higher forms of life, survival depends on the availability of inorganic and organic nutrients for use either directly or as precursors of other compounds necessary for the formation and maintenance of body structures and physiology functions. If an appropriate blending of nutrients is not available, normal formation of structure and function may be disrupted.

The study of such disruptions leading to abnormal development is the domain of teratology. Abnormal variation in structure and function may result from alterations in an organism's biological or external environment during the developmental period. Manipulation of nutrients has proved to be a valuable and in teratology from its inception and has provided insights important for the basic understanding of development. Clinically relevant information has also resulted from such research.

Identification of nutritional factors as teratogens in humans usually requires information regarding the nutritional status of a population as well as recognition and knowledge of other factors relevant to prenatal development. In most studies involving humans, the detailed data needed for suitable statistical analysis are not available and the interpretation of results relies heavily on information obtained from experimental animals.

The production of congenital anomalies by deficiencies of vitamin A and riboflavin marked the beginning of experimental teratology. The use of rigorously controlled animal experiments in teratology and their statistical evaluation have recently been discussed at length. Careful experimental design, proper sampling techniques, precision in collection of quantitative data, and appropriate statistical evaluation are fundamental to the identification of sources of biological variability and the mechanisms of abnormal development.

This section summaries the present knowledge of the importance of nutrients during the prenatal period in mammals. The extensive literature on nutrition during the neonatal and early postnatal period has been included.

11.1 MAJOR NURTRIENTS

11.1.1 General Malnutrition

11.1.1.1 War and famine

It is curious that much of the information available on the health and well being of population has been collected during periods of human history when the value of human life was at its lowest points. The effects of general malnutrition on populations under conditions of war and famine have been reviewed by Hytten and Leitch. During World War II when food supplies in the Netherlands were extremely limited for a period of 8 months between 1944 and 1945, amenorrhea and irregularity of menstrual cycles were common, producing a marked decrease in birth rate. The acute shortage of food resulted in higher incidences of miscarriage, abortion, stillbirth, neonatal death, and malformation than in the prewar period when the population was well nourished.

Antonov has described the appalling effects of hunger, accompanied by bitter cold and extreme physical exertion, during the bombing and siege of Leningrad in World War II. He noted a high incidence of stillbirths and premature births during this period. Birth weight was low, neonatal mortality was high, and newborn infants were very apathetic. He did not report an increase in congenital malformations.

11.1.1.2 Clinical studies

Clinical studies on the effect of maternal nutrition on the condition of newborn infants have yielded conflicting results. However, it does appear that the diet of pregnant women can affect development of fetus. A definite correlation between the quality of

the mother's diet during the prenatal period and condition of the infant at birth was reported by Burke et al. Infant weight and length correlated with the quantity of dietary protein. Jeans et al. have reported similar findings and also found an increase in the incidence of premature births in poorly nourished women.

Retarded postnatal bone growth and cardiovascular abnormalities have been reported in clinical investigations of infants malnourished before birth. Wilson et al., reported that 10% of infants with fetal growth retardation and physiological problems after birth resulting from congenital anomalies.

Primrose and Higgins collected data on nutrition education and subsequent nutritional supplements from a group of low-income pregnant women in Montreal. After improved nutrition, the incidence of stillbirths, perinatal mortality, and neonatal mortality was lower in the Province of Quebec than in Canada as a whole.

11.1.1.3 Significant of low birth weight

Available studies in humans have not provided convincing proof that general material malnutrition during pregnancy increases the incidence of gross congenital malformations; however, other parameters of fetal development, such as birth weight, have provided an indieation of the presence of congenital anomalies not apparent at birth. Low birth weight is one way of determining the condition of the newborn. It may be an indication of general malnutrition and has been correlated with congenital anomalies and perinatal mortality. However, low birth weight is also correlated with socioeconomic level and race, which are, in turn, also correlated with malnutrition. In human studies, it is seldom possible to differentiate between these factors.

11.1.1.4 Animal studies

Total food intake estimation has been studied extensively in animals. Chow and Lee restricted food intake of pregnant and lactating rats to 50 or 75% of the amount normally eaten and observed permanent growth stunting in the offspring. Similar reductions in growth have been observed in animals receiving restricted diets during the gestation period alone. Malformations have not been observed due to food restriction, however it has been postulated that metabolic derangements occur and some permanent anatomical aberrations have been seen. Skeletons of offspring form undernourished female rats were smaller and less mature than controls

even after more than 3 months of ad libitum feeding. Prenatal undernutrition was also reported to cause perineurial damage even after nutritional rehabilitation.

The cellular growth of fetuses from undernourished mothers has received considerable attention in recent years. Maternal undernutrition during the gestation period results in a reduction in cell number in tissues undergoing rapid cellular proliferation at the time of the nutritional insult. Decreased numbers of cells have been observed in the placenta and various fetal organs in humans as well as animals.

11.1.1.5 Brain development and behaviour

The effect of prenatal and early postnatal undernutrition on the growth and development of the brain and its functional maturation has received considerable attention, and the literature is too extensive to be reviewed here. Prenatal undernutrition does not cause gross malformations of the brain; however, abnormalities of development have not been noted.

Winick and Noble showed that if dietary restriction occurs during the period of hyperplastic growth of an organ, there is a reduction of cell number, but if it occurs during the hypertrophic growth phase, there is a reduction in cell size. The decrease in cell size is reversible by nutritional rehabilition; however, the reduction in cell number appears to be permanent. The brain, like other organs, is affected by undernutrition. Since the development of different brain regions occurs over a considerable time period, and many different on the timing of the nutritional insult. In addition to cellular growth, the weight and cortical thickness of the brain are decreased as well.

The chemical composition of the brain is also affected by undernutrition. Reduced myelination accompanied by a decreased concentration of cerebrosides, cholesterol, and other components of myelin have been reported. Alterations in brain enzyme activities also occurs, the most notable of which are the enzymes of the cholinergic system which remain altered long after animals have been rehabilitated nutritionally. The effect of undernutrition on the chemical development of the brain is markedly dependent on the period of brain growth and development when inadequate food intake occurred.

Functional aspects as well as anatomical and chemical parameters of the brain have also been investigated in undernourished animals and humans. It is now well established that malnutrition during critical periods of development brings about changes in the behavior of

animals. The malnourished animal responds differently to some environmental stimuli than does the normal animal and in some cases is less able to learn even after nutritional rehabilitation. Increasing the environmental stimulation in addition to nutritional supplementation may influence the behavior of previously undernourished animals and may overcome some of the effects of nutritional deprivation on behavioral as well as on biochemical parameters.

Although much is known about the effects of nutritional deprivation on anatomical and biochemical changes in the brain, causal relationships with behavior, and especially with human intellectual capacity, have not been elucidated at this time.

11.1.1.6 Fasting

Fasting during pregnancy has teratogenic effects in some strains of mice, but similar reports have not been published for other species. Exencephaly as well as vertebral and costal malformations in 19-day fetuses of mice of Strain 129 occurred after subjecting animals to total fasts of 24 or 30 hr during the 7^{th}, 8^{th}, 9^{th}, or 10^{th} days. Fasts of 40 hr were incompatible with pregnancy. Small quantities of glucose and amino acids counteracted these effects. The teratogenicity of fasting in mice has also been confirmed in other strains.

McClure concluded that the pathogenesis of embryonic mortality caused by fasting results from a failure of normal hypophyseal gonadotrophic function. The effects of fasting on embryonic development were affected by maternal age and weight and were decreased by intermittent fasting before pregnancy. Felig and lynch reported that concentrations of blood ketone acids, plasma glucose, and insulin rose more rapidly in pregnant women who fasted for 8 hr during the second trimester of pregnancy than in nonpregnant controls.

