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Utilization of nitrogen by the animal organism: V. Influence of caloric intake and methionine-supplementation on the protein metabolism of albino rats fed rations low in nitrogen and containing varying proportions of fat

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NOTE TO USERS

UTILIZATION OF NITROGEN BY THE ANIMAL ORGANISM V. INFLUENCE OF CALORIC INTAKE AND METHIONINE-SUPPLEMENTATION ON THE PROTEIN METABOLISM OF ALBINO RATS FED RATIONS LOW IN NITROGEN AND CONTAINING VARYING PROPORTIONS OF FAT

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TABLE OF CONTENTS

Page

Page

Page

Page

LIST OF TABLES IN BODY OF THESIS

Page

LIST OF FIGURES IN BODY OF THESIS

Page

INTRODUCTION

The second World War emphasized, as never before, the drastic effects of food shortages in occupied countries on the health, vitality, and morale of populations. The nutritional problems encountered in the rehabilitation of starving citizens of these liberated countries were of a magnitude never before envisaged. They not only challenged nutrition workers but also served as an impetus to many organizations in their studies of global nutrition. One of these groups, the International Food Conference at Hot Springs, Virginia, set up as an objective the goal of "freedom from want of food, suitable and adequate for the health and strength of all peoples." (Boudreau, 1943). Formulation of plans for emergency relief and for an international nutrition program following widespread disruption of agricultural productivity in times of war was the natural outcome of this and similar conferences.

Emergency situations of a nutritional nature also are met in times of peace. Shipwrecks, plane crashes, and floods leave their imprint on the pages of history, and present situations where the quantity and quality of foodstuffs in a limited ration must be given careful thought. Fat, protein, and carbohydrate as individual

constituents of a dietary mixture assume greater importance in their abilities to maintain the body when the normal food intake must, of necessity, be decreased.

Following the discovery of vitamins as essential dietary units, emphasis shifted from the dietary role of the three so-called major foodstuffs to the part played by the "little things in nutrition." During the last war, however, researches disclosed that knowledge available regarding the interplay of protein, carbohydrate, and fat in nutrition did not permit good dietary planning. Provision of a combination of foodstuffs that will support individuals in maximal well-being in times of stress is a problem of great importance in the maintenance of both military and civilian personnel. More information on the interrelationships of proteins, carbohydrates, and fats in supporting nutrition is greatly needed.

Work reported in 1947 by Swanson, Willman, Brush, and Clark from the Nutrition Laboratory at the Iowa State College showed that the excretion of nitrogen was markedly depressed in rats maintained on a low nitrogen diet when dried whole eggs were incorporated into the ration. Certain other protein supplements, including the tissues of the rat itself, did not produce this effect. Dietary incorporation of the essential amino acids in egg proteins, both individually and collectively, indicated that

methionine, in a large part, was responsible for the phenomenon. Analyses of whole carcass, liver, and muscle of adult rats in these experiments suggested that this effect of methionine on nitrogen metabolism represented more than a supplementation of amino acids arising from the degradation of body protein. Brush et al. (1947) write as follows in this connection:

> Continued loss in body weight, the lack of change in the total nitrogen content of the carcass, the constancy of the ratio of methionine nitrogen to total nitrogen of the carcass, and the increment in hepatic tissue when methionine is fed to the depleted animal point to the possibility that this amino acid does not act in the general maintenance of body tissues but in the synthesis of functional proteins and important metabolites (p. 409).

Under the condition of nitrogen starvation described in these experiments, the animal apparently was forced to utilize its own tissues as a source of metabolites essential for that minimal metabolism associated with life. The depression in nitrogen excretion occuring when methionine was added suggested that methionine was the critical moiety for which the animal was raiding its own tissues in an attempt to maintain these vital metabolic processes.

In 1946, other interesting observations were made (Swanson, 1946). It was found that when the food intake was restricted, dietary fat played a striking role in

preventing the breakdown of body tissue in a nitrogen-starved animal. In the absence of dietary protein, the quantity of fat in the ration definitely was related to the rate of protein catabolism when the caloric intake of the animal was reduced to less than 50 per cent of the normal ingestion. When methionine was added to these diets containing minimal quantities of fat, protein catabolism again was altered profoundly. For example, when the caloric intake was restricted to 25 per cent of the normal ingestion, methionine fed in quantities supplying 4 mg. of nitrogen per day reduced the catabolism of body protein to a level characteristic of that of rats receiving limited calories supplied by a diet containing 20 per cent fat. Thus, fat and methionine seemed to have similar functions in regulating the rate of protein catabolism.

These phenomenal alterations in the expected course of protein catabolism could not be explained satisfactorily from the work thus far conducted in the Nutrition Laboratory at the Iowa State College, or from researches reported in the literature. The present project, therefore, was undertaken with three objectives in mind. First, it seemed important to verify preliminary results under more precise experimental conditions. With ad libitum feeding, the appetite of an animal soon begins

to reflect the absence of nitrogenous constituents in the diet. Force-feeding provides a method whereby the caloric intake can be maintained at the level desired, and dietary modifications can be carefully controlled. Second, the study was initiated in an attempt to elucidate, in part at least, the mechanism of the action of dietary fat in the regulation of nitrogen metabolism in an animal partially depleted of its body reserves of nitrogen and force-fed a protein-free diet at normal and restricted caloric intakes. Third, it was hoped that some light might be thrown on the interesting connection of methionine and fat in protein metabolism.

REVIEW OF LITERATURE

NITROGEN BALANCE AS A TOOL FOR NUTRITIONAL RESEARCH

Historical

As early as the time of Hippocrates (460-370 B. C.), there existed a belief in the occurrence of one specific "universal nutrient substance" in the diet (Mendel, 1928). This idea persisted until the early part of the nineteenth century. It remained for the rapid development of organic chemistry to dispel this belief in a single universal food principle and to throw new light on the true nature of the foodstuffs.

It was Prout who first postulated that all organized bodies consisted of three great staminal principles..... saccharina, oleosa, and albuminosa. These substances were all found in milk, which Prout considered as the prototype of a perfect food. To him, it seemed logical to assume that inasmuch as "all the more perfect organized beings feed upon other organized beings, their food must necessarily consist of one or more of the three staminal principles" (Mendel, 1928, p. 12).

Prout's theory of nutrition did not indicate any special differences in the nutritive functions of these organized bodies, now accepted as carbohydrate, fat, and

protein. To Magendie, the father of experimental medicine, we owe the first clear-cut distinction of the nutritional significance of nitrogenous and non-nitrogenous compounds (1816). From his research, he concluded that the nitrogen of tissues is derived from food nitrogen, and that non-nitrogenous foods are not converted into nitrogenous components of the organism. This finding and the observation of the French chemist, Pierre Bertholet, that nitrogen was a constant constituent of all animal tissues (Sahyun, 1948, p. 5), formed the basis for widespread interest in the fate of nitrogenous compounds in metabolism. The further observation that nitrogen was a component of excreta, and the suggestion that its presence there could be used as a measure of protein metabolism paved the way for extensive studies which followed.

The recognition of the importance of nitrogenous foods in nutrition ushered in a new era of progress. In 1839, the Dutch chemist, Mulder, recognized the resemblance between albuminous substances that could be extracted from animal and plant tissues respectively. In The chemistry of animal and vegetable physiology, he wrote:

> In both plants and animals a substance is contained, which is produced within the former, and is imparted through their food to the latter. To both, its uses are numberless. It is one of the most complicated

> substances, is very changeable in composition under various circumstances, and hence is a source of chemical transformations, especially within the animal body, which cannot even be imagined without it. It is unquestionably the most important of all known substances in the organic kingdom. Without it no life appears possible on our planet. Through its means the chief phenomena of life are produced.
>
> It is present in all parts of plants, in roots, stems, leaves, fruits, and in their several saps. It is contained in very unlike parts of the animal body. In plants it assumes three different forms, in which it is either soluble in water, or insoluble in water, or soluble in alcohol. In animals it also exists in various forms, being either soluble or insoluble in water. In the insoluble form its structure is variable. It forms different compounds with sulfur, with phosphorus, or with both, . . . and hence the differences it presents in appearances and physical properties. This substance has received the name of <u>protein</u>, because it is the origin of so many dissimilar bodies and is itself therefore a primary substance (p. 291).

To the teachings of Justus von Liebig above all others is due the prominence which was attached in the last century to the nutritive role of proteins. In the <u>Familiar letters on chemistry</u>, Liebig expressed his concept of the dominant importance of protein as follows:

> Everywhere throughout organized nature, where animal life is developed, we find the phenomena of life depending on the presence of albumen. The continuance of life depending on its presence in blood, it is indissolubly connected in this nutrient fluid.
>
> In so far as the notions of formation, nutrition, or the nutritive property are

> inseparable from that of a substance, whose properties and composition are collected in the word albumen; only those substances are in a strict sense, nutritious articles of food, which contain either albumen, or a substance capable of being converted into albumen (1851, p.346).

To Liebig, various physiological functions appeared to proceed at the expense of albuminous tissue structures; thus, the source of energy liberated when work was performed was referred by him to the muscle protein. Although many of Liebig's early teachings long have been disproved, his influence has stimulated progress in scientific knowledge even to the present time.

It remained for Boussingault, a French contemporary of Liebig, to establish the principles of the balance test as a measure of nitrogen utilization. In 1839, he published a study in which the food, urine, feces, and milk of a lactating cow were collected and analyzed for nitrogen. So highly impressed was he by the importance of the nitrogenous constituents in the rations of domestic animals, that he rated feeds largely in accord to their content of nitrogen. He realized, however, that the non-nitrogenous constituents were not without value (Boussingault, 1844).

Bidder and Schmidt (1852) applied in refined form the technique conceived by Boussingault many years earlier, reporting in 1852 as a result of work with cats,

that food nitrogen ingested could be accurately accounted for in the urine and feces excreted. Their results appear in the classical publication, Die verdauungssafte und der stoffwechsel.

To Carl Voit, a great research physiologist, we owe the opening of a new era in the science of nutrition.... the quantitative and, therefore, the scientific era. After perfecting the nitrogen balance technique, he conducted extensive observations on man and animals. Voit gave painstaking care and attention to the elaboration of methodology in metabolic research which still serves as a model. His work and writings emphasize the metabolic interchangeability of the nutrients within limits, and the way one nutrient may spare another because of like functions they serve.

In a study of the metabolism of nitrogen in dogs during starvation, Voit (1866) observed that withholding the food resulted in a relatively low excretion of nitrogen if ample fat stores existed in the body, for fat was supplying the internal needs of the subject. Later, after the depletion of stored fat, the nitrogen excretion increased; then, body protein was being used to provide for that portion of the total metabolism which body fat had formerly served. Voit found also, in these experiments, that exercise during starvation produced increased

excretion of nitrogen, since the demands of activity augmented energy metabolism which was being supported solely by body protein. However, dogs receiving no protein but ample amounts of fat and carbohydrate to cover their caloric needs for exercise did not show an increased excretion of nitrogen. These findings were in direct contrast to those of Liebig, and contradicted the theory that protein was the fuel for muscle contraction.

In 1867, Voit reported the nitrogen balances of dogs receiving diets of meat only. He observed that nitrogen equilibrium could be maintained at varying levels of protein intake. However, following a shift from one level to another, there was invariably a time lag before the attainment of equilibrium at the new level. A dog accustomed to a relatively low protein intake, when given a higher protein intake, showed a positive nitrogen balance for a few days indicating that protein storage occurred before equilibrium at this higher level of intake was finally established. Likewise, a shift from a higher to a lower but still adequate level of protein intake resulted in protein loss for a few days. This and other experiments of similar character led Voit to his theory of the existence of two types of body protein. He visualized "organ protein" as the stable form of protein forming the essential structures of the body and not

readily available for metabolic needs. In contrast to organ protein, a "circulating" or "storage" type was readily mobilized for metabolism in periods of need. Plasma proteins and that loosely bound to the cells of certain organs made up this type of protein.

Thus, in the hands of Voit and his students, nitrogen balance studies became the powerful instrument of metabolism work. Numerous papers written by Voit and his coworkers appeared after 1860 in the Zeitschrift fur biologie, of which Voit was the editor.

The determination of fecal nitrogen was an integral part of the refined nitrogen balance technique. Max Rubner, a student of Voit, devised a method for testing the availability of protein in various food sources. By determining the difference between food nitrogen and fecal nitrogen, Rubner (1879) found that the ingestion of low residue foods such as eggs, meat, cheese, and milk resulted in fecal losses of only 2.5 to 5.0 per cent of the nitrogen consumed. From foods containing large quantities of roughage, such as carrots or coarse black bread, as much as 40 per cent of the original nitrogen was found in the feces. Thus, the differences in the nutritive efficiency of proteins of different foodstuffs seemed to be related in a large part to factors influencing their digestion.

In 1890, Felix Hirschfeld reported that heavy exercise is not associated with greater nitrogen excretion as compared to the resting state, provided the increased caloric needs are amply covered by non-nitrogenous foods. In stressing the importance of meeting caloric needs, Hirschfeld emphasized an important point which frequently had led to erroneous conclusions concerning protein requirements. Earlier workers had not grasped the significance of energy balance as well as nitrogen balance in determining the overall picture of metabolism.

Hundreds of such nitrogen balance experiments were performed from 1860 to 1900. Atwater and Langworthy (1897) compiled the then existing data in a publication for the United States Department of Agriculture under the title, A digest of metabolism experiments.

Further improvement in the details of executing a balance experiment have come in connection with studies on the "biological value" of proteins. The respective researches of Mitchell, Murlin, Allison, and Swanson and their collaborators are particularly noteworthy in this connection (Mitchell and Carman, 1926; Murlin, Edwards, Fried, and Szmanski, 1946; Allison, Anderson, and Seeley, 1947; Brush, Willman, and Swanson, 1947).

Relation of Amino Acids to Nitrogen Equilibrium

The development of knowledge regarding the chemical composition of proteins led gradually to the concept of amino acids as the "building stones" of the protein molecule (Fischer, 1901). Nutrition studies which paralleled these investigations demonstrated the importance of these building blocks in the maintenance of nitrogen balance.

Gelatin was the only protein substance recognized in the nineteenth century as being inadequate for the support of nutrition. Voit, in his Ueber die bedeuntung des leimer bei der ernahrung which appeared in 1872, wrote that gelatin as the sole source of nitrogen in the diet did not maintain nitrogen equilibrium in dogs. In the same year, Otto Nasse made the interesting discovery that the aromatic amino acid, tyrosine, was the basis for the color reaction of proteins with Millon's reagent. Munk (1894), in a discussion of the nutritive properties of gelatin and its failure to serve as the sole dietary source of protein, listed in detailed outline the various ways in which it differed from other proteins. Among them, he included the failure of gelatin to give the Millon reaction, indicating that it lacked tyrosine. These, then, were the first scientific writings to hint that differences in the nutritive value of proteins might be ascribed to their amino acids as basic components.

Kauffmann (1905) subsequently conducted an extremely interesting experiment in which he found that gelatin supplemented with tyrosine, cystine, and tryptophan in the proportions that they occur in casein supported nitrogen equilibrium in dog and man.

The work of Fischer and Kossel (Sahyun, 1948, p. 33) suggested that proteins were broken up into amino acids before absorption in the intestine. Loewi (1902) reasoned that if this were true, ingestion of the cleavage products of meat protein should maintain nitrogen equilibrium in the same way as the ingestion of the intact protein. He fed a dog pancreas tissue which had been self-digested until its protein had been converted into amino acids as indicated by the almost complete disappearance of the Biruet reaction. Fat and carbohydrates were also given with the digest. Nitrogen equilibrium was obtained and even nitrogen retention accomplished.

Subsequently, Henriques hydrolyzed protein by splitting it with trypsin and erepsin, then treated it with 20 per cent sulfuric acid (Sahyun, 1948). The resulting material consisted entirely of amino acids with no admixture of polypeptides. This substance supported the organism in nitrogen equilibrium. He also observed for the first time that in the absence of a single amino acid, i.e., tryptophan, nitrogen equilibrium could not be attained.

Abderhalden, Ewald, Fodor, and Rose (1915) in later experiments proved that nitrogen equilibrium and nitrogen retention could be established in a dog when the diet contained instead of protein, the following mixture of pure amino acids: glycine, d-alanine, l-serine, l-cystine, d-valine, l-leucine, d-isoleucine, d-phenylalanine, l-aspartic acid, l-arginine, l-tryptophan, l-proline, l-histidine, l-lysine, l-glutamic acid, and l-tyrosine. This work laid the foundation for future experiments in which certain amino acids were demonstrated to be essential for the maintenance of nitrogen balance and suggested to future investigators that semi-synthetic diets might be used in the study of nitrogen utilization.

"Endogenous Nitrogen"

The studies of Folin (1905) on the plane of protein nutrition and its relation to the composition of the urine are classic in the field of protein metabolism. He distinguished between a constant catabolism of nitrogen, not dependent on the level of protein consumed, and a variable catabolism of nitrogen, related to the dietary intake of this nutrient. The dietary magnitude of these phases was measured by estimating them in separate experimental periods in which the animal was fed a nitrogen-rich diet or one extremely low in nitrogen. As a result of these

analyses, Folin postulated a theory of protein metabolism as follows:

> There must be at least two kinds of protein metabolism . . . the two forms of catabolism are essentially independent and quite different. One kind is extremely variable in quantity, the other tends to remain constant. The one kind yields chiefly urea and organic sulphates, no creatinin and probably no neutral sulphur. The other, the constant catabolism, is largely represented by creatinin and neutral sulphur, and to a lesser extent by uric acid and ethereal sulphates . . . . It is quite clear that the metabolic processes resulting in the end products which tend to be constant in quantity appear to be indispensable for the continuation of life . . . . I would therefore call the protein metabolism which tends to be constant, tissue metabolism, or endogenous metabolism, and the other, the variable protein metabolism, I would call the exogenous . . . metabolism (1905, p. 122-123).

This concept of an independent "endogenous" metabolism was challenged soon after Folin presented it. Osborne and Mendel (1914) could not harmonize inevitable "wear and tear" of tissues with the fact that animals could be maintained for long periods of time when a diet was fed that contained incomplete protein and which became adequate for growth upon the addition of certain amino acids. They interpreted the maintenance requirement as the call of the body for specific amino acids for definite physiological functions. In their opinion, therefore, the tissues would be degraded only to liberate essential amino acids not

available from food sources. Mitchell and Hamilton, in 1929, wrote that if this were true, theoretically, the "endogenous" metabolism could be depressed below that characteristic of a period of low nitrogen feeding by feeding complete proteins.

Sherman (1920) too, believed, that the catabolism of tissue could be decreased by dietary means. He was aware that the nitrogen of mixtures of amino acids incomplete in regard to maintenance requirements might have a favorable effect on nitrogen balance. He proposed that hydrolysis of proteins constituted the initial step in protein metabolism, and that hydrolysis might be checked or even reversed by the increased concentration of amino acids that follows the digestion and absorption of protein. A mixture of amino acids would check hydrolysis, then, in a measure commensurate with its completeness because any one amino acid could be effective only at the point at which it was liberated from the "catabolizing protein molecule."

Mitchell and coworkers, on the other hand, accepted the assumption of Folin regarding the independence of the two types of metabolism. From studies of the influence of proteins and amino acids on the excretion of urinary nitrogen (Burroughs, Burroughs, and Mitchell, 1940),

these workers were unable to lower consistently the endogenous output of urinary nitrogen of mature rats. They felt justified, therefore, when formulating a plan for estimating the biological value of proteins to base it on the concept of a constant endogenous metabolism.

Other investigators, however, continued to question the constancy of endogenous metabolism. In 1935, Borsook and Keighley presented a theory of the "continuing nitrogen metabolism" which they defined as "the nitrogen metabolized on any one day that was already present in the tissues." It was thus distinct from exogenous nitrogen, and bore no relation to the endogenous or "wear and tear" metabolism postulated by Folin. The continuing metabolism could be estimated after establishing equilibrium on a diet low in sulfur and by measuring the sulfur excreted in the urine during well defined experimental periods. To these workers, the frequently observed lag in attaining nitrogen balance when passing from one level of nitrogen intake to another and the constancy of the free amino nitrogen content of the tissues, despite extreme variations in diet and nutritional state, were evidence of the synthetic processes normally in operation. In a man in nitrogen equilibrium, the continuing metabolism constituted more than one-half the total urinary nitrogen

and was a function of previous dietary history.

At the University of Rochester, Whipple and his coworkers have contributed much to our knowledge of protein metabolism. They demonstrated the existence of reserve stores of proteins in the liver by means of the plasmapheresis technique, using dogs maintained on a low protein diet (Robscheit-Robbins, Miller, and Whipple, 1943). These reserve protein stores could be drawn on for the production of plasma protein and hemoglobin, Fairly large peptide units being used for the formation of functional substances. Their work demonstrates clearly a dynamic relationship between food proteins, plasma proteins, reserve proteins in the liver and tissue proteins, a concept very much in agreement with that of a "metabolic pool" as elaborated by Schoenheimer (1942).

That amino acids from exogenous sources participate actively in tissue metabolism was irrefutably demonstrated by Schoenheimer and his associates (Schoenheimer, 1942) in work with isotopic compounds. They showed that dietary amino acids might enter directly without transformation into tissue structure, or that by transamination reactions, their nitrogen might be transferred to deaminated molecules forming new amino acids. This was illustrated by the feeding of one labelled amino acid; isotopic nitrogen was present in all other amino acids removed

from the tissues, except lysine. Thus, Schoenheimer and his coworkers concluded that automatic and non-interruptable processes of synthesis and degradation occurred, and that amino acids liberated by the opening of peptide bonds mixed freely with others to form a metabolic pool of constituents whose origin was indistinguishable. They could not reconcile such reactions with the theory involving an independent "endogenous" metabolism. They pointed instead to the existence of a dynamic equilibrium between tissue proteins and the surrounding nutrient media.

If the concept of Folin in respect to the existence of two independent and different forms of catabolism is true, the ingestion of protein following the administration of a nitrogen-low diet should result in an increment in the quantity of nitrogen excreted in the urine. Swanson and coworkers (Willman, Swanson, Stewart, Stevenson, and Brush, 1945; Swanson, Willman, Brush, Brown, and Stewart, 1947), however, demonstrated that this did not always occur, and thereby raised the question, from another angle, of the validity of the hypothesis of a constant, independent "endogenous" metabolism.

Marshall demonstrated in 1943 that the addition of small quantities of dehydrated egg protein to the nitrogen-low diet fed to rats partially depleted of their nitrogen reserves decreased, rather than elevated, the

amount of nitrogen excreted in the urine. This surprising observation subsequently was confirmed by Allison and his coworkers (Allison and Anderson, 1945). The phenomena was noted by Swanson et al. (1946) only when proteins of high quality were fed. When diets containing an equivalent amount of protein from pork, rat muscle, casein, or gelatin were given, the usual increases in urinary nitrogen output over the endogenous level were noted. A daily dose of 30 mg. dl-methionine was as effective as 400 mg. of nitrogen derived from egg protein in causing a depression in the excretion of nitrogen.

These data were interpreted to mean that egg proteins supply nitrogenous metabolites that are more efficiently utilized than those arising in the catabolism of body tissues coincident with existence on a protein-free diet and that methionine is an amino acid of key importance in conserving the tissues of the body.

THE INFLUENCE OF CALORIC RESTRICTION ON PROTEIN METABOLISM

General Effects of Caloric Restriction

The influence of caloric restriction on metabolism has been the object of investigation by many workers in recent years. The U. S. Army Nutrition Laboratory, following the analysis of United States and Canadian army rations, reported in 1941,

> Simple caloric deficiency is the greatest single menace to health and efficiency. Emphasis on nutrient value should stress calories all the time. If sufficient calories are eaten in the form of a variety of foods of good biological value, then all other nutrients will automatically be taken care of (p. 58).

McCay, Cromwell, and Maynard (1935) observed retarded growth, increased life span and greater ultimate body size in the albino rat after feeding diets restricted in calories. When fed only enough calories to maintain these animals at a stationary weight, that at weaning (McCay, Maynard, Sperling, and Barnes, 1939; McCay, Ellis, Barnes, Smith, and Sperling, 1939), these rats were found to be less susceptible to lung disease and to have a lower incidence of tumors. However, extensive calcification of the kidneys was observed, with heavily calcified cartilages, and calcification of the aorta near the base of the heart.

Ershoff and Adams in 1946 observed a significant reduction in the total number of granulocytes in the blood of female rats fed a synthetic ration at a reduced caloric intake. Since this effect was corrected by treatment with folic acid, the results may have been due to a deficieney of this vitamin. O'Doherty and White (1947) stated that a decrease in the number of lymphocytes also occurs.

In a study of the effects of an extensive experiment on semi-starvation on 32 human volunteers who lived on a European famine diet for six months, Keys (1946) reported interesting observations when calorie intakes were restricted to 1760 calories per day, as compared to an intake of 3150 calories which had been required in the preliminary 3 month control period to maintain nitrogen balance. That the most striking deficiency in the test diet was calories was indicated by the fact that it supplied 49 gms. of protein, 1.3 mg. thiamin, 21 mg. niacin, 0.7 mg. of riboflavin, 30 mg. of iron, 0.76 gm. of calcium, and 1.24 gm. of phosphorus. Although the loss in weight was 37 lb., representing 24 per cent of the control body weight, the actual loss of body tissue was considerably more. At the end of semi-starvation, there was an average of 12 to 16 lb. of excess water in the body indicating a real loss of approximately 50 lb. per man.

The total blood volume changed considerably less in proportion to the change in body weight and there was an actual increase in plasma volume; in other words, hydremia occurred as well as edema.

The loss in strength in these experiments for a single muscular contraction amounted to about 30 per cent, but the loss in capacity to continue work which would ordinarily result in exhaustion in a few minutes was close to 80 per cent. The speed of small muscular movements, however, was considerably less affected.

There was a notable shrinking of the heart as seen by X-ray examination, and a small decrease in blood pressure. The bradycardia observed was considered, at least in part, as a protective adaptation conserving the heart and economizing on calories.

The subjects were always cold, and evidenced a pronounced fall in skin temperature with a decline in basal metabolism and blood circulation. More distressing than these symptoms, however, were the unremitting hunger, weakness, depression, and a sense of being old. Related to these was the lack of sex drive and interest, and a strong tendency toward neurosis. The men were profoundly depressed, markedly hypochrondiacal, and moderately hysterical. Exhaustive tests and measurements in the intellective area

showed no loss in intelligence, memory, reasoning power, or comprehension, although there was evident mental as well as physical lethargy. Visual functions were completely unaffected while hearing actually was slightly improved.

In rehabilitation, Keys observed that calories were of overwhelming importance. Within reasonable limits, every increase in the number of calories was associated with an increased rate of recovery. If the total caloric intake was low, i.e., less than 2500 calories per day, supplementary protein seemed to be valuable. With a good caloric intake, extra protein beyond that in the diet itself did not appear to be especially beneficial.

Caloric Intake and Nitrogen Utilization

Caloric restriction seems also to have a definite effect on the utilization of dietary protein for growth. Bosshardt, Paul, O'Doherty, and Barnes (1946) observed in the adult animal that a restriction of the caloric intake resulted in a decrease in the utilization of protein nitrogen. The authors relate this finding to the sparing action of carbohydrate on urinary nitrogen excretion, as observed by Lusk in 1928.

In a discussion of results of earlier work in which an increased growth utilization of casein was observed

when liver fractions were added to the experimental diets (Bosshardt and Barnes, 1946), Bosshardt and his coworkers stated:

> The possibility exists that, in protein evaluation studies involving ad libitum feeding, differences between proteins may be exaggerated because of variations in the ill-defined and often overlooked appetite aspects of the diet, which result in marked differences in total food intake and thus in caloric intake. Our results . . . may have been due, in part at least, to an increased food intake resulting from the correction of an unrecognized dietary deficiency (p. 228).

Swanson and her coworkers at the Iowa State College (Stevenson, Swanson, Willman, and Brush, 1946) have demonstrated that when a nitrogen low ration is fed to rats, a definite relationship exists between the level of dietary fat and the rate of protein catabolism, providing the caloric intake is reduced to 50 per cent or less of the energy requirement. Based on determinations of nitrogen balance, these studies were conducted with male, albino rats. When high fat diets were fed, an increase in the quantity of urinary nitrogen excreted occurred with each successive reduction in calories, thereby giving a measure of the extent to which the animal was forced to draw upon its own tissues for its energy needs as the caloric value of the ration decreased. When the low fat diet was administered at the two lower levels of caloric intake,

marked changes in the pattern of metabolic processes were indicated. When the high fat and low fat rations were fed and the calories restricted to 50 per cent of the normal intake, the low fat group excreted 305 mg. more nitrogen in the urine than did the high fat group. At the 25 per cent level of caloric intake, the differences in the excretion of nitrogen by the two groups was 374 mg.

Allison and Anderson (1945) also have observed in a group of three dogs that an increase from 80 to 100 calories per kilogram of body weight per day resulted in a decreased excretion of urinary nitrogen. A corresponding decrease in the amount of protein necessary to maintain nitrogen balance was reported. In a later study (Allison, Anderson, and Seeley, 1946), the authors defined the nitrogen balance index as the rate of change of nitrogen balance with respect to absorbed nitrogen. Their data indicated that within limits a variation of the non-protein caloric intake altered the urinary nitrogen excretion without a change in the nitrogen balance index. This effect has been interpreted by Bosshardt, Paul, O'Doherty, and Barnes, (1946), as one of decreasing the endogenous nitrogen metabolism of the animal.

The latter experiments of Allison and his coworkers (1946) indicated that the nitrogen balance index in adult dogs was not affected until the caloric intake was reduced to below 50 per cent of that considered by them to be adequate

for optimal protein utilization. A reduction of the caloric intake to 25 per cent of that adequate for optimal protein utilization resulted in a marked decrease in the nitrogen balance index. At no level of protein intake was positive nitrogen balance maintained when only 25 per cent of the adequate caloric intake was offered.

Bosshardt and his collaborators (Bosshardt, Paul, O'Doherty, and Barnes, 1948) comment on the work of Allison's group as follows:

> These results would suggest that when the non-protein caloric intake is reduced systematically two types of response are encountered. In the first type, which is observed when the caloric restriction is relatively small, the nitrogen balance index remains essentially constant, although the "endogenous" metabolism changes. In the second type, where a more severe caloric restriction is imposed, there is a marked decrease in the nitrogen balance index (p. 78).

In a study designed to study the influence of caloric intake on the growth utilization of dietary protein, Bosshardt and his colleagues (Bosshardt, O'Doherty, and Barnes, 1946) observed that decreases in caloric intake resulted in decreases in protein efficiency ratios when the protein consumption of the experimental animals was maintained constant. In these studies, it was interesting, however, that there was a range of caloric intake that maintained maximal protein utilization. Increasing the caloric consumption beyond this was without effect on the protein efficiency ratio.

The work of these investigators emphasizes the importance of controlling the non-protein caloric intake in protein experiments.

Bosshardt and his coworkers comment further that when poor proteins such as wheat gluten are supplied in ordinary isocaloric diets the voluntary food intake is so severely inhibited by the poor protein, that suboptimal caloric intake results (Bosshardt, Paul, O'Doherty, and Barnes, 1948). Therefore, a part of the discrepancy between methods of protein evaluation may be due to a self-imposed caloric deficiency which might cause an exaggerated decrease in the utilization of low quality proteins. They suggest further, that, in determinations of protein quality, the non-protein caloric intake be maintained at a level at which optimal values will be obtained.

Studies with different species of animals indicate a definite relation between the retention of nitrogen and the energy value of the ration. In the mouse (Bosshardt and Barnes, 1946; Bosshardt, Paul, O'Doherty, and Barnes, 1948), in the rat (Stevenson et al.; Willman et al.; Swanson, 1947), in the dog (Allison and Anderson, 1945), and in man (Johnson et al., 1947; Schwimmer and McGavack, 1948; Schwimmer, 1947), nitrogen retention is decreased when the caloric value of the ration is decreased.

In experiments with human subjects, Rose (1949) again emphasizes the relation of the energy value to the nitrogen requirement. More energy was needed to keep a subject in positive nitrogen balance when he consumed an amino acid mixture or an acid-hydrolyzed protein than when an equivalent amount of intact protein was eaten. Rose, in these experiments designed to determine the amino acid requirements of man, therefore, followed the practice of administering 55 calories per kilogram of body weight per day.

Caloric Restriction and Endocrine Activity

Caloric restriction seems also to affect the elaboration of endocrine secretions. Brush and Rusch (1948) observed decreased ovarian function when the caloric intake was reduced. Estrous ceased in all mice restricted to 6 calories per day, but was normal in mice allowed 10 or more calories of the control ration. Injection of estradiol benzoate resulted in estrous function, thus indicating that the production of estrogens had been decreased by the restriction of the energy intake.

Selye (1936) and Selye *et al.* (1940) have described an increase in the weight of the adrenal gland when the caloric value of the ration is restricted. This phenomenom has been observed at the Iowa State College* when

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protein free diets of low caloric value are fed. The increase in the size of the adrenal gland in these studies has been ascribed to increased protein catabolism.

An increase in the activity of the adrenal cortex has been reported by Boutwell, Brush, and Busch (1948) when dietary calories are low. These authors interpret their observations, and those of Long et al. (1940) on increased gluconeogenesis when calories are restricted in mice, as an indication of a stimulation of the adrenal cortex, since gluconeogenesis is a measurable criterion of pituitary-adrenal cortical activity. The increased increment in the livers of these mice maintained on diets low in calories probably arises in part from non-carbohydrate sources such as amino acids and protein. These adrenal-controlled glycogen precursors can be designated as "building block reserve." In contrast, the rate of cortical steroid secretion in the well-fed mouse is such that only neglible amounts of this reserve are converted to carbohydrate, as was shown by low contents of glycogen in the liver.

Decreased gonadtropnic activity and cessation of the estrous cycle has been shown to occur in female rats fed subnormal food intakes (Mason and Wolfe, 1930). In stomach tube feeding experiments, Reinecke, Ball, and Samuels (1949) also found that either caloric restriction or protein restriction caused a reduction in gonadtrophic function

with a loss of body weight and atrophy of the genital organs. All were restored to normal by injections of gonadotrophic hormones. In addition, Ingle (1938) observed involution of the thymus gland in caloric deficiency. Although the animals maintained on restricted caloric intakes in the experiments of Reinecke et al. (1949) were in anestrus, it is significant to note that they responded normally to physiological amounts of estradiol. This demonstrated that the end organ response was not impaired, and that cell proliferation was not incompatible with caloric restriction. The reduction in the manufacture of pituitary hormones when calories are restricted should be viewed, probably, as a protective mechanism of nature.