11.1.1.7 Protein deficiency

When general malnutrition or undernutrition is imposed during pregnancy, protein, a crucial nutrient, becomes limited. Few studies have differentiated between energy restriction, protein deficiency, and protein-energy undernutrition. The relation of dietary protein levels to reproduction in the rat was first studied with purified diets by Nelson and Evans. No congenital malformations were observed but there was a high incidence of resorptions with low protein diets

(containing 0, 2.5 and 5% protein). Injections of estrogen and progesterone maintained pregnancy although maternal weight loss was still high. Adrenocortical steroid or adrenocorticotropic hormone (ACTH) administration has been used to maintain pregnancy in protein-deficient rats, apparently acting through mobilization of maternal tissue protein. In contrast to the rat, mortality in the pig is less severely affected by protein-free diets during the gestation period. Plasma-growth hormone is elevated in protein-restricted pigs as in protein-deprived children.

An extensive series of studies on prenatal maternal protein deficiency in rats has been carried out by Zeman and her colleagues. Prenatal deficiencies of protein resulted in decreased birth weight, birth length, and liver and kidney weight and in increased heart, brain, and thymus weights in relation to body weight as compared with controls. The cell number of these protein-deprived fetuses was also less than that of controls and appeared to be irreversibly decreased.

New born young form protein-restricted rats and kidneys with less well-differentiated glomeruli, proportionately more connective tissue, fewer collecting tubules, and reduced kidney function. Both morphological and functional differences appeared to be irreversible by postnatal nutritional supplementation. Morphological abnormalities were also observed in other tissues, particularly the intestine and skeleton. Many of the alterations appeared to be related to persistent hormonal changes in the offspring.

11.1.1.8 Amino acids

Deficiency—Relatively few studies have been conducted on the effects of deficiencies of specific amino acids on prenatal development in mammals. Pike observed cataracts in the offspring of rats given tryptophan-deficient diets. Zamenhof and his colleagues, studying prenatal brain development in rats, found that omission of tryptophan, lysine, or methionine produced decreases in birth weight and cerebral weight, cerebral cell number, and protein content. The effects were similar to those observed with protein-deficient diets. The omission of one essential amino acid (except of lysine) in diets containing amino acid mixtures resulted in a high number of resorptions. If methionine, valine, or isoleucine was omitted, birth weight and placental weight were decreased. Persaud and Kaplan studied the effects of hypolycin-A, a leucine analogue, injected intraperitoneally

on fetal development of rats. Most fetuses at term had malformations which included gastroschisis, encephalocele, syndactyly, and stunting. The experiment suggests the importance of leucine for fetal development.

Excess—Excessive amounts of single amino acids also have deleterious effects on fetal development. Large amounts of lysine or leucine in the diet resulted in retarded fetal growth. Excess leucine also has been found to cause resorptions and malformations. Hyperphenylalaninemia induced in rhesus monkeys by excessive dietary phenylalanine resulted in offspring with reduced learning behavior. Woolley and van der Hoeven have observed similar effects in newborn mice. Luse et al. fed pregnant rats a high level of phenylalanine and gave them daily intraperitioneal injections of the amino acid. They found fetal death, resorption, and congenital malformations in the offspring. In humans, maternal phenylketonuria may result in spontaneous abortion, intrauterine growth retardation, mental retardation, microcephaly, and other congenital malformations, including skeletal anomalies, cardiac defects, esophageal atresia, agenesis of spleen, and lung abnormalities. Allan and Brown reported that one phenylketonuric mother, who had three retarded children while on an unrestricted diet, gave birth to a normal infant when she took a low phenylalanine diet during a subsequent pregnancy.

11.1.1.9 Carbohydrates

In contrast to protein, there is little information on the effect of deficiencies or excesses of total dietary carbohydrate on prenatal development. Galactose in excessive amounts has been shown to be deleterious to the developing mammal in utero. Bannon and co-workers fed pregnant rats a diet containing 25% galactose and found histological abnormalities of the lens in fetuses. Segal and Bernstein extended these studies and found a high incidence of cataracts in the new born of pregnant rats fed a diet containing 40% galactose. Using similar conditions. Haworth et al. reported retarded body and brain growth and reduced numbers of brain cells in offspring of galactose-fed females.

These findings in experimental animals are consistent with clinical observations of congenital galactosemia, which leads to mental retardation and lens opacities. Results from the animal experiments suggest that the effect of congenital galactosemia in humans may be initiated in utero, and women known to heterozygous for the galactosemia gene should take a diet low in lactose during pregnancy.

11.1.1.10 Lipids

Deficiency—Although malformations have not been reported as a result of dietary lipid deficiency, the lipid composition of the maternal diet can have profound effects on the composition and metabolism of the fetus. A fat-deficient diet will not support normal pregnancy or lactation, and young neonates do not survive due to a lack of essential fatty acids (EFA), especially linoleic. Alling et al. reported that a low level of EFA in the diet of rats for two generations caused low birth weight in the offspring and lower concentration of cerebrosides in their brains than in controls.

Excess—When diets containing high levels of cholesterol were fed to pregnant rodents, there was high fetal mortality and low fetal weight in rabbits, but not in rats. Drugs used to increase and decrease the blood cholesterol level have both been shown to be teratogenic in experimental animals.

11.2 VITAMINS

11.2.1 Fat-soluble Vitamins

11.2.1.1 Vitamin A

Deficiency—The first experimental production of a congenital malformation by environmental means of a vitamin A-deficient sow were born without eyeballs. In addition to various eye defects, vitamin A deficiency in pregnant sows produced other anomalies such as accessory ears, subcutaneous cysts, cleft lips, and misplaced kidneys. Palludan has expanded on the early work of Hale in pigs and analyzed the morphogenesis of the eye in embryos from both normal and vitamin A-deficient sows.

Warkany and his colleagues have studied in great detail the teratogenic effects of vitamin A deficiency in the rat. Eye abnormalities consist of colobomas, eversion retinal abnormalities, defects of cornea, and "open eye"; a fibrous retrolenticular membrane frequently occurred in place of the vitreous body. Additional defects were abnormalities of the genitourinary tract including fused kidneys, absence of male accessory glands, and lack of vaginal development, as well as keratinizing metaplasia in epithelia derived from the embryonic urogenital sinus in fetuses. Cardiovascular problems consisted of defects in the interventricular septum and various aortic arch anomalies. Vitamin A deficiency during the prenatal period also resulted in diaphragmatic hernia. After analyzing the anomalies resulting from prenatal vitamin A deficiency, Wilson and his colleagues

concluded that the malformations were dependent on the period of active organogenesis at the time of the nutritional insult. Roux et al. found that urogenital and eye anomalies could result even with vitamin A deficiencies and were so mild that the mother appeared normal.

In rabbits, mild-deficiencies of vitamin A resulted in infertile ova and degeneration of fertilized ova prior to implantation. Deficiencies of the vitamin during late gestation resulted in resorption, abortion, fetal ocular abnormalities and hydrocephalus in newborn and postnatal young.

Although the teratogenicity of vitamin A deficiency has been described extensively in a number of species, the mechanism of action remains obscure. Clausen has provided some evidence that the role of vitamin A in sulfation may be related to the effects on myelination.

Excess—Cohlan first observed that hypervitaminosis A in pregnant rats resulted in congenital malformations in the offspring. Gross anomalies of the skull and brain occurred in 54% of the offspring when 35,000 IU of vitamin A was given mothers form the 2nd, 3rd, or 4th to the 16th day of gestation. Of the numerous studies published since, the most notable are the extensive series of experiments by Giroud and his collaborators. They confirmed the observation of Cohlan on the teratogenicity of vitamin A and showed that the types of anomalies depended on the gestational stage at the time of vitamin A administration. The most common malformations were anencephaly and cleft palate, but malformations of the face and eye, anophthalmia, spina, bifida, syndactyly, and malformations also occurred. Giroud and his collaborators have extended their findings to the histological level, describing ureterohydronephrosis, encephalocele, and meningocele. Masi et al. have shown that excess vitamin A also results in dental malformations. In addition to the rat, hypervitaminosis A has been shown to produce congenital malformations in other species. Giroud and Martinet have produced malformations in the mouse with relatively small doses of the vitamin. Congenital malformations have also been produced in the guinea pig, rabbit, hamster, and pig

Mechanism of action—Anatomical studies and experiments using tritiated thymidine indicate that the abnormal morphogenesis induced by vitamin A resulted from a loss of synchrony in differential growth rates of tissues. DNA synthesis in neuroepithelia cells is abnormal,

and there is lengthening of the cell cycle, leading to a decreased rate of cell proliferation. Nanda observed that vitamin A retards the growth of the palatal processes so they do not come in contact with each other at the required time, leading to cleft palate.

Abnormal metabolism of sulfated mucopolysaccharides has been suggested as another mechanism for the teratogenic action of vitamin A possibly involving the general effect of the vitamin on membranes of cells and intracellular organelles. Takekoshi has suggested that the teratogenecity of vitamin A involves the thyroid gland.

The ester form of vitamin A, retinyl acetate, has been used in the majority of experiments, but retinoic acid is also teratogenic. It is about 40 times more active than other vitamin A compounds in producing congenital malformations and is rapidly eliminated from the body. Kochhar utilized retinoic acid to relate specific embryonic stages to the production of limb abnormalities and suggested that retinoic acid had a disruptive effect on spatial organization of mesenchymal cell condensations, resulting in abnormally shaped cartilage models.

Postnatal Effects of Prenatal Hypervitaminosis A—Learning ability in rats subjected to mild teratogenic doses of vitamin A during gestation was impaired, although few malformations were observed. Other behavioral deficits have been reported when excess vitamin A was given on days 14 and 15 or on days 17 and 18 of gestation.

Human—A few cases of the teratogenic effects of vitamin A deficiency or excess have been reported in humans. Sarma reported the birth of a premature baby with microcephaly and anophthalmia form a woman deficient in vitamin A. McLaren observed that intrauterine vitamin A deficiency as well as other nutritional deficiencies may be responsible for eye abnormalities and impaired vision in children. A child with congenital urogenital anomalies was reported born to a woman who took excessive amounts of vitamin A during pregnancy (25,000 IU daily for the first 3 months and 50,000 IU daily thereafter). Gal and co-workers also found that maternal serum vitamin A levels postpartum were significantly higher in mothers of infants with central nervous system (CNS) malformations than in mothers of normal babies. Fetal liver vitamin A levels were consistent with this finding. Insufficient information is available on the relationship of vitamin A to both gross congenital malformations and subtle postnatal deficits in the human. Fundamental research is needed on placental transfer of vitamin A

to the fetus; the relationship of maternal, fetal, and neonatal serum levels to maternal dietary vitamin A; and the neonatal and postnatal condition of the infant.

11.2.1.2 Vitamin D

Deficiency—Since vitamin D is required for the absorption and utilization of calcium, deficiency of the vitamin during pregnancy results in abnormal development of the fetal skeleton. Even if calcium and phosphorus are present in subobtium amounts in the diet, fetuses have nearly normal concentrations of these elements if sufficient quantities of vitamin D are available during pregnancy. Warkany found that the offspring of rats given a rachitogenic diet had skeletal abnormalities at birth which resembled those of rickets. Wallis reported similar findings in cattle. Vitamin D deficiency in association with low calcium intake in humans leads to the development of fetal rickets and in some cases neonatal tetany and enamel hypoplasia of the teeth. Mellanby and Coumoulso have provided some evidence that these conditions may lead to poor dentition and high susceptibility to dental caries. Recently, the newly identified active metabolites of vitamin D have led to new treatments for genetic diseases involving vitamin D.

Excess—Vitamin D in excessive amounts in the diet of pregnant rats produced offspring of low birth weight and with low concentrations of calcium and phosphorus. In rats, hypercalcemia does not normally occur with excessive administration of vitamin D. A decrease in wet weight, ash weight, calcium and phosphorus content of the carcass and fetal bones with impairment of osteogenesis resulted when large dosages of vitamin D were given to pregnant rats. Abnormal bone development, with thick periosteum, thin compact bone and multiple fractures with impaired fracture healing persisted during the postnatal period in such rats as early as the fifth day of life.

Hypervitaminosis D during pregnancy results in retarded growth in the offspring that persists throughout life, becoming more marked with time. Ornoy et al. reported that excessive vitamin D_2 caused fundamental damage to fetal osteogenic tissues. In these studies, however, no attempt was made to control the possible effect of maternal hypervitaminosis D on location so that there may also have been impaired nutrition during the suckling period. Potvliege concluded that the pregnant rat is less susceptible to toxic effects of vitamin D than in her nonpregnant counterpart.

In rabbits, hypervitaminosis D during pregnancy produced hypercalcemia in the mothers and anomalies of the aorta in the fetuses which resembled supravalvular aortic stenosis syndrome (SASS) in children. Cranial, facial, and dental anomalies were observed in these rabbit fetuses and are also seen in children with SASS. There was also hypoplasia of the mandible, congenital absence of teeth, microdontia, dysgnathia, enamel hypoplasia, and malocclusion. Peculiar facies, premature closure of the cranial bones, strabismus, odd-shaped ears, and low birth weight were also noted. The level of vitamin D in maternal and fetal blood was much higher in rabbits treated with high levels of the vitamin thar in controls, showing that transplacental passage took place. Serum calcium levels were also higher in fetuses as well as in the maternal animals.

Human—Idiopathic infantile hypercalcemia, a genetic disease in children, appears to be similar in many of its features to the effects of excessive vitamin D in the pregnant rabbit. Vitamin D metabolism appears to be abnormal in children with the disease. Goodenday and Gordan studied children ranging in age from 6 weeks to 16 years born to mothers who received vitamin D therapy for hypoparathyroidism. The children were normal and none had any of the cardiovascular or craniofacial stigmata associated with infantile hypercalcemia. The large amounts of vitamin D given to these hypoparathyroid women did not produce hypercalcemia in the mother and thus differed from experimental animals in which fetal anomalies occurred.

11.2.1.3 Vitamin K

Vitamin K is essential for normal clotting of the blood and deficiencies result in hemorrhage and death. Schofield described the sweet clover disease in cattle which causes a deficiency of vitamin K due to the ingestion of dicoumarol, a vitamin K analogue. Cattle ingesting dicoumarol produced calves which die of hemorrhage. "The active well-developed calf born of a cow fed for 13 days on damaged sweet clover hay dies of hemorrhage 28 hours after birth.

Rabbits given a vitamin K-deficient diet had a high rate of abortion. When dicoumarol was given for only 2 days, fetuses died in utero. Lower levels of dicoumarol resulted in newborn rabbits with very low prothrombin and a tendency to hemorrhage, although the mother appeared normal. Quick showed that pups of dogs given dicoumarol at therapeutic levels in the last week of pregnancy had a

reduced prothrombin level at birth, although that of the mother was normal.

Since dicoumarol is used in the treatment of clotting diseases (thromboembolic diseases) in humans, considerable concern has been expressed as to whether this drug has any deleterious effects on the growth and development of the human fetus if given to pregnant women. Such treatment may continue over a long period of time, especially in patients with mitrial valve prosthesis. Several cases of fatal anomalies have been reported in the infants of women undergoing such treatment. In one, the infant was blind and mentally retarded. In another, there were hypoplastic nasal structures. In a third report, fetal or neonatal death occurred. Other studies, however, suggested that oral anticoagulants of the coumarin type are not teratogenic or deleterious to the fetus if the therapy is properly managed.

11.2.1.4 Vitamin E

Vitamin E was discovered by Evans and Bishop in experiments investigating the relationship of nutrition to reproduction in rats. Animals given a diet later known to be deficient in vitamin E were sterile. Cheng and her collaborators were able to maintain pregnancy in the rat and produce teratogenic effects through the administration of small amounts of vitamin E. When vitamin E-deficients rats were given 2 mg of DL-α-tocopherol acetate by stomach tube on the 10^{th} day of gestation, the resorption rate was high and 37% of the live fetuses showed one or more malformations at term. Anomalies included exencephalus, umbilical hernia, scoliosis, club feet, cleft lip, syndactyly, anencephalus, and kinked tail.