Samuels summarizes the effects of caloric restriction on endocrine systems in a recent publication (1948). He states that reproduction, growth, and basal metabolic rate are under the control of hormones of the pituitary gland, either directly, or indirectly through the influence of these hormones on other glands. He states:

> There is the possibility that the effects of caloric restriction observed in earlier experiments may have been due to effects of caloric and protein deficiency on the pituitary gland, since this gland is one of the first to show changes in this case, and the experiments were conducted by limiting the total food intake on a mixed diet (p. 101).

Thus, the animal's body adjusts to the limitation either in the amount of protein or to the total number of calories

available, apparently by reducing production of hormones in the pituitary gland. Limitation of gonadal function is usually observed first, followed by a reduction in growth rate, and in the production of adrenal and thyrotrophic hormones. Limitation in the production of these substances decreases the protein and calorie demand and thus adapts the organism to the limitations of its dietary environment.

FAT AND NITROGEN UTILIZATION

Historical

The importance of fat in nutrition has long been recognized. Lusk (1923) has described the work of early investigators in this field.

Voit in 1901 first demonstrated a definite relationship during starvation between the fat stores of the body and the length of life of animals. He showed that a rabbit with an "original" fat content of 7 per cent lived 19 days and lost 49 per cent of his body protein. Another rabbit with an original fat content of only 2.3 per cent lived 9 days, while the loss of body protein amounted to 35 per cent. Autopsy of these animals revealed a very small amount of fat in the tissues, indicating that toward the end, life had been maintained almost exclusively by the combustion of protein. Voit concluded that stores of fat in the body spare body protein.

In a fasting animal which still contained body stores of fat, Voit observed also that the ingestion of 100, 200, and 300 gm. of fat scarcely influenced the protein metabolism. Bartmann in 1912, however, noted that if fat were given to the extent of 150 per cent of the energy requirement, it was readily absorbed and spared protein to a

maximum of 7 per cent.

Schulz reported that in starvation, there is an increase in the quantity of fat in the blood. Rosenfeld observed further that the amount of fat in the liver increased to 10 per cent in starving animals. If carbohydrate and protein was ingested, the concentration of fat in the liver fell to 6.2 per cent. When the fasting animal is given fat, the lipid content of the liver may rise as high as 25 per cent. However, if carbohydrate is given simultaneously, the liver does not retain this fat, it being deposited elsewhere.

That fat may be synthesized from dietary carbohydrate was indicated by Lowes and Gilbert in 1852. Many workers have confirmed this finding, i.e., Rubner (1857), Morgulis and Pratt (1913), Osborne and Mendel (1924), Longenecker (1939), and Hoaglund and Snider (1939). Longenecker showed that this synthesis occurred when either high protein or high carbohydrate diets were fed. Less oleic acid and more palmitic and arachidonic acids were synthesized when these animals were fed a high fat ration than when the diet contained no fat.

Role of Dietary Fat in Nutrition

Fat-deficiency Syndrome

That fat is an essential nutrient in the diet was suggested as early as 1927. In that year, the first reports

of actual deficiency symptoms caused by the lack of dietary fat appeared in the literature.

Fat deficiency disease is characterized by restricted growth, caudal necrosis, kidney lesions, and early death (Evans and Burr, 1927; Burr and Burr, 1929). These symptoms could be cured or prevented by the dietary supplementation of the fat-free diet with linoleic acid or linolenic acid (Evans and Lepkovsky, 1932; Burr, Burr, and Miller, 1932). One-half drop of linoleate was effective in these experiments in curing scaliness of the feet, tail, and skin, and in restoring growth.

Quackenbush and his coworkers (1938, 1939, 1940) reported a detailed description of the deficiency disease in 1938, emphasizing that in the chronic stages acute dermatitis occurred, with involvement of ears, hind paws, and entire periphery. The lips and forepaws of these animals were generally parchementized, with ringlets of scales appearing on the tail which sometimes sloughed off, leaving shiny, denuded surfaces. No denudation of the torso occured, but it was covered with minute adherent scales. These symptoms which occurred within 8 to 10 weeks could be cured by the administration of unsaturated fats or esters of linoleic acid. These workers characterized the four stages of the lesions, and called the syndrome "rat acrodynia"

Subsequent workers established the quantitative requirement for the essential unsaturated fatty acids (Martin, 1939) and added arachidonic acid to the list of essential substances (Turpeinen, 1937). Deuel, Greenberg, Calbert, Savage and Fukin (1950) have indicated that the rat requires 20 mg. of linoleic acid per day for optimal growth. Other studies demonstrated the inability of the rat to synthesize the essential unsaturated fatty acids (Burr et al., 1930, 1931; Hume et al., 1940; and Turpeinen, 1938). Finally, Schoenheimer (1942, p. 14) in experiments with deuterium proved beyond doubt that linolenic acid was an indispensable moiety.

In human beings, a similar set of symptoms has been reported (Brown et al., 1938) when fat is absent from the diet. A marked drop in the arachidonic and linoleic acid contents of the blood, an increased concentration of serum protein, and a slight tendency toward leucopenia were observed. In a study of the nutritional state of the civilian population of Stettgart, Germany during the second World War, Ruffin and French (1946) reported a craving for fat in the undernourished and starving. Follicular hyperkeratosis, pigmentation, pseudoacrocyanosis, and fine desquamation of the skin were observed, although these could not be related to a deficiency of fat alone. Many of the diets contained as little as 15 to 20 grams of fat

from all sources. Similar observations were made by Davidson, Wickie, and Reiner (1946).

Studies by Burr and his associates (Brown, Hansen, Burr, and McQuarrie, 1938) have shown that blood lipids reflect the absence of dietary fat. There is a moderate decrease in the degree of unsaturation of serum fatty acids (Evans and Lepkovsky, 1932; Hansen and Burr, 1933; Hansen and Brown, 1937), as indicated by lowered iodine numbers. Hematuria and abnormal respiratory quotients (Brown et al., 1938), decreased fatty acid transport to the liver (Barnes, Rusoff, and Burr, 1942), and decreased ability to synthesize essential fatty acids (Burr and Beber, 1934) are characteristic also. That resistance to acute infection and artificial fever might be a result of fat deficiency was suggested by other workers, i.e., McQuarrie and Stoesser (1935); Stoesser (1935); Spesman and Arnold (1937); and Hoelzel (1937). Sinclair (1940) reported careful experiments indicating that the essential fatty acids were important in fat storage suggesting at the same time, that the requirement for essential fatty acids may be greater when animals are fed a high fat diet than when a low fat diet is fed. Burr et al. (1940) has investigated the comparative curative values of the unsaturated fatty acids in fat deficiency.

That a deficiency of fat may lead to impaired

reproductive performance was reported by Quackenbush et al. (1942) and supported earlier observations of Evans, Lapkovsky, and Murphy (1934). Failure of normal partuition, excessive placental and uterine hemorrhage, loss of weight, and anemia all were cured when supplements of cottonseed oil, ethyl linoleate, or ethyl arachidonate were fed. In the male rat, atrophy of the testes occurred when the diet contained no fat.

Essential nature of fat per se

It is a common belief that additional fat in the diet is of no specific nutritional value if adequate calories and essential unsaturated fatty acids are provided. A number of recent reports, however, have indicated that fat should be regarded as an essential foodstuff. The role played by fat cannot be explained entirely by the essential fatty acids which it supplies or by its high caloric value.

When rations supplied between 38 and 62 per cent of the total caloric intake in the form of fat, experiments in the laboratory of Deuel and his coworkers (1947) indicated that rats maintained better growth, exhibited better reproductive performance and lactation, and showed greater physical capacity, than when less dietary fat was fed. After subjecting the animals to a caloric restruction permitting only minimal growth during a 12-week period

following weaning, Scheer et al. (1947) observed that with the institution of ad libitum feeding, subsequent growth was best in those rats fed the high fat diets. Marked resumption of growth was characteristic of the rats given diets containing 10, 20, or 40 per cent fat, whereas it practically stopped in the animals fed minimal amounts of fat. In later experiments, Scheer, Cody and Deuel (1947) reported that weight loss was less rapid, mortality lower, recovery on the reinstitution of ad libitum feeding quicker and reproductive performance superior in young adult rats receiving diets containing fat than when no fat was fed.

In still other experiments, Scheer, Straub, Fields, Meserve, Hendrick and Deuel (1947) demonstrated that although the fat content of the carcasses was slightly higher when rats were fed high fat diets than when they received diets containing minimal amounts of fat, the increased body weight could not be accounted for on the basis of fat alone, and that, in actuality, the fat content of the body was not quantitatively related to that of the diet.

Other workers have noted similar beneficial effects of feeding high fat diets on the growth of rats. French (1947) observed that rats on a low protein regime grew more rapidly and efficiently when fed a diet containing 30 per cent of fat than when 2 per cent dietary fat was offered. Three experiments from the same laboratory demonstrated

that an increase in the fat content of isocaloric diets from 2 to 30 per cent resulted in an increase in weight as well as in a decrease in heat production (Forbes, Swift, Thacker, and French, 1946). Also, growth of rats fed, ad libitum, diets containing 8 per cent corn oil or lard was 20 per cent superior to that of animals whose rations contained no added fat, but were supplemented with ethyl linoleate (Pearson and Panzer, 1949).

The usual opinion that carbohydrate serves as the most effective fuel for muscular contraction has been questioned seriously following the report by Deuel, Meserve, Straub, Hendrick and Scheer (1947) that increased work capacity occurred in rats living on a high fat diet. Samuels, Gilmore, and Reinecke (1949) recently confirmed the earlier observations of Deuel demonstrating that rats fed diets containing 80 per cent of the calories as fat survived longer and accomplished a significantly greater amount of work during a subsequent period of fasting than animals which received diets containing no fat, but the same proportion of protein or carbohydrate.

Deuel and his collaborators (1950) have described experiments in which fat-deficient rats were given injections of the growth hormone of the pituitary gland and were fed dietary supplements of methyl linoleate, linoleic acid and cottonseed oil. When 20 mg. of linoleic acid were fed,

prompt growth response resulted; however, the administration of 10 per cent cottonseed oil to rats receiving linoleate at an optimum level resulted in a further acceleration of growth. These workers suggest that these findings may indicate a beneficial effect of the oil to be ascribed to some factor other than linoleic acid.

Another feature of the metabolism of fats is their protein-sparing action when they are incorporated in a low fat diet supplying an inadequate number of calories. When the caloric intake was 100 or 75 per cent of that required, no changes were noted by Stevenson et al. (1946) or by Willman, Brush, Clark, and Swanson (1947) in the quantity of urinary nitrogen excreted by rats receiving protein-free diets in which the fat content varied from 0 to 20 per cent. The removal of fat from the ration, however, doubled the excretion of urinary nitrogen when the ration provided only 25 per cent of the needed calories. As a result, the nitrogen balances when the protein-free diets were given at 25 per cent of the caloric requirement were considerably less negative when animals were fed the fat-containing diet than when they were fed diets from which this foodstuff was missing. These findings have been confirmed by Schwimmer and McGavack (1948) in an extension of the study to the human being.

The recent report of Pearson and Panzer (1949) is of

interest in this connection also. These workers reported that the fecal and urinary excretions of phenylalanine, lysine, valine, and methionine were significantly less in rats receiving corn oil than in those whose diets contained no added fat. This variation could not be ascribed to a lack of the essential fatty acids, since the rats in both groups received a supplement of ethyl linoleate each day sufficient to meet the daily requirements.

There are other circumstances in which fat seems to meet metabolic needs more satisfactorily than carbohydrate. Dugal and coworkers (1945) found that the survival of rats kept at an environmental temperature of -40° C. on a high fat diet was substantially greater than the survival of those on a low fat regime. At these low temperatures, the rats voluntarily selected a diet containing as much as 50 per cent of the calories in the form of fat. A lower fat content was preferred at higher environmental temperatures.

Unidentified Factors in Fat of Nutritional Significance

A Dietary Factor Concerned with Carbohydrate Metabolism. A metabolic abnormality in rats on a fat-deficient diet in which carbohydrate is converted into fat to a degree not found in normal animals has been described by Wesson and coworkers (1927, 1931, 1933). The known vitamins and essential amino acids studied by these investigators

at that time were not effective in correcting or preventing this condition. Neither was linoleic acid effective, when fed in large amounts and over a relatively long period of time (Wesson and Burr, 1931), although it was curative for the symptoms of fat-deficiency disease as characterized by Burr and his colleagues (1929, 1930, 1932). When certain fats are fed to rats whose carbohydrate metabolism is abnormal, a more nearly normal condition is obtained (Wesson, 1927). This finding has been confirmed by Burr and Beber (1932).

In these experiments by Wesson and his collaborators, the liquid fat fraction of lard was found to be more than 10 times as active in correcting the metabolic abnormality than the solid fat fraction. While relatively large amounts of the liquid fat fraction were rapidly effective, the same amount of ethyl stearate was inactive. Small daily amounts of the liquid fat or of the solid fat fraction (6.67 mg) prevented the appearance of the metabolic disorder, but did not affect the stunting and emaciation as compared to that which occurred in rats fed the fat-deficient diet only.

The authors suggest that the abnormal formation of fat from carbohydrate by the rat on a fat-deficient diet was not brought about because of a need of fat for catabolism, but because of a deficiency of some metabolic factor in the food supplied. Since the saponifiable fraction of

liquid fat was active, it was indicated that the dietary factor was not only resistant to saponification, but also acid in nature.

Factor in Lard Distillate. Kaunitz and his coworkers (1950) have recently reported the presence of a possible new factor in a fraction of lard distillate. When this factor is added to an already adequate diet, additional growth is stimulated. Full reports on this observation have not yet been published.

Growth Factor in Summer Butter. The presence of a growth factor in summer butter has been reported independently by Schantz, Bontwell, Elvehjem, and Hart (1940) in this country and Boer and Jansen (1941) in Denmark. In the Wisconsin experiments rats showed superior growth on butter fat as compared to corn oil when lactose was the sole source of carbohydrate. Interestingly, when the carbohydrate of the ration was supplied by either dextrose, sucrose, dextrin, or starch, the superiority of butterfat disappeared and corn oil gave rates of growth comparable or even slightly better than butter fat.

Deuel, Movitt, Hallman, and Mattson (1943), however, observed no differences in the growth of weanling rats over a twelve week period when they were fed mineralized skimmed milk powder, vitamin supplements, and butter as compared with corn, cottonseed, olive, peanut, margarine, or soybean

oils. These observations were confirmed by X-ray determinations of tibia length.

Workers in the Danish laboratory postulated that the new fat-soluble nutritional essential was vaccenic acid (Boer and Jansen, 1944). Although the findings of these workers were clear cut and well substantiated by their experimental data, other investigators have been unable to confirm their findings (Nath et al., 1949; Lassen and Bacon, 1949).

Jack and Hinshaw (1947) suggest that another fraction of milk fat, known as the -53° C. filtrate fraction, may be responsible for the growth promoting activity of milk fat. This factor was shown to be much superior to whole milk fat itself. Elvehjem and his coworkers have confirmed this finding (1947, 1948).

Nutrition and Levels of Fat in the Diet

That excessive quantities of fat in the diet may be toxic, however, has been suggested by several investigators. In 1925, Bloor reported that when fat supplied more than 80 per cent of the energy requirement, incomplete combustion occurred with formation of acetone and ketone bodies. He believed that the organism was overtaxed in its effort to catabolize fat, and as a result, these substances were formed and excreted (Bloor, 1943). Sako (1942) observed

a lowered resistance to pneumococci in mice which had received diets containing 50 per cent of fat as compared to animals fed 5 or 23 per cent fat. Increased erthrocyte destruction (Loewy, et al., 1943) and reduced ability to work (Higgins, 1930) also have been reported when excessive amounts of fat were ingested.

When a diet containing less than 10 per cent of the calories from fat was ingested, retarded growth occurred and smaller quantities of free cholesterol and cerebrosides were stored in the body (Williams et al., 1945). Increasing the calories derived from fat from 40 to 70 per cent did not affect the distribution of lipids in the body.

That the nutritive value of dietary fats may vary has been suggested by workers from several laboratories. Forbes, Swift, Buckman, Schopfer, and Davenport (1944) fed isocaloric diets containing 2, 5, 10, and 30 per cent of fat to growing rats; they found increasing weight gains in the order of the increasing fat contents of the diet. When corn oil supplied the source of fat in skim milk diets, however, Boutwell, Geyer, Elvehjem, and Hart (1945) observed a reduction in weight gains with increasing levels of this source of fatty acids, i.e., 25, 30, and 35 per cent of corn oil. Lower levels of the oil were beneficial. A similar effect of corn oil has been reported by Barki, Collins, Elvehjem, and Hart (1950).

That the optimal level of fat in the diet may vary with different sources of fat was suggested by Hoagland and Snider in 1940. When a series of vegetable fats were fed at 5, 30, and 55 per cent levels in isocaloric diets, the growth of rats was improved when the levels of hydrogenated lard, leaf lard, neutral lard, oleo, and peanut oil were increased up to 55 per cent. Increased quantities of cottonseed oil above 30 per cent resulted in reduced growth. In additional studies on cottonseed oil and margarine fat, Deuel, Meserve, Straub, Hendrick, and Scheer (1947) found that optimal growth occurred in male rats when the ration contained from 30 to 40 per cent of margarine or 20 per cent cottonseed oil.

It should be recognized, however, that the animal organism, and particularly the human body, can adjust to wide differences in the relative proportion of the three major foodstuffs, carbohydrate, fat, and protein, in the diet. When high fat or high protein diets were fed to animals which had been on a sotck diet in which carbohydrate predominated, there was at first a diarrhea with considerable loss of unchanged food material (Samuels, 1946). However, after a few days, this diarrhea ceased, and the efficiency of the gastrointestinal tract becamé equal to that of the rats when they received the original diet.

This author explains that if an animal is continued on a given diet high in one major food factor, the proportion of the digestive enzymes in the pancreatic juices undergoes a change in favor of the predominating foodstuff. In some manner, the metabolism of the secretory cells is changed so that more of the needed enzyme is produced. Acetone bodies always appear in the blood and urine in increased quantities when the animal is fed a high fat diet or is fasted. However, if the high fat diet is fed an additional period, there is a drop in the acetone level. This would suggest that adjustment to the high fat diet requires time.

Samuels, Gilmore, Reinecke (1948) also observed a difference in the peripheral utilization of glucose by rats fed high fat diets. This difference could not be accounted for by any difference in metabolic rate or in the use of circulating acetone bodies. The relatively long time required for adjustment to the high fat diets suggested to these authors that again there was a fundamental change in the enzyme systems of active cells which enabled these cells to use more of the foodstuff that predominated in the diet. This hypothesis is supported by evidence that requirements for vitamins which are parts of coenzyme systems is affected by the type of diet fed.

That there is a difference between sexes in the

utilization of fat has been observed by several investigators. Wynn and Haldi (1944) have reported that both males and females stored more fat when given high fat diets than they did when they were fed low fat rations. This report has been confirmed by Loeb and Burr (1947) in studies on rats, guinea pigs, and mice. On the contrary, Boycott has observed that when animals of both sexes are fed high fat diets, the carcasses of females were lower in fat than those of males. Neither was there any difference in the fat content of animals of either sex when they were reared on high fructose and high glucose diets (Bachmann, 1938).

After force-feeding of fasting ketonuric rats, Butts and Deuel (1933) reported that diacetic acid was eliminated more freely in females than by males. Mendel and Anderson have observed that males store more perirenal fat than females (1930); females store more genital fat. These latter findings were confirmed by Reed, Yamaguchi, Anderson, and Mendel (1930), and by Haldi, Giddings, and Wynn (1941).

Interrelation of Fat, Protein, and Carbohydrate

Although much is known concerning the functions of fat, it has been regarded from a practical standpoint, as being interchangeable with carbohydrate on a metabolizable energy basis. Swift and Black (1949) have reviewed a series of experiments conducted in their laboratory to study

the interrelations of fat and carbohydrate as constituents of a normal diet.

In the first series of experiments conducted by the body balance procedure, it was reported that acceptability of the diets increased as the fat content increased from 2 to 30 per cent (Forbes, Swift, Elliott, and James, 1946). In 1946, Forbes, Swift, Elliott, and James recorded that the digestibility and the retention of food nitrogen were highest when the diet contained 30 per cent of fat. The experiment on which this conclusion was based has been repeated under somewhat varying conditions. From these studies, Forbes and his collaborators concluded that the fat content of the diets was without positive influence on the utilization of nitrogen (Swift and Blake, 1949).

In a second experiment, conducted with a much higher level of 10 of the vitamins, the influence of different quantities of dietary fat, i.e., 2, 10, and 30 per cent, was compared (Forbes, Swift, James, Bratzler, and Black, 1946). Statistically significant results were obtained for increases in body gains of fat and energy, and decreases in heat production as the fat content of the diet was raised.

In a third experiment, conducted to determine the influence of the level of protein in the diet on the manner in which energy was utilized in the high fat diets, the protein content of the rations were reduced from the usual

22 per cent level to 7 per cent (French, Black, and Swift, 1948). The metabolizable energy values of the daily food were virtually equal when the fat content of the diets was either 2, 5, 10, or 30 per cent. However, the heat production or energy expense of utilization at both planes of nutrition, diminished in the increasing order of the fat contents of the diets.

Interrelation of Fat and Vitamins in Metabolism

Thiamin. In 1928, Evans and Lepkovsky first showed that the quantity of thiamin needed to maintain rats could be reduced when the ration contained large quantities of fat. These workers reported later (Evans and Lepkovsky, 1929; Evans and Lepkovsky, 1931; Evans, Lepkovsky, and Murphy, 1934) that fat exerted a definite sparing effect on the requirement of the rat for thiamin. Typical symptoms of the vitamin deficiency failed to develop when the thiamin-deficient diet contained 50 per cent lard. This relationship was studied further by Salmon and Goodman (1937), Kemmerrer and Steenbock (1933), and by Stirn, Arnold, and Elvehjem (1939). These investigators showed that the livers and muscle tissues of the rats fed the high fat diet contained no more thiamin than did those of rats fed the vitamin-low basal ration. The livers of these animals were, however, low in co-carboxylase. This

finding suggested that while the animals were unable to build up or maintain their reserves of thiamin, they were still able to obtain energy from the metabolism of fat for growth purposes. The findings were confirmed in another report (Arnold and Elvehjem, 1939).

Whipple and Church (1936) showed that the fat reserve of animals maintained on a thiamin-low ration increased when thiamin was added to the ration. They suggested that in thiamin deficiency, the rat preferentially utilizes fat as opposed to carbohydrate, thereby wiping out fat reserves.

Riboflavin. That the fat content of the diet exerts a definite influence on the riboflavin requirement of the rat was shown as early as 1941 (Mannering, Tipton, and Elvehjem). In 1945, Boutwell, Geyer, Elvehjem, and Hart observed that weanling rats fed, ad libitum, rations containing lactose and corn oil grew at a rate inferior to those receiving butterfat in the diet. It was concluded that the substitution of corn oil for butterfat resulted in a decreased synthesis of the B vitamins by the intestinal flora. Mannering et al. in 1944 had also observed that the apparent requirement of the rat for vitamins of the B complex could be altered by removing dietary fat, but that the kind of carbohydrate in the ration was an important factor also.

Czaczkes and Guggenheim (1946) found that the fat

content of the diet exerted a significant effect on the growth of rats given a low protein diet. There seemed to be an inverse relationship between the fat content of the diet and the amount of riboflavin which would maintain the vitamin at a functional level in the organs and in the urine. These authors suggested that the different requirements for riboflavin as observed in these experiments were due to differences in the amounts of riboflavin which were synthesized in a form available to the organism in the intestinal tract.

Pyridoxine. A relationship between the requirement for pyridoxine and the amount of essential fatty acids in the diet has been demonstrated by Birch in 1938. Pyridoxine was found effective in the cure of dermatitis, i.e., "rat acrodynia". These findings have been confirmed by other workers (Schneider, Steenbock, and Platz, 1940; Salmon, 1941; Quackenbush, Kummerow, and Steenbock, 1942).

Since cures of dermatitis did not parallel the degree of saturation of the fats used, early workers did not believe the curative properties were due to a fatty acid (Hogan and Richardson, 1934). The full relationship was not clear at that time. Sarma, Snell, and Elvehjem (1947) have recently added new information to this problem in their report that supplementary oleic acid may cause growth inhibition in diets containing suboptimum amounts of pyridoxal or pyridoxamine.

Pantothenic Acid. Although pyridoxine and pantothenic acid fed singly to rats maintained on a diet low in unsaturated fatty acids did not effect a cure of the "rat acrodynia," the feeding of these two vitamins improved the skin condition (Quackenbush, Kummerow, and Steenbock, 1941). This finding has been confirmed by Richardson, Hogan, and Itschner (1941). Interestingly, pyridoxine and pantothenic acid when fed with ethyl linoleate cured the acrodynia, but did not affect the scaly condition of the tail and hind paws similarly. The authors interpret this finding as suggestive of the need for an additional factor.

Biotin. Oleic acid has been shown to replace biotin in the bioassay of amino acids with Lactobacillus casei. This was especially true when it was used in connection with a non-fatty surface active agent such as serum albumin (Williams and Fieger, 1949).

Niacin. In studies with rats maintained on niacin-deficient diets, Salmon (1947) has observed that growth was restricted if the diet contained 30 per cent of fat and a low content of casein. He suggests that fat may exert a sparing action on the nicotinic acid requirement of the rat.

Choline. Choline is recognized as an effective agent in the prevention and cure of fatty livers induced by the

feeding of a high fat diet (Best, Hershey, and Huntsman, 1932). Best and his collaborators reported that choline prevented the deposition of neutral fat (glycerides) and to a lesser extent, the cholesterol fat in the so-called "cholesterol fatty liever" (Best, Channon, and Ridout, 1934). In curative experiments choline accelerated the removal of cholesterol esters as well as glycerides from the liver.

In 1937, McHenry reported the existence of an apparent relation between choline and thiamin in the production of fatty livers. He found that it was difficult to produce fatty livers in rats fed choline-free diets unless ample thiamin was supplied in the diet. When the diet contained more than 40 per cent of fat, there was a significant increase in liver fat in the absence of added thiamin. At lower levels of fat intake, fatty livers did not occur, even in the absence of choline, unless thiamin was available. Work by Boxer and Stetten (1944) indicated that the effect of thiamin was essentially an indirect one resulting from its action in stimulating appetite and thus increasing the need for lipotropic factors. The relation of choline to liver fat will be discussed further in a following section of this manuscript.

Vitamin B12. Bosshardt, Paul, and Barnes (1949)

have established an interesting relationship between vitamin B_{12} and dietary fat. When animals were fed a purified diet containing 20 per cent protein and all the known B vitamins with the exception of B_{12}, a reduction of the fat content of the ration to 0.5 per cent, with a corresponding increase in carbohydrate, resulted in a marked decrease in the growth of male weanling mice. This growth retardation was completely counteracted by vitamin B_{12}. In the absence of B_{12} the addition of fat to this diet resulted in gradual increases in growth until approximately 40 per cent of dietary fat was present. Fat levels in excess of 40 per cent were detrimental regardless of whether or not B_{12} was given.

Bosshardt and his coworkers suggest that these data indicate a sparing action of vitamin B_{12} on fat, similar to the well-known sparing action of thiamin.

It is interesting in these experiments that when varying concentrations of B_{12} were given to mice fed the low fat diet, a straight-line log dose-response curve was obtained.

When animals in the experiments of Bosshardt and coworkers were kept on a high protein, low fat, B_{12}-deficient regime, they lost weight. The addition of fat or B_{12} partially counteracted this effect, normal growth resulting

when both were fed in the ration.

Choline, Vitamin B_{12}, and Methionine. Jukes and Stokstad (1949) observed that the administration of either choline or vitamin B_{12} markedly increased the rate of growth of chicks maintained on a choline-vitamin B_{12} deficient ration. Maximum growth was not obtained, however, unless choline and B_{12} were fed at the same time.

Jukes and Stokstad also observed that following a deficiency of both methionine and vitamin B_{12} induced in chicks by feeding a diet consisting mainly of ground peas, gelatin, and glucose, supplementary methionine was more effective than vitamin B_{12} in stimulating growth. Growth remained decidedly suboptimal, however, unless both substances were given simultaneously.

Stekol and Weiss (1949) recently reported experiments in which diets deficient in cystine, methionine, choline, and vitamin B_{12} were fed to rats. These rations contained S^{35} homocystine and 8 per cent casein. Rats grew for about 2 weeks, then declined in weight and died with severe kidney necrosis and fatty livers. The addition of vitamin B_{12} permitted slow growth for several weeks, and no deaths were observed. If a portion of the homocystine in the amino acid diet was replaced by cystine, the rats nearly tripled their rate of growth in the presence of B_{12}.

When S^{35} homocystine and bromobenzene were injected into adult rats maintained on an 8 per cent casein diet and choline, much larger amounts of p-bromophenylmercapturic acid with a greater total radioactivity were eliminated in the urine than when choline was omitted from the diet. Similarly, on an amino acid diet containing homocystine and choline, the injection of S^{35} homocystine and bromobenzene yielded larger amounts of the mercapturic acid than when choline was not present in the diet. The total radioactivity of the mercapturic acid was greater also in the presence of choline in the diet.

Schaefer, Salmon, and Strength (1949) reported that the incidence and severity of renal injury in weanling rats fed diets low in choline and methionine were significantly decreased by supplementing the diet with a vitamin B_{12} concentrate or crystalline vitamin B_{12}. Under the conditions of these experiments, 30 micrograms per kgm. of diet of the vitamin could replace one-half of the supplementary choline or methionine required for protection against kidney damage. When rats were given subprotective levels of choline and vitamin B_{12} in addition, their weight increased significantly, but the addition of vitamin B_{12} to an adequate level of choline did not produce an increase in weight gain. The authors suggest a possible interrelationship between vitamin B_{12}, choline, and methionine.

Lipotropic Factors. The term "lipotropic" was first used to describe the action of choline in the prevention and cure of fatty livers (Best, Huntsman, and Ridout, 1935) and has since referred to other substances which also prevent or remove an accumulation of excess fat in the liver.

Several distinct types of fatty livers may result from a number of causes. These are:

1. Fatty livers induced by dietary means such as:
   a. Ingestion of a low protein, low choline diet
   b. Ingestion of extremely a high fat diet
   c. Ingestion of excessive amounts of cholesterol
   d. Ingestion of certain alcohol extracts of raw meat and raw dried liver
   e. Ingestion of certain of the B complex vitamins
2. Fatty livers induced in dogs by removal of the pancreas
3. Fatty livers resulting from starvation
4. Fatty livers resulting from ingestion of certain poisons (such as carbontetrachloride)
5. Fatty livers induced by hormones (anterior pituitary extract)

The condition is characterized by the infiltration and deposition of excessive quantities of fat. The fat deposit is divided largely between neutral (glyceride) fat and bound sterol fat in the form of cholesterol esters. Fatty livers differ in type essentially in the ratio of glyceride to sterol fat. Thus, in the fatty livers

induced, for example by high fat feeding, low protein diets, or choline deficiency, the pathological structure consists of a predominance of glyceride fat and a relatively small amount of cholesterol fat. In fatty livers induced by an excess of cholesterol, cholesterol and sterol esters may predominate over glycerides.

Following the discovery of the lipotropic activity of choline and related compounds, it was soon discovered that other types of compounds also possessed lipotropic activity. Prominent among these were certain proteins, such as casein (Best and Huntsman, 1935). Recently, also, methylsulfonium compounds such as dimethylthetin and dimethyl- - propiothetin have been recognized as a new class of methyl donors (Maw and du Vigneaud, 1948; Dubnoff and Borsook, 1949). Activity has been ascribed also to two separate fractions derived from the pancreas, i.e., Dragstedt's lipocaic (Dragstedt et al., 1936) and the anti-fatty liver fraction, AFL, of Entenman, Chaikoff and Montgomery (1944).

The discovery of the lipotropic effect of casein led naturally to a study of its component amino acids. Best and Huntsman (1935) suggested that the action of casein in removing fat from the liver might be due to the metabolic conversion of certain of its amino acids to betaines, or perhaps choline (Channon and Wilkinson, 1935). The

following year a significant advance in the problem was made by the report of Tucker and Eckstein (1937) of the lipotropic nature of the amino acid, methionine.

In later studies on the three sulfur-containing amino acids, methionine, homocystine, and cystine, du Vigneaud and his group (du Vigneaud, Chandler, Moyer, and Keppel, 1939) discovered that homocystine could replace methionine as a growth factor for rats only if small amounts of choline were present in the diet. They concluded that the effect of choline was not due to its lipotropic activity, but to the donation of a methyl group by choline to homocystine, thereby converting it to methionine. The validity of this assumption later was confirmed through experiments using isotopically labeled compounds. These experiments established the occurrence of a reversible transmethylation reaction between methionine and choline (du Vigneaud, Cohn, Changler, Schenck, and Simmonds, 1941).

Hormonal Control of Fat Metabolism

Fat and the Pituitary Gland. For several years the anterior pituitary gland has been recognized as taking part in the control of liver fat. Injection of pituitary extract in rats produced a fatty liver (Best and Campbell, 1936). Conversely, the liver fat content of hypophysectomized animals was found significantly low in experiments

by Shipley, Chudzik, and Gyorgy (1948).

It was shown by tracer studies using deuterium that the fat present in hepatic cells after the administration of pituitary extracts came from peripheral deposits, and that the fat which appeared during the feeding of a diet low in lipotropic factors did not arise from this source (Barrett, Best, and Ridout, 1938; Steffe and Salcedo, 1944). Such findings indicate that the dietary and hormonal effects do not proceed along metabolic pathways that are closely parallel.

In studies on the growth rate of rats fed restricted calories, Deuel, Greenberg, Calbert, Savage, and Fukui (1940) observed an immediate growth response when fat deficient rats were given an injection of growth hormone and 20 mg. of linoleic acid. This was in contrast with the more tardy response observed when methyl linoleate was fed alone.

Although it had been reported that hepatic cirrhosis may follow hypophysectomy in dogs, it was subsequently demonstrated that these lesions were correlated with hypothalamic damage and not with the absence of the pituitary (Graef et al., 1944). Samuels et al., (1942) have shown that hypophysectomy in the rat reduced the concentration of fat in the livers of animals given either a high fat or a high carbohydrate diet.

Fat and the Adrenal Gland. Hartman, Brownell, and Thatcher (1947) have obtained evidence that there may be a specific hormone (fat factor) in the adrenal cortex which differs from the other adrenal steroids and which promotes fatty metamorphosis of the liver.

Evidence that the adrenal cortex is involved in the control of liver fat is based largely on the observation that adrenalectomy prevented fatty livers (McKay, 1937). MacKay has reported that fat seems to be mobilized from the liver more rapidly in rats fed a low protein diet if the animals were adrenalectomized.

Samuels, Gilmore, and Reinecke (1948) explain the increase in the concentration of sugar in the blood of rats previously fed high fat diets by the stimulation of glucose formation from glycogen due to the release of epinephrine and by the stimulating effect of exercise on the release of 11-oxygenated steroids from the adrenal. They suggest that if one foodstuff is to constitute the major source of energy, it would appear that chances for survival under fasting and stress would be greatest if a high fat diet were eaten.

Fat and the Thyroid Gland. That thyroidectomized animals show a striking decrease in the quantity of fat present in the liver as compared to control animals has been reported by Shipley, Chudzik, and Gyorgy (1948).

Apparently, thyroidectomy counteracted the effect of an anti-lipotropic diet; the diets fed to the animals contained 8 per cent of casein, 38 per cent of vegetable oil, and 50 per cent of sucrose. A similar reduction in the concentration of total fatty acids in the liver below the normal level has been reported by Artom following thyroidectomy of animals of various species kept on a standard laboratory ration, i.e., dogs, rabbits, and guinea pigs.

Thyroxine was without effect on the fat content of the liver of rats fed a high carbohydrate, low protein, fat-free diet (Forbes, 1945). The ingestion of high fat diets limited the rise in oxygen consumption following a subcutaneous injection of from 5 to 10 mg. of thyroxin, thus protecting against its action. The metabolism of animals fed an adequate diet continued to rise until it was 150 per cent above normal while that of the animals in the high fat group rose to a maximum of only 70 per cent above normal.

METHIONINE IN PROTEIN METABOLISM

Historical

The discovery of methionine by J. G. Mueller (1921) occurred more than a century after that of cystine, and more than 40 years after cystine had been recognized as a constituent of protein. The presence in preparations of natural leucine of a sulfur-containing impurity, which, in part at least, undoubtedly was methionine, was noted early by Emil Fischer (1900), but apparently this observation was not followed up. It is interesting that Mueller's discovery was not the result of research directly aimed toward unknown forms of protein-sulfur. Rather, it was the product of studies on bacterial growth in meat infusions and the result of judicious follow-up of a casual observation on the loss of a nutritional factor by charcoal treatment. Ironically the new substance proved to be different from the unknown growth factor (Mueller, 1922) which led to its discovery. The clarification of the structure of the compound is a result of the work of Barger and Coyne (1928) and it is to them that we owe its present name.

Among early experiments, two are of particular interest because they influenced the train of thought for so many

years. The work of Osborne and Mendel (1911) and of Sherman (1925) indicated that cystine was the sulfur-containing amino acid indispensable for growth. Then, Jackson and Block in 1931 showed that methionine could replace cystine as an essential dietary component for this fundamental process. Final proof of the essentiality of methionine came from the Illinois laboratory (Womack, Kemmerer, and Rose, 1937) came from investigations in which highly purified amino acids served as the sole source of dietary nitrogen. The data showed that methionine could replace cystine in the diet, but that cystine could not replace methionine.

Methionine as a Provider of Organic Sulfur

Cystine

The work of Osborne and Mendel (1914) and of Sherman and his coworkers (1925) indicated that cystine was an essential amino acid. In 1931, Jackson and Block reported the almost incredible observation that methionine was able to serve in lieu of cystine in promoting the growth of animals kept on a cystine-deficient diet. This aroused considerable interest in the methionine-cystine relationship.

The body can manufacture cystine from methionine. However, the reverse reaction does not appear to take place. The albino rat fed a diet devoid of these two sulfur-containing amino acids cannot grow. If methionine is added, the animal responds with normal growth. However, if no methionine is present, no matter how much cystine or cysteine are added to this diet, the animal does not grow and eventually dies. Thus methionine is necessary for the growth of the rat, but the animal can get along without cystine, if sufficient methionine is present. Under the latter conditions, the total amount of cystine in the body increases as growth occurs. Tarver and Schmidt (1942) proved this beyond doubt in their experiments with labeled methionine using radioactive sulfur. Cystine was

isolated from the tissues rats containing S^{35}. This work solved the question of the conversion of methionine to cystine, but left an even more intriguing one as to how the conversion took place.

Various hypotheses were advanced concerning the mechanism of the conversion. Subsequent testing of these hypotheses led to the present day concept that methionine is converted to cysteine by first being demethylated to homocysteine; the homocysteine then condensed with serine, splitting out water to yield cystathionine. The cystathionine was then cleaved by an enzyme in the liver to yield cysteine.

Methionine Homocysteine Serine Cystathionine

Cysteine

Thus, the methionine furnished only the sulfur for the synthesis of cysteine in the body; the serine furnished the carbon chain and nitrogen, according to this hypothesis. That this reaction was one which actually occured was proved in subsequent experiments employing the tracer technique (du Vigneaud et al., 1944). Approximately 80 per cent of the sulfur of cystine present in a new coat of hair grown during the time that labeled methionine was fed to young rats had been derived from dietary methionine. However, the cystine contained no significant amounts of

the isotopic carbon in the methionine administered.

Taurine

The conversion of methionine sulfur to taurine sulfur was conclusively proved by Tarver and Schmidt (1942) in experiments with S^{35} in which dogs were used as experimental animals. Fifty-six per cent of the methionine sulfur was incorporated into tissue protein. Of this amount 35 per cent was found in the carcass, 24 per cent in the liver, 16 per cent in the gastrointestinal tract, and 9 per cent in the kidney. Smaller fractions were found in the genitourinary tract, lungs, skin, hair, and bile.

Methionine as a Provider of Labile Methyl Groups

Choline

Another important function of methionine, in addition to its role as a potential source of body cysteine and cystine, is its ability to provide labile methyl groups to the organism. The process of transferring labile methyl groups *in toto* from one organic compound to another *in vivo* is known as transmethylation.

That methyl groups from methionine may be transferred to choline in experiments with deuterium-labelled methyl moieties has been demonstrated (du Vigneaud *et al.*, 1941). When methionine labeled in the methyl group was fed to animals maintained on a methionine-free choline diet,

choline containing the deuterium was isolated from metabolic products proving unequivocally that methionine could furnish methyl groups for the synthesis of this compound in vivo. Choline was synthesized by a linking of the methyl group obtained from methionine with ethanolamine arising from the reduction of glycine or the decarboxylation of serine.

Creatine

That the methyl of methionine takes part in the synthesis of the important metabolite, creatine, also has been demonstrated clearly by isotopic studies. Creatine is another material whose exact biological functions are not all known. Its universal occurrence in body tissues, however, is evidence of its importance. It appears to be present in muscle more abundantly than elsewhere and there, as creatine phosphate, is essential for muscle contraction.

Du Vigneaud, Chandler, and Moyer (1941) in tracer experiments studied the migration of methyl groups in vivo from dietary methionine and dietary choline to creatine in body tissues and to creatinine in the urine. Employing liver slices, Borsook and Dubnoff (1940) found that only the methyl from methionine, in contrast to all other methyl containing compounds tested, was used for the formation of creatine. Just as ethanolamine proved to be the acceptor for the methyl group in the synthesis of choline,

guanidoacetic acid, glycocyamine, was established as the intermediary acting as the acceptor for the methyl group in the synthesis of creatine.

Symptoms of Methionine Deficiency

Since the discovery of threonine in 1938 by W. C. Rose and coworkers, making it possible for the first time to maintain and grow rats on a highly purified diet, methionine has been classed as an essential amino acid, not only for the growth process, but also for the maintenance of nitrogen equilibrium. As defined by Rose (1942), an essential amino acid is "one which cannot be synthesized by the animal organism, out of materials ordinarily available, at a speed commensurate with the demands for normal growth." Methionine is not only essential for the rat, but also is indispensable for man, the dog, the chick, and the mouse.

Several reports in the literature attest to the fact that the omission of methionine from a mixture of the essential amino acids in an otherwise complete diet results in a negative nitrogen balance (Brush, 1947; Cannon *et al.*, 1948; Rose, 1942). Elvehjem and his coworkers (1950) recently reported an interesting observation. They noted that when rats were force-fed in contrast to *ad libitum* feeding, positive nitrogen balance was maintained for

three weeks when methionine was the only dietary deficiency. This work, if confirmed, will have implications in studies relating to the maintenance of nitrogen equilibrium as a measure of the essentiality of amino acids.

In the experiments of Elvehjem and his collaborators (1950), striking differences were observed between the control group and the normal animals, fed a ration deficient only in methionine. There was bleeding of the feet and of the tissues around the mouth in the latter group. The livers were fatty and exhibited a very low xanthine oxidase activity.

Other less specific deficiency symptoms have been reported by other investigators. Sydenstricker and coworkers (1946) reported corneal vascularization in rats fed diets low in either lysine, methionine, or protein. Albanese and coworkers (1946) observed marked drops in the concentration of hemoglobin in the blood of rats maintained on methionine-deficient diets for 100 days or more. There was no decrease in the number of red cells, however. Since the casein hydrolysate used, when fortified with tryptophan and cystine failed to correct this condition, the hypochromic anemia was assumed to have been due to a deficiency of methionine.

Glynn, Himsworth, and Neuberger (1945) showed that a mild methionine deficiency in rats caused anorexia, arrest

of growth, and sometimes wasting of body tissues. Severe deficiency led to hypoproteinemia and macrocytic anemia. This condition was remedied by supplementary methionine, but not by cystine.

Physiological Role of Methionine

Detoxification of Organic Compounds

Lewis and coworkers (1935) have demonstrated the importance of the sulfur-containing amino acids in the detoxification of aromatic halogens administered to experimental animals. Miller and Whipple (1940) have reported that methionine, or cystine plus choline, protects against the hepatoxic effects of chloroform anesthesia in protein-depleted dogs. Smith (1946) by administering methionine increased the survival time of rats exposed to methyl chloride. The development of cirrhosis and nephrosis in rats exposed to carbon tetrachloride was prevented by methionine in these experiments, but it gave no protection to the normal animal. Similarly, methionine has been shown to be beneficial to benzene-poisoned rats and arsenical-poisoned dogs only if they are protein-deficient (Goodell, Hanson, and Hawkins, 1944).

Renal Hemorrhage

Mueller, Cox, and Sloat (1946) in experiments with rats fed casein and casein hydrolysates supplemented with

methionine observed beneficial effects on renal hemorrhage. Although the exact way in which methionine exerted its effect was not clear, the authors suggest that the total effect is the maintenance of normal renal tissue.

Therapeutic Effects on Burns, Fractures, and Wounds

Numerous reports in the literature suggest a beneficial effect of methionine on wound healing in protein-depleted animals. No similar action, however, has been shown to occur in the normal animal. Lofalio, Morgan, and Hinton (1941) showed that a subcutaneous injection of methionine in protein-depleted rats resulted in a marked shift of the curve of wound healing toward normal as measured by tensile strength. This amino acid was also found to accelerate the speed of healing following excision of skin from the backs of these animals. The authors suggest that these effects should be attributed to the -SH groups, indicating that the -SH enzymes exert their influence during the early stages of healing, shortening the lag period and hastening proliferation.

Beneficial effects of methionine in relation to decreasing the nitrogen loss following thermal burns have been reported by Croft and Peters (1945). A supplement of dietary methionine sharply reduced the urinary output of nitrogen in these experiments.

Nitrogen Sparing Effect

Albanese and coworkers (1944) report that while the addition of methionine increased the rate of growth in rats and the magnitude of nitrogen retention in dogs, in man, however, it was without effect on nitrogen retention. Observations were made on four different groups: (1) depleted surgical patients fed intravenously, (2) normal adults fed at the maintenance level, (3) normal adults who had been protein-depleted for three weeks or more then fed only an amount of nitrogen equal to their endogenous loss, and (4) normal infants. In no one of these four groups did the addition of extra amounts of the essential sulfur-containing amino acid increase nitrogen retention. These results in humans have been confirmed by other investigators (Benditt, Woolridge, and Stepto, 1948).

In an investigation of the therapeutic effect of protein feeding after severe burns, the English workers, Croft and Peters (1945) found that a supplement of dietary methionine sharply reduced the urinary output of nitrogen and improved the physiological condition of the experimental animals subjected to hot water. They advanced the theory that the liver "raids" tissue proteins in search of needed building blocks, and excretes unwanted amino acids. This prodigal destruction did not occur

when methionine was supplied.

White and Lewis (1935), Stekol (1935), and Miller (1940) independently recorded a surprising depression in the excretion of urinary nitrogen after feeding methionine to animals on a low nitrogen diet. This body sparing action was subsequently confirmed by Miller (1944), Willman et al., (1945), Allison, Anderson, and Seeley (1945).

A similar effect was noted by Swanson and coworkers (Swanson, Everson, and Stewart, 1946) when fat was removed from a low nitrogen diet and the caloric intake restricted. These workers found that methionine prevented the enormous loss of tissue that occurred when fat was eliminated from the diets furnishing one-fourth of the needed calories.

Urea Formation

That the nitrogen sparing effect of methionine was reflected in the partition of nitrogen in urine was reported simultaneously by workers in this country (Allison, Anderson, and Seeley, 1947), and by workers in Europe (Leuthardt et al., 1947). According to Allison, "the decrease in the excretion of nitrogen in the urine which accompanied the addition of methionine to casein is due to a decrease in the excretion of urea and not of ammonia, creatinine, or creatine. Thus, methionine affects the

metabolic paths involved in the synthesis of urea, altering the ratio between the excretion of ammonia and urea." That this phenomenon was also true in rats was reported by Hoover, Stewart, and Swanson (1949) the following year. These workers showed, in addition, that methionine was more effective in decreasing the excretion of urea than were whole egg proteins or any of the other essential amino acids. This effect was shared by homocystine and the methionine analogs, ethionine, methionine sulfone, and methionine sulfoxide.

Relation to Vitamins

Data have been reported recently indicating a specific metabolic relationship between methionine and certain of the vitamins. Elvehjem and his coworkers (1946) observed that the content of riboflavin in the livers of rats fed protein-free diets decreased. With the addition to the diet of methionine, the hepatic stores of riboflavin returned to approximately normal values. Roblin and his coworkers (1945), in a study of the relation of methionine and sulfonamides in bacterial growth, suggested a possible relationship between methionine and para- amino benzoic acid in metabolism.

Relation to Enzyme Systems

A definite relationship between methionine and various enzyme systems has been indicated by the work of Elvehjem

and his coworkers (1950). These workers reported a marked drop in the level of xanthine oxidase in the hepatic tissues of rats fed diets deficient in methionine. Methionine feeding restored the level of xanthine oxidase to normal.

Animals fed a high fat, protein-deficient ration showed a pronounced elevation of serum alkaline phosphatase (Tuba, Cantor, and Richards, 1949). Hough and Freeman (1942) have demonstrated that methionine reversed the effect of protein deficiency. Alkaline phosphatase was shown also to be related to fat metabolism by Hough et al., who interpreted the lowered serum alkaline phosphatase levels when methionine was added as a return to normal of fat mobilization (1943).

Such many and varied functions of methionine suggest that it is of fundamental importance in the metabolism of the animal organism. Its specific role in maintaining adequate nutrition in spite of an inadequate diet is continuing to receive much attention.

Additional Studies with Radioactive Methionine

Studies using radioactive methionine have further clarified the function of this essential amino acid. Tarver and Morse (1948), in a study of the rates of release and incorporation of labeled methionine in tissue protein in vivo, reported that after 24 hours, the

concentration of this amino acid was greatest in the proteins of the intestinal mucosa, and decreased in the order of kidney, plasma, liver, spleen, brain, skin, hair, and muscle. In additional observations on intestinal mucosa, these investigators found that the maxima of uptake occurred in the region of the junction of the duodenum and jejunum, the junction of the jejunum and ileum, and in the terminal segment of the large intestine. The value for the protein of the red blood cells, at first lower than that of any other organ rose continuously, while all tissues and plasma exhibited a downward trend. These results have been confirmed by Maass et al. (1949).

Du Vigneaud and his coworkers (1949) fed methionine with a high concentration of C^{14} in the methyl group by stomach tube to rats. Highest concentrations of this amino acid were observed in the kidneys, liver, and adrenal glands. They suggested that the overall picture of the metabolism of the methyl group is one of active participation in both degradative and oxidative, synthetic and transmethylation reactions.

In an investigation of the fate of labelled methionine in normal and cystinuric dogs, Tarver and Schmidt (1947) found higher concentrations of S^{35} in cystinuric animals, indicating that an abnormality in the rate of formation or

excretion of cystine in the cystinuric animal. In further experiments with the hepatectomized dog (Tarver and Schmidt, 1947), it was found that in the absence of the liver, this animal incorporates methionine into its tissue proteins at the same rate as the normal dog. This process proceeds in the kidney, pancreas, and intestinal mucosa and results in cystine sulfur.

Thus, the liver is not essential for the conversion of methionine sulfur to cystine sulfur. However, it is interesting to note that the synthesis of globulins and albumin was greater in the normal animal than in the hepatectomized dog.

Inhibitory Effects of Methionine

In vivo

Recent experiments of Allison and his coworkers (1948) indicate that excess methionine in the diet of rats may have adverse effects. These workers fed 4.8 per cent of dl-methionine in a casein diet, and noted a loss of weight and negative nitrogen balance. Dl-methionine in excessive amounts not only spared no nitrogen, but actually increased nitrogen loss in normal human beings. Kade and Shepard (1949) report that 2.0, 2.5, and 3.0 per cent dl-methionine inhibited growth and protein utilization when added to an 8 per cent casein diet fed to rats.

Later experiments by Rothe and Allison (1949) indicated that a marked lack of fat stores was evident in rats fed 4.8 per cent methionine. Glycine and arginine were able to counteract the loss of weight in part. An increase in the excretion of creatinine and creatine was also observed. The weights of both the liver and kidney increased. The increment observed in weights of the adrenal and thyroid glands was less marked.

In Vitro

In vitro experiments have resulted in interesting observations when methionine is added. Krebs (1946) observed that when methionine was added to liver slices with ammonium salts, less urea was formed than when the ammonium salts alone were added. This same effect was produced with guinea pig liver slices. Leuthardt and coworkers (1947) in Europe have confirmed these results.

Bernheim and Bernheim (1949) reported a similar effect with rat liver slices. Methionine inhibited urea production from ammonium chloride in the presence of pyruvate and ornithine. This effect was more pronounced with guineapig liver slices. The authors concluded that methionine binds magnesium because the addition of this metal overcame the inhibition. The formation of citrulline from ornithine was specifically inhibited by methionine.

Excretion of Methionine

In studies on medical students, Tidwell et al., (1947) observed that during a three-day experimental period, the excretion of methionine was not affected by the ingestion of a low protein diet. It did decrease, however, during fasting, or when a high fat diet was fed for the same period.

In a later study with rats, Johnson et al., (1947) observed that methionine intake or greatly varying levels of protein did not affect methionine excretion. No difference in sulfur and nitrogen excretion was noted when methionine supplements were added to the diet. These authors, however, report that the intraperitoneal injection in rats of a test supplement of methionine on the day the urine was collected gave small but signficant increases in the urinary excretion of the amino acid.

Tidwell (1949)reports further that with 15 per cent or more of fat in the diet, rats excreted less methionine than when fed a diet containing 5 per cent fat or a normal stock ration. Likewise, significantly less methionine was excreted when the animals were fasted for 3 days. Feeding of a choline supplement to the fasting animal increased methionine excretion to a level characteristic of that when protein was present in the ration.

Pearson and Panzer reported a greater excretion of methionine when no fat is present in the diet than when corn oil was added. Less methionine was found in the feces when corn oil was fed, than when the rats received no dietary fat. They suggested that 8 per cent corn oil in the diets of these animals enhanced the efficiency with which methionine was absorbed and utilized.

Implications of a Specific Function of Methionine in Protein Metabolism

In 1941, Harris and Kuhn (1941) in studies with bacteria found that methionine antagonized the action of sulfonamide. In their presence, Escherchia Coli neither oxidized, decarboxylated or deaminated this amino acid. Sulfonamides inhibited the synthesis of methionine, found to be dependent in these experiments on the level of para-amino benzoic acid in the nutrient medium. Methionine, in the absence of sulfonamides, preserved the respiration of resting cells, decreased the induction period prior to growth, and stimulated the synthesis of respiratory enzymes. The authors concluded, "the role of methionine is neither to supply nitrogen, nor energy for growth; this suggests that its role is a special one, probably concerned with anabolism."

The finding by Brush et al.(1947) that methionine decreased urinary nitrogen losses and spared body tissues to

a marked degree when rats were fed protein-free diets, led these workers to the conclusion, "this amino acid . . . act s . . . in the synthesis of body proteins and important metabolites.

Similar results have been observed by Miller (1944). Methionine exerts a marked protein-sparing action as was shown by a sharp decrease in organic sulfur in the urine, equivalent to only 15 to 40 per cent of the dietary amino acid sulfur. Miller has suggested that these observations may be of fundamental importance in explaining the action of methionine in preventing experimental cirrhosis, in lowering the incidence of experimental hepatoma, and in decreasing susceptibility to liver poisons. Explanation might be found in a decrease in the sulfur containing enzymes or in their lowered activity (or both) following reduction in the essential sulfhydrul activators such as glutathione. This hypothesis is in accord with Englehardt's view (1942) that cell proteins are primarily enzymes and the recent demonstration by Barron (1943) that free sulhydryl groups are associated with full activation in many of the oxidative enzyme systems involved in carbohydrate metabolism.

Stekol, in a study of mercapturic acid synthesis in 1935, found that a marked fall occurred in the urinary

nitrogen 4 days after ingestion of bromobenzene, dl-methionine being fed on the fifth day. He reported . . "that dl-methionine arrests the breakdown of tissue...caused by bromobenzene feeding after the synthesis and excretion of p-bromophenylmercapturic acid have been completed." It seems reasonable that the fall in urinary nitrogen is identical with the protein-sparing action of methionine shown in other experiments.

In experiments with chicks maintained on rations deficient in vitamin B_{12}, and similar diets to which B_{12} was added, Charkey et al., (1950) have observed strikingly low levels of methionine in the blood as compared to the other amino acids. Since examination of the amino acid composition of both chicken muscle and of the diets showed much higher relative contents of methionine than that found in the blood of these animals, the authors interpret their results as being suggestive of a unique relationship of methionine in metabolism, particularly as to the role of vitamin B_{12} in amino acid utilization. It was interesting to note in these experiments that the addition of methionine to the rations containing vitamin B_{12} resulted in improved growth and feed utilization. Since the addition of a vitamin B_{12} supplement resulted in a decrease in the concentration of methionine and other amino acids in the blood, the authors suggest that vitamin B_{12} may exert a function in enhancing the utilization of circulating amino acids for building fixed tissues.

PLAN OF THE EXPERIMENT

The nature of the protein-sparing action of dietary fat which follows its incorporation into a nitrogen-low diet, has been examined in the present investigation. Special emphasis, also, has been given to a study of the protective role exerted by methionine on protein metabolism when it supplements a fat-free ration. These problems have been investigated when the energy value of the experimental diets were optimal and suboptimal.

Male, albino rats were used as the experimental animals. The various control and test groups and the respective diets fed are indicated below, together with the number of rats employed.

I. CONTROL GROUPS

A. Normal Animals Fed the Laboratory Stock Diet
   a. Full caloric intake ..... 30 rats
   b. Restricted caloric intake ..... 6 rats

B. Animals Fed the Low Nitrogen Diet
   a. High fat ..... 6 rats
   b. Low fat ..... 12 rats

II. GROUPS FED THE UNSUPPLEMENTED NITROGEN-LOW DIETS

A. High Fat Diet
   a. Full caloric intake ..... 39 rats
   b. Restricted caloric intake ..... 18 rats

B. Low Fat Diet
   a. Full caloric intake ..... 50 rats
   b. Restricted caloric intake ..... 18 rats

III. GROUPS FED THE METHIONINE-SUPPLEMENTED NITROGEN-LOW DIETS

A. High Fat Diet
   a. Full caloric intake ..... 21 rats
   b. Restricted caloric intake .. 21 rats

B. Low Fat Diet
   a. Full caloric intake ..... 21 rats
   b. Restricted caloric intake .. 24 rats

The animals in each group were distributed among the specific studies to be described in which various phases of protein metabolism were investigated.

The respective nitrogen balances of the various experimental groups were determined. In earlier studies (Swanson, et al. 1946) in which the sparing action of fat was demonstrated, it was difficult to control the food intake due to the loss in appetite evoked by the ingestion of the nitrogen-poor rations. It was desirable, therefore, to verify the results of preliminary observations under more precise conditions, i.e., controlling the food intakes byforce-feeding so that adequate caloric intakes could be maintained throughout the experimental period.

It seemed that a comparison of the partition of nitrogenous constituents of the urine under the various dietary regimes would reflect changes in metabolism occurring under these conditions and give an indication of the adjustments the

organism was forced to make. This approach promised to be fruitful because preliminary studies already had revealed that the nitrogen-sparing action of methionine was reflected by a decrease in both the quantity and absolute amount of urea present in the urine (Hoover, Stewart, and Swanson, 1949). Analyses were made therefore of the respective quantities of total nitrogen, urea, ammonia, allantoin, amino nitrogen, and creatinine excreted in theurines of the various groups. In addition, since other investigations have shown that the feeding of high fat diets alters the patternof urinary amino acids (Tuba et al., (1949), an estimation of the excretion of the components of this fraction of the total nitrogen seemed desirable. Chromatographic analyses were made, therefore, of the urinary amino acids.

Simultaneous analyses of nitrogenous constituents in blood and urine have proved valuable in many studies of a nutritional nature. The concentrations of urea and amino nitrogen were determined, therefore, in blood. Since a suggestion had appeared in the literature that alkaline phosphatase was concerned with fat metabolism (Hough et al., 1943), concentrations of this enzyme in the blood of the various experimental groups was investigated.

It was questioned whether or not a disturbance in the metabolism of carbohydrate was associated with the removal of fat from the ration under the specific experimental conditions imposed by the present investigation. As an initial approach to this problem, glucose tolerance and the deposition of glycogen in the liver were studied.

It is generally accepted that the seat of many processes involved in protein metabolism is in the liver. It was felt that an analysis of the occurrence of certain constituents in the livers of the rats fed the various test diets might be useful in the interpretation of the part played by fat and methionine in the regulation of protein metabolism. Therefore, the concentrations of moisture, fat, total nitrogen, riboflavin, and niacin were measured.

The histological study of organs provides valuable information as to structural changes intissues occurring under various conditions. To aid in the interpretation of other analytical data obtained in this investigation, microsections of the liver, kidney, and adrenal glands were stained with hematoxylin and eosin. Other sections were stained with selective fat stains, Scarlet Red and Nile Blue Sulfate, and with a glycogen stain, Carmine Red.

EXPERIMENTAL PROCEDURE

SELECTION AND CARE OF EXPERIMENTAL ANIMALS

Male, albino rats of Wistar stock, strain A, inbred for 99 generations by brother and sister matings, were used in these experiments. From weaning time at 28 days until the beginning of the experimental period, they were housed in groups of two in round wire-meshed cages and allowed free access to the diet of the stock colony and distilled water. Body weights were recorded each week.

All animals were in good physical condition at the beginning of the experiment as judged by their general appearance, alertness, muscle tone, and freedom from respiratory disease. When they were approximately six months old ($\pm$ two weeks), they were separated into groups of six animals each so that the average weights of each group were 330 $\pm$ 3 gm. Littermates were not used in these studies. Animals within the groups usually varied in weight by plus or minus 20 gm. from the average. This method of screening insured that the animals would be approximately 300 gm. in body weight at the end of the depletion period on the low nitrogen diet, when force-feeding was initiated.

RATIONS

Stock Diet

All animals were maintained on the standard stock colony diet of the Nutrition Laboratory of the Iowa State College, i.e., either Steenbock XV or Steenbock XVII, from the time of weaning until the time that the experiment was initated when the rats were 6 months old. These diets differed only in that dried milk was incorporated into the latter in the place of liquid milk fed as a separate supplement.

Ingredients of the basal stock ration are shown in Table 1. This diet was supplemented with either 5 gm. of ground beef, 10 gm. of cabbage, or 10 gm. of carrots daily. Three times a week the animals were given 2 drops of cod liver oil.

Experimental Diets

The basal nitrogen-low diets containing either 20 per cent or no fat were fed throughout the balance experiment. The composition of these diets is shown in Table 2. The quantity of food offered in these experiments was determined in preliminary work in which the food intakes of animals fed the high and low fat diets was carefully determined in experiments employing a large number of animals over a

TABLE 1. COMPOSITION OF THE STEENBOCK XVII CONTROL DIET

Ingredient	Quantity
	gm.
Yellow cornmeal	560
Crude casein	50
Linseed oil meal	160
Ground alfalfa leaf	20
Pabst yeast, unirradiated	95
Irradiated yeast	5
Wheat germ	100
Klim	163
Sodium chloride	5
Calcium chloride	5
Trace elements	---

period of two weeks. The animals maintained on the high fat ration ate, on the average, 12.0 gm. per day per 300 gm. body weight; those fed the low fat diet ate 15.0 gm. per 300 gm. body weight per day. The animals were offered the basal diets ad libitum during the first 14 days of the experiment, then force-fed the respective experimental rations throughout the remainder of the experiment, or until the dietary modification was introduced. Each day, 24 ml. of the semi-liquid homogenized diets were fed, the high fat diet containing 12.0 gm. of solids, the low fat diet, 15.3 gm. These amounts of the basal diets provided the same daily caloric intakes for the groups fed the high energy rations.

Preparation of the Dry Diets

Basal Diets

A standard procedure was used in the preparation of all diets. The butterfat used in the high fat ration was prepared by melting several pounds of butter which had been purchased from the local market. When the fat was thoroughly melted, the milk solids which floated on the top were carefully removed by skimming. The clear liquid was filtered through a clean cheese cloth placed in a hot water funnel above a receptacle. It was stored in the refrigerator until ready for use.

TABLE 2. COMPOSITION OF LOW NITROGEN BASAL DIETS

Ingredients	High fat low nitrogen diet	Low fat low nitrogen diet
	gm.	gm.
Dextrin*	730	930
Butterfat**	100	-
Lard***	100	-
Osborne and Mendel salt mixture****	40	40
Ruffex*****	20	20
Sodium chloride******	10	10
Total	1000	1000
Per cent nitrogen	0.056	0.051
Calories/gm.	4.85	3.81

*Purchased from Fisher Scientific Co.
**Purchased on the local market as butter and prepared in the Nutrition Laboratory
***Swift's lard, purchased on the local market
****Osborne, T. B., and Mendel, L. B., J. Biol. Chem., 37, 557-601 (1919)
*****Purchased from Eimer and Amend, New York
******Purchased from Fisher Scientific Co.

In the preparation of the high fat diet, the desired amounts of butterfat and lard were melted together. The quantities of the dry ingredients needed in a formula were weighed on the Torsion balance and tabled together carefully on a large sheet of cellophane. The dry ingredients were transferred to the Hobart mixer, the melted fat added, and all were mixed at medium speed for 20 minutes. This uniform mixture was transferred then to containers for storage in the refrigerator until ready for use.

The procedure for the preparation of the low fat diet was essentially the same, with the exception that no butterfat or lard was added, an equivalent weight of dextrin being substituted for these constituents.

Methionine-supplemented Diets

Because of the recent evidence that metabolic mechanisms involved in the processes of fabrication and maintenance of tissue proteins require the simultaneous availability of essential dietary components, it was felt desirable to incorporate the methionine supplements directly into the dry diets. Thus, variables which may have been introduced from delayed supplementation were eliminated. Four mg. of methionine nitrogen, equivalent to

44 mg. of methionine, per 300 gm. rat per day, or 2.78 gm. of methionine per 1000 gm. low nitrogen diet, were substituted for an equivalent quantity of dextrin in the preliminary mixing of the diet. The methionine was ground in the mortar with a small quantity of the dextrin, then with larger quantities. The remaining dextrin was added to the methionine mixture, along with the other constituents of the diet. The entire mass was thoroughly mixed in a Hobart mixer.

Vitamin Mixture

The vitamin mixture supplementing the experimental rations was prepared carefully, quantities that would supply 1000 daily doses being made at each mixing. To prevent possible destruction of certain vitamins and the gumming of the ingredients which occurred after long exposure to the air, it was necessary to complete the preparation of the mixture in the shortest possible time. To accomplish this, the ingredients were mixed in a special diet room, free from strong sunlight and on a dry, clear day. The process was started in the morning and completed before the end of the day, with storage of the mixture in the freezing unit. Small quantities of the mixture were removed for use as needed. The composition of the mixture is shown in Table 3.

TABLE 3. COMPOSITION OF THE VITAMIN MIXTURE

Vitamins	Dose per day
Thiamin	40 mcg.
Riboflavin	60 mcg.
Pyridoxine	40 mcg.
Inositol	10 mg.
Para-aminobenzoic acid	10 mg.
Nicotinic acid	0.5 mg.
Calcium pantothenate	0.1 mg.
Ascorbic acid	1.0 mg.
Choline chloride	5.0 mg.
Biotin	1.0 mg.
Rice Bran polish concentrate*	100.0 mg.
Alpha tocopherol solution**	0.75 mg.
Cod liver oil***	0.50 mg.

*Prescription Products Division, Borden Co., New York
**Five gm. alpha tocopherol added to 333.4 gm. Wesson oil
***Squibb's medicinal, from Judisch Bros., Ames, Iowa

In the ad libitum feeding period, the standard vitamin mixture was offered to the animals in small, round, pyrex cups and was, in general, immediately eaten. When forced feeding was initiated, the vitamins were incorporated into the liquid diets.