The percentage of abnormal young was dependent on the composition and amount of vitamin E contained in the diet. Fetal tocopherol levels, however, did not correlate with the vitamin E intake of the mothers. Gortner and Ekwurtzed were unable to repeat the finding of Cheng concerning teratogenic effects of Vitamin E; vitamin E-deficient diets caused only minor effects on fetal development.

Kenney and Roderuck chronically depleted rats of vitamin E and found that by giving tocopherol at a critical time in development, they could produce a syndrome resembling eclampsia in rats. Gestation was prolonged and most of the young were stillborn. Maternal mortality at parturition was high, but the dead fetuses did

not appear to be grossly abnormal. Fetal deaths and resorption also occur in mice as a result of Vitamin E deficiency.

11.2.2 Water-soluble Vitamins

11.2.2.1 Ascorbic acid

Deficiency—Unlike the other Vitamins, ascorbic acid is required by only a few animal species, namely, man and other primates, the guinea pig, and certain fishes and tropical birds. Information on the effect of ascorbic acid deficiency during pregnancy in mammals is limited to a few studies with guinea pigs. Mouriquand and co-workers reported that a vitamin C-deficient diet resulted in spontaneous abortions in the guinea pig, especially between the 26th and 30th days of gestation.

Rivers et al. studied the effects of ascorbic acid deficiency histologically and in relation to its biochemical role in collagen synthesis. They measured levels of ascorbic acid, proline and hydroxyproline and examined mucopolysaccharide, collagen, and elastin components histologically. Collagen synthesis was impaired and histological anomalies were present in mucopolysaccharides, collagen, and elastin. Since the mothers were given the deficient diet only between days 20 and 35, they themselves showed no outward signs of vitamin C deficiency.

Although scurvy has been known for hundreds of years, surprisingly little has been reported for ascorbic acid deficiency in human pregnancy. The relationship between vitamin C intake and serum levels during pregnancy was studied in the Vanderbilt cooperative study of maternal and infant nutrition. In general, serum levels decreased during pregnancy except in women who had a high level of ascorbic acid intake. There was no difference in congenital malformations between groups with the highest and lowest serum levels, but an increased frequency of premature birth was associated with the lowest intake on vitamin C and women with the lowest serum concentrations.

Excess—Massive doses of vitamin C during pregnancy have been shown to be detrimental in several species. High levels of vitamin C intake resulted in infertility and fetal mortality in guinea pigs, although no toxic effects were seen in the pregnant females. Mouriquand and Edel substantiated these findings and also observed abortions. Samborskaya and Ferdman reported stillbirths and abortions in guinea pigs, rats, and mice. In women, large doses of vitamin C have also

been reported to terminate early pregnancy; however, no control group was used.

11.2.2.2 B-complex Vitamins

Riboflavin

Deficiency—Following the reports of hale on congenital malformations in vitamin A-deficient pigs, Warkany and Schraffengerger reported that riboflavin deficiency in pregnant rats caused malformations of the newborn. Malformations consisted of shortness of the mandible and the long bones, fusion of the rigs and sternal centers of ossification, syndactylism, brachydactylism, and cleft palate. In later work, dentofacial changes, mainly micrognathia, cleft palate and associated dental anomalies, hydrocephalus, and eye defects, have been observed.

The teratogenic effects produced by dietary riboflavin deficiency in rats was soon confirmed by Giroud and Boisselot. Bologna and Biccioni showed that if a riboflavin-deficient diet was given 40 days before pregnancy, about half the newborns were abnormal, but if the diet was given to females 60 days before mating, no young were produced. Even extremely mild deficiency of riboflavin was shown to be teratogenic. Using glactoflavin, a riboflavin antimetabolite, Nelson and her colleagues produced acute transitory riboflavin deficiency in pregnant rats. They found skeletal anomalies and soft tissue defects not previously reported in the cardiovascular and urogenital system and the diaphragm and epidermis. In mice, Kalter and Warkany used galactoflavin in combination with a riboflavin-deficient diet to produce anomalies in mice and found that the type of frequency of malformations were dependent on the strain used.

Shepard et al. have investigated the development of limb malformation sin riboflavin-deficient, galactoflavin-treated embryos using histological and histochemical techniques. The use of antimetabolites to produce vitamin deficiencies is very useful in the clarification of temporal relationships involved in abnormal development, but they may have toxic effects in addition to their antivitamin action.

In pregnant pigs, riboflavin deficiency resulted in young that were dead at birth or died within 48 hr thereafter, with "enlargement of the front legs due to gelatinous edema in the connective tissue.

Mechanism of action—Noting the similarities in the malformations resulting from riboflavin and folic acid deficiencies,

Miller et al. investigated the effect of riboflavin deficiency on flavin and folate levels in whole embryos and in fetal and maternal livers. Their results suggested that galactoflavin teratogenicity may be due to a low level of flavin adenine dinucleotide (FAD) during differentiation. Maternal liver folate and citrovorum factor were reduced by 50% after 16 days of a riboflavin-deficient diet, but it was not possible to correlate the folate levels with the teratogenicity of the riboflavin deficiency.

Aksu et al. examined the succinic and nicotinamide adenine dinucleotide (NADH) oxidase systems (the terminal electron transport systems) in riboflavin deficient, galactoflavin-treated rats. Activity of the oxidase systems was lower than control values at 12 through 16 days of gestation in embryos and fetuses, but not in the placenta and maternal heart. They concluded that the teratogenic effect of riboflavin deficiency results from a reduction in the activity of electron transport system. Shepard and Bass, however, concluded that the basic mechanisms for limb defects occurred at the local cell level and not from generalized physiologic impairment of the embryo.

Potier de Courcy and colleagues have extensively investigated chemical and biochemical changes in the maternal fetal organism resulting from riboflavin deficiency. They found a reduction in glycogen, protein, DNA, RNA and alkaline phosphatase in the placenta and fetus. The calcium concentration, while normal in 13-day embryos, was reduced at 21 days. Sodium and potassium concentrations were normal, as were zinc levels.

Riboflavin in human pregnancy—The course of pregnancy labor, condition of the newborn, and neonatal development of infants was studied in a group of pregnant women found to have clinical riboflavine deficiency. There was an increase in the incidence of prematurity, stillbirths, and vomiting; no effects were seen in toxemia, infections, or hemorrhagic complications. Riboflavin deficiency depressed lactation, but birth weight and the incidence of malformations were unaffected.

11.2.2.2.1 Thiamine

Nelson and Evans gave pregnant rats a thiamine-deficient diet beginning on the day of mating or for 1 to 3 weeks prior to pregnancy. The deficiency resulted in a very high rate of resorptions and still births, and living young were of low birth weight. Maternal body weight decreased and maternal mortality increased. These effects appeared largely as a result of reduced food intake as a consequence

of the vitamin deficiency. Injections of estrone and progesterone maintained pregnancy even though food intake remained restricted.

Brown and Snodgrass fed female rats from weaning to maintain a diet low in thiamine, but gave adequate thiamine during pregnancy. The rats produced litters, but they were smaller tan controls.

In the pregnant sow, Ensminger and co-workers reported that a thiamine-deficient diet resulted I a high rate of stillbirths, weak new born young, and poor maintenance of pregnancy. In humans, congenital beriberi was seen in the newborn infants of pregnant women with the thiamine deficiency.

11.2.2.2.2 Niacin

Most of the work on the importance of niacin (nicotinic acid) during prenatal development has been done with the use of an antimetabolite, 6-aminonicotinamide (6-AN). Multiple congenital abnormalities resulted when this antagonist was fed to pregnant rate. When 6-AN was given for 2- or 3-day periods during pregnancy, a high incidence of embryonic mortality and resorption, and a low incidence of abnormal young were observed. Malformations included defects of the skeleton, central nervous system, eye, urinary system, trunk, thyroid, and thymus gland. Single injections of 6-AN resulted in a high incidence of hydrocephalus as well as ocular, urogenital, and vascular anomalies. Chamberlain and Nelson also observed skeletal defects, cleft palate, cleft lips, and exomphalos with single injection. Chamberlain has also used 6-AN to study the development of cleft palate late in gestation and to study the role of niacin in the rat embryo using in vitro studies.