In preparing the vitamin mixture, the water soluble ingredients were divided into two sub-groups on the basis of physical properties. Handling was thereby simplified considerably. Dextrin was added so that the quantity fed daily was contained in 500 mg. The quantities needed for 1000 doses and the division of the components of the mixture into the two groups are shown in Table 4.

The vitamins of group A were mixed first in the following manner. A quantity of dextrin, 375 gm. was weighed on the Torsion balance. Choline chloride and rice bran polish were rapidly weighed on a Torsion Balance into a tared 250 ml. beaker. Biotin, which has been purchased in small ampules containing known amounts of the vitamin, was quantitatively transferred to the mixture of choline chloride and rice bran polish with15 ml. of 50 per cent alcohol. After thorough mixing, 75 gm. of dextrin were introduced and the mixture stirred until a smooth brown paste was formed. It was then quantitatively transferred to a pyrex plate with 10 ml. of 50 per cent alcohol.

TABLE 4. QUANTITIES OF VITAMINS USED IN THE PREPARATION OF 1000 DOSES OF VITAMIN MIXTURE

Group	Components of vitamin mixture	Quota per rat per day	Per 1000 doses
			gm.
A	Choline chloride	5 mg.	5.0
	Biotin	1 mcg.	0.00
	Rice Bran polish concentrate	100 mg.	100.0
B	Thiamine	40 mcg.	0.040
	Riboflavin	60 mcg.	0.060
	Pyridoxine	40 mcg.	0.040
	Inositol	10 mcg.	10.000
	Para-amino benzoic acid	10 mg.	10.000
	Calcium pantothenate	0.10 mg.	0.100
	Nicotinic acid	0.5 mg.	0.500
	Ascorbic acid	1.0 mg.	1.000
C	Dextrin		A quantity sufficient to bring dry weight of 1000 doses to 500 gm.

The paste was dried before a large fan set at high speed. The mixture was stirred frequently until it had lost its gloss and had become hard and glue-like in quality.

The desired quantities of the vitamins of group B were weighed on the analytical balance and ground with 25 gm. of dextrin inthe mortar. This mixture was incorporated into the dry mixture containing the vitamins of group A. The mass was rubbed to remove all lumps, pushed immediately through a medium sieve, and spread evenly on a large tray to dry. When the powder was dry, the remaining 275 grams of dextrin were added and thoroughly mixed with it. This made a total weight of 500 grams for every 1000 doses.

If more than 1000 doses were needed, the lots were prepared separately, tabled together, and stored as one batch in the freezer. The inadvisability of making a lot larger than 1000 doses has been demonstrated earlier in our laboratory. It was found that this larger quantity dries slowly, has a tendency to absorb moisture rapidly if allowed to stand too long, and is difficult to manipulate in the process of preparation.

A teaspoon calibrated to deliver 500 mg. of the vitamin mixture was used to measure daily doses. However, during that interval of the experimental period when the mixture was incorporated into the liquid diet, 500 mg. for each 12 or 15 grams of the high or low fat diets respectively was weighed out on the Torsion balance and homogenized directly with the diets.

Preparation of the Semi-liquid Diets

Animals, when fed low nitrogen rations, show a loss of appetite that is followed by decrements in body weight. In order to prevent the decreased food intake, to keep the animals from scattering their food, to control the daily food intake, and to provide the proper number of calories to each animal, the experimental diets were made into homogenates which could be introduced directly into the stomach of the animal.

A standard procedure was developed for homogenizing the diets, with special precautions taken to insure a mixture that would supply the dietary constituents quantitatively. Before the experiment was initiated, the density most desirable for force-feeding was determined. It was necessary to formulate a mixture that could be manipulated with a syringe and which would deliver the suspension accurately without any settling of the solid components.

High Fat Semi-liquid Diet

A mixture of 360 gm. of the high fat diet and 450 ml. of distilled water provided a solution of desirable density. This proportion of diet and water produced a total volume of 720 ml. and provided 12 gm. of diet per day for 30 rats in a volume of 24 ml.

TABLE 5. QUANTITIES* OF DRY DIET AND WATER USED IN THE PREPARATION OF THE SEMI-LIQUID DIET

Dry diet	Quantities used			Total volume	Ml. per rat per day
	Dry diet	Vitamin mix	Distilled water		
	gm.	gm.	ml.	ml.	
High fat	360	15	450	720	24
Low fat	458	15	420	720	24

*Sufficient to feed 30 rats/day

In preparing the homogenate for feeding, 360 gm. of the dry diet were weighed on the Torsion balance. Four hundred and fifty ml. of distilled water were added to the dry diet in a mixing bowl. After preliminary mixing with a spoon, one-half of this mixture was poured into the Waring Blendor and homogenized for one-half minute. The remainder of the mixture was then quantitatively transferred to the Waring Blendor, and the entire solution homogenized for an additional minute. The material adhering to the upper part of the blendor was scraped down, and the mixture again blended for one-half minute. In order to remove air bubbles, the mixture was stirred thoroughly with a long thick glass rod before it was transferred to three half-pint containers for storage.

Low Fat Semi-liquid Diet

The large quantity of dry diet required to make the low fat diet isocaloric with the high fat diet made homogenization impractical. Therefore, after the required quantity of dry diet and vitamin mix had been measured, this diet was prepared by the addition of small quantities of the total volume of distilled water, i.e., 420 ml. with stirring until a smooth mixture was obtained. After addition of the total quantity of water, the liquid mixture was stirred for an additional five minutes

to insure even consistency. It was then transferred to labeled half-pint containers and stored in the refrigerator.

At the time that dietary modifications were to be initiated, the following alterations in the method of preparation of the diets were made to insure that the same quantities of methionine and vitamins were supplied as had been given in the first period of the experiment. As was discussed earlier, when diets at the normal level of caloric intake were fed, the quantity of methionine required to supply 4 mg. nitrogen per 56 calories was substituted for an equivalent amount of dextrin. When the caloric intake was restricted to 25 per cent of the normal intake, four times this amount of methionine per 300 gm. rat per day was incorporated with four times the quantity of vitamin mix. Thus, while the animals were fed only one-fourth of the fuel food intake administered in period I they received the same amount of methionine and vitamins as the animals on full food intake.

Since the level of feeding was maintained at 56 calories per day per 300 gram rat, the amounts of food, methionine and vitamins varied from animal to animal, but were the same on the basis of body weight. Since the animals were selected carefully at the beginning of the experiment so that the weights would be very similar, these differences were not great (See Table 6).

TABLE 6. CALORIES AND VOLUMES OF LIQUID DIETS FED IN RELATION TO THE WEIGHT OF THE ANIMAL

Weight of rat	Volume of liquid diet		Total calories per day
	Per feeding	Per day	
gm.	ml.	ml.	cal.
201 - 210	8.4	16.8	39.2
211 - 220	8.8	17.6	41.0
221 - 230	9.2	18.4	42.9
231 - 240	9.6	19.2	44.8
241 - 250	10.0	20.0	46.7
251 - 260	10.4	20.8	48.5
261 - 270	10.8	21.6	50.4
271 - 280	11.2	22.4	52.3
281 - 290	11.6	23.2	54.1
291 - 300	12.0	24.0	56.0
301 - 310	12.4	24.8	57.9
311 - 320	12.8	25.6	59.7
321 - 330	13.2	26.4	61.6

Precautions Observed

In preparing these semi-liquid diets, special precautions were taken to obtain a homogenate free from air bubbles. The quantity of each blended diet prepared on a given day was transferred quantitatively into a graduate cylinder, covered, and allowed to stand overnight in the refrigerator. The next morning the volume of the diet in the cylinder was observed when it was removed from the refrigerator and after it had reached room temperature. These volumes were compared to the original volume the previous evening before the cylinders were placed in the refrigerator. There was little, if any variance throughout the experiment, as shown in Table 7.

The half-pint jars containing the diets were stored in the refrigerator until the time of use. Since it was necessary to warm the diets twice daily, the use of small containers which held approximately the daily quota of the diet minimized evaporation and possible alteration of the caloric density of the ration. Fresh quantities of the diet were mixed every third day in order to allow the mixture to stand overnight before it was fed. Two jars were placed in the lower part of the refrigerator, directly beneath the freezing unit, and the remainder of the jars in the freezing compartment. One day before the

TABLE 7. VOLUMES OF LIQUID DIETS OF HIGH AND LOW FAT CONTENTS AFTER STANDING OVERNIGHT IN THE REFRIGERATOR

Diet	Quantity of dry diet	Volume of water added	Volume of solution after standing overnight
	gm.	ml.	ml.
High fat	360	450	720
			720
			720
			715
			720
			720
			720
			724
			720
			720
Low fat	458	420	722
			720
			725
			720
			730
			720
			720
			718
			720
			720

diet was to be used, it was partially thawed by removal from the freezing unit to the area beneath the unit. It was felt that this method insured that no alteration of the vitamin, methionine or other components of the ration occurred through mold or bacterial growth.

The diet was removed from the refrigerator approximately one-half hour before feeding time and brought to room temperature by placing the jar in a pan of lukewarm water. It was stirred with a glass rod at regular intervals during the warming process. Care was exercised against the use of hot water, since the diet had a tendency to cook if hot water were used.

Caloric Value of the Solid and Semi-liquid Diets

In preliminary work, the energy provided by fat and carbohydrate was calculated using figures reported by Rubner. These were found to agree closely with actual caloric intakes as computed from results of the caloric determinations in the bomb calorimeter (Table 8).

The caloric value of the four rations was determined by the Department of Engineering at the Iowa State College.

The caloric values of both the basal dry diets and of the semi-liquid diets were determined. In preparing the

TABLE 8. ACTUAL AND CALCULATED ENERGY VALUES OF DIETS

Diet	Estimated values*	Actual values**			
		Fresh diet	Oven-dried diet	Liquid diet***	Caloric density
	cal./gm.	cal./gm.	cal./gm.	cal./gm.	cal./ml.
High fat	4.85	4.60	4.87	4.86	2.33
Low fat	3.81	3.66	3.90	3.87	2.33

*Based on Rubner's values, 4.1 for carbohydrate, 9.0 for fat

**As determined by the Department of Engineering, Iowa State College

***Mixture from syringe with catheter attached

dry diets for analysis, portions of the high and low fat diets as prepared for ad libitum feeding were dried overnight in an air oven at 80° C. The caloric value of one day's portion of the semi-liquid diets was analyzed. To secure a sample, three 24 ml. aliquots of each liquid diet were shot from the syringe with the catheter attached. These aliquots were dried first on a steam bath, and then placed in the air oven overnight. Then the three aliquots were tabled together and ground in a mortar, thoroughly mixed, and appropriate samples taken for analysis.

Results as shown in Table 8 indicate not only that the calculated values agree closely with the actual caloric determinations, but also, that the method of preparation and feeding of these diets was accurate.

FORCE-FEEDING TECHNIQUE

The forced feeding technique was introduced in this experiment so as to control the daily intake of food and calories accurately. A method developed by Samuels in 1948 was used with modifications introduced by the author.

A veterinary syringe, No. I. D. 105, with a capacity of 20 ml., complete with a six-inch No. 8 French catheter was used. It was checked as to its accuracy in delivering diet of the consistencies used in this experiment by introducing fifty ml. of the liquid diet into a 50 ml. volumetric flask and checking the meniscus against the calibration line.

The syringe was cleaned with warm Dreft solution, rinsed with 70 per cent alcohol and then dried. It was filled with the diet and bubbles were forced out. After the catheter had been attached, liquid was forced through the catheter to fill it; the metal band on the syringe was then set at the desired mark. The syringe was placed on a tray at a convenient height for the operator's right elbow.

In feeding the animal, it was held upright in the left hand with one third of its body length above the cage. The thumb was placed on the mandible with the second finger resting on the head between the eyes. The

animal's left back leg was held to his side with the fourth and fifth fingers of the left hand. The front leg was held between the thumb and the second finger. This position is similar to that assumed when one attempts to remove a cork from a bottle with the thumb and third finger.

After the rat's mouth had been forced open by gentle pressure on the mandible with the thumb of the left hand, the catheter was moistened by slipping it quickly into a vessel of distilled water. It was then inserted into the mouth on the left side of the upper front teeth. With a gentle motion, the catheter was pushed along the roof of the mouth and down the esophagus. Preliminary trials had shown that the tube could be inserted four and one-fourth inches from the rounded tip, and the catheter had been marked at this point.

After the catheter had been introduced successfully, the syringe was lifted carefully from its support with the right hand; the plunger of this instrument was placed against the operator's chest. It was pushed gently toward the operator until the barrel of the syringe touched the metal band, thus delivering the desired amount of liquid diet into the animal. Then, the catheter was carefully and quickly removed from the animal's mouth.

When the last rat in each group had been fed, the syringe and catheter were washed with warm Dreft solution and rinsed with 70 per cent alcohol.

The animals became accustomed to the method of feeding after two or three feedings and were easily handled. After fourteen days of ad libitum feeding, this method of feeding was used exclusively until the end of the experiment.

Each animal was fed twice each day at 8 A.M. and at 8 P.M. an amount of the liquid diet. Twelve ml. per 300 gm. rat were administered at each feeding. This amount supplied 56 calories per day per 300 gm. rat to both the high fat and low fat groups, since the rations had been prepared in isocaloric proportions.

Preliminary experiments were designed to study the quantities of liquid diets which could be administered at either two or three feedings so that an adequate caloric intake would be maintained. It was concluded from this study that the albino rat of the Wistar strain as grown in our laboratories, could not tolerate over 13.5 ml. of food per feeding at two feedings per day, or 9 ml. of food in three feedings per day.

METHOD OF SACRIFICING THE ANIMALS

Description of the Instrument

The instrument used to sacrifice the animals was one which had been specially designed by the author for this purpose. It was devised to work on ordinary 110 voltage electric current and provided an extremely satisfactory method of preparing the animals for necropsy.

Two pieces of ordinary electric wiring, 3½ yards in length were used. One end of the two intertwined pieces was attached to an electric plug. At the other end were two metal pincers with springs. Two feet from the end to which the plug was attached, a circuit indicator was placed. This was a rectangular metal box on which were placed an off-and-on switch and a red light as safety measures. When the current was passing through the device, the red light indicated this. A chromium plate with vertical slits protected the light.

After the 10 hour fast prior to necropsy, the animal to be sacrificed was placed on a table. The electrical instrument had been previously attached to the circuit, and the light switch permitting flow of current to the metal box turned on. The rat was held securely with the left hand while pincers were attached. The

second finger of the left hand was placed on the neck at the base of the skull; the three remaining fingers and thumb held the body on the left and right sides, respectively. With the right hand, one pincer was placed on the anus and the other directly into the mouth. In the latter case, one portion of the pincer was connected inside above the teeth and the other beneath the jaw on the outside. By so attaching the clamps, the animal could not free itself by use of the feet.

Immediately after attaching the animal in this way, the switch on the apparatus was turned on, and the current allowed to run through. Three short shocks of four seconds each with two intervals of three seconds each were usually sufficient to stun the animal to a state in which it offered no resistance in the few seconds before the organs could be removed.

Reaction of the Animal

Immediately on receiving the first four-second shock, the animal leaped a few inches into the air, clinched itself tightly, feet pulled to its sides and muscles strained. Breathing ceased during this interval but was resumed when the current was broken for the next three-second interval.

This reaction occurred to a much lesser extent during the second shock interval of three-seconds. After the third shock, the animal's muscles were completely relaxed, it was still breathing, and seemed in all respects to be completely normal. The only outward sign of reaction was an exudate of sperm, which occurred in both normal and experimental animals.

The pincers were quickly removed, and the animal was placed on a necropsy board. Its four feet were securely fastened by means of metal pins through the cartilagenous region. At this time, the animal was very much alive, breathing normally, yet still relaxed.

In preliminary work, an attempt was made to ascertain what changes might have been produced by this method of stunning the animal. A study of liver weights, adrenal weights, liver glycogen, liver fat, histology of the liver, kidneys, and adrenals with both hematoxylin and eosin, glycogen, and special fat stains did not reveal any differences between the animals stunned by this method and those sacrificed with ether. Gross examination of the heart, lungs, muscles and other organs did not reveal further differences. The exudate of sperm reported above has been observed also by other investigators and seems to be a specific reaction of the organism to an electric shock.

Accurate timing of the procedure with an interval timer turned on simultaneously with the electric instrument revealed that the entire procedure required only 15 to 20 seconds from the time the pincers were attached until the animal was placed on the necropsy board.

EXTIRPATION AND SUBSEQUENT TREATMENT OF TISSUES

To minimize post-mortem changes, rapid removal and immediate treatment of all tissues were essential.

An incision was made along the linea alba from the anus toward the diaphragm. The abdominal wall was then cut transversely to expose the intestinal tract. Care was taken to avoid severing the larger blood vessels, or disturbing the liver or other internal organs. The thoracic cavity was opened by making a V-shaped incision through the ribs. This flap when pulled back exposed the heart.

From three to five ml. of blood were removed from the right ventricle by the use of a 5 ml. syringe equipped with a stainless steel No. 21 gauge needle. The blood was gently forced from the syringe into a 15 ml. centrifuge tube on the surface of which two drops of a solution containing a small quantity of sodium oxalate, 10 ml. sodium fluoride, and 1 mg. thymol had been previously

dispersed and dried. The tube was stirred with a fine glass rod, covered with tin foil, and immediately placed in the refrigerator.

As soon as the sample of blood was removed, the animal was killed by cutting the diaphragm. The liver was removed by severing the hepatic vessels, and promptly blotted with filter paper to remove surface blood. The tip of the longest lobe and a cross section of the largest lobe were placed in a weighing bottle for the estimation of moisture. The remaining portions were transferred to previously weighed 125 ml. stoppered Erlenmeyer flasks. The kidneys and adrenal glands were then removed, and cleared of adhering fat. The adrenal glands were placed in weighed bottles. The kidneys were divided into halves and their general appearance noted.

To maintain the original composition of the tissues, the samples were extirpated, weighed, and treated as quickly as possible. Care was taken to avoid drafts and fluctuations in temperature. In this respect, invaluable assistance was given by three other workers who handled the tissues and blood as they were removed. Preliminary work of a series of 10 weighing bottles by one of these workers and the author indicated that no discrepancy was introduced when the author later weighed the dried samples.

The physical condition of animals at the end of the experiment and before necropsy was noted and recorded. Systematic examination of the esophagus, trachea, stomach and intestinal tract was made to ascertain any effects of forced feeding. Fat depots in various parts of the body, and the condition of the lungs, liver, kidney, and adrenal glands were observed, as were the skin, tail, and gait of the animal. Forms on which this data was recorded are shown in the appendix.

NITROGEN BALANCE TECHNIQUE

General Plan of the Balance Test

The procedure used in studies of nitrogen balance was essentially that employed by Brush et al., (1946) in the Nutrition Laboratory of the Foods and Nutrition Department at the Iowa State College. Certain modifications introduced by later workers in the laboratory were incorporated.

When an animal is fed a protein-free ration it first excretes nitrogen lavishly in the urine; then after a few days it becomes more conservative in its output. Finally, a more or less constant plane of urinary nitrogen excretion is attained. It was important in the present investigation to determine the interval in which the animal maintained itself in this latter more-or-less steady state in order to deleneate the time when the two balance tests could be conducted. Only by having the animal in this "steady state" could one be sure that any changes induced by experimental or dietary manipulation were "real".

Other workers in this laboratory had ascertained the urinary nitrogen eliminated at successive intervals in the test period using experimental animals maintained on the low nitrogen diet with ad libitum feeding. This work had indicated that a preliminary adjustment period of 14 days

permitted the animal fed a low nitrogen diet to arrive at the interval when nitrogen metabolism reached a definite plateau. They had adopted the following procedure for testing the utilization of a dietary source of nitrogen:

Nitrogen-low feeding period

Preliminary depletion period	14 days
Collection period	7 days

Nitrogen-feeding period or dietary modification

Adjustment period	4 days
Collection period	7 days

To ascertain whether the excretion of nitrogen remained the same under the conditions of the present experiment with forced feeding, these experiments were repeated. Evidence was obtained which indicated that an 18-day period of protein deprivation was required for the depletion of the body stores of protein and the maintenance of the excretion of nitrogen at a constant plane. the final plan, extending over 32 days, is shown below:

Nitrogen-low feeding period

Preliminary adjustment period	18 days
Collection period	4 days

Nitrogen-feeding period or other dietary modification

Adjustment period	4 days
Collection period	5 days

The timing of the various periods in the metabolism test, the nature of the diets fed and the feeding employed are shown schematically in Figure 1. In order to reduce the

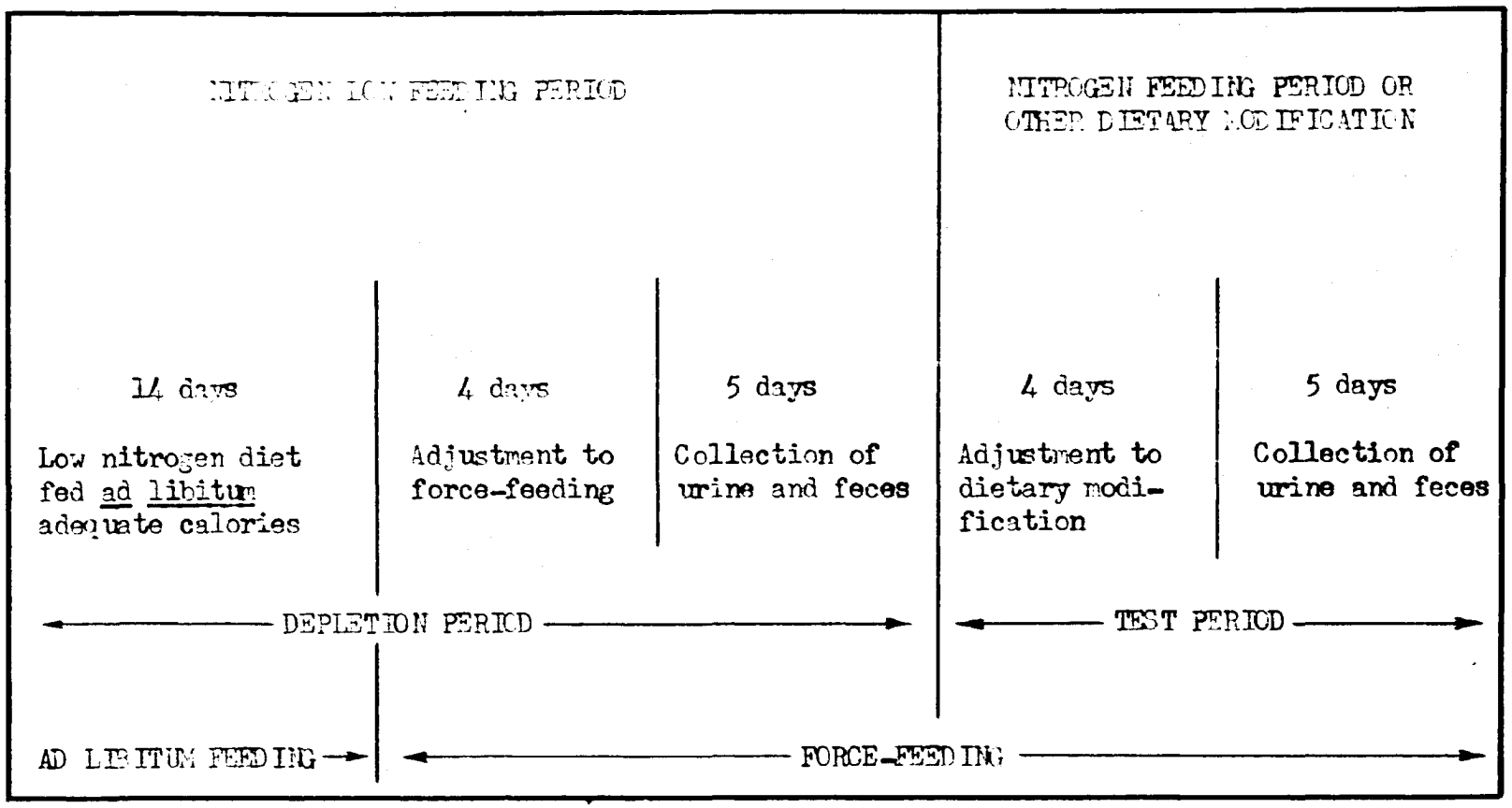

FIGURE 1. THE NITROGEN BALANCE TEST

time needed for the completion of the metabolism test the actual collection periods were shortened from seven days to five days.

In Figure 2 are shown data collected by the author which formed the base for this plan. It demonstrates the constancy of nitrogen metabolism in the interval covering the first and second collection periods when the animals were fed the low nitrogen diets containing either 20 per cent or no fat. No supplementation was offered during the entire 32 days of the balance test.

Adjustment to Force-feeding

Preliminary experiments showed that the animal adjusted to the force-feeding procedure more rapidly if only a fraction of the total daily intake were given at first, with a gradual increase to the daily allotment. On the evening preceding the initiation of force-feeding, food cups were removed from the cages, allowing a 10-hour interin which the stomach might be cleared of its contents. On the first day, one-half of the daily quota of food was given as a homogenate by the force-feeding method with the other being offered as solid food. This amount of liquid food was administered in two feedings of 6 ml. each. On the second day, two-thirds of the quota was fed as semi-liquid food and one-third as solid. The full food intake was offered on the third day as semi-liquid food.

Collection of Urine and Feces

On the evening before each collection period and at the end of each collection period, food was offered at 5 P. M. instead of the customary 8 P. M. feeding time, in order to allow sufficient time for digestion and to insure that collection would be made only of uring reflecting the metabolism of food received by the animals during the collection period. On the morning of each collection period, the animals were fed the food homogenates which had been mixed with small quantities of ferric oxide as a fecal marker. Each rat was placed in a wide-meshed metabolism cage which rested on a Pyrex glass plate lined with seven nitrogen-free filter papers. On these papers, which had been acid treated to prevent loss of ammonia, the feces and the major portion of the urine fell and dried. The animals were fed throughout the first 5-day collection period the same number of calories which they had received during the adjustment period, i. e., 56 caolries per day per 300 gm. rat.

On the morning of the twenty-third day, the first morning of the feeding of the dietary modification, the animals receiving one-half the high and low fat diets were divided into four groups and force-fed either (1) the same diets at the same caloric intake as in period I, or (2) the same diets at 25 per cent of this caloric intake. The remaining

half the total number of rats was divided into four groups and were placed on these experimental regimes described above; in this instance, however 44 mg. of methionine were added daily to the high and low fat diets offered at the two planes of energy intake. After a 4-day period for adjustment to these procedures, nitrogen balances as described earlier, were determined for the following 5-day period.

Urine and fecal collections were completed on the morning of the 32nd day. It was necessary, however, to collect feces until all which represented the nitrogen feeding period had been excreted, as shown by the appearance of the colored feces marking the first post-experimental day. The animals were then sacrificed, samples of blood were taken, and the organs removed for analysis.

The Treatment of Urine Samples

As discussed earlier, urines were collected on high quality filter papers containing only traces of nitrogen. These had been soaked overnight in a 10 per cent solution of glacial acetic acid in 95 per cent ethyl alcohol and allowed to dry in the air. Seven were placed on the pyrex plated under each metabolism cage, and one was removed each day during the collection period. On the final day, the last three papers were removed. Feces were brushed free of hair and dust daily and placed in 125 ml. Erlenmeyer flasks con-

taining 50 ml. of 20 per cent hydrochloric acid. The papers were scraped clean of hair and other particles and placed in wide-mouthed, one liter Erlenmeyer flasks containing 200 ml. of 20 per cent hydrochloric acid. All containers were covered with pyrex beakers of an appropriate size.

At the end of each collection period, each cage with its pyrex plate was quantitatively washed with warm distilled water applied under pressure, and the washings were transferred to the Erlenmeyer flask containing the papers corresponding to the particular cage. The acid extract from the filter papers was poured quantitatively through a Buchner funnell fitted into a two-liter suction flask. The papers were carefully transferred to the funnel and washed with lukewarm water. The Erlenmeyer flask was thoroughly rinsed and its contents added to the flask containing the other portion of urine. Contents of the suction flask were transferred quantitatively to a one-liter volumetric flask, and after cooling to room temperature, these flasks were made up to volume. Portions of these samples were transferred to 350 ml. pharmacy bottles, covered with a layer of toluene, and stored in the refrigerator until the time of analysis.

Treatment of Feces

In order to demarcate sharply the feces that represented the metabolic and digestive processes of any 5-day period

of collection, the nitrogen low diet was colored red with ferric oxide (0.1 gm. per 100 gm. of diet) on the first day of each period. The feces representing the food ingested on this day were, therefore, red in color. The first red feces excreted and all following feces were collected daily and brushed free of food and hair. On the morning of the beginning of the adjustment period, food colored red was fed again. The excretion of red feces marked the food eaten the day after the collection period terminated. Collections, therefore, continued until red feces appeared; these were added to the flasks representing the period.

The fecal material was placed in 125 ml. Erlenmeyer flasks containing 50 ml. of 20 per cent hydrochloric acid and covered with small pyrex beakers. When the collection was completed from each period, the suspension was digested in an autoclave at 15 lbs. pressure for 2 hours. The digest was rubbed through a fine sieve, transferred quantitatively to a 250 ml. volumetric flask, and made up to volume. After thorough mixing, the material was transferred to an 8 oz. pharmacy bottle and stored until the time of analysis.

To test whether the procedure described insured quantitative recovery of the nitrogen excreted by the animals, preliminary experiments had been designed which simulated

the experimental conditions. Aliquots of a standard solution of ammonium sulfate were sprinkled over a metabolism cage, set up as described in the collection procedure, over a period of 5 days. Comparable aliquots were placed directly in an Erlenmeyer flask containing 200 ml. of 2 per cent hydrochloric acid. Filter papers were removed daily, brushed free of particles, and placed in flasks. At the end of the five day period these were analyzed for nitrogen. Data confirming the accuracy of this procedure are shown in the appendix.

ANALYTICAL PROCEDURES

The methods used in the analysis of tissues and metabolic materials are described in the Appendix. Procedures for the analysis of the following are recorded therein:

A. Nitrogen Balance

- Urine
- Feces
- Food

B. Nitrogen Partition of Urine

- Total nitrogen
- Urea
- Ammonia
- Amino nitrogen
- Allantoin
- Creatinine

C. Chromatography of Urine Amino Acids

One dimensional chromatogram
Two dimensional chromatogram

D. Organ Analyses

Liver
Total nitrogen
Moisture
Fat
Riboflavin
Niacin

Adrenal weights

E. Blood Analyses

Urea
Amino nitrogen
Serum alkaline phosphatase

F. Carbohydrate Metabolism

Glucose tolerance test
Glycogen

G. Histological Analyses

Liver - hematoxylin and eosin stain
Liver fat - Scarlet red and Nile blue sulfate stain
Liver glycogen - Carmine stain

TABLE 9. NUMBER OF EXPERIMENTAL ANIMALS USED IN THE VARIOUS ANALYSES

Diet	Calories per day	Group A	Group B	Group C	Group D	
		Nitrogen balance, nitrogen partition, blood urea and amino nitrogen, organ analyses	Glucose tolerance and chromatography	Serum alkaline phosphatase and glycogen	Histology	Vitamins
Unsupplemented nitrogen-low diet						
High fat	56	6	6	3	9	6
Low fat	56	6	6	3	9	6
High fat	14	6	6	3	9	6
Low fat	14	6	6	3	9	6
Nitrogen-low diet plus methionine						
High fat	56	6	6	3	9	6
Low fat	56	6	6	3	9	6
High fat	14	6	6	3	9	6
Low fat	14	6	6	3	9	6
Control diets						
Control	*	6	6	3	20	6
Control	**	6	-	3	6	6
High fat***	56	6	-	-	6	6
Low fat***	56	6	-	-	6	6

*Stock diet ingested ad libitum, 16 gm./day
**Ingestion of stock diet restricted, 4 gm./day
***Fed the low nitrogen diet for 23 days

DISCUSSION OF RESULTS

Mean data only are reported in the main body of this dissertation. However, individual data relating to each analysis appear in the Appendix. If the reader is interested in the range of values over which any observation extends, inspection of these tables will give him this information. Any desired tables can be located from the listing of tables contained in the Appendix.

NITROGEN BALANCE AND MORTALITY OF RATS

As had been observed in earlier experiments reported in 1946 by Swanson and co-workers, nitrogen balances seem to indicate that fat and methionine have a common role in the regulation of the rate at which protein catabolism proceeds in a nitrogen-starved animal. Under the exacting conditions imposed by the present experiment with the energy value of the ration maintained constant by force-feeding, the early findings have been confirmed, for the most part, in a striking fashion.

Experimental results are presented in Table 10. Six rats are represented in each experimental group. In studying the data, it should be kept in mind that the nitrogen balances in Period I represent the more or less

TABLE 10. MEAN NITROGEN BALANCES OF RATS FED LOW NITROGEN DIETS OF VARYING FAT CONTENTS (6 RATS PER GROUP)

Diets fed in		Calories per day per 300 gm. rat		Nitrogen balances	
Pd. I	Pd. II	Pd. I	Pd. II	Pd. I	Pd. II
				mg./5 da.	mg./5 da.
High fat	High fat	56	56	-284	-288
Low fat	Low fat	56	56	-259	-290
High fat	High fat	56	14	-310	-514
Low fat	Low fat	56	14	-280	-1465
High fat	High fat Me*	56	56	-328	-215
Low fat	Low fat Me*	56	56	-251	-236
High fat	High fat Me*	56	14	-250	-528
Low fat	Low fat Me*	56	14	-261	-495

*Abbreviation for methionine

"steady" plane of nitrogen catabolism that is eventually reached when test animals are transferred from a good protein diet to one deficient in this nutrient. Also, it should be recalled that when dietary modifications were made, they were introduced in the adjustment period prior to Period II (See Figure 1). Any differences observed, therefore, between data representative of Periods I and II can be attributed to the dietary manipulation.