Ruffo and Vescia fed a nicotinic acid deficient diet to rats without an antagonists and found a marked reduction in the number of young born to rats. Using a diet deficient in tryptophan as well as niacin (since the rate can convert tryptophan to niacin), Fratt et al. also observed a high rate of fetal resorptions but did not se congenital malformations. Malformations in rats were, however, reported by Greengard et al. from a seemingly similar diet, although a description of the diet was not given. They were able to prevent the effects by giving adrenocortical steroids.

11.2.2.2.3 Vitamin B_6

Deficiency—In 1948, Nelson and Evans published the first report on the essentiality of pyridoxine in the pregnant rat as a part of their investigations on the role of nutritional factors in reproduction.

Using the antagonist desoxypyridoxine, they produced pyridoxine deficiency and observed a very high incidence of resorptions. There was a reduction in the number of young per litter and birth weight; the percentage of fetuses dead at parturition was high. Since injection was estrone and progesterone of gonadotropic hormones could maintain pregnancy, they thought that resorptions resulted from abnormal gonadal function. Davis et al., using much higher levels of desoxypyridoxine, reported congenital malformations resulting from vitamin B_6-deficiency. The malformations observed include digital defects, cleft palate, omphalocele, micrognathia, and exencephaly.

Eberle and Eiduson have studied pyridoxine deficiency in pregnant rats without the use of pyridoxine antagonist. They concluded that the fetus was protected from B6 deficiency during pregnancy. Other workers, however, found that feeding a pyridoxine-deficient diet to pregnant rats during the last 2 weeks of gestation and during lactation resulted in the birth of some young that had convulsions as early as 3 days of age. Plasma transminases and pyridoxal phosphate of brain were low in B_6-deficient pups.

The level of pyridoxine in the diet prior to pregnancy is important. Rats given a pyridoxine-deficient diet prior to pregnancy and supplemented during the course of pregnancy gave birth to young with birth weights as low as or lower than offspring born to mothers receiving desoxypyridoxine during pregnancy. Korner and Nowak monitored the degree of pyridoxine deficiency using the tryptophan loading test and the erythrocytic glutamate-oxalacetate transaminase (EGOT) activation test. They studied the effect of B6 deficiency during period of gestation, or gestation and lactation, on the biochemical development of the rain in fetal and neonatal rats. Deficiencies of B_6 during gestation and lactation resulted in poor survival, retarded weight gain, reduced activity, and a higher incidence of errors in a maze test and even death by convulsive seizure.

The concentration of gamma-amino butric acid (GABA) and activities of glutamate-decarboxylase and dopa-decarboxylase were lower in the brains of these animals. Other differences in brain chemistry included lower concentrations of pyridoxine, cerebrosides, and protein. In other studies, Moon and Kirksey studied cellular growth during prenatal and early postnatal periods in progeny of pyridoxine-deficient rats, but made no mention of congenital anomalies in their newborn young. It should be noted that most of these studies did not differentiate between deficiency during the

periods of pregnancy and lactation, i.e., between nutrition of the unborn fetus and the suckling young.

Excess— Schumacher et al. studied the effect of a high intake of pyridoxine during pregnancy and found no effect on the young, little size, growth to weaning or requirement for pyridoxine after weaning.

11.2.2.3 Folic acid

Experimental animals—Folic acid deficiency in pregnancy has been studied primarily with the use of folate antagonists. The first experiment in which congenital malformations were produced by folate deficiency used only an intestinal antibiotic to suppress vitamin synthesis by the intestinal microflora in combination with a folate-deficient diet. A mild deficiency was produced by Giroud and Lofebvres-Boisselot, resulting in cleft lip, coloboma, hydrocephalus, ectopia of thoracic and abdominal organs, and eye abnormalities.

In an extensive series of publications, Nelson and her colleagues reported in detail on the wide variety of congenital malformations using the antagonist x-methyl pteroylglutamic acid (x-methyl PGA) in conjunction with a folate-deficient diet. The timing of the insult was important in determining if the number of resorptions was high and the type of congenital abnormalities produced.

Abnormalities included edema and anemia, cleft palate, facial defects, syndactyly, skeletal abnormalities, and anomalies of the urinary system, lungs, and eyes, defects of the cardiovascular system were also seen. Short transitory subjection of the pregnant rat to this experimental regimen produced offspring with a high incidence of multiple abnormalities. Malformations in 70 to 100% of the full-term young resulted from only 48 hr of deficiency between day 7 and day 12 of gestation. Tuchmann-Duplessis and co-workers have also used x-methyl PGA to produce congenital malformations in the mouse and cat.

Several groups have studied nucleic acid synthesis in relation to congenital malformations resulting from folate deficiency. Aminopterin, a folate antagonist, depressed DNA but not RNA levels in the fetal liver. Potier de Courcy, however, found significant decreases in fetal protein, DNA, and RNA of the whole body, liver, brain, and placenta. Administration of DNA, orotic acid, or ascorbic acid to pregnant rats did not alter these effects. The lower DNA content did not correlate with DNase activity and was attributed to an inhibition of DNA synthesis rather than to increased catabolism.

Johnson and his colleagues have utilized 9-methyl-PGA in combination with folate deficiency and found reduced levels of ATP, ADP, and AMP compared to untreated rats. These observations might be related to the increased oxygen consumption observed by Netzloff et al., in similarly treated embryos. Alterations in nonspecific isoenzyme patterns of a number of enzymes were reported by Johnson and Chepenik. Studies of phosphomonoesterases in fetal limbs showed marked depression of activity in fetuses from O-methyl-PGA-treated females. Changes in enzyme patterns correlated with anomalous chondrogenesis and osteogenesis.

Humans—Folic acid is an essential nutrient for the developing human embryo and adult. The folate antagonist aminopterin has been used as an abortifacient agent. If given too late or in insufficient quantity, abortion was not produced and the result was a malformed child. Whether folate deficiency in pregnant women is teratogenic is still controversial. Folic acid deficiency occurs in 3 to 22% of pregnant women, but concrete evidence linking it to malformations is lacking.

Fraser and Watt reported that 5 of 17 women with megaloblastic anemia gave birth to infants with serious birth defects. The Hibbards observed folate deficiency in 62% of mothers of malformed infants as compared with 17% in mothers of normal infants, using elevated urinary excretion of formimino glutamic acid (FIGLU) after a histidine load as a sing of folate deficiency. They concluded that an association did exist between malformations of the nervous system and folate deficiency and suggested that a genetic factor might be involved that influences folate metabolism. Other investigators, however, did not find a correlation between malformation and folate status as measured by plasma levels and erythrocyte morphology.

11.2.2.3.1 Pantothenic acid

Pantothenic acid, the active metabolic form of which is coenzyme A, is important for embryonic development. Nelson and Evans first showed that dietary deficiency of the vitamin during pregnancy resulted in resorptions and small litters with underdeveloped young. Lefebvres-Boisselot reported exencephaly, anophthalmia or microphthalmia, edema, and hemorrhage in pantothenate-deficient animals. Other anomalies included limb abnormalities, malformations of the brain and renal system, and abnormal intrauterine growth without malformation at parturition.

Fetal abnormalities occurred in the absence of deficiency signs in the mother. Analysis of pantothenic acid in maternal and fetal livers showed that when maternal liver pantothenate fell to 40%, fetal death occurred, but with less severe depression of pantothenate levels, malformations resulted. In one study on the 10th day of gestation (a critical time for development of brain and eye malformations), the level of maternal liver pantothenate in pantothenate-deficient rats was only 16% lower than normal. Biochemical examination indicated that the content of protein, DNA, and RNA was low in liver, brain, and total body of fetuses from deficient mothers at term. The brain, however, which showed the least depression of these values, had the highest incidence of malformations. The placenta showed similar changes, but the maternal liver was normal with respect to these biochemical parameters.

Nelson et al. used a pantothenate antagonist, Ω-methyl-pantothenic acid, in combination with a deficient diet and observed cerebrum and eye defects, digital hemorrhages and edema, interventricular septal defects, anomalies of the aortic arch pattern, hydronephrosis and hydroureter, clubfoot, cleft palate, and tail and dermal defects. High doses of ascorbic acid partially compensated for the effects of the deficiency.

The postnatal effects of pantothenate deficiency during the first 14 days of gestation included motor incoordination, deficient righting reflexes, motor spasms, and poor head orientation. These abnormalities persisted into adulthood.

Hurley and her colleagues studied the effect of pantothenic acid deficiency during pregnancy in the guinea pig and found that deficiency during the 9th and 10th (last) weeks of gestation resulted in abortion or death of the mother. Liver fat and pantothenic acid levels of the newborn were also affected. The experiments suggested that the greatest need for pantothenic acid during the fetal development of the guinea pig is during the period shortly before birth, which the concentration of pantothenic acid and coenzyme A in fetal liver rises sharply in this species.

Ullrey et al. reported that pantothenic acid-deficient diets in pregnant pigs resulted in abnormal prenatal development, infertility, resorptions, and abortions.

11.2.2.3.2 Vitamin B_{12}

In the rat, the abnormality resulting from vitamin B_{12} deficiency during prenatal development is hydrocephalus. Because of the

association of folic acid and vitamin B_{12}, much early work is confounded by the inability to separate these two deficiencies. The hydrocephalus which occurred under thee conditions appeared to be a result of vitamin B_{12} deficiency. Woodard and Newberne showed that the etiology of hydrocephalus produced by vitamin B_{12}-deficient diet is the same with or without added x-methyl folic acid.

Newberne and Young have studied the long-term effects of the deficiency in the progeny. In offspring of rats receiving a diet marginally deficient in vitamin B_{12}, the birth weight was low and body weight remained significantly lower than normal after 21 days, 3 months, and 1 year. Lover enzyme activities and B_{12} concentration were also low in newborns.

11.2.2.3.3 Choline

Ensminger and his colleagues have studied that importance of choline on prenatal development in the sow. Most of the offspring died when the maternal diet was deficient in choline Kinked tails as well as fatty livers, hemorrhages of the kidney, and other kidney abnormalities were observed during the postmortum and microscopic examination of the newborn. In mice, offspring born to mothers made choline-deficient during pregnancy had a high incidence of postnatal mortality. Full-term fetuses of rats given a choline-deficient diet and their mothers had fatty livers.

Kartzing and Perry studied the postnatal effects of a prenatal dietary deficiency of choline in rats and reported that the progeny were hypertensive at 35 to 58 days of age. By 86 to 108 days, however, the blood pressure in these animals returned to normal. These findings may be related to alterations in kidney function or structure. The influence of dies varying in methionine and choline content with and without vitamin B12 has been investigated in the developing rat embryo by Newberne et al. While marginally deficient newborn offspring appeared clinically normal, biochemical measurements indicated that brain cells were smaller and there were irregularities in protein synthesis.

11.3 MINERAL ELEMENTS

11.3.1 Major Elements

11.3.1.1 Calcium

The effects of calcium deficiency during pregnancy are similar to those of vitamin D deficiency. One of the earliest experiments

demonstrating the importance of maternal nutrition for fetal development involved calcium. In 1911, Hart et al., reported that when cows were fed only wheat during pregnancy, the offspring were stillborn or immature and the weak calves did not live. When the ration was supplemented with bone meal, the calves were normal. Abnormal effects are not seen in the fetus until the calcium deficiency is prolonged. Even under conditions of calcium deficiency in the mother, fetal development and even calcification may be normal. Release of maternal skeletal calcium occurs under such conditions by stimulation from the parathyroid glands.

Severe calcium deficiency over a prolonged period of time will interfere with the normal development of the embryo. Bawden and Osborne have shown that the calcium concentrations of the litter was not depressed when pregnant rats were given a calcium-deficient diet, but there was a highly significant difference between the ash content of the femurs of calcium-deficient females and those of controls. Itoh et al., reported similar findings in pregnant sows on normal and calcium-deficient rations. Comar has studied the transfer of calcium from maternal to fetal tissues.

The ossification and calcification of the fetal skeleton are dependent on the ratio of calcium to phosphorus, in addition to the amount of calcium in the maternal diet. In humans, proper ossification of the fetus is dependent on an adequate supply of dietary calcium and phosphorus.

Excessive calcium in the diet is also deleterious to the young of pregnant animals. Fairney and Weir reported that the offspring of hypercalcemic rats (produced by feeding a high calcium diet) were abnormal. Hypercalcemic mothers produced small liters with offspring of low birth weight, poor growth, and short sparse fur with patchy alopecia.

11.3.1.2 Magnesium

The rapidity with which magnesium deficiency is produced is similar zinc deficiency. Hurley and Cosens observed embryonic death and malformations in rat fetuses with severe magnesium deficiency. Milder deficiencies resulted in live embryos that were small, edematous, pale, and malformed, with low plasma protein and severe anemia. In maternal animals with mild deficiencies of magnesium, packed cell volume (PCV) and hemoglobin were normal; however, in term fetuses, hemoglobin, PCV, and red blood cell count were markedly reduced. Cohlan et al. and Dancis et al. also observed a

fall in the maternal plasma magnesium, decreased magnesium concentration in the fetus, and fetal anemia in magnesium-deficient rats during pregnancy. Hurley has reviewed the relationships of magnesium deficiency to human pregnancy.

11.3.1.3 Sodium and potassium

Surprisingly, few studies have been reported on sodium deficiency in prenatal development. In Early work, Orent-Keiles and co-workers studied the effect of a very low intake of sodium in rats. Mating of sodium-depleted rats produced very few young, and those that survived to full-term were extremely small in size. However, when pregnant rats were given a diet low in sodium for 1 week prior to mating and throughout gestation, litter size, resorptions, and fetal weight at term were not significantly affected, nor were fetal levels of total sodium or potassium levels. A high sodium intake by the mother also had no effect in these studies.

Dancis and Springer studied fetal homeostasis in rats in relation to potassium and sodium deficiency of the maternal diet. In both deficiencies, the young were of low body weight at term, but potassium concentration in fetal plasma did not change, although it was decreased in fetal tissues. Sodium deficiency, on the other hand, produced proportional depression of plasma sodium in the fetus. Maternal hyperkalemia induced hyperkalemia in the fetus as well.

In ewes, sodium depletion resulted in lower than normal levels of sodium in fetal plasma and amniotic fluid and higher than normal volume of allantoic fluid. In mice subcutaneous injections of sodium chloride at levels of 1900 or 2500 mg/kg at 10 or 11 days of gestation produced fetal death and malformations.

11.3.2 Trace Elements

11.3.2.1 Iron

The most noteworthy effect of iron deficiency in both animals and man is anemia characterized by low levels of hemoglobin. Iron deficiency during pregnancy results in hypochromic microcytic anemia in the mother and newborn young. The severity of anemia in the offspring depends on the degree of iron deficiency on the maternal diet.

All showed that the degree of iron deficiency in the offspring of female rats given a diet lacking in iron increased with successive pregnancies. O'Dell and co-workers fed a diet more complete than that used by Alt and reported that pregnant rats were mildly anemic,

but their offspring were severely anemic, weak, and almost entirely nonviable although they were not grossly malformed. In humans, it has also been reported that infants of anemic mothers frequently shared the iron deficiency and were anemic. The more severe the anemia of the mother, the more severe that of the infants at birth. Sisson and Lund have shown that anemia in newborns is important in the etiology of anemia in later infancy.