When either the high fat or the low fat basal diet was fed in quantities that met the energy requirements of the animals, the mean nitrogen balances were nearly constant in Periods I and II. Losses in body tissue were represented by negative balances of 284 mg. and 288 mg. in the two periods when the high fat diet was administered; of 259 mg. and 290 mg. when the low fat diet was given. The difference between the two latter figures is of the order of the standard deviation from the mean balance of some 300 nitrogen balances of rats previously fed this same nitrogen-free diet. It is not considered significant. Therefore, in the following discussion, the balances of the rats given the full caloric intakes of both diets will be considered as the base value for comparison. In fact, these values are nearly identical with the mean balance of groups in Period I, i.e., -278 mg. Therefore, further

interpretations of the data in Table 10 will be made on differences between group means in Period II.

When the calories provided were restricted to one fourth of the normal ingestion, the catabolism of nitrogen in rats given the high fat diet was increased 1.8 times, the nitrogen balances of these animals changing from -288 mg. to -514 mg. in the 5-day period. When the low fat diet was administered to animals with the restricted energy intake, however, the nitrogen balance fell from -290 mg. to -1465 mg. The omission of fat from the diet, therefore, approximately trebled the rate of catabolism of these animals that had become accustomed to living on a protein-free ration. Body tissue undoubtedly was called upon to serve as a source of energy in both instances, but why the exclusion of fat should result in so catastrophic a breakdown of tissue when both groups received the same number of calories, is difficult to comprehend.

The nitrogen-sparing action of methionine is clear-cut in these experiments. In the animals fed the high fat diet at full caloric intake, the feeding of 4 mg. of methionine nitrogen per day decreased the destruction of body tissue by 73 mg. over a period of five days, thus being reflected in a change from -288 mg. to -215 mg. in

the nitrogen balances. It is interesting that the addition of methionine to the low fat diet did not result in as marked a saving of body tissue as occurred when it supplemented the high fat diet. The body nitrogen spared in the two groups was 54 and 73 mg., respectively.

When the caloric intake was restricted, the dietary addition of the amino acid to the high fat diet did not influence the course of protein catabolism, indicated by negative balances of 528 mg. and 514 mg. in the supplemented and unsupplemented groups, respectively. In sharp contrast, methionine in the diet proved surprisingly effective when fat was not present in the basal ration, changing from a negative balance of 1465 mg. to one of 495 mg. This latter balance was nearly identical to that of the animals whose restricted diets contained fat and no methionine (-514 mg.), and similar to that of the normal stock animal whose daily food was restricted to one-fourth of its normal intake, i.e., -372 mg.

The analysis of the nitrogen lost from body tissue by the animals fed the restricted low fat diet and the same diet supplemented with methionine presents an interesting story. If the nitrogen representing that excreted by animals fed the low fat diet only is converted into its equivalent of body tissue 49 gm. of body tissue had been

catabolized. The calculation of this equivalent is based on the analyses of Brush et al. (1947). This group of animals actually lost an average of 43 gm. body weight in the 5-day period. On the other hand, when the nitrogen lost in the urine is calculated in terms of body tissue for the group fed methionine, only 16 gm. tissue should have been lost. It is indeed striking that these animals actually showed a loss in weight of only 15 gm. How was this phenomenal protection of body tissue brought about by a single amino acid, only one of the many which must be present for protein synthesis? Does it not indicate that methionine fills a vital need in the metabolic framework? The observation that it is able when present alone in a diet containing no other source of dietary protein would certainly suggest this explanation.

The sparing action of methionine on the course of protein catabolism in this particular group of rats is phenomenal, and it is most interesting to speculate why it exerts a greater influence under caloric restriction than it does when full calories are administered. Does it mean that in the presence of unusually large quantities of endogenous amino acids, it supplements the metabolic pool, permitting the resynthesis of either body tissue or functional metabolites? Could it be, in some way,

related to an enzyme system concerned with the synthesis of tissue protein? Perhaps its presence actually prevents the raiding of body tissue to obtain this essential metabolite. Possible explanations for the observations will be sought in later sections of this manuscript.

It is interesting that the data obtained in this experiment are very similar to those obtained in an earlier experiment in which the food intake was controlled on the basis of the ad libitum ingestion in the pre-period. These results of the two balance tests may be compared in Table 11. In Experiment 1, the levels of calorie intakes were 56 and 14 calories per day as based on analyzed values; in Experiment 2, approximately 47 and 12 calories per day as estimated from food tables.

The balance tests in Table 10 seem to indicate that the metabolic disturbance induced by administering a low fat diet is confined to the group whose calorie intake was restricted. The ration does not seem to have exerted a deleterious influence when full calories were offered. Nevertheless, a hint came in this experiment that the rats receiving no fat in their rations are not in as good nutritional state as those consuming fat. Of special interest is the high mortality of animals fed the low fat diet.

TABLE 11. FAT AND METHIONINE IN NITROGEN METABOLISM IN TWO DIFFERENT EXPERIMENTS

Diets fed in period II	Caloric intake per day	Nitrogen balances in Pd. II	
		Experiment I*	Experiment 2**
		mg./5 da.	mg./5 da.
High fat	full	-288	-231
Low fat	full	-290	-201
High fat	restricted	-514	-418
Low fat	restricted	-1465	-724
High fat Me***	full	-215	-185
Low fat Me	full	-236	-196
High fat Me	restricted	-528	-397
Low fat Me	restricted	-495	-436

*Present study
**Conducted in 1946; food intake controlled on basis of ad libitum consumption in Period I
***Abbreviation for methionine

Thirty-one per cent of the animals used in the balance experiment conducted during the spring of 1949 died 16 to 20 days after the feeding of the low fat diet was initiated (Table 12). In another series run in the summer of 1949, 48 per cent of the rats died. In a third experiment, conducted in the fall of that year, 50 per cent of the animals expired. No animals in groups simultaneously fed the high fat diet were lost in any of these experiments (Table 12).

It seemed necessary, therefore, to ascertain whether or not this mortality was a reflection of the absence of fat in the low nitrogen diet, or an artefect induced either by the force-feeding method or other factors. It seemed possible that the greater density of the low fat diet was responsible. It may have increased the bulk of food present at one time in the stomach and thereby the digestive load. Although preliminary tests had indicated that the rat can tolerate as much as 13 ml. of homogenate per feeding, the possibility existed that dividing the daily quota into three portions instead of two would decrease the mortality rate. An experiment was therefore set up in which the low fat diet was administered in three feedings per day instead of two. Also the bulk of the high fat diet was increased by the addition of ruffex, maintaining

TABLE 12. MORTALITY OF RATS FED THE LOW NITROGEN DIET

Test	Diet*	Time of year	Total no. of rats	Deaths	
				No.	Per cent
I	Low fat	Spring, 1949	48	15	31
	High fat		48	0	—
II	Low fat	Summer, 1949	27	13	48
	High fat		12	0	—
III	Low fat	Fall, 1949	12	6	50
	High fat		12	0	—
IV	Low fat	Winter, 1950	12	3	25
	Low fat**		12	2	17
	High fat		12	0	—
	High fat plus ruffex		12	0	—

*All diets supplied 56 calories per day per 300 gm. rat
**3 feedings per day

at the same time, the same caloric intake as that received when the low fat diet was administered, and providing a density which equalled that of the low fat diet. The results of this test are recorded in Table 12 (Test IV).

It is clearly evident again, that animals died only when the diet containing no fat was fed. Deaths were not eliminated by administering the food in three portions per day. Neither did the addition of ruffex to the high fat diet result in the death of animals fed this diet.

It seems fairly certain, therefore, that the omission of fat under the conditions of this experiment was reflected in a high mortality rate. Those rats that were able to survive the first 20 days of the experiment were also able to withstand the oncoming metabolism period. No rats were actually lost after the dietary adjustment was made.

That the low fat diet exerted a detrimental effect on rats fed full calories, again will be indicated by other data which show that the course of metabolism was altered seriously.

PARTITION OF NITROGEN IN URINE

Following early observations that the partition of nitrogenous constituents in the urine could be used as a measure of the metabolism of both "endogenous" and "exogenous" nitrogen (Folin, 1905), considerable interest has developed in this technique. The procedure already has been applied in part to the study of the nitrogen-sparing action of methionine on body protein by several groups of investigators (Hoover, Stewart, and Swanson, 1949; Allison, Anderson, and Seeley, 1947; and Leuthardt, Fahrlander, and Nielson, 1947).

Data relating to the partition of nitrogen in the urine excreted by the various experimental groups of rats studied in the present investigation have yielded interesting results. Findings are presented in Tables 13 and 14. In period I in which rats received the high and low fat diets in adequate quantities, the urinary excretion of the various nitrogenous constituents was essentially the same in the two groups, both in terms of absolute and relative quantities (Table 13). In period II (Table 14), however, when dietary modifications were introduced, certain striking alterations in metabolic pathways were apparent.

The quantity of allantoin nitrogen excreted, both on the total and relative basis, was slightly greater when

TABLE 13. PARTITION OF NITROGEN IN URINES OF RATS IN PERIOD I WHEN FED A LOW NITROGEN DIET OF VARYING FAT CONTENTS (MEAN VALUES: 6 RATS PER GROUP)

Diet in Pd. I	Cal. per day	Total nitrogen	Allantoin N		Creatinine N		Amino N		Urea N		Ammonia N		Sum of nitrogenous constituents
		mg.	mg.	%	mg.	%	mg.	%	mg.	%	mg.	%	mg.
Unsupplemented nitrogen-low diet in Pd. II* **													
High fat	56	195	70	35	23	11	13	4	114	50	6	3	227
Low fat	56	169	63	39	22	14	10	6	77	47	.8	.5	173
High fat	14	193	67	36	21	10	5	2	83	45	3	.3	182
Low fat	14	196	63	34	18	9	7	4	91	47	.7	.4	182
Nitrogen-low diet plus methionine in Pd. II* **													
High fat	56	195	64	33	17	9	9	5	92	47	.7	.5	183
Low fat	56	166	59	36	17	14	5	3	76	46	.8	.5	156
High fat	14	193	35	35	18	9	4	2	87	45	.7	.4	145
Low fat	14	175	64	37	25	14	15	9	70	48	.7	.4	178
Control diets													
Control	*	2050	64	3	24	1	11	.5	1927	94	21	1	2038
Control	**	1261	63	5	25	2	6	.4	1084	86	76	6	1225

*Stock diet ingested ad libitum, 16 gm./day

**Ingestion of stock diet restricted, 4 gm./day

***The entire lot of rats was divided into 8 groups on the basis of the dietary regime to which they were subjected in Period II. The data in this table relate to the nitrogenous excretion of each of these groups in period I.

TABLE 14. PARTITION OF NITROGEN IN URINES OF RATS FED A LOW NITROGEN DIET OF VARYING FAT CONTENTS (MEAN VALUES: 6 RATS PER GROUP)

Diet in Pd. II	Cal. per day	Total nitrogen	Allantoin N		Creatinine N			Amino N		Urea N		Ammonia N		Sum of nitrogenous constituents
		mg.	mg.	%	mg.	%		mg.	%	mg.	%	mg.	%	mg.
Unsupplemented nitrogen-low diet														
High fat	56	180	80	45	14	8	4	2.0	79	44	5	0.3		182
Low fat	56	180	61	35	18	10	11	6.0	85	47	2	1.0		177
High fat	14	459	71	15	14	3	9	2.0	330	72	42	9.0		466
Low fat	14	1389	81	6	28	2	4	0.3	875	63	349	25.0		1337
Nitrogen-low diet plus methionine														
High fat	56	188	64	34	17	9	11	6.0	68	36	20	10.0		180
Low fat	56	164	62	37	4	2	8	5.0	61	37	16	10.0		151
High fat	14	450	72	16	23	5	9	2.0	207	46	135	30.0		446
Low fat	14	450	76	18	27	6	14	3.0	234	52	97	22.0		448
Control diets														
Control	*	2050	64	3	21	1	10	0.5	1927	94	21	1.0		2043
Control	**	1261	63	5	25	2	6	0.4	1084	86	76	6.0		1254

*Stock diet ingested ad libitum, 16 gm./day
**Ingestion of stock diet restricted, 4 gm./day

rats were fed the high fat diet at full caloric intake than when the low fat ration was offered. The low fat-fed rats resembled the normal stock animals in respect to the quantity of this constituent excreted, i.e., 61 mg. vs. 64 mg. per 5-day collection period.

Restriction of the food given the stock rats was not reflected in a change in the excretion of allantoin. However, in the experimental groups, a high excretion of allantoin was characteristic when dietary calories were restricted, 71 mg. being excreted by rats fed the high fat diet and 81 mg. by rats receiving the low fat diet.

When methionine was added as a supplement to the high fat diet fed at full caloric intake, the quantity of allantoin excreted was reduced to that excreted by the group fed the low fat diet and by the stock control animals. The action of methionine was less marked when dietary calories were reduced.

The excretion of creatinine was essentially the same in period II for groups maintained on adequate amounts of the high and low fat diets. When a caloric restriction was imposed, creatinine excretion seemed not to be affected in animals which received dietary fat. However, if fat was absent from the diet, the excretion of this compound was doubled. It appears as if a decreased caloric intake

is associated with an increased output of creatinine when the low fat diet was fed. This finding suggests that urinary creatinine is arising from the breakdown of body tissue because these rats were excreting 1389 mg. of nitrogen in this balance test.

If the quantity of urinary creatinine is taken as an index of breakdown of body tissue, then it would seem that methionine exerted a beneficial effect when added to the low fat diet at full caloric value; rats fed this diet excreted only 4 mg. creatinine in the 5-day test period as compared to 18 mg. excreted by rats fed the high fat ration supplemented with methionine. It should be recalled that methionine feeding improved the nitrogen balance somewhat in this group.

Why no reduction in creatinine excretion occurred when the same methionine supplemented diet was restricted in caloric value is worthy of speculation. Despite the fact that urinary excretion of nitrogen has dropped from 1389 mg. to 450 mg. as the result of the feeding of methionine, creatinine remains at the same concentration in the urines of the unsupplemented and supplemented groups.

The data relating to the excretion of amino nitrogen in the urine show interesting trends. Approximately three times the quantity of this nitrogenous constituent was

excreted by the animals receiving the low fat diet at the full caloric intake as was excreted by animals fed the high fat ration. This same trend was reflected in the proportion of amino nitrogen to total nitrogen. Methionine-feeding of the low-fat rats reduced the amino nitrogen excreted by approximately one-third. The feeding of this amino acid to animals force-fed the high fat diet however, increased strikingly the amount of amino nitrogen in the urine.

When the caloric restriction was imposed, the urinary nitrogen supplied by amino groups was unaffected by methionine feeding to animals receiving the high fat diet, 9 mg. being excreted by both the unsupplemented and supplemented groups. However, more than three times the quantity of this constituent appeared in the urine when rats were fed the low fat ration supplemented with methionine, as compared to that excreted by rats fed the low fat diet with no supplement. The significance of this observation is not apparent.

An investigation of the individual amino acid constituents of the amino nitrogen fraction was pursued by means of the chromatographic method of analysis. With the use of one dimensional chromatograms, the following amino acids were tested in the urine hydrolysate of animals fed the high and low fat diets: phenylalanine, tyrosine,

glutamic acid (and glutamine), histidine, norleucine, arginine, methionine, serine, hydroxyproline, leucine, threonine, lysine, glycine, cysteine, norvaline, aspartic acid, and cystine.

Tyrosine, arginine, cysteine, cystine, glycine, lysine, aspartic acid, isoleucine and norleucine were excreted by animals fed the high and low fat diets. Greater quantities of these amino acids were found, however, in the urines of rats on the low fat diet at full level of caloric intake than on either the high fat diet fed at this level of caloric intake or the restricted calorie groups. Quantities of amino nitrogen of lesser magnitude than those found in the urines of animals fed the low fat diet were observed when this diet was supplemented with methionine. The urines of the rats given unsupplemented low fat diet contained in addition to the amino acids listed above arginine, cysteine, lysine, aspartic acid, glycine, and glutamic acid and glutamine. The appearance of these amino acids in the urine was questionable when the other diets were fed.

The most striking observation resulting from the chromatographic analyses was one which had totally been unexpected. No methionine was found in any of the urines of the experimental animals. This observation was confirmed

in two dimensional chromatograms where the pure amino acid was allowed to migrate simultaneously and in the same position with the samples. The complete absence of methionine from the urinary nitrogen gives added support to the hypothesis that, in animals fed a low nitrogen diet there is a conservation of this essential metabolite. The finding that increased quantities of specific amino acids were excreted when methionine was not present supports the idea that the body raids its tissues to secure this substance for the support of vital tissue reactions. Also it may be possible that methionine functions at a key position in the synthesis of amino acids into body protein.

Perhaps, even more impressive than the findings described up to this point are those relating to the excretion of urea and ammonia in the urines of the experimental animals. In this study, the normal rat fed an adequate diet excreted 94 per cent of its total nitrogen as urea nitrogen. When the protein-free diets of high and low fat contents were fed, the relative quantity of urea fell to approximately 45 per cent of the total nitrogen excreted in the second period of the balance test. Experiments with ad libitum and force-feeding feeding of the low nitrogen diet have indicated the caloric protection provided by force-feeding results in a somewhat reduced per cent of urea

nitrogen*.

The excretion of urea nitrogen when rats were fed adequate amounts of the high and low fat diets was 79 and 85 mg. respectively in the 5-day balance test. When calories were restricted, greater quantities of urea were excreted by both groups of animals. There was a tremendous increment in total nitrogen excreted by rats maintained on the low fat diet, i.e., 1389 mg. was accounted for in part by the urea fraction of 875 mg.

The feeding of methionine decreased the quantity of urea excreted when both protein-free diets were fed. This finding is in accord with the observation of others (Hoover and co-workers, 1949, and Allison et al., 1947).

At the lower level of caloric intake, the body sparing action of methionine was more pronounced. There was a marked decrease, i.e., 330 mg. vs. 207 mg., in the quantity of urea excreted by the high fat animals fed the supplemented and the unsupplemented rations, respectively. When the amino acid was added to the calorie-poor low fat diet, the fall in the excretion of urea nitrogen was still more impressive, dropping from 875 mg. to 234 mg. in the 5-day period. This decrease in urea gives further proof of the

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*Hoover, C. A., Unpublished data, files, Nutrition Laboratory, Iowa State College.

very marked body sparing action of methionine under the present experimental conditions, and reflects the reduced quantity of amino acids to be deaminated.

The effect of methionine on the elimination of urea is even more marked than that of fat in the diet. In this regard it is interesting to note that when calories were restricted, the presence of this amino acid in the low fat ration fed to rats induced the same effect on urinary nitrogen as that induced either when fat only was present, or when fat and methionine both were present, i.e., 450 mg., 459 mg., and 450 mg. respectively. The presence of fat in the ration was associated with a decrease of 545 mg. of urea nitrogen when calories were restricted as compared to one of 641 mg. when methionine supplemented the low fat diet.

Rats fed the two basal experimental diets excreted minimal amounts of ammonia in the urine, i.e., high fat group, 5 mg. per 5 days; low fat group, 2 mg. per 5 days. The relative proportion of ammonia in the total urinary nitrogen of the rats given no fat was the same as that in the stock rats (1.0 per cent); that in the urines of the fat-fed rats was somewhat lower (0.3 per cent).

There was some increase in the excretion of ammonia in the urine when methionine was added to the low and high

fat diets at full caloric intake.

When only one-fourth of the normal number of calories were fed, phenomenal alterations in the size of the ammonia fraction occurred. When the high fat diet was offered, the ammonia fraction accounted for 9 per cent of the total nitrogen in period II, as compared to 0.3 per cent in period I; when the low fat diet was given, the respective proportions were 25.0 per cent and 1.0 per cent. That this shift to ammonia excretion was related to the caloric restriction imposed may be shown by a comparison of data obtained in periods I and II of the balance test. Reducing the caloric value of the low fat diet changed the excretion from 0.7 mg. to 349 mg. of urinary ammonia in animals fed the unsupplemented low fat diet (Tables 13 and 14).

Methionine again seems to exert an effect when restricted calories were fed. It reduced the excretion of ammonia from 349 mg. to 97 mg. in the low fat group. The reverse effect seemed to occur in animals fed the high fat diet at this low level of caloric intake, the ammonia fraction increasing from 42 mg. to 135 mg., in the unsupplemented and supplemented groups respectively.

It was not surprising to find that the rats receiving restricted calories excreted more urea than the full-fed animals. Use of body tissue for energy called for

deamination of an increased quantity of endogenous amino acids. However, that elimination of fat from the diet should speed up catabolic processes was surprising. Perhaps the load was greater than the urea-synthesizing enzyme systems could take care of, and it was necessary to establish a new pathway for the elimination of the metabolic residues in the blood. As a result, a large proportion appeared as urinary ammonia. The presence of glutamine, glutamic acid and aspartic acids in the urines of these rats as shown by the qualitative chromatogram is suggestive also.

The data regarding the influence of methionine can not be interpreted with the information now available.

The ratios of ammonia to urea in Table 15 are interesting in this connection also, and may be useful in later studies.

CONSTITUENTS OF BLOOD

Urea and Amino Nitrogen

When adequate calories are fed the respective concentrations of amino nitrogen and urea were determined in the bloods of the various experimental groups. The data are presented in Table 16.

Stock animals in the post absorptive state in this

TABLE 15. PROPORTIONS OF AMMONIA NITROGEN TO UREA NITROGEN IN THE URINES OF RATS FED A LOW NITROGEN DIET

Diet	Cal./day/ 300 gm. rat	Ratio of ammonia nitrogen to urea nitrogen	
		Period I	Period II
Unsupplemented nitrogen-low diet			
		per cent	per cent
High fat	56	5	5
Low fat	56	1	3
High fat	14	4	13
Low fat	14	1	40
Nitrogen-low diet plus methionine			
High fat	56	8	30
Low fat	56	1	30
High fat	14	8	50
Low fat	14	1	41
Control diets			
Control	*	--	1
Control	**	--	7

*Stock diet ingested ad libitum, 16 gm./day
**Ingestion of stock diet restricted, 4 gm./day

TABLE 16. MEAN CONCENTRATIONS OF AMINO NITROGEN AND UREA NITROGEN IN BLOOD OF RATS FORCE-FED LOW NITROGEN DIETS OF VARYING FAT CONTENT (6 RATS PER GROUP)

Diet in period II	Cal./day/300 gm. rat in period II	Amino nitrogen	Urea nitrogen
		mg./100 ml.	mg./100 ml.
Stock control	**	17	15
High fat	56	26	6
Low fat	56	47	2
High fat Me*	56	20	5
Low fat Me	56	31	3
Stock control	***	14	17
High fat	14	34	6
Low fat	14	18	31
High fat Me	14	27	8
Low fat	14	18	16

*Abbreviation for methionine
**Stock diet ingested ad libitum, 16 gm./day
***Ingestion of stock diet restricted, 4 gm./day

experiment maintained a blood level of amino nitrogen of 17 gm. per 100 ml. of blood. It may be recalled that the animals had been fed the high and low fat diets for a period of 23 days before the dietary modification was introduced. At the end of this interval values characteristic of the amino nitrogen in the blood of these groups were 16 and 11 mg. per 100 ml. respectively.

Although the concentration of amino nitrogen remained normal (stock controls, 17 mg. per cent) during the period of protein depletion when the high fat diet was fed, further maintenance of animals on this ration for an additional period of 9 days was reflected in an elevation of the quantity of this constituent, i.e., to 26 mg. per 100 ml. of blood. The increment when the low fat was fed in adequate quantities was much higher than when fat was present, i.e., 26 mg. per cent vs. 47 mg. per cent. This latter value was also approximately four times greater than that at the end of 23 days. In the preceding section, it was noted that many rats reared on the low-fat ration died. Possibly, in those rats, concentrations of the constituent may have reached values early in experimental history that were not concomitant with life. The animals surviving may have been those that were able to tolerate the low-fat diet more successfully and thereby maintained a lower concentration

of amino nitrogen for a longer period than did the rats that succumbed. This is merely interesting speculation.

The feeding of methionine reduced the concentration of amino nitrogen by one-third in the blood of the rats fed the full calorie-low fat diet. It also effected some decrease in the quantity of amino acids in the blood of rats receiving fat and full calories; the concentration was still well below that in the blood of rats given no fat. Indeed, it approaches the normal figure. Here may be a condition that shows why supplementary dietary methionine does not reduce the negative nitrogen balance in the low-fat rats to the same extent that it does in the high-fat rats.

It may be interesting to examine the urea figures for the same group of rats at this point.

The concentration of urea nitrogen in the blood of the normal controls was 15 mg. per cent. This value was reduced markedly by feeding the low-nitrogen diets for a period of 23 days, being only 4 mg. per cent and 8 mg. per cent respectively in the blood of rats fed the high and low fat diets at the end of this time.

Maintenance on the high fat ration for an additional nine days did not materially change the concentration of urea in the blood of the animals receiving fat. It did, however, result in a lowering of blood urea in the rats

given no fat. Only 2 mg. per cent was present.

Values of urea below normal are observed in hepatic insufficienty whereas amino acid nitrogen may be high. This is the picture in the experimental groups just described. The observations suggest an impairment of liver function resulting in a decline in the synthesis of urea. That the integrity of the hepatic tissue reflects the quality of the diets fed will be presented in a later section. If enzymes for the elaboration of urea have been lost, then it would seem that methionine does not exert its protective influence on nitrogen metabolism by functioning in these enzyme systems, because the concentration of urea in the blood did not even approach normal when methionine supplemented either the high or low fat diet providing the full caloric requirement.

When Inadequate Calories Were Fed

The concentration of amino nitrogen in the blood was not lowered appreciably when the quantity of stock diet fed was one-fourth the *ad libitum* consumption. The constituent in the blood of the rats given fat increased, however, when the calories were restricted to one-fourth the adequate intake. There was some decrease in the value that was associated with a slight increment in urea nitrogen when

methionine was added to the ration.

The data might suggest that the lowering of the caloric intakes of the rats fed no fat resulted in a metabolic improvement. Amino nitrogen changed from 47 to 18 mg. per cent; urea from 2 to 31 mg. per cent. The author questions the validity of this assumption, however, on the basis that the terrific catabolic load imposed by removing fat from the ration. The quantity of urinary nitrogen excreted changed from 459 to 1389 mg. as a result of the omission. Of this, 879 mg. were urea nitrogen. It is possible, therefore, that the quantity of urea nitrogen in the blood represents incomplete clearance. The decreased quantity of amino nitrogen from that in the blood of the rat receiving no fat and high calories may be due to the fact that a metabolic mechanism had been established for the excretion of a large proportion of amino acid nitrogen as ammonia.

Methionine added to the high fat diet did not materially change the picture from that when the ration was offered at full caloric value.

The feeding of methionine seems to have a profound effect on the protein catabolism of animals fed the fat-free diet and restricted in caloric intake. The effect of this amino acid was evident in a reduction of 50 per cent in the quantity of urea nitrogen in the blood, as compared to that

present in the blood of animals fed the unsupplemented low fat ration, the concentration in this case, 16 mg. per 100 ml. being the same as that found in the blood of the normal rat. The concentration of amino nitrogen also was normal. This time, the author believes a truly beneficial effect of supplementary methionine feeding is reflected in the blood because catabolism has been reduced, 450 mg. of nitrogen appearing in the urine against 1389 mg. when the unsupplemented low calorie diet was offered. The decreased concentration of urea nitrogen in the blood of these animals was paralleled by striking decreases in the excretion of urea and in the excretion of ammonia, reductions of 641 mg. and 252 mg. respectively.

These data are highly suggestive then, that in rats the feeding of as little as 4 mg. of methionine nitrogen as a supplement to a low protein diet prevents the breakdown of body tissue. Conversely this may be stated by saying that it supports the hypothesis that the body raids its tissues for an essential metabolite, i.e., methionine, to perform functions which are vital to life, throwing away in the process other amino acids liberated in the disintegration of the protein molecule.

Serum Alkaline Phosphatase

The concentration of alkaline phosphatase in the serum of rats has been reported to be related to the levels of fat and protein in the diet (Hough and Freeman, 1942; Hough, Monohan, Li, and Freeman, 1943). It was apparent from the work of these investigators that fat metabolism was related to the serum phosphatase activity and that the phosphatase-decreasing action of proteins was associated with the methionine content of the diet. Cantor (1949) has shown a direct correlation between the activity of serum alkaline phosphatase and the amount of fat ingested. When calories were restricted in the diet of adult male rats, the fall in serum alkaline phosphatase was shown to be proportional to the amount of fat consumed rather than to the lowered caloric intake. Tuba, Cantor, and Richards (1949) have presented evidence to show that the increased phosphatase values when diets low in protein are fed to rats represent a response to interference in the mobilization of fat, and that the addition of labile methyl groups either in the form of methionine or protein favorably affects the concentration of this enzyme.

The concentrations of serum alkaline phosphatase found in the present investigation are summarized in Table 17.

TABLE 17. MEAN CONCENTRATIONS OF SERUM ALKALINE PHOSPHATASE IN THE BLOOD OF RATS FED A LOW PROTEIN DIET (3 RATS PER GROUP)

Diet in period II	Cal./day/300 gm. rat in period II	Units of serum alkaline phosphatase
Unsupplemented nitrogen-low diet		
High fat	56	11
Low fat	56	19
High fat	14	16
Low fat	14	19
Nitrogen-low diet plus methionine		
High fat	56	8
Low fat	56	21
High fat	14	15
Low fat	14	27
Control diets		
Control	*	20
Control	**	31

*Stock diet ingested ad libitum, 16 gm./day
**Ingestion of stock diet restricted, 4 gm./day

The concentration of this constituent in the blood of animals fed the high fat diet was lower than that in the blood of rats fed the low fat diet in all instances, not only when full calories were administered, i.e., 11 vs. 16 units, but also when a caloric restriction was imposed, i.e., 16 vs. 19 units. Caloric restriction did not seem to affect the serum alkaline phosphatase concentrations in the blood of animals fed the low fat diet, the values being the same in both instances.

When methionine was added as a supplement to the high fat ration, the concentration of serum alkaline phosphatase was lowered when full calories were offered. This amino acid, however, did not affect the alkaline phosphatase values when a caloric restriction was imposed; the values were 16 and 15 units for the unsupplemented and the methionine-supplemented high fat rations, respectively. On the other hand, the addition of methionine to the diets of animals fed the low fat diets maintained the concentration of this enzyme when full calories were administered and increased it when a caloric restriction was imposed.

Two definite statements seem possible, however, from the data. In comparing the concentrations of alkaline phosphatase in the blood of the experimental animals to that in the blood of the control groups, (1) the feeding of a low

protein diet high in fat lowered the concentration of alkaline phosphatase in the blood of rats and (2) with the restriction of caloric intake increased the concentration of this enzyme in the blood of animals maintained on the stock ration and on the low protein rations containing high and low percentages of fat.

The possible reason for divergence of these results from those reported in the literature may lie in the method of feeding. The caloric intake was maintained at 56 calories per day throughout the experimental period by force-feeding. The diets in all instances were protein-free, whereas in the experiment reported by Tuba et al. (1949) a ration containing 9.1 per cent casein was fed.

CARBOHYDRATE METABOLISM

Glucose Tolerance Test

Reports in the literature indicate that a fundamental change in the metabolism of peripheral tissues occurs when a ration high in fat is fed (Samuels, 1946), as shown by a decrease in the rate of fall of blood sugar after intravenous injection of glucose. These experiments suggested that studies of the glucose tolerances of the various groups of rats employed in the present experiment would be of interest.

In Figure 3 are shown the curves representing the average utilization by nine experimental groups of 0.35 gm. per 100 gm. body weight (8.75 per cent solution of glucose) force-fed after 10 hours of starvation. The glucose tolerances of the normal stock control group are depicted by the heavy line in each figure.

Glucose Tolerances in Rats Fed the Unsupplemented Diets

The mean concentration of glucose in the blood of the fasting normal rat was 90 mg. per cent; that of the rats fed the synthetic ration of high fat content at full caloric intake, 93 mg. per cent. The fasting blood sugar levels in the other three experimental groups ranged from 130 mg. per cent to 150 mg. per cent. The peak of the curve in all five groups was reached at the 30 minute interval.

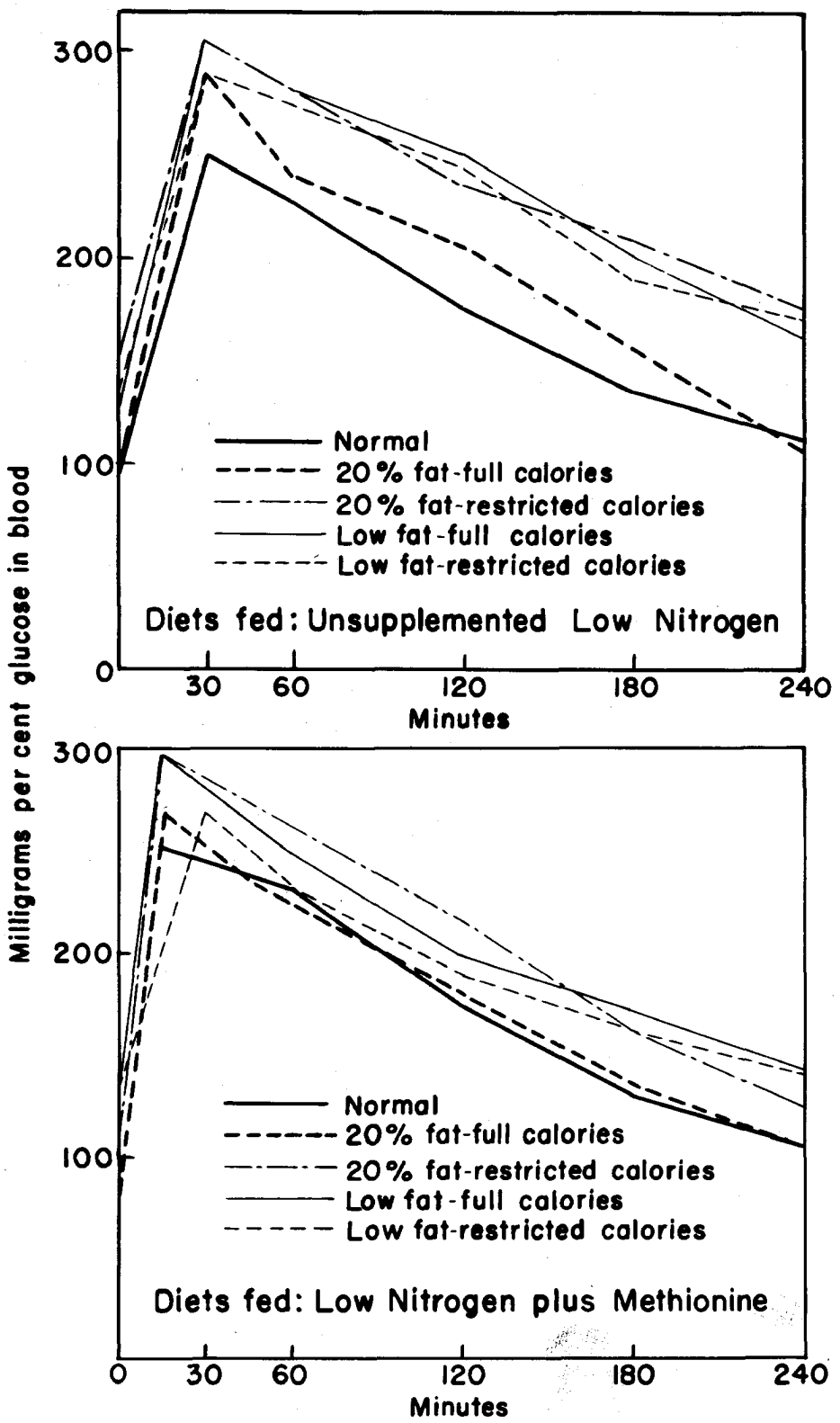

FIGURE 3. GLUCOSE TOLERANCE CURVES OF RATS FED VARIOUS MODIFICATIONS OF A LOW NITROGEN DIET

Thirty minutes after the administration of the test dose, in the animals given the fat diet in sufficient quantities to meet their energy requirement, the concentration of glucose in the blood was 40 mg. per cent higher than that of the blood of the normal control animal deriving its calories largely from carbohydrate. The concentration of glucose in the blood of these rats returned to the fasting level, however, after a 4 hour interval.

The omission of fat from the ration fed to animals in this experiment resulted in a marked distortion of the ability of these animals to utilize glucose. The concentration of sugar in the blood of these animals, when in the fasting state, was higher than that of the animals fed fat, and the peak reached at the half-hour interval after the administration was higher. Even after a four-hour interval, the curve of the animals maintained on the low fat ration at full calories had not returned to normal.

Caloric restriction also seemed to influence adversely the utilization of glucose. Concentrations of the nutrient in the bloods of the animals in both groups were somewhat higher at the early intervals of the test than were those of the rats fed the high calorie-low fat diet, but the general shape of the curves were the same. At the end of the four-hour interval, the concentration of blood glucose was not near normal, being 155 mg. per cent as against 110 mg. per dent in the normal control.

Methionine influenced the glucose tolerance curve of all groups of rats favorably. The change induced in the glucose tolerance of rats that had received a full caloric intake of the high fat diet was especially marked. Their glucose tolerance curve could nearly be imposed on that of the normal rats. The amino acid also substantially improved the distorted curve shown by animals fed the ration low in fat, but high in calories.

The beneficial effect of supplementary methionine when offered to rats restricted in caloric intakes was also notable. The curves showing the ability of animals previously fed the high fat and low fat rations at one-fourth of the caloric intake were very much closer to that of the stock control animal than was the case when these diets were unsupplemented. The dietary addition, however, possibly was most beneficial to the animals receiving no fat in the ration.

Glycogen

The relation between the amount of glycogen deposited in the liver and the ability of the animal to tolerate a test solution of glucose have been utilized in this study as a measure of the extent to which carbohydrate mechanisms have been affected by the various dietary regimes. Samuels Reinecke, and Ball (1942), have reported that the rate of disappearance from the liver of glycogen in the fasting animals is much less if the animal is fed a diet of high fat content

than if it is maintained on a high carbohydrate regime. Similiar observations have been reported by McKay, Carne, Wick, and Visscher (1941) and Guest (1941).

After a fasting period of 10 hours, the concentrations of glycogen in the livers of the experimental and control groups were determined. These data are shown in Table 18.

Greater amounts of glycogen were found in the livers of animals maintained on the high and low fat diets at full caloric intake than in the normal animal. It is interesting in this connection that the fasting blood glucose values discussed in the preceding section were also much higher than those of the normal animals.

When a caloric restriction was imposed, the concentration of glycogen in the livers of the normal animals increased greatly, being 0.3 per cent and 1.9 per cent for the normal animal at full calories, and the normal restricted in calories, respectively.

When the caloric intake of the animals fed the high fat ration was restricted, a similar increase in liver glycogen was observed. However, when fat was omitted from the ration, of rats in this experiment, the concentration of liver glycogen fell to 0.7 per cent, as compared to 2.5 per cent glycogen observed in the animals fed the adequate caloric intake.

The significance of this latter observation is not clear. Animals in this group had very little or no fat in the fat depots of the body. It seems reasonable that with a caloric restriction, and omission of fat from the ration, that available stores of glycogen were rapidly being depleted and that

TABLE 18. CONCENTRATIONS OF GLYCOGEN IN THE LIVERS OF RATS FED A LOW NITROGEN DIET (3 RATS PER GROUP)

Diet in period II	Calories per day per 300 gm. rat	Glycogen in the liver
Unsupplemented nitrogen-low diet		
		per cent
High fat	56	1.8
Low fat	56	2.5
High fat	14	2.2
Low fat	14	0.7
Nitrogen-low diet plus methionine		
High fat	56	1.7
Low fat	56	2.4
High fat	14	1.8
Low fat	14	2.5
Control diets		
Control	*	0.3
Control	**	1.9

*Stock diet ingested ad libitum, 16 gm./day
**Ingestion of stock diet restricted, 4 gm./day

this may have reflected the mobilization and use of the last reserves of muscle glycogen. Animals in this group were cold to touch, and, much less active than comparable groups restricted in calories.

The concentrations of glycogen in the livers of animals which had been fed the methionine-supplemented rations, however, were essentially the same as those of the unsupplemented group, (Table 18). When a caloric restriction was imposed, however, the glycogen present in the livers of animals fed the high fat ration decreased, changing from 2.2 per cent to 1.8 per cent respectively, for the unsupplemented and supplemented rations. It is very interesting, that the addition of this amino acid to the low fat diet caused a return to the concentration of glycogen characteristic of rats fed the low fat diet and adequate calories. A similar return to normal had been observed in the glucose tolerances of these animals when methionine was added as a supplement to the restricted calorie diets.

BODY WEIGHTS

At the end of the experiment (See Table 19), the weights of rats maintained on the high and low fat diets were essentially the same, as illustrated by mean values of 248 and 249 gm. respectively. When the caloric intakes of these two series of animals were restricted, however, both groups of animals, on the average, lost 24 gm. of weight.

TABLE 19. MEAN WEIGHTS OF LIVERS OF RATS FED A LOW NITROGEN DIET AND THEIR RESPECTIVE CONCENTRATIONS OF MOISTURE AND FAT (6 RATS PER GROUP)

Diet	Cal./day/ 300 gm. rat	Body weight	Weight of liver			Moisture		Fat		
			Fresh	Dry	Dry-fat-free	Total	Per cent	Total	On wet basis	On dry basis
		gm.	gm.	gm.	gm.	gm.	%	gm.	%	%
Unsupplemented nitrogen-low diet										
High fat	56	248	8.56	2.74	1.64	5.82	68.0	1.10	13.1	40.3
Low fat	56	246	7.34	2.25	1.47	5.10	69.6	0.78	10.6	34.3
High fat	14	223	5.60	1.61	1.30	3.99	71.3	0.30	5.4	19.0
Low fat	14	223	4.87	1.28	1.12	3.42	72.8	0.16	3.3	12.2
Nitrogen-low diet plus methionine										
High fat	56	247	8.36	3.10	1.80	5.29	63.3	1.24	14.8	40.6
Low fat	56	239	9.32	3.27	1.43	6.06	65.0	1.83	21.2	55.4
High fat	14	222	3.30	1.00	0.85	2.32	71.1	0.13	3.8	13.2
Low fat	14	231	5.24	1.57	1.30	3.67	70.5	0.27	5.2	21.0
Control diets										
Normal	*	300	8.87	2.84	2.49	6.03	68.3	0.36	4.0	12.6
Normal	**	285	5.77	1.82	1.49	3.95	68.4	0.33	5.8	18.3
High fat	56***	264	7.47	2.24	1.62	5.23	70.0	0.62	8.3	27.7
Low fat	56***	276	8.50	2.51	1.72	5.99	70.5	0.78	9.1	30.8

*Stock diet ingested ad libitum, 16 gm./day
**Ingestion of stock diet restricted, 4 gm./day
***Low nitrogen diet for 23 days

Supplementation of the high fat diet with methionine seemed ineffectual in reducing the loss in weight when calories were restricted, and did not affect body weight of animals maintained on full calories.

When the low fat diet was supplemented with methionine at the lower level of energy intake, the rats weighed 8 gm. more, on the average, than they did when fed the unsupplemented diet. Also their weight approached that of animals fed 56 calories of the same diet. Within this low fat group fed restricted calories, one animal actually gaimd weight when methionine was added to its diet. It is this group whose nitrogen balance was so materially improved by the addition of the amino acid to the diet.

ORGAN ANALYSES

Moisture and Fat in Liver

In studies of animals maintained on protein-free rations for 7, 14, and 21 days, Seifter, Harkness, Rubin, and Muntwyler (1948) observed a dimunition of liver protein accompanied by a steady fall in the water content of the liver and a steady rise in liver fat. They attribute the reduction of the nitrogen content of the fat-free solid fraction of the liver to an increase in liver glycogen.

In the present experiment, the concentration of moisture was the same in both the normal animal fed adequate

quantities of the stock colony diet, and the experimental group fed the high fat rations, i.e., 68 per cent. It was somewhat higher (70 per cent) in the low-fat group. When methionine was added to the high and low fat rations, however, a decrease in the moisture content of the livers was noted. These values were 63 per cent for the animals fed the high fat ration at an adequate caloric level and 65 per cent for groups fed the low fat ration. In all experimental groups, a restriction of the caloric intake of the animals resulted in an increase in the moisture content of the livers. This did not occur in the series of rats receiving only one-fourth of their normal quota of stock diet. These values were essentially the same for both the supplemented groups and the unsupplemented groups (See Table 19).

It is generally accepted that maintenance on a low protein ration results in increased fat deposition in the liver. In the present experiment, greater quantities of hepatic fat were found in the groups fed adequate calories of the two low nitrogen rations of varying fat content than were observed in the normal animal. These values, on the wet basis, were 13, 11, and 4 per cent, respectively; on the dry basis, 40, 34, and 13 per cent. The addition of methionine to the diet of the animals fed full calories of the high fat diet did not result in an increase in the quantity of fat present in the liver. However, its addition to

the similar low fat ration caused the hepatic fat to rise to 55 per cent on the dry basis. Reisen, Schweigert, and Elvehjem (1946) have presented evidence to indicate that the increase in liver fat which accompanies protein restriction in the diet is not due to a decreased intake of methionine per se. When they incorporated methionine into the low protein diet at a level equivalent to the methionine content of a 20 per cent casein ration, a lowering of the concentration of liver fat was not observed.

When the caloric intake of the ration was restricted, appreciably smaller quantities of fat were found in the livers of the high and low fat groups, as compared to the concentrations of hepatic fat in animals fed full calories. These values were 40 per cent on the dry basis in the high fat group and 34 vs. 12 per cent in the low fat group. The addition of methionine to the high fat diet seemed beneficial. Again, however, its presence increased the hepatic fat from 12 to 21 per cent (dry basis) in the low fat group.

Weights of Liver

Analyses of the water and fat contents of the livers of the animals in the various test groups have made possible the description of the liver on the dry fat-free basis. The material remaining represents for the most part nitrogenous tissue and glycogen. The data in Table 19 showthat the quantity of hepatic tissue diminished appreciably

TABLE 20. CONCENTRATIONS OF NITROGEN IN THE LIVERS OF RATS FED A LOW PROTEIN DIET

Diet	Calories per day	Nitrogen in the liver***				
		Total	Wet basis	Dry basis	Fat-free dry basis	Glycogen-free, fat-free, dry basis****
		mg.	%	%	%	%
Unsupplemented nitrogen-low diets						
High fat	56	218	2.56	7.98	13.54	14.70
Low fat	56	181	2.46	8.22	12.22	14.11
High fat	14	202	3.61	12.60	15.54	18.00
Low fat	14	186	3.66	12.40	15.00	17.04
Nitrogen-low diets plus methionine						
High fat	56	182	2.18	5.92	10.00	10.91
Low fat	56	224	2.40	6.91	13.41	18.62
High fat	14	126	3.82	13.26	15.36	15.94
Low fat	14	127	3.85	14.11	15.67	17.41
Control diets						
Control	*	318	3.57	11.32	12.93	12.90
Control	**	223	3.90	12.21	15.00	16.15

*Stock diet ingested ad libitum, 16 gm./day
**Ingestion of stock diet restricted, 4 gm./day
***6 animals per analyses
****3 animals per analyses

during the administration of the protein-free high fat diet at an adequate caloric intake (2.5 gm. in the normal rat vs. 1.6 gm. in the experimental rat). There was even less hepatic tissue when the low fat ration was fed. Supplementary methionine induced a 12 per cent increment in the quantity of moisture-free fat-free tissue in the rats given the high fat diet but not in those given the low fat ration.

In the group restricted as to calories and fed the high fat diet supplemented with methionine, a serious reduction in the amount of tissue occurred (1.30 gm. to 0.85 gm). This again is the group whose nitrogen balance was not benefited by the supplementary feeding. In contrast, an increment in liver nitrogen was found in the restricted low fat group when the amino acid was added; this accompanied a sparing of body tissue of 970 mg. in the 5 day period.

Nitrogen in Liver

A decrease in the concentration of protein in the liver has been reported by Seifter *et al.* (1948) when animals were maintained on protein-free diets with adequate caloric intake. This change occurred progressively over a period of 21 days and paralleled a decrease in the activity of certain enzyme systems in the liver. The rate

of decrease was greatest in the 1st week of protein restriction. After 21 days, no further loss of liver protein concentration was observed as compared with analyses conducted on rats maintained on the protein-free rations for two weeks.

In the present study, a sharp decrease in the concentration of nitrogen in the liver was observed when animals were fed either the low or high fat rations. On a wet basis, these values were 2.56 and 2.46 for the high and low fat rations respectively, as compared to 3.57 per cent for the normal control animal at full caloric intake. When the caloric intake was restricted, an increased proportion of nitrogen was found, these values being 3.61 per cent and 3.66 per cent for the two diets. Supplementation of the high fat diet with methionine resulted in a lowering of the relative concentration of nitrogen in the liver when the animals were fed the high fat ration with adequate calories, and a slight increase when caloric intake was restricted. The values in the latter instance are approximately the same as those found when the normal aimal is offered a restricted food intake.

When the nitrogen content of the hepatic tissue is analyzed on a dry basis, the alteration in the concentration of hepatic nitrogen becomes more striking. The percentages are approximately 8 for the high and low fat groups, as compared to 11.32 for the normal stock control rats.

The addition of methionine to the diets of the animals fed the high and low fat rations at full caloric intakes resulted in a sharp reduction of the nitrogen in the liver on a dry basis. Although the proportions of hepatic nitrogen in the animals fed the high and low fat diets at 85 per cent of the full caloric intake were about the same as that of the normal controls restricted in food intake, when methionine was added to these diets, the nitrogen content of the liver on a dry basis was increased to 13.26 per cent and 14.11 for the animals fed the high and low fat diets at restricted calories with additional methionine. It is striking in this instance, that the nitrogen concentration is increased as compared to the control group fed restricted calories, and decreased far below that of the normal control fed full calories when methionine is added as a supplement to the diets of the animals fed the high and low fat rations at full caloric intakes.

When the nitrogen concentration in the liver is examined on a fat-free dry basis, values for the experimental groups are similar to those of the control animals with one exception. In the group fed the high fat ration at full caloric intake and supplemented with methionine, the percentage of nitrogen in the liver is considerably less than that of the normal animal or that of animals fed the low fat ration, i.e.,10.00 per cent as compared 12.93 and 13.41 per cent.

When the hepatic nitrogen is examined on a glycogen-free, fat-free, dry basis, the same relationship holds true as has been discussed under the fat-free, dry basis. In this latter case, the percentage of hepatic nitrogen seems distorted still, suggesting the possibility that a different type of tissue is present when methionine is fed as a supplement to a high fat ration fed at the full caloric intake.

Concentrations of Vitamins in Hepatic Tissue

It has been well established that in states of inanition and during periods of restricted protein intake, animals suffer a loss of tissue protein, but the significance of this loss in relation to vitamin concentrations has not been elaborated clearly.

In the last few years, however, a relationship has been shown to exist between protein intake and the liver concentrations of certain B vitamins. Thus, Sarett and Perlzweig (1943), Unna and coworkers (Unna, Singer, Kenslery, Taylor, and Rhoads, 1944), and Reisen, Schweigert, and Elvehjem (1946) have shown that the concentration of riboflavin in the liver is decreased in rats maintained on a low protein diet. On the other hand, Flinn and coworkers (1946) have reported that maintenance of rats on a protein-free ration caused a increase in the concentration of liver

TABLE 21. CONCENTRATIONS OF TOTAL NITROGEN, RIBOFLAVIN AND NICOTINIC ACID IN THE LIVERS OF RATS FED A LOW NITROGEN DIET

Diet	Cal. per day	Total nitrogen	Riboflavin		Niacin	
			Total	Ratio: Ribo. to Nit. x 10^{-5}	Total	Ratio: Niac. to Nit. x 10^{-5}
		mg.	mcg.		mcg.	
Unsupplemented nitrogen-low diet						
High fat	56	218	118	54	803	37
Low fat	56	181	54	30	679	37
High fat	14	202	112	55	676	33
Low fat	14	186	94	51	423	23
Nitrogen-low diet plus methionine						
High fat	56	182	93	51	718	39
Low fat	56	224	123	55	955	43
High fat	14	126	61	48	391	31
Low fat	14	127	70	55	732	39
Control diets						
Control	*	318	201	63	1131	36
Control	**	223	106	48	771	35

*Stock diet ingested ad libitum, 16 gm./day
**Ingestion of stock diet restricted, 4 gm./day

nicotinic acid, pantothenic acid, pyridoxine, biotin, and vitamin B_c in chronically undernourished animals.

More recently, Seifter, Harkness, Rubin, and Muntwyler (1948) have reported that maintenance of rats on a protein-free diet caused a decrease in the concentration of liver riboflavin and nicotinic acid as compared to pair-fed controls. These decreases exceeded the loss in liver nitrogen.

In the present study, striking differences were observed between the concentrations of these vitamins in the livers of control animals maintained on the stock ration and those of the experimental groups. These data are shown in Table 22.

Riboflavin

The livers of the normal control animds fed adequate food intakes contained 201 mcg. of riboflavin. When this quantity of riboflavin was compared to the total nitrogen present in the liver, the ratio of riboflavin to nitrogen was 63×10^{-5}.

When the high fat ration was fed to the experimental animals at full caloric intake, a marked reduction in the concentration of riboflavin in the liver was observed. The livers of these animals contained only 118 mcg. of total riboflavin, approximately one-half of that in the tissue of the normal controls. This finding is in accord with the decreased concentrations of riboflavin reported by other

workers when a low protein ration is fed. The ratio of riboflavin to total nitrogen in this instance also decreased being 54 x 10^{-5}. Comparison of this value with the normal ratio indicates that the riboflavin has been lost at a more rapid rate than the nitrogen.

The concentrations of riboflavin in the livers of animals fed the low fat ration, however, were strikingly lower than those of the normal animal. It also was much lower than those of the animals which had received the high fat rations at full caloric intake. In this latter instance, the value, 54 mcg., paralleled a decrease in the total nitrogen of the liver from 218 to 181 mg. The loss in the concentration of riboflavin exceeded the loss in liver nitrogen as shown by the ratio of total riboflavin to total nitrogen which fell to 30 x 10^{-5}.

When the caloric intake of the animals fed the high fat ration was decreased, the concentration of riboflavin in the liver was essentially the same as that found when these animals were fed full calories. The ratio in this case was also essentially the same. A slight decrease, i.e., 112 mcg. to 94 mcg. was observed when fat was removed from the ration but the ratio of riboflavin to total nitrogen was the same as had been found in groups maintained on adequate caloric intakes.

Since the riboflavin intakes of the animals maintained on the protein-free rations of high and low fat content were the same, both when full calories and restricted calories were administered, it would seem that the level of this vitamin in the liver was independent of the intake, and depended rather on the level of tissue protein. This appeared to be the case in general except for the rats fed the full caloric intake on the low fat diet. The observation is supported by similar findings reported by other investigators (Seifter, Harkness, Rubin, and Muntwyler, 1948; Reisen, Schweigert, and Elvehjem, 1946; Flinn, Pilgrim, Gregg, and Axelrod, 1946; Hegsted, Mills, Elvehjem, and Hart, 1941).

When methionine was added as a supplement to the high fat ration, a slight decrease in the concentration of riboflavin in the livers of these animals was observed. However, since the total nitrogen content of these livers was also decreased, the ratio 51×10^{-5}, was essentially the same as had been observed with the unsupplemented groups.

The addition of methionine to the rations of the animals fed the low fat diet produced a phenomenal increase in both the total riboflavin deposited and the total nitrogen present. The increase in riboflavin was over two times the concentration of the vitamin in the livers of the unsupplemented group, i.e., 123 mcg., and 54 mcg., respectively.

The ratio of riboflavin to total nitrogen increased to that observed in animals fed the high fat ration at full caloric intake, and approached that of the normal stock animal, i.e., 55, 54, and 63 mg. respectively. Similar observations relating to the increased riboflavin content of livers when methionine is added to the ration have been reported by Reisen, Schweigert, and Elvehjem (1946), and Unna, Singher, Kensler, Taylor, and Rhoads (1944).

When the energy value of the ration was restricted, it was interesting to note that decreased concentrations of riboflavin were found when methionine was added to the low and high fat rations, i.e., 127 mcg. and 126 mcg., respectively. The ratios of riboflavin to total nitrogen, however, were the same as had been observed when full calories were administered to the animals receiving the high fat diet and to the ratio of riboflavin to total nitrogen observed in the livers of the normal animal restricted in calories.

Since riboflavin is an integral part of the coenzyme necessary for the activity of d-amino acid oxidase, and since the riboflavin concentration in the livers of these animals decreased, it would appear quite possible that the changes observed might reflect a change in the coenzyme concentration of the liver. It seems likely, too, that a loss of enzyme protein per se may have occurred.

In recent years, the concentration of liver protein has been shown to bear a direct relationship to the activity of enzyme systems in the liver. In this respect, Potter and Klug (1947) reported that the livers of animals maintained on a diet high in carbohydrate and low in protein, or a diet high in fat and low in protein possessed diminished succinoxidase activity and decreased capacity for the oxidation of octanoate and citrate. Axelrod, Swingle, and Elvehjem (1942) on the other hand, reported an increase in succinoxidase activity of livers of animals maintained on a restricted food intake for three weeks.

Miller, in a more recent study (1948) has reported that the activites of a number of enzymes, including catalase, alkaline phosphatase, xanthine dehydrogenase, and cathepsin, are decreased in the livers of rats fasted for a period of 7 days. The observed loss of enzyme activity paralleled or exceeded the loss of liver protein; the authors condluded that the decrease in activity represented a loss in enzyme protein per se. Similar results were obtained in rats maintained on a protein-free diet (Miller, 1948).

A quantitative relationship has been shown to exist between protein intake and liver arginase activity by Lightbody and Kleinman (1939). These authors demonstrated that the liver arginase activity was directly related to the protein intake and the length of time the animals were maintained on a particular dietary regime. It was interpreted

as expressing an adaptation of the arginase content of the liver to a need for the enzyme as determined by the amount of protein which the animal was required to metabolize.

In the study of Seifter et al. (1948), the diminution in liver protein was accompanied by a steady fall in the water content and a steady rise in the liver fat. Protein restriction also resulted in a decrease in the activities of arginase and d-amino acid oxidase, this paralleling the decrease in concentrations of nicotinic acid and riboflavin. The diminution in the amounts or activities of these enzymes in all cases exceeded the decrease in the concentration of liver potein.

In the latter experiment, actual levels of the activities of d-amino acid oxidase and arginase were found to be directly related to the concentration of protein in the liver, and to the levels of riboflavin and niacin. This relationship occurred even though the intake of the vitamins for all groups was maintained constant, and at an adequate level.

In summary, it may be stated that marked losses of the riboflavin present in the liver occurred when the eight experimental diets were fed. Omission of fat from the full calorie diet increased the extent of losses by more than 100 per cent. Methionine feeding was very effective in improving the riboflavin concentration. In all cases except when the low fat diet was given at full caloric value, the

losses in riboflavin paralleled the losses in hepatic nitrogen. The addition of methionine to the low fat diet brought about a return of the riboflavin-nitrogen ratio characteristic of the rats fed fat. At reduced caloric intake, methionine was ineffective in causing an increase in either the hepatic stores of nitrogen or riboflavin of rats of these experiments.

Niacin

When protein-low rations are fed, decreases in the liver concentration of nicotinic acid, as well as in liver protein were observed by Seifter et al. (1948).

The quantity of nicotinic acid in the livers of the control animals fed the full caloric intake was 1131 mcg. The ratio of this vitamin to the total nitrogen in the liver was 36×10^{-4}. When the protein-fre high fat ration was administered at full energy intake, the concentration of niacin was sharply diminished, falling to 803 mcg. It is interesting that this decrease paralleled a decrease in the total nitrogen of the liver, resulting in a ratio of niacin to total nitrogen of 37×10^{-4}. When the low fat diet was fed at the full caloric intake, the quantity of niacin in the liver of these rats was much lower than had been found in the high fat group, i.e., 679 mcg. Since the nitrogen content of the livers of animals fed the low fat ration was also decreased, the ratio of niacin to total nitrogen of the liver was essentially the same as had been observed in both the normal

animal and the animal fed the high fat ration, 37 x 10^{-4}.

When the caloric intake of the animals fed the high fat ration was restricted to 25 per cent of the normal ingestion, a decrease in the level of niacin in the liver was observed, i.e., 676 mcg. The ratio of niacin to total nitrogen decreased slightly in this instance, being 33 x 10^{-4}. However, when the low fat diet was administered at a restricted caloric intake, a sharp and striking drop occurred in the concentration of niacin, i.e., 423 mcg. as compared to 676 mcg. for the animals fed dietary fat. The ratio of nitrogen to niacin was correspondingly decreased, falling from 37 x 10^{-4} in the animals fed full calories to 23 x 10^{-4} in the low fat, calorie-restricted group.

Supplementation of the high fat ration fed at the full caloric intake resulted in a decreased concentration of niacin in the liver, although the ratio of niacin to total nitrogen remained about the same as that of the unsupplemented group, 39 and 37 x 10^{-4} respectively. The addition of methionine to the low fat ration gave clear-cut results, and accounted for an additional 276 mcg. of niacin in the livers of these animals. The level of niacin shifted from 679 mcg. in the livers of the unsupplemented group to 955 mcg. in the livers of animals fed methionine. The ratio of niacin to total nitrogen was slightly higher than had

been observed before, being 43 x 10^{-4}.

Restriction of the caloric intake of the animals fed the high fat ration, and supplementation of this diet with methionine resulted in a marked decrease in the concentration of niacin, it changing from 676 mcg. to 391 mcg. The ratio of niacin to total nitrogen was, however, the same for both groups, being 33 for the unsupplemented group and 31 for the animals fed methionine. A marked decrease in the nitrogen content of the livers of these animals accounted for the similarity of the niacin-nitrogen ratios.

A phenomenal effect of methionine was shown in the marked increase in the niacin found in the livers of the animals fed no fat, this amino acid accounting for more than 300 mcg. of additional niacin. In this instance, the niacin concentration changed from 423 mcg. to 732 mcg. in the supplemented group fed restricted calories. It is interesting here that the ratio of niacin to total nitrogen, 39 x 10^{-4} was essentially the same as that of the normal control animal, 36 x 10^{-4}, and the ratio of the animals fed the high fat ration supplemented with methionine, 39 x 10^{-4}.

Again, a direct relationship seems to exist between the total nitrogen and niacin concentrations in the livers of the animals in the present study, with one exception. This time, it is the rats that have been deprived of calories and fat that show an abnormal hepatic

niacin-nitrogen ratio. In the case of riboflavin, it was the rats fed full calories of the same diet that suffered most.

If vitamins are present in a combined form and as components of enzyme systems, a fall in the ratio suggests a loss of an enzyme system. Such observations have been made by Seifter et al. (1948) and by Robinson and his co-workers (1947) with respect to nicotinic acid.

Weights of Adrenal Glands

Interesting changes in the weights of the adrenal glands of animals maintained on the experimental rations were observed in this study. These data are shown in Table 22.

The normal stock control animal fed a normal food intake was observed to have adrenal glands which weighed on the average 37.9 mg. When this weight was compared to the body weight of the animals, the resulting ratio was 13×10^{-5}.

When the high fat and low fat rations were fed at full caloric intakes, the adrenal glands were slightly enlargened, being 39.7 and 41.7 mg. for these groups respectively. The supplementation of the high and low fat diets of these animals resulted in a still greater hypertrophy of the adrenal glands.

TABLE 22. WEIGHTS OF ADRENAL GLANDS OF RATS FED A LOW NITROGEN DIET

Diet	Calories per day	Body weight	Weight of adrenal gland	Ratio: adrenal weight to body weight x 10-5
		gm.	mg.	
Unsupplemented nitrogen-low diet				
High fat	56	251	39.7	16
Low fat	56	240	41.7	18
High fat	14	208	41.8	23
Low fat	14	249	28.8	16
Nitrogen-low diet plus methionine				
High fat	56	242	43.8	18
Low fat	56	233	45.2	19
High fat	14	220	41.2	19
Low fat	14	236	39.9	18
Control diets				
Control	*	292	37.9	13
Control	**	279	52.9	19
High fat***	56	264	41.3	15
Low fat	56	276	47.7	17

*Stock diet ingested ad libitum, 16 gm./day
**Ingestion of stock diet restricted, 4 gm./day
***Fed the low nitrogen diet for 23 days

When the caloric intakes of the animals fed the high and low fat rations were restricted, the weights of the adrenal glands were essentially the same of those of the animals fed the full caloric intake, both in the supplemented and unsupplemented groups (See table 22). However, due to the greater loss in body weights by these animals, a higher ratio of adrenal gland weight to body weight was observed. In the unsupplemented group these ratios were 23×10^{-5} and 16×10^{-5} for the high and low fat groups, respectively; in the groups fed methionine, they were 19 and 18×10^{-5}.

It is interesting that restriction of the diet also affected the weights of the adrenal glands of the control animals. The adrenal weights were 52.9 mg. in this group, and the ratio of the adrenal gland weights to body weight was 19×10^{-5}. These findings suggest strongly that when the animals were fed the high and low fat rations, enlargement of the adrenal glands occurred at full caloric intake; when calories were restricted, a further hypertrophy took place. Selye et al. (1940) has reported that caloric restriction results in an increase in the weight of the adrenal gland.

It would seem from the data in Table 22 that a slight protective effect has been exerted by methionine in the group of animals restricted in calories and fed the high

fat ration. The adrenal weights of the unsupplemented group and supplemented group were 41.8 mg. and 41.2 mg., respectively. Because of a greater body size in the supplemented animals, the ratio of the adrenal weight to the body weight is smaller for the methionine-fed group, i.e., 23 vs. 19 x 10^{-5}. This seems to be the only case, however, in which methionine exerts a beneficial influence.

It has been reported, and now generally accepted, that one of the primary functions of the adrenal cortex is the regulation of the metabolism of carbohydrate and protein. Insufficiency of the cortical hormone is characterized by a depletion of the carbohydrate of the blood and tissues; cortical excess shows its influence in greater than normal amounts of glucose and glycogen in the blood and tissues.

It is interesting to correlate this effect of the adrenal with the increased amounts of glycogen found in the livers of the experimental animals. A slight amount of adrenal enlargement was observed in all of the groups; between 2 and 9 times the amount of glycogen found in the livers normal animals were present in the livers of the experimental animals. Distorted glucose tolerance curves, indicating abnormal carbohydrate metabolism, in animals in the experimental groups would also indicate that disturbances occurred. In this instance, methionine feed-

ing returned the ability of the animal to tolerate a test dose of glucose to near normal, whereas, it was ineffectual in correcting the enlargement of the adrenal glands or in causing a significant reduction in liver glycogen.

HISTOLOGY OF THE LIVER

Appearance of Cells: Hematoxylin and Eosin Stain

Reports in the literature dealing with the normal histology of the albino rat are very limited. Divergencies in the histological methods used, the age, sex, genetic history of the animals, and differences in the diets used in these experiments make it difficult to compare data from published observations. Therefore, for the purpose of the present study, the microscopic anatomy of tissues taken from 6-month old albino male rats in the Wistar stock colony of the Nutrition Laboratory of the Iowa State College has been considered as the control animal. The histological procedures used in preparing the tissue sections have been carefully controlled so that comparisons between organs from the control group of rats and those from the experimental animals would be valid. Any variations of the tissues from those of the normal group, therefore, are considered to be due to alterations induced by the experimental diets.

In an earlier section of this manuscript, it was stated that animals were sacrificed by first stunning them with an electric shock. It was felt desirable to study the organs of animals sacrificed by this method

and those anesthetized by the use of ether. Sections of the livers from animals prepared for necropsy in these two ways were carried through the histological procedures described in the appendix.

Careful examination of tissues which had been stained with hematoxylin and eosin, Scarlet Red stain, Nile Blue Sulfate stain, and Carmine Red stain failed to indicate great differences in these two groups of animals. Photomicrographs of these slides are shown in Figures 4A and 4B, 8A and 8B, 12A and 12B, and 16A and 16B. Cellular structure may be somewhat sharper in the sections taken from the rats sacrificed by means of an electric shock, Figures 4A and 4B.

It was concluded, therefore, that the method used in this study, that of stunning the animal with an electric shock, did not alter the appearance of the liver. Neither did additional studies on the adrenal glands, kidneys and other chemical analyses[1] on the blood of these animals reveal any differences.