11.3.2.2 Copper

11.3.2.2.1 Deficiency

Lambs—The importance of copper for prenatal development arose from investigations of a disease in lambs called enzootic ataxia or "swayback," in Western Australia. The disease is characterized by spastic paralysis especially of hind limbs, severe incoordination, and in some cases blindness in newborn lambs. The brain of the affected animal is smaller than normal., with collapsed cerebral hemispheres and shallow convolutions. The cerebella are particularly small and there is marked demyelination throughout the brain. Enzootic ataxia in sheep results primarily from copper deficiency during the gestation period. The copper content of the livers of affected lambs was low, as was the copper content of the blood, milk, and liver of maternal ewes. Copper administration of the pregnant ewe prevents the disease.

Palsson and Grimsson reported a conditional identical to enzootic ataxia in lambs born to ewes fed seaweed, and Roberts et al. reported another hypocuprotic disease in lambs that is similar but not identical, to swayback.

Cancilla and Barlow have studied the ultrastructure of the central nervous system in lambs with swayback. They reported an increased size of nerve cells, progressive lack of Nissl substance, abnormalities of the Golgi apparatus, increase in neurofibrils, and an alteration of neuronal and astrological processes.

Excessive sulfate and moylbdate intake will also produce swayback by inducing a conditioned copper deficiency in the pregnant ewe. Low levels of copper were observed in the brain, along with a deficiency of cytochrome oxidase in the motor neurons. It is not clear, however, whether the activity of cytochrome oxidase can be correlated with incidence of swayback.

Experimental animals—In rats, copper deficiency during pregnancy resulted in a very high incidence of fetal death and

resorption. When rats were fed a copper-deficient diet for 1 month before mating, very few young survived to term. In studies with less severe copper deficiency, fetuses survived to term but were anemic, nonviable, edematous, and had characteristic subcutaneous hemorrhages. In many cases, copper-deficient offspring showed localized ischemia of the extremities, such as the feet and tip of the tail, and had a high incidence of skeletal anomalies and abdominal hernia. Histological sections of the skin also indicated marked changes including a decreased number of hair follicles.

In the guinea pig, Everson observed anomalies in the nervous system similar to those in lambs with enzootic ataxia. Newborn guinea pigs of mothers made copper-deficient during the periods of growth and gestation had a high incidence of ataxia, growth abnormalities, and gross malformations of the brain. Anomalies included agenesis of the cerebellum, soft and translucent cerebral cortical tissue, missing or abnormal cerebellar folia, and decreased myelin development. Phospholipid determination of whole brains were consistent with histological finding of myelination. Skeletal anomalies were also observed by Asling and Hurley.

An interaction between a mutant gene in mice (crinkled) and copper metabolism has recently been reported. Feeding mothers a high copper diet during the periods of gestation and lactation doubled the survival time of the mutant mice to 30 days. High dietary copper also prevented the characteristic lag in pigment development, thinness of epidermis, and paucity of hair bulbs in the mutant. Another interaction between a mutant gene copper metabolism has since been reported. Mice homozygous for the mutant gene (quaking) exhibit a high-frequency axial tremor. These mice also have a low concentration of copper in their brains. Copper supplementation during the gestation and suckling period reduced the frequency of tremors and brought brain copper levels to normal.

Humans—The kinky hair syndrome in infants in a fetal X-linked recessive genetic disease characterized by slow growth and progressive cerebral degeneration. Danks and his colleagues have demonstrated reduced intestinal absorption of copper as the underlying cause of the disorder. The primary defect appears to be the inability to transport copper across the serosal cell membrane.

Mjolerod et al. have reported a case in which a young woman was treated with penicillamine during pregnancy and gave birth to a child with connective tissue anomalies. Symptoms included lax skin,

hyperflexibility of the joints, vein fragility, varicosities, and impaired wound healing. They concluded that the anomalies were the result of the penicillamine treatment. Penicillamine is used to remove copper from patients with Wilson's disease, and it is possible that the treatment produced copper deficiency in the fetus. However, penicillamine also chelates and removes other trace elements such as zinc.

11.3.2.3 Iodine

Deficiency—The only known function of iodine is in relation to its role in thyroid hormone, and deficiencies are thus related to hypothyroidism. Iodine deficiency is the fetus results in cretinism, while in the adult it is associated with goiter. Cretinism was recognized as early as the 16th century, and its connection with goiter was realized by the beginning of the 19th century. In the middle of the 19th century, it was proposed that endemic goiter and (by implication) endemic cretinism were the result of iodine deficiency. Since then, the causative connection between iodine deficiency and endemic goiter has become clear, but that between iodine deficiency and endemic cretinism is less so.

The cretinous child is mentally and physically retarded with a potbelly, large tongue, and facies resembling those of Down syndrome. Other characteristics include shortness of stature, retarded dentition, delayed epiphyseal development, and skin that is coarse and myxedematous. Deaf-mutism or impaired speech may also be present. Fierro-Benitez and his associates reported on the pattern of cretinism in an area of highland Ecuador where iodine deficiency is severe; however, there was considerable variability in the data.

The most convincing evidence that iodine is causally related to endemic cretinism is the decline in its incidence after the introduction of iodine prophylaxis. In certain regions where there was a high rate of endemic goiter, a high incidence of cretinism, and low iodine content of the soil, the institution of iodized salt resulted in a decline in the incidence of endemic cretinism as well as endemic goiter. Pharoah et al. provided additional confirmatory evidence in which severe iodine deficiency was prevented by intramuscular administration of iodized oil in the New Guinea highlands. To determine whether endemic cretinism resulted from iodine deficiency, alternate families were treated with either iodized oil or saline solution. A follow-up study over 4 years revealed 26 endemic cretins out of a total of 534 children born to mothers who had not received iodized oil. In

comparison, only seven cases of endemic cretinism occurred among 498 children born to mothers treated with iodized oil. In six of the seven cases, the mother was pregnant with the trial began. In those who did not receive the iodized oil, mothers of five of the cretins were pregnant at the beginning of the trial. These data suggest that severe iodine deficiency in the mothers resulted in neurological damage during fetal development.

In addition to the importance of iodine deficiency in the development of endemic cretinism, it is possible that other factors, genetic or environmental, may interact with iodine deficiency to produce the condition. Differential responses to low levels of intake may also be involved.

In field animals, Hart and Steenbock in 1918 reported the birth of hairless pigs with hypertrophied thyroids. Stillborn or weak young also occurred.

Excess—Excessive iodine intake in pregnant women is also deleterious to the developing fetus. Several cases of congenital goiter and hypothyroidism due to maternal ingestion of excess iodine have been reported. Carswell et al. reported neonatal deaths and mental retardation in such cretinous survivors. Similarly, cretinism resulting from prenatal treatment with sodium iodine. I has been well documented. In such cases, the condition presumably developed through fetal thyroid damage by radioactive iodine therapy. Smith et al. have made similar observations in dogs.

11.3.2.4 Manganese

The first reports that manganese was an essential element for animals also showed that it was necessary for the prenatal development of mammals. Orent and McCollum found that offspring of manganese-deficient rats were unable to survive the postnatal period, but cross-fostering experiments showed that postnatal mortality was caused by debility of the offspring rather than by failure of lactation in the mother. Progeny of manganese-deficient rats were characteristically uncoordinated, with lack of equilibrium and retraction of the head. This same ataxic condition has also been reported in other mammals including the pig, guinea pig, and mouse. The critical period for the production of ataxia with manganese deficiency is between day 14 and 18 of gestation in the rat and it is irreversible. Development of body righting reflexes is delayed, presumably through abnormal development of the inner ear. The lesion responsible for the abnormal equilibrium and body

righting reflexes of manganese-deficient offspring appears to be the defective development of otoliths in the vestibular portion of the inner ear, resulting in missing or completely absent otoliths. Similar observations have been made in guinea pigs and rats.