Examination of the microsections of the liver from the control group fed the Steenbock xvii diet revealed large octagonal shaped hepatic cells arranged in cords

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[1] Hoover, C.A. Unpublished data, Files, Nutrition Laboratory, Iowa State College

Explanation of Figure 4

Hematoxylin and Eosin Stain

4 A. Diet: Normal, sacrificed with ether

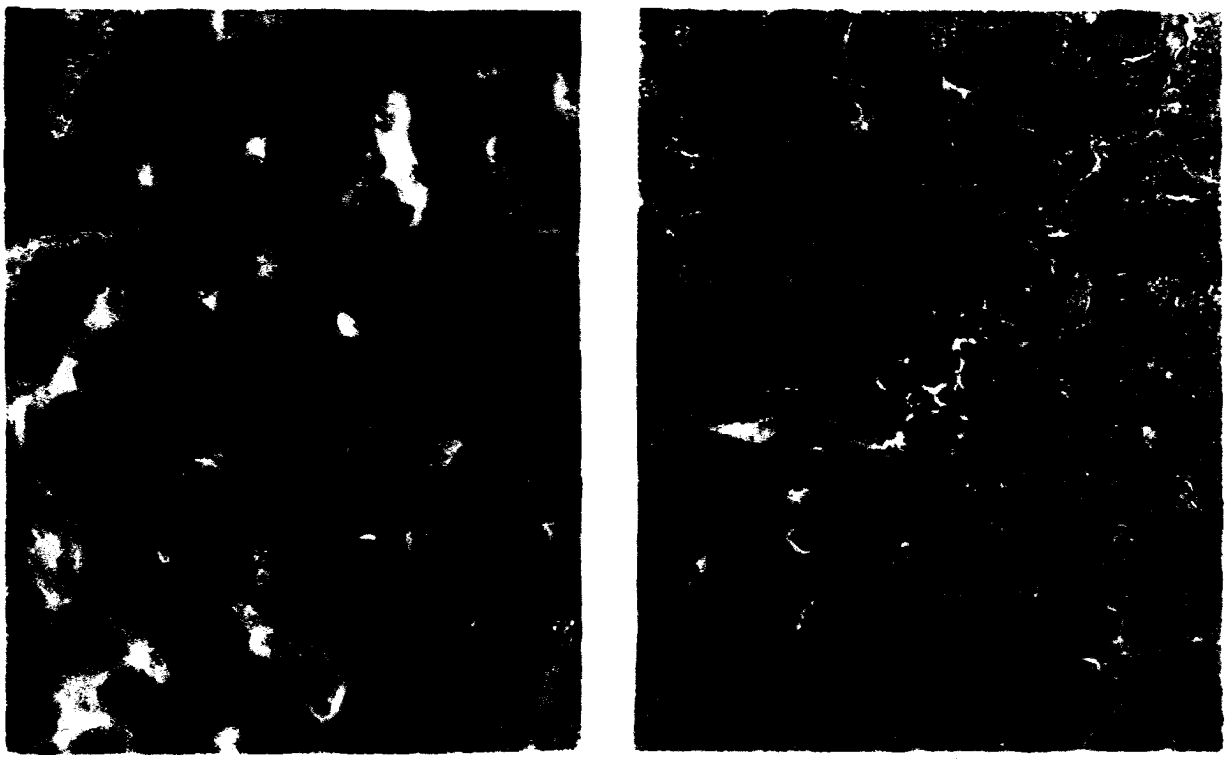
Large octagonal-shaped hepatic cells arranged in radiating cords; cytoplasm is faintly granular, cell outlines are distinct, and the nuclei stain dark blue.

4 B. Diet: Normal, sacrificed with an electric shock

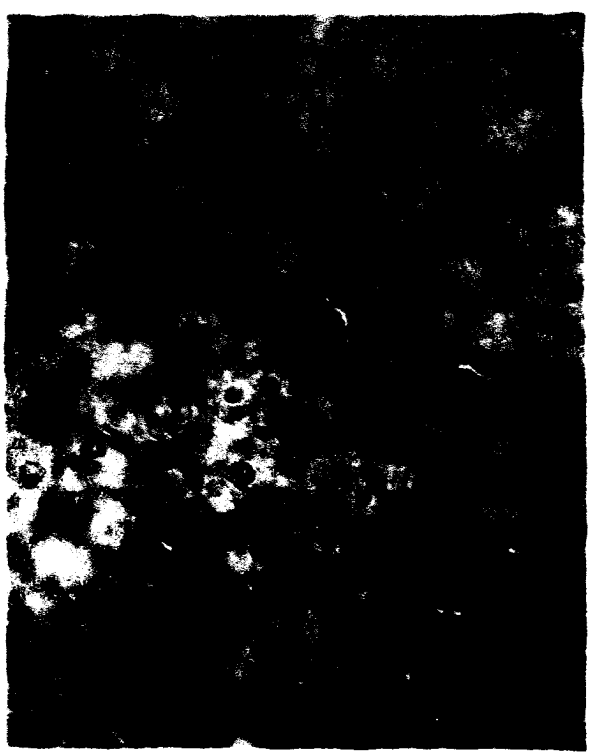
Large octagonal-shaped hepatic cells arranged in radiating cords; cytoplasm is faintly granular, cell outlines are distinct, and the nuclei stain dark blue.

4 C. Diet: Normal, fed one-fourth of the food intake

Large areas of faintly staining hepatic cells, found sometimes around the central vein, at other times at the periphery of the lobule; cell walls in these areas are sometimes indistinct; the nuclei stain faintly; cells are swollen, obliterating the sinusoids.



A B



C

FIGURE 4. HEPATIC TISSUES OF RATS: H AND E STAIN

radiating from the intralobular vein (Figure 4B). The cytoplasm of these cells was faintly granular, cell outlines were distinct, and the nuclei stained dark blue. There was no evidence of pathological changes.

This picture, however, was not characteristic of that observed in rats fed the stock colony ration at one-fourth of the normal food intake (Figure 4C). There were large areas of faintly staining cells, found sometimes around the central vein, at other times at the periphery of the hepatic lobule. The cell walls in these areas were sometimes indistinct, the nuclei stained faintly if at all. There were swollen hepatic cells and swollen liver sinusoids which obliterated the parenchyma. The presence of faint or no cellular outlines in many cases even with the nuclei present, suggested the possibility of the formation of an abnormal cytoplasm, such as might occur in hyaline degeneration. Shrunken nuclei, irregular nuclear outlines, and lack of proper staining characteristics may indicate that a disturbance of protein metabolism had occurred in the hepatic tissue of this group of animals.

In areas thus described which were adjacent to normal hepatic cells, there was a transition, indicating various stages in the progression of the altered cellular picture. This observation and the fact that results were consistent

within the group would indicate that the condition observed in the livers of the animals maintained on one-fourth of the normal food intake was a true experimental finding, not an artefact.

It is interesting that although the integrity of the cytoplasm was maintained in these cells, the tissue did not take the stain in a manner characteristic of the sections taken from livers of the normal animals. Extensive search, however, revealed no evidence of pathological changes. Since the caloric intake of this group of animals was restricted by offering only one-fourth of the total food normally eaten, a deficiency not only of protein, fat, and carbohydrate, but also of vitamins and minerals may have occurred. This type of liver change, therefore, is interpreted as having been induced by the dietary manipulation.

Livers from animals (Figure 5A) which were maintained on the high fat diet for 23 days, the period prior to the introduction of the dietary modification, showed still another type of liver change, being different both from that of the normal animal fed a normal caloric intake, and from that of the normal animal fed a restricted quantity of food. In this case, the hepatic cells were swollen, approximately two times that of the normal rat. In many

Explanation of Figure 5

Hematoxylin and Eosin Stain

5 A. Protein-free diet:
20 per cent fat,
full calories
fed for 23 days.

Swollen cells, cytoplasm sparse, nuclei intact; greater damage at the periphery of the hepatic lobule.

5 B. Protein-free diet:
No fat, full calories
fed for 23 days.

Swollen cells, with intact nuclei, cytoplasm clumped together within the cell; cellular changes occur mainly at the center of the hepatic lobule, around the central vein.

A

B

FIGURE 5. HEPATIC TISSUES OF RATS FED A LOW NITROGEN DIET FOR 23 DAYS: H AND E STAIN

instances, the cytoplasm was completely missing, or lumped together in parts of the cell. Distinct cell nuclei, however, were present, and were found in or near the central portion of the hepatic cells. The tissue still maintained its characteristic staining qualities. This type of cellular alteration was usually more pronounced at the periphery of the hepatic lobule, the liver structure being more nearly normal around the central vein.

When fat was omitted from the nitrogen low diets, essentially the same picture occurred as when fat was present in the diet (Figure 5B). In this instance, however, the cytoplasm seemed more clumped and cell outlines less distinct.

In hepatic tissue from animals fed the low nitrogen diet of high fat content for an additional 9 days, that is, until the time of necropsy, this same type of cellular alteration occurred, but to a more marked extent (Figure 6A).

After the animals had been fed an additional 9 days on the low fat ration, microsections of their livers indicated that the described changes had progressed to a greater extent than had occurred in the previous period (Figure 6B).

It is interesting here to speculate as to the cause

of the swollen hepatic cells. It is possible that the clear areas in the cytoplasm may have been once occupied by fat droplets and/or glycogen. In the former instance, however, one would have expected that the nuclei of the cells would have been found near the cellular membrane, having been forced to the periphery of the cell by the fat droplet. As stated earlier, this was not the case. In the latter instance, the use of 10 per cent formalin as a fixative would not have been expected to dissolve the glycogen present in the tissue, thus accounting for the spaces within the hepatic cells. On the other hand, however, quantitative analyses of both the fat and glycogen in the livers of the control animals and experimental groups revealed that quantities of these constituents were present which exceeded those amounts found in the normal rat. Photomicrographs showing the deposition of fat and glycogen reveal the same differences (Figures 10, 11, 14, 15, 18 and 19).

When the caloric intake of the animals fed the high and low fat diets was restricted to 25 per cent of the normal ingestion (Figure 6C and 6D) the changes induced by feeding the experimental diets were like those discussed in relation to the normal animal restricted in calories (Figure 4C). Again the indistinct cell walls and

Explanation of Figure 6

Hematoxylin and Eosin Stain

6 A. Protein-free diet: 20 per cent fat, full calories.

Swollen cells, with intact nuclei; cytoplasm seems lumped together within the cell; condition is most extensive around the periphery of the hepatic lobule.

6 B. Protein-free diet: no fat, full calories.

Same as 6 A., but not as extensive.

6 C. Protein-free diet: 20 per cent fat, restricted calories.

Normal; some had same appearance as that shown in Figure 7 C.

6 D. Protein-free diet: no fat, restricted calories.

Areas of faintly staining hepatic cells, found sometimes around the central vein and at other times at the periphery of the lobule; cell walls are sometimes indistinct; nuclei stain faintly, if at all.

Explanation of Figure 7

Hematoxylin and Eosin Stain

7 A. Protein-free diet: 20 per cent fat supplemented with methionine, full calories.

Same as Figure 6 A., but improved.

7 B. Protein-free diet: no fat supplemented with methionine, full calories.

Same as Figure 6 B., but greatly improved.

7 C. Protein-free diet: 20 per cent fat supplemented with methionine, restricted calories.

Some livers in this group were normal; other tissues had the appearance of that shown.

7 D. Protein-free diet: no fat supplemented with methionine, restricted calories.

Normal.

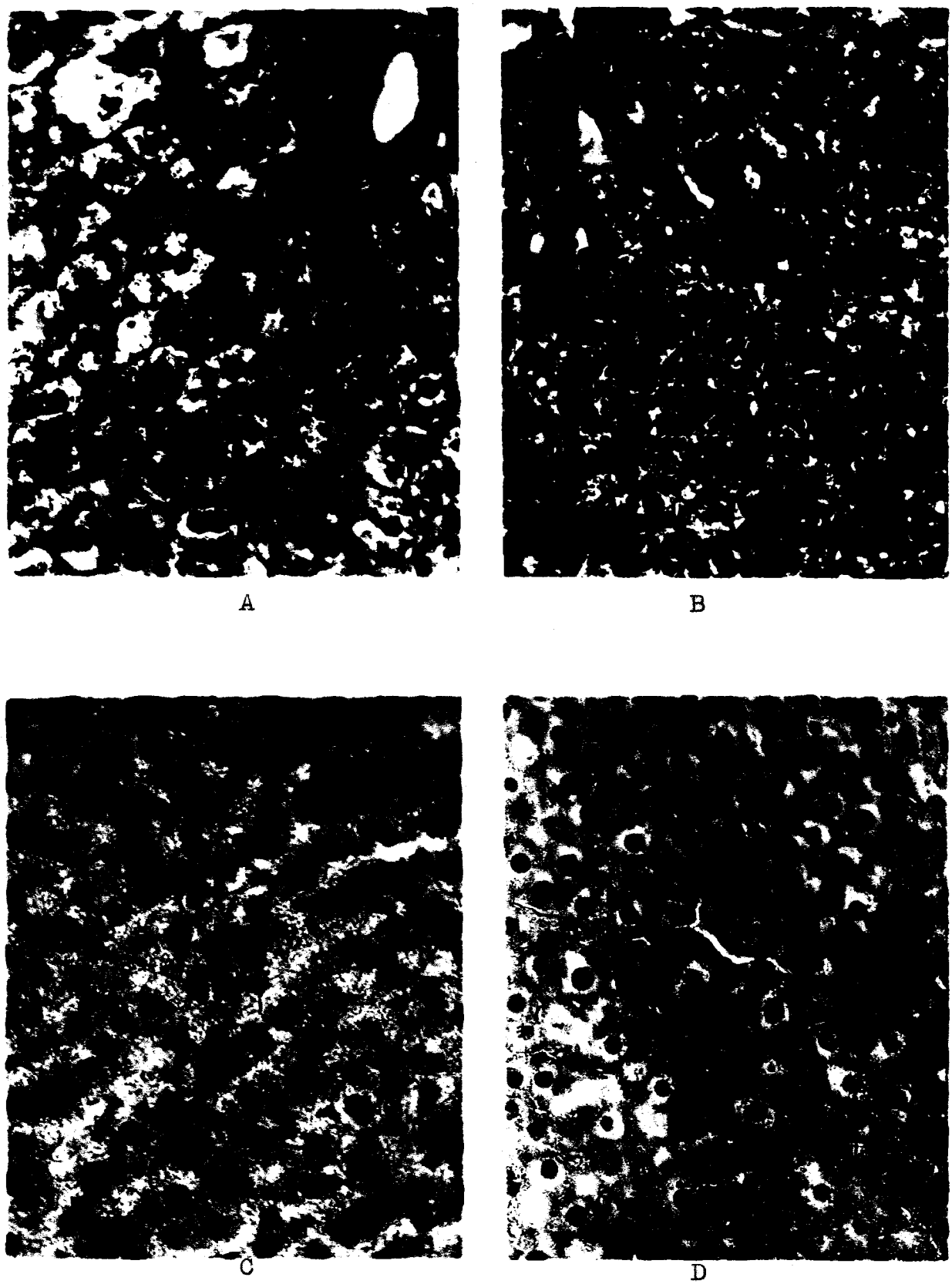


FIGURE 6. HEPATIC TISSUES OF RATS FED A LOW NITROGEN DIET: H AND E STAIN

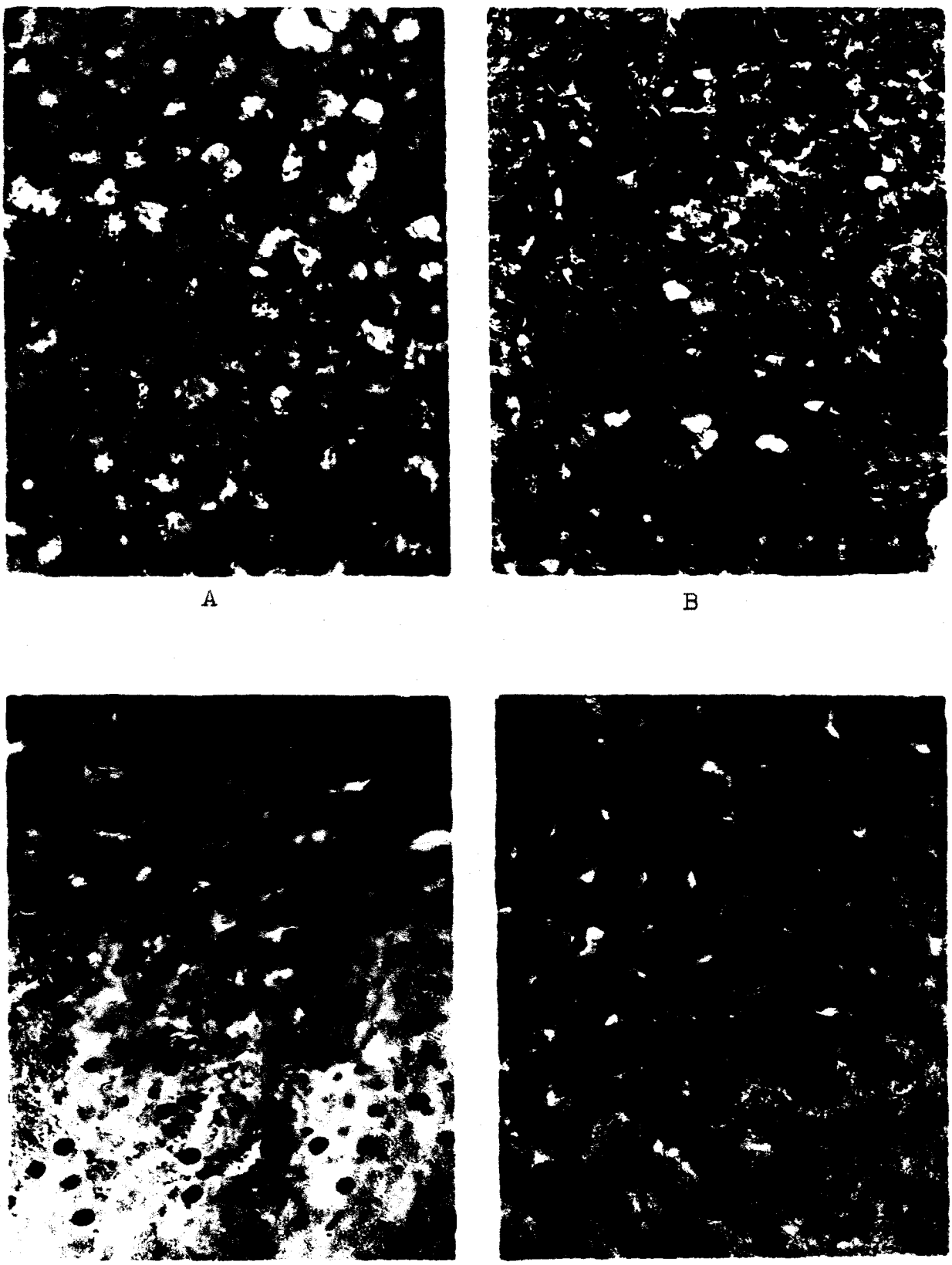


FIGURE 7. HEPATIC TISSUESOF RATS FED A LOW NITROGEN DIET SUPPLEMENTED WITH METHIONINE: H AND E STAIN

faintly staining nuclei were present. Not all of the animals receiving fat showed this picture, but faintly staining areas were found in all of the livers of animals receiving the fat-free ration.

The supplementation of diets with methionine (Figure 7A) produced striking phenomena. When full calories were fed to the animals in the high and low fat groups, methionine feeding again seemed to improve the appearance of the liver cells (Figures 7A and 7B). The cells now were nearly normal in appearance and the cell membranes were clear-cut and distinct (Figure 7A). A few globules of fat within the cell could be distinguished. Improvement was apparent in the over-all appearance of the tissue also when methionine supplemented the low fat diet (Figure 7B). The cytoplasm seemed to be evenly distributed throughout the cell. Fat globules within the cell were fairly numerous.

The photomicrographs showing the condition of the hepatic cells before and after the supplementation of the low calorie high fat diet with methionine (Figures 6C and 7C) indicate that methionine did not exert a beneficial effect. The picture, however, is not typical of all of the animals in these groups. Hepatic tissues of rats fed the high fat low calorie ration were like

that of the normal animal in some cases (Figure 7C), and in other instances, it did not seem to be improved by the methionine supplement (Figure 6C).

The hepatic tissues from animals, when methionine was added to the high and low fat diets with a caloric restriction imposed, were identical in appearance to those of the normal (Figures 7C and 7D). No faintly staining areas were found, the hepatic cellular cytoplasm and nuclei stained characteristically. Liver sinusoids were also present in their characteristic arrangement. Observations were consistent from animal to animal in the group of rats fed the fat-free ration (Figure 7D).

Thus it would seem that altered cellular metabolism resulted from a restriction of calories in both the diets containing no fat and fat. Methionine exerted a protective action in preventing the occurrence of the changes described.

An additional indication of the fact that no pathological changes occurred in these animals was found from a study of the livers of rats fed the high and low fat rations for an interval of 23 days (Figures 5A and 5B). Had pathological changes occurred, it would have been logical to expect the presence of "scar tissue," resulting from the ingestion of necrotic cells by phagocytes, and

replacement of the tissue subsequently by a form of connective tissue. Careful examination of the liver sections taken from rats fed the experimental diets for 23 days revealed no scar tissue. Examination of the livers of the animals fed an additional 10 days, (Figures 6A and 6B), a group in which one would expect to find more "scar tissue" as the experiment progressed, also gave negative results.

These observations lend additional support to the conclusion that the changes observed in the livers of animals maintained on the experimental diets and their dietary modifications are due to an altered intracellular metabolism induced by ingestion of the diet.

Glycogen in the Liver

In both the normal animal fed the full caloric intake (Figures 8A and 8B) and the animal restricted in calories (Figure 8C), glycogen deposition in the liver was even. However, when the experimental diets were fed, interesting differences in the way this organ constituent was deposited occurred.

When the high fat ration was fed, (Figures 9A, 10A), the glycogen was unevenly distributed throughout the hepatic lobule, but with greater quantities at the

Explanation of Figure 8

Glycogen Stain

8 A. Diet: Normal, full calories, sacrificed with ether.

Practically no glycogen (0.3%).

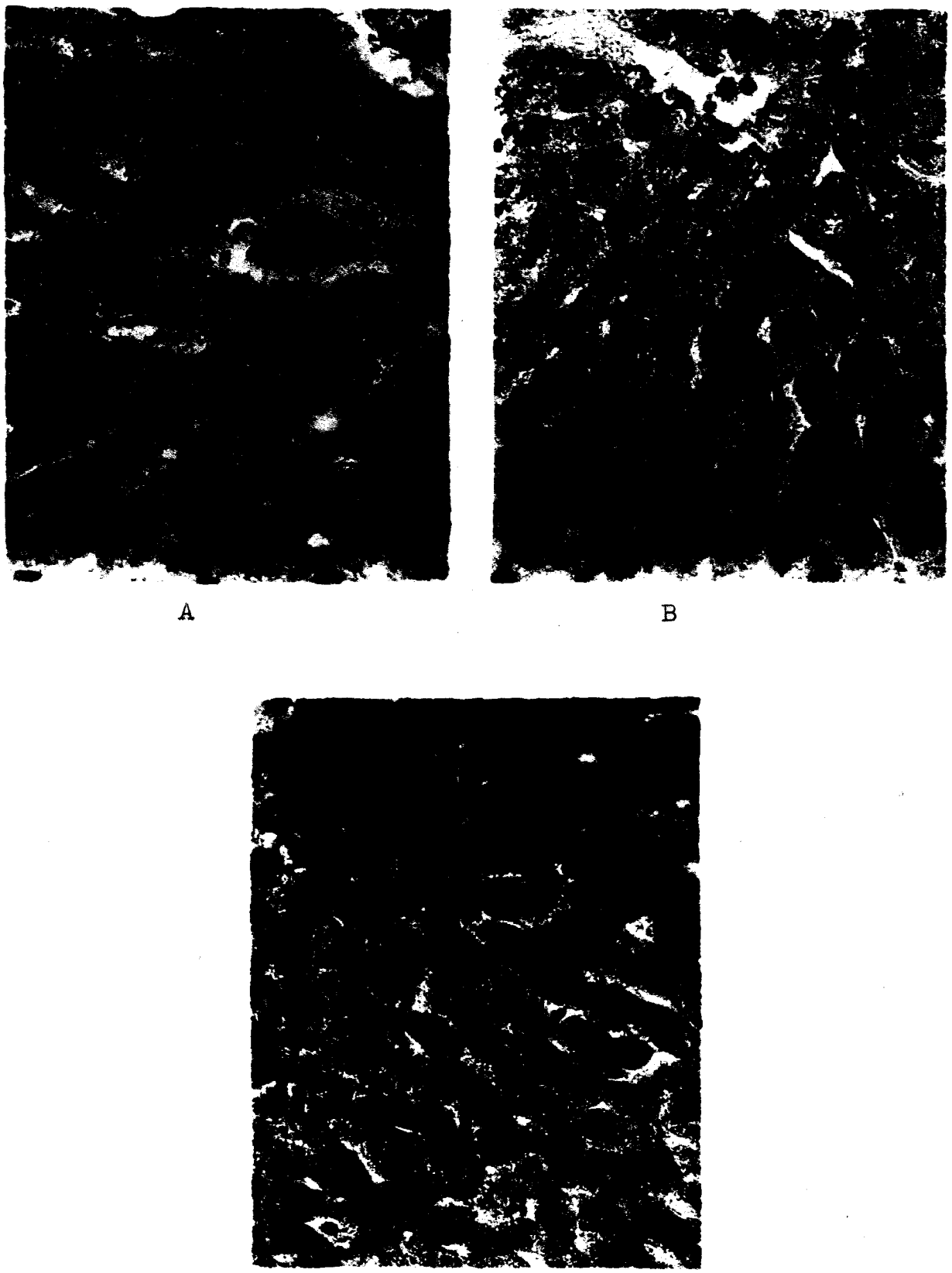
8 B. Diet: Normal, full calories, sacrificed with an electric shock.

Practically no glycogen (0.3%).

8 C. Diet: Normal, restricted calories.

Even glycogen deposition in the hepatic lobule (1.9%).

In these and the following photomicrographs showing glycogen deposition, glycogen can be recognized by small dark specks within the cell.



A B C

FIGURE 8. GLYCOGEN IN HEPATIC TISSUES OF NORMAL CONTROL RATS: CARMINE STAIN

periphery of the lobule. This same phenomenon was observed when the low fat diet was fed to rats at the full caloric intake (Figures 9B, 10B).

When calories were restricted, even though greater quantities of glycogen were deposited in the livers of both the control and experimental animals, the deposition, for the most part, was even (Figures 10C and 10D).

The supplementation of the high and low fat rations with methionine, resulted in decreased deposition of glycogen in the livers of the animals fed these diets. Both when full calories were fed, and when the caloric intake was restricted, the deposition of this constituent was, for the most part, even (Figures 11A, 11B, 11C, and 11D).

Fat in the Liver

Microsections of the liver were stained with two selective fat stains, Scarlet Red and Nile Blue Sulfate. With scarlet red, neutral fats stain darkest red; then follow the cholesterine esters and cholesterine fatty acid mixtures. Other lipids stain lightly or not at all. With Nile Blue Sulfate, neutral fats stain rose red, cholesterine stains rose violet, and lipoids and fatty acids stain dark blue.

Explanation of Figure 9

Glycogen Stain

9 A. Protein-free diet:
20 per cent fat,
full calories,
fed for 23 days.

Uneven glycogen deposition,
occurs mostly at the peri-
phery of the hepatic lobule.

9 B. Protein-free diet:
no fat, full calories
fed for 23 days.

Uneven glycogen
deposition.

A

B

FIGURE 9. GLYCOGEN IN HEPATIC TISSUES OF RATS FED LOW NITROGEN DIETS OF HIGH AND LOW FAT CONTENT FOR 23 DAYS: CARMINE STAIN

Explanation of Figure 10

Glycogen Stain

10 A. Protein-free diet: 20 per cent fat, full calories.

Greater glycogen deposition at central vein, uneven. (1.8%-Analyzed)

10 B. Protein-free diet: no fat, full calories.

Even glycogen deposition in the hepatic lobule. (2.5%-Analyzed)

10 C. Protein-free diet: 20 per cent fat, restricted calories.

Even glycogen deposition in the hepatic lobule. (2.2%-Analyzed)

10 D. Protein-free diet: no fat, restricted calories.

Even glycogen deposition in the hepatic lobule. (0.7%-Analyzed)

Explanation of Figure 11

Glycogen Stain

11 A. Protein-free diet: 20 per cent fat supplemented with methionine, full calories.

Even glycogen deposition.
(1.7%-Analyzed)

11 B. Protein-free diet: no fat supplemented with methionine, full calories.

Even glycogen deposition.
(2.4%-Analyzed)

11 C. Protein-free diet: 20 per cent fat supplemented with methionine, restricted calories.

Uneven glycogen deposition, mostly at the periphery of the hepatic lobule.
(1.8%-Analyzed)

11 D. Protein-free diet: no fat supplemented with methionine, restricted calories.

Even glycogen deposition.
(2.5%-Analyzed)

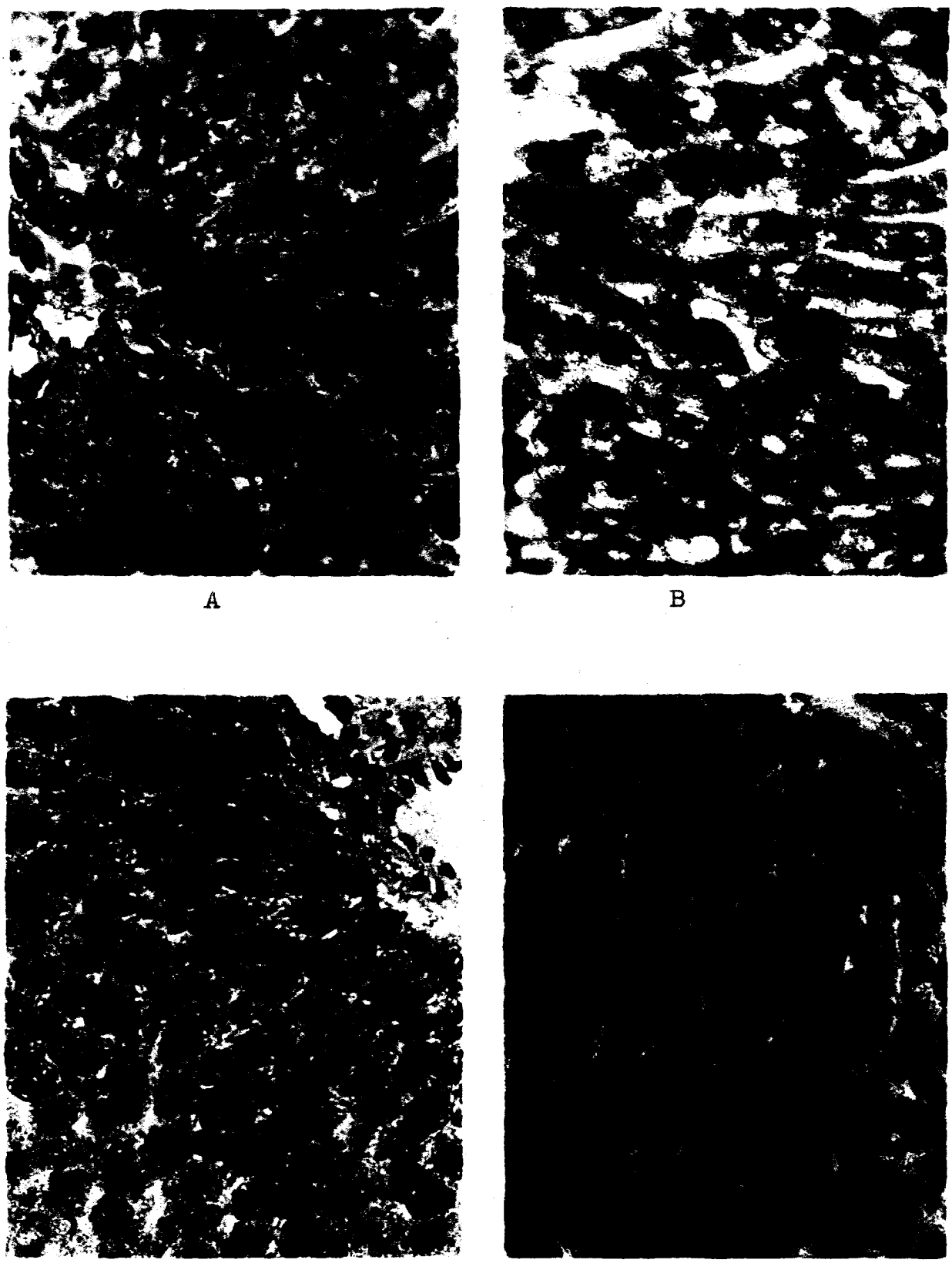
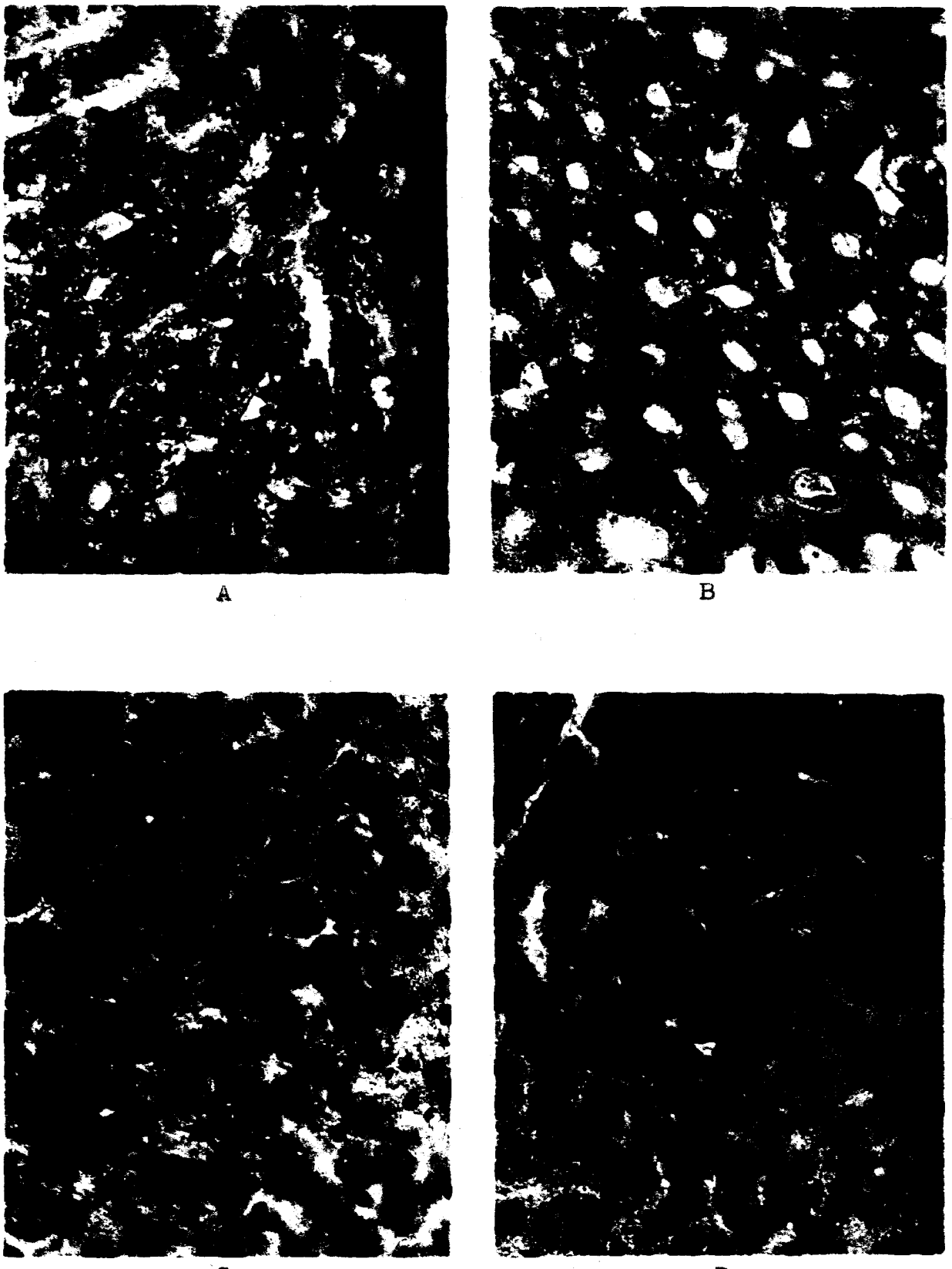


FIGURE 10. GLYCOGEN IN HEPATIC TISSUES OF RATS FED LOW NITROGEN DIETS OF HIGH AND LOW FAT CONTENT: CARMINE STAIN



A B C D

FIGURE 11. GLYCOGEN IN HEPATIC TISSUES OF RATS FED LOW NITROGEN DIETS OF HIGH AND LOW FAT CONTENT SUPPLEMENTED WITH METHIONINE: CARMINE STAIN

Scarlet Red Stain

In the normal stock colony animal, only small amounts of fat were present in the liver, i.e., 4 per cent (Figures 12A and 12B). When the caloric intake of the normal rat was restricted to 25 per cent of the normal ingestion, the fat content of the liver increased to 6 per cent (Figure 12C). These small quantities of fat are shown as dark droplets in Figure 12.