Abnormal synthesis of mucopolysaccharides may be the underlying cause for the failure of otolith development in manganese-deficient animals. In vitro studies of fetal cartilage showed reduced and delayed uptake of ^{35}S in tibias and reduced uptake in the macular cells of the inner ear in manganese-deficient fetuses. Furthermore, a nonmetachromatic otolithic matrix was observed that did not contain ^{35}S.

Prenatal manganese deficiency also results in abnormalities of the skeleton including disproportionate growth and tibial epiphyseal dysplasia.

The mutant mice called pallid also have the ataxic condition caused by abnormal development of the otoliths seen in manganese deficiency. This congenital ataxia in mice homozygous for the pallid gene can be prevented by giving a diet containing high levels of manganese to the pregnant female. The failure of otolith development in pallid mice also seems to be due to abnormal synthesis of mucopolysaccharides as evidenced by reduced uptake of ^{35}S and may be related to ultrastructural changes.

In mink, a mutant gene exists that is analogous to pallid in the mouse. In pastel mink, approximately 25% of the animals exhibit an ataxic condition as a pleiotropic effect of the gene. Erway and Mitchell have demonstrated that this condition results from the reduction or absence of otoliths and that it can be prevented by manganese supplementation of the pregnant female. The discovery of analogous genes interacting with manganese in at least two species as well as the copper-crinkled and copper-quaking examples discussed above suggest that similar interactions may occur in other species including the human.

Hurley and Bell have studied manganese deficiency during gestation in mice with different genetic backgrounds. The response to a low dietary intake of manganese varied considerably, depending on the genetic strain, although, except for the pallid mutant, the usual level of dietary manganese was adequate for normal development. Thus, the recommended dietary level of a nutrient in this case manganese, is probably sufficient to prevent signs of deficiency for most individuals. However, the responses of individuals

at low or borderline levels will depend on their genetic background. Similar relationships between genetic and nutritional factors may account for differences in the incidence of cretinism in areas of low iodine intake.

11.3.2.5 Zinc

Experimental animals—Congenital malformations produced by zinc deficiency in mammals were first reported in rats by Hurley and Swenerton. When normal well-fed female rats were mated and given a zinc-deficient diet throughout pregnancy, half of the implantation sites were resorbed at parturition, live young weighed about half of controls, and all fetuses showed gross congenital malformation. When given a quantity of zinc-supplemented diet identical to that eaten by the zinc-deficiency mothers, all the offspring were normal. The teratogenic effect of dietary zinc deficiency has since been confirmed by several laboratories. Short transitory periods of zinc deficiency during pregnancy were also shown to be teratogenic. Zinc deficiency induced from day 6 to 14 of pregnancy resulted in about half the young being abnormal. When the deficiency was given for only the first 10 days of pregnancy 22% of the full term fetuses were malformed. Warkany and Petering have shown that with a deficiency period limited to 3 days (From the 10^{th} to 12^{th}), a small but significant percentage of young were abnormal and had malformations of the brain.

Zinc deficiency resulted in anomalies in every organ system, but the highest incidence occurred in the nervous system. In juvenile animals, the characteristics lesion of zinc deficiency is hyperplasia of the esophageal mucosa, which shows up as a typical histological abnormality.

Examination of zinc-deficient animals for changes in enzyme activities involved in carbohydrate metabolism has not aided in understanding the mechanism where by zinc deficiency produces malformations. Examination of the effects of zinc deficiency on nucleic acid synthesis has been more fruitful. In zinc-deficient embryos at 12 days for gestation, the uptake of tritiated thymidine was much lower than in normal embryos, suggesting that DNA synthesis was depressed. The reduction in uptake of ^{3}H-thymidine into DNA in zinc-deficient embryos is greatest in the head region, which is where the highest incidence of malformations occurs. Zinc repletion of zinc-deficient where the highest incidence of malformations occurs. Zinc repletion of zinc- deficient mothers reversed the lower

incorporation of ^{3}H-thymidine to normal levels. Falchuk et al. have shown that zinc is required for normal for normal cell cycles stages in *Euglena gracilis*.

Recently, Dreosti and Hurley have reported that the activity of thymidine kinase, an enzyme involved in DNA synthesis in rapidly proliferating tissues, is depressed in zinc-deficient embryos. Duncan and Hurley observed that the activity of thymidine kinase is depressed in zinc-deficient embryos as early as the 9th day of pregnancy; this is one of the earliest known alterations in the zinc deficient embryo and provides additional support that changes in nucleic acid synthesis are involved in the teratogenic effects of zinc deficiency.

The effect of dietary zinc deficiency in rats is rapid. Plasma zinc concentration in pregnant rats fell approximately 40% after only 24 hr on a zinc deficient diet. The pregnant rat apparently cannot mobilize zinc from material tissues in amounts sufficient to supply the requirements for normal fetal development. Although zinc cannot be mobilized under conditions of zinc deficiency, Hurley and Swenerton observed that when calcium was also lacking form the diet (resulting in a breakdown of bone), zinc was also released. The simultaneous deficiency of calcium with zinc alleviated the teratogenic effect of zinc deficiency in intact but not in parathyroidectomized rats. This indicated that conditions bringing about resorption of bone increase the availability of skeletal zinc.

Similar findings occurred when the breakdown of maternal tissues was increased by feeding pregnant mothers protein-deficient diets in addition to zinc. The percentage of implantation sites affected was lower in rats receiving a diet deficient in both protein and zinc than in those receiving a diet deficient in zinc alone.

In mild states of zinc deficiency containing less than 9 ppm zinc during pregnancy, the level of zinc in the diet correlated with the incidence of fetal death and malformation. The total weight of the litter and the fetal weight at term correlated with the level of dietary zinc up to 14 ppm. However, the incidence of malformations did not correlate with fetal zinc content or maternal plasma zinc at term, although a correlation did exist during the second trimester with plasma zinc.

The effect of transitory zinc deficiency during pregnancy was investigated by feeding mothers zinc-deficient diets for a given time period, followed by zinc replacement. When normal pregnant rats were given a zinc-deficient diet from day 6 to 14 of gestation, the

maternal plasma zinc fell rapidly during the deficiency period and quickly returned to normal with zinc supplementation. There was a high rate of stillbirths, low birth weight, a high incidence of congenital malformations, and very poor survival to weaning, with most of the postnatal mortality occurring in the first week. The plasma zinc of the young was normal, as were also the maternal plasma and milk, suggesting that postnatal zinc nutriture was adequate. These results indicate that a short period of zinc deficiency during prenatal life resulted in irreversible changes which subsequently affected postnatal development.

The rapid effect of zinc deficiency on the developing embryo is apparent after only 3 days of feeding the mother a zinc-deficient diet. Abnormal changes, including abnormal cleavages, were seen in the preimplantation blastocysts on day 3 (sperm positive = day 0). By day 4, only 18% of the eggs from zinc-deficient females could be characterized as normal blastocysts, while 97% from controls were normal.

The observation of chromosomal aberrations in zinc-deficient animals is also consistent with the idea that zinc deficiency results in abnormal nucleic acid synthesis. In cytogenetic studies, chromosome spreads from both fetal live and maternal bone marrow of zinc-deficient animals showed chromosomal aberrations, especially gaps and terminal deletions in significant incidence.

Humans—Acrodermatitis enteropathica is an autosomal recessive genetic disease characterized by severe skin lesions on the extremities and around body openings, alopecia, and diarrhea. Human milk is of therapeutic value in the treatment of the disease, and the onset of symptoms is frequently associated with weaning infants form breast milk to cows' milk. Moynahan and Barnes have reported low plasma zinc levels in patients with this disease and the successful treatment of the disorder with oral zinc. A large proportion of the zinc in human milk has recently been demonstrated to be bound to a small molecular weight fraction, in contrast to the zinc in bovine milk where it is associated with high molecular weight fractions. This difference may account for the therapeutic properties of human milk.

It has been proposed that zinc deficiency may play a role in human congenital malformations. Teratogenic effects of excess amounts of some metals have recently been reviewed by Ferm.