When the high fat diet was fed to the experimental animals for a period of 23 days (Figure 13A), the quantity of fat in the liver was strikingly increased as compared to the control animal (Figures 12A and 12B). The fat consisted mainly of cholesterine esters and neutral fat and was deposited, for the most part, at the periphery of the hepatic lobule.

When the low fat ration was fed to rats for 23 days (Figure 13B), the fat deposited consisted largely of neutral fat, and was found in all parts of the hepatic cell.

In the group of animals maintained on the experimental rations for a period of 32 days, still greater quantities of fat were deposited in the liver of animals fed the high fat diet (Figure 14A) than had been observed in the control

Explanation of Figure 12

Scarlet Red Fat Stain

12 A. Diet: Normal, full calories, sacrificed with ether.

The very dark spherical particles and droplets are neutral fat. Tiny dark specks within the cells are cholesterine esters.

12 B. Diet: Normal, full calories, sacrificed with an electric shock.

Same as 12 A.

12 C. Diet: Normal, restricted calories.

The fat appears as small droplets between the cells and as tiny dark specks within the cells.

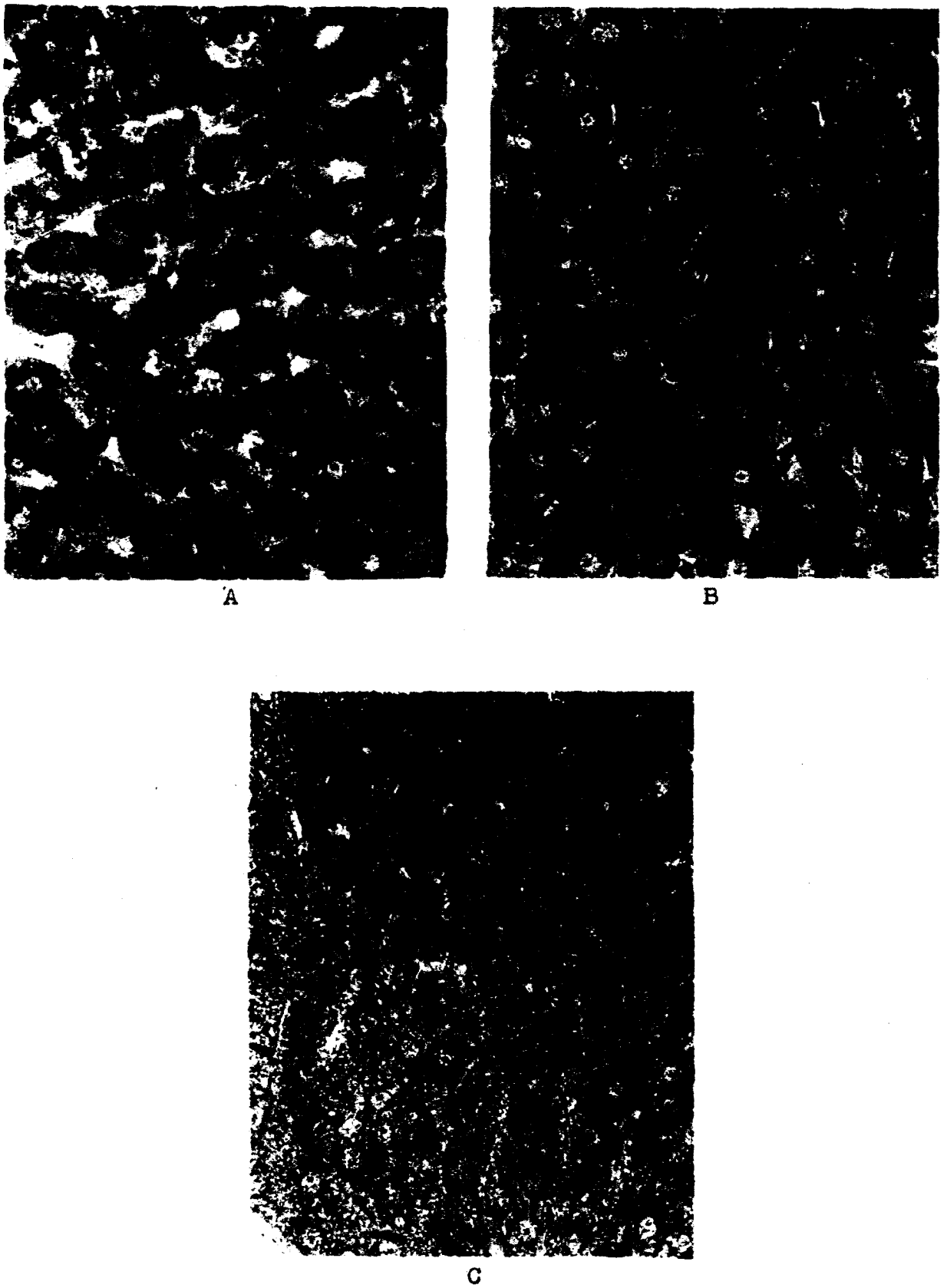


FIGURE 12. FAT IN HEPATIC TISSUES OF NORMAL CONTROL RATS: SCARLET RED STAIN

Explanation of Figure 13

Scarlet Red Fat Stain

13 A. Protein-free diet:
20 per cent fat,
full calories,
fed for 23 days.

The darkest droplets are neutral fat; lighter ones are cholesterine esters. Very light staining particles are lipins.

13 B. Protein-free diet:
no fat, full calories,
fed for 23 days.

Same as above.
Very great increase in neutral fat droplets.

A

B

FIGURE 13. FAT IN HEPATIC TISSUES OF RATS FED LOW NITROGEN DIETS OF HIGH AND LOW FAT CONTENT FOR 23 DAYS: SCARLET RED STAIN

groups or the animals fed the experimental rations for 23 days (Figure 13A). When the former diet was fed at full caloric intake, the fat present in the liver consisted mainly of cholesterine esters, although large amounts of neutral fat and lipins were present. (See Figure 14A). In the livers of animals fed the low nitrogen ration containing no fat (Figure 14B), the fat deposits were neutral fat, with smaller amounts of cholesterine esters.

When the animals receiving the high and low fat diets were restricted in caloric intake, the concentrations of fat in the liver were 5 and 3 per cent respectively (See Figures 14C and 14D). Only occasional fat droplets could be seen.

The addition of methionine as a dietary supplement increased the quantities of fat in the livers of both the animals fed the high and the low fat rations at full caloric intake, these values being 15 and 21 per cent respectively. The deposited fat consisted largely of cholesterine esters when the high fat diet was fed (Figure 15A), and of neutral fat when the low fat diet was administered (Figure 15B).

When the caloric intake of the animals was restricted to one-fourth of the normal ingestion and methionine supplemented the diet (Figures 15C and 15D), the quantity

Explanation of Figure 14

Scarlet Red Fat Stain

14 A. Protein-free diet:
20 per cent fat,
full calories.

Greater quantities of neutral fat are found in this section than in others.

14 B. Protein-free diet:
no fat,
full calories.

Fat droplets in this section are mainly cholesterine esters.

14 C. Protein-free diet:
20 per cent fat,
restricted calories.

Very small quantities of lipins.

14 D. Protein-free diet:
no fat,
restricted calories.

Very small quantities of neutral fat.

The darkest droplets are neutral fat; medium staining particles are cholesterine esters; lighter particles are cholesterine fatty acid mixtures and lipins.

Explanation of Figure 15

Scarlet Red Fat Stain

15 A. Protein-free diet: 20 per cent fat supplemented with methionine, full calories.

Striking increase in the quantity of cholesterine esters and lipins.

15 B. Protein-free diet: 20 per cent fat supplemented with methionine, full calories.

Great increase in neutral fat and cholesterine esters.

15 C. Protein-free diet: 20 per cent fat supplemented with methionine, restricted calories.

Mostly lipins, some cholesterine fatty acid mixtures as indicated by small dark droplets.

15 D. Protein-free diet: no fat, supplemented with methionine, restricted calories.

Some neutral fat, larger quantities of lipins and cholesterine fatty acid mixtures.

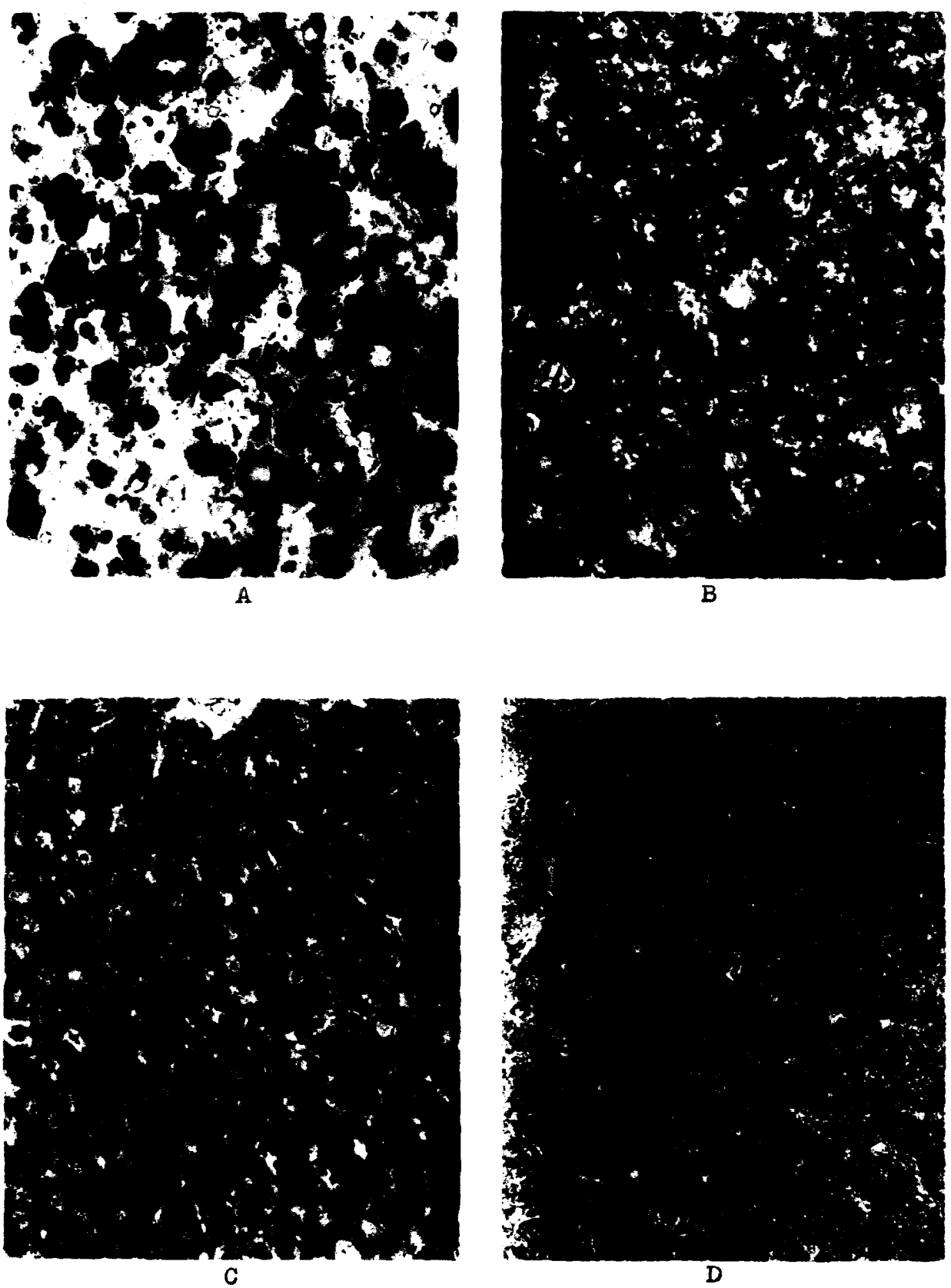


FIGURE 14. FAT IN HEPATIC TISSUES OF RATS FED LOW NITROGEN DIETS OF HIGH AND LOW FAT CONTENT: SCARLET RED STAIN

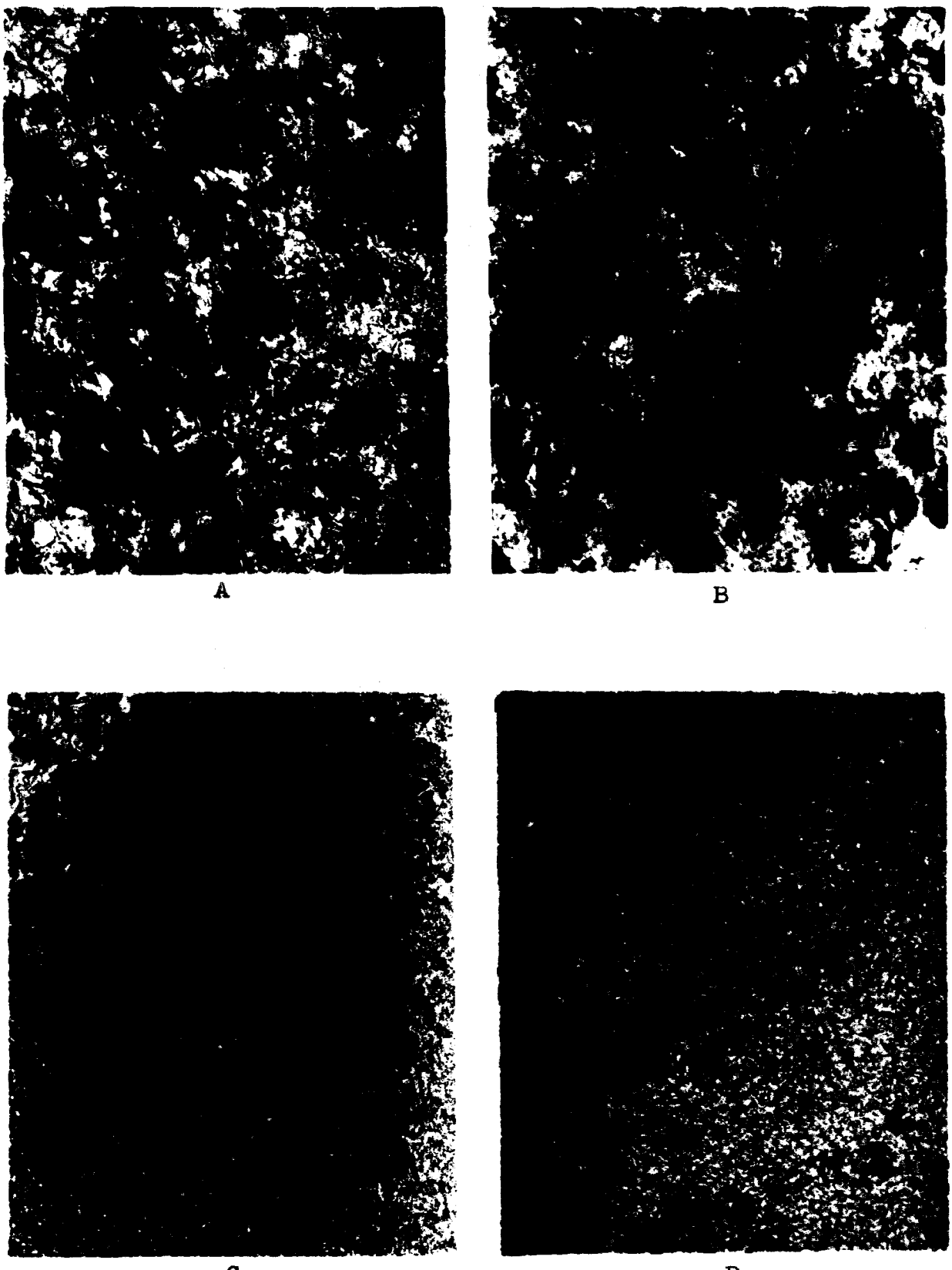


FIGURE 15. FAT IN HEPATIC TISSUES OF RATS FED LOW NITROGEN DIETS OF HIGH AND LOW FAT CONTENT SUPPLEMENTED WITH METHIONINE: SCARLET RED STAIN

of fat in the liver decreased markedly and approached that of the normal animal (Figure 12C). The fat deposited seemed largely to be lipoidal fat.

Nile Blue Sulfate

The findings when Nile Blue Sulfate was used to selectively stain the liver microstains were essentially those of the observations reported for Scarlet Red. Therefore, these will not be discussed at length (See Figures 16, 17, 18 and 19).

It is interesting that in the animals fed the supplement of methionine and offered the low and high fat rations (Figures19A and 19B) at full caloric intake, large quantities of neutral fat were deposited as compared to the other groups. No neutral fat was present in the livers of animals restricted to one-half of the normal food intake (Figures 18C, 18D, 19C and 19D).

Explanation of Figure 16

Nile Blue Fat Stain

16 A. Diet: Normal, full calories, sacrificed with ether.

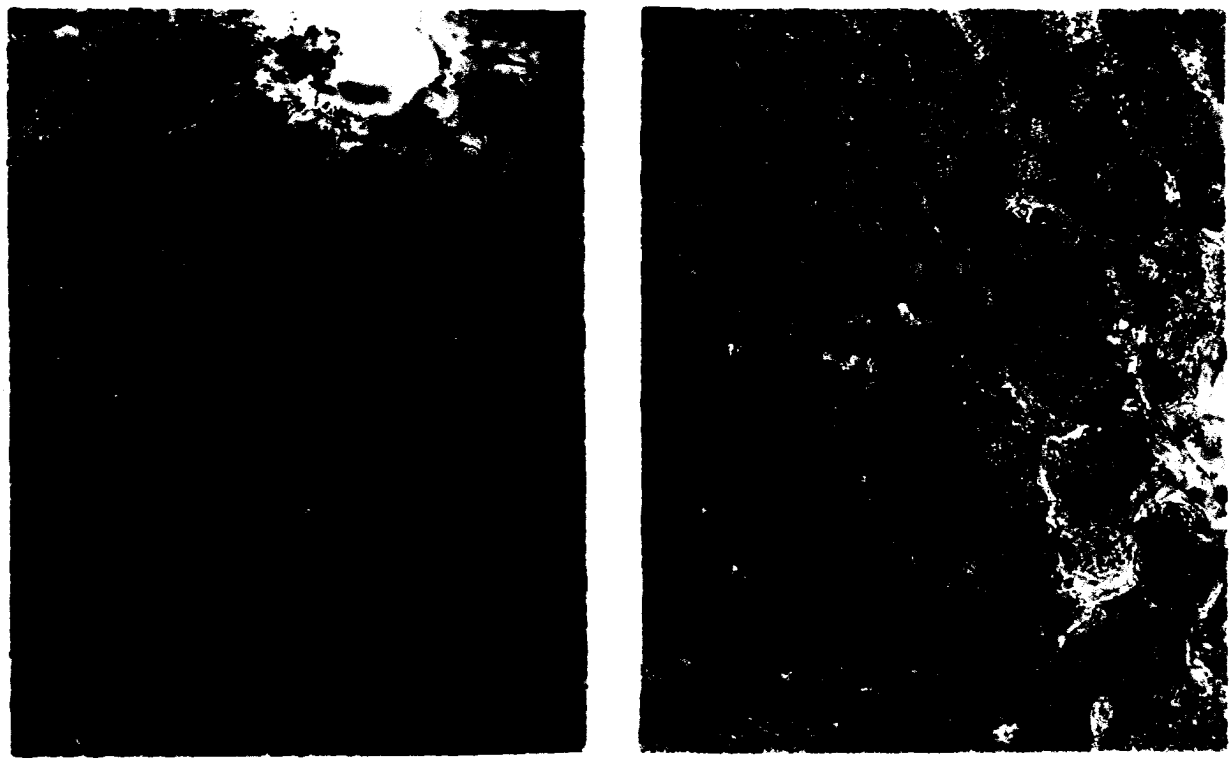
Small quantities of lipoids and fatty acids found between cells.

16 B. Diet: Normal, full calories, sacrificed with an electric shock.

Small quantities of fatty acids and lipoids as indicated by dark blue droplets.

16 C. Diet: Normal, restricted calories, sacrificed with an electric shock.

Neutral fats and cholesterine predominate.



A B

C

FIGURE 16. FAT IN HEPATIC TISSUES OF NORMAL CONTROL RATS: NILE BLUE SULFATE STAIN

Explanation of Figure 17

Nile Blue Sulfate Fat Stain

17 A. Protein-free diet: 20 per cent fat, full calories, fed for 23 days.

Large quantities of neutral fat found at periphery of hepatic lobule.

17 B. Protein-free diet: no fat, full calories, fed for 23 days.

Mostly lipoids and fatty acids, no neutral fat.

A

B

FIGURE 17. FAT IN HEPATIC TISSUES OF RATS FED LOW NITROGEN DIETS OF HIGH AND LOW FAT CONTENT FOR 23 DAYS: NILE BLUE SULFATE STAIN

Explanation of Figure 18

Nile Blue Sulfate Fat Stain

18 A. Protein-free diet: 20 per cent fat, full calories.

Small quantities of neutral fat, lipoids and fatty acids; greater amounts of cholesterine near periphery of hepatic lobule.

18 B. Protein-free diet: no fat, full calories.

Greater quantities of neutral fat; less cholesterine.

18 C. Protein-free diet: 20 per cent fat, restricted calories.

Mostly lipoids or fatty acids as indicated by dark droplets. Very small amounts, however.

18 D. Protein-free diet: 20 per cent fat, restricted calories.

Greater quantities of neutral fat; more evenly dispersed through hepatic cells.

Explanation of Figure 19

Nile Blue Sulfate Fat Stain

19 A. Protein-free diet: 20 per cent fat, supplemented with methionine, full calories.

Small droplets of neutral fat, cholesterine, and fatty acids. Finely dispersed throughout the cell.

19 B. Protein-free diet: no fat, supplemented with methionine, full calories.

Here neutral fat as indicated by darkest droplets, and cholesterine predominate. Found in larger quantities at hepatic periphery.

19 C. Protein-free diet: 20 per cent fat, supplemented with methionine, restricted calories.

Some neutral fat between cells; more cholesterine shown as dark specks within cells.

19 D. Protein-free diet: 20 per cent fat, supplemented with methionine, restricted calories.

Cholesterine is finely dispersed throughout cells, as indicated by dark specks, some fatty acids also (dark blue droplets).

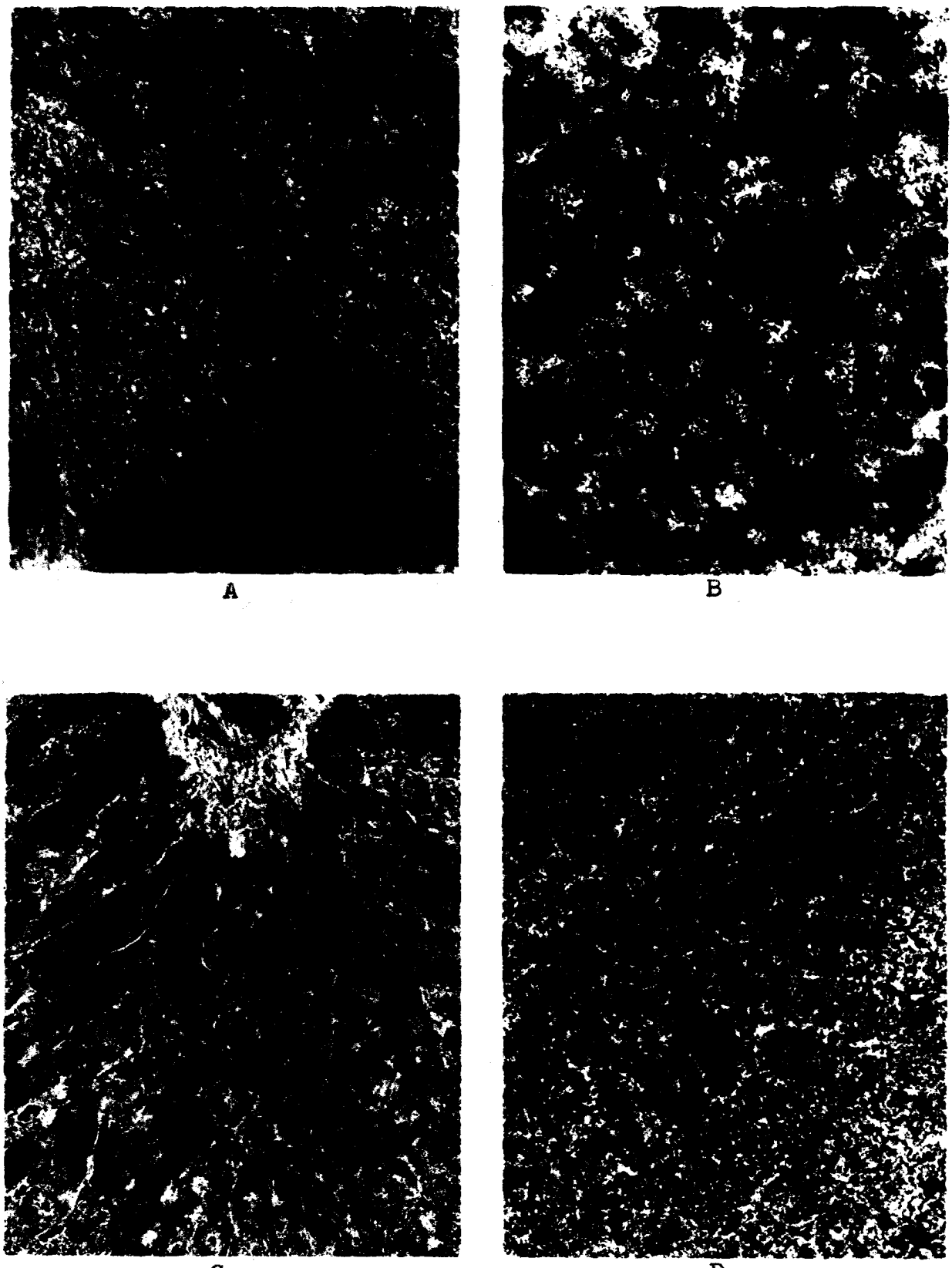


FIGURE 18. FAT IN HEPATIC TISSUES OF RATS FED LOW NITROGEN DIETS OF HIGH AND LOW FAT CONTENT: NILE BLUE SULFATE STAIN

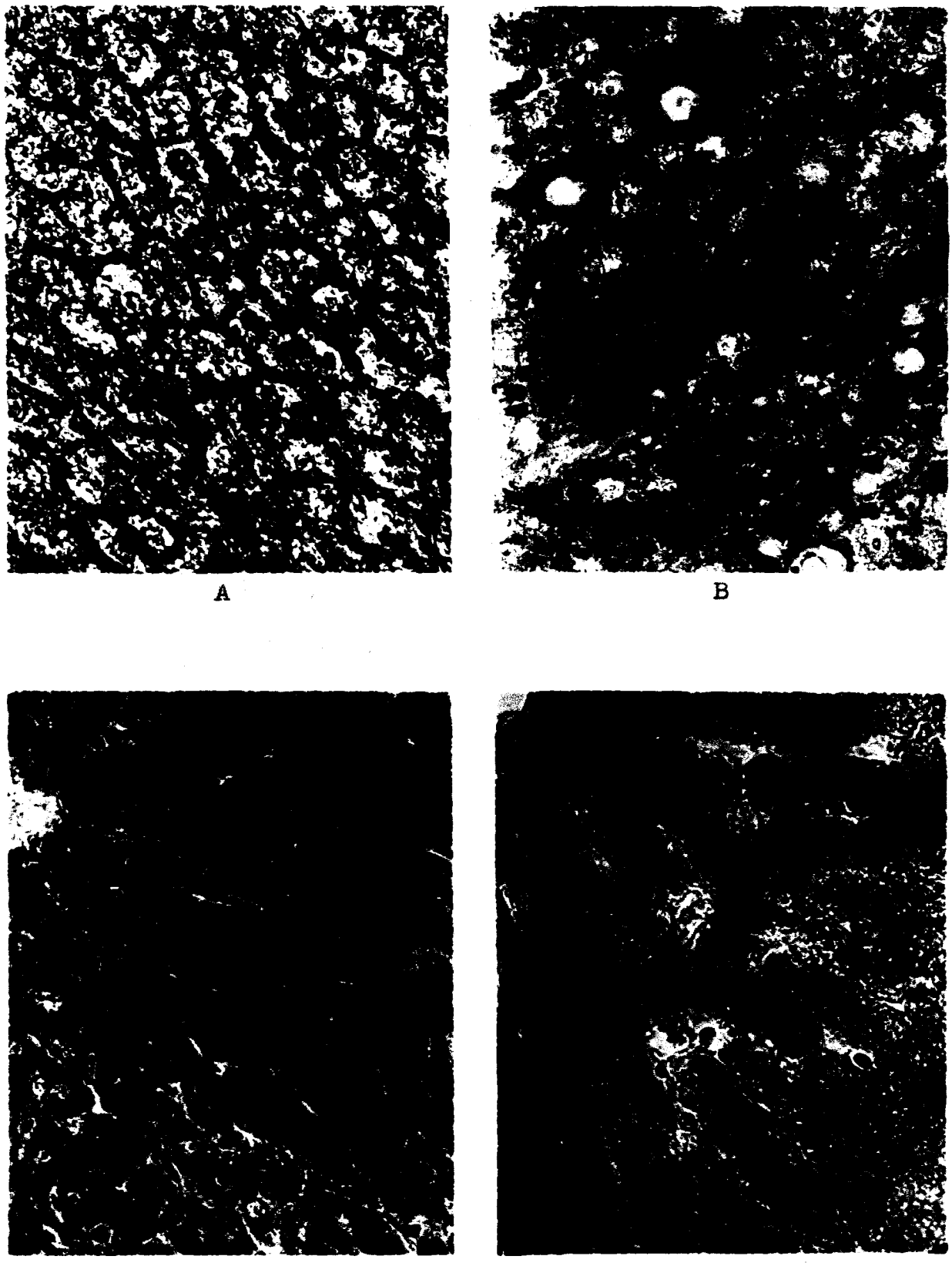


FIGURE 19. FAT IN HEPATIC TISSUES OF RATS FED LOW NITROGEN DIETS OF HIGH AND LOW FAT CONTENT SUPPLEMENTED WITH METHIONINE: NILE BLUE SULFATE STAIN

Summary of Histological Findings

The overall appearance of the liver suggests strongly that no pathological changes were induced by feeding the respective high and low fat rations, but that the changes observed were due to an altered intracellular metabolism. Although the cytoplasm appeared clumped and large spaces were found within the cells when hepatic tissues from these groups were stained with hematoxylin and eosin, the observations on tissues from comparable sections from the same animal indicate that these spaces had been formerly occupied by fat and glycogen. The quantitative analyses of fat and glycogen support this assumption, revealing that from 3 to 5 times more fat, and from 3 to 8 times more glycogen were present in the hepatic tissues of rats fed the high and low fat rations as compared to those fed the normal diet. This would account for the swollen condition of the individual cells as shown by hematoxylin and eosin staining. It seems evident, however, that when methionine was added to the experimental rations, that the general appearance of the liver was improved. The cytoplasm seemed less clumped, fewer spaces were found within the cells, cellular components were more evenly distributed, and the cellular membrane seemed stronger, as indicated not only by hematoxylin and eosin staining, but

also by the fat and glycogen stains. It seems possible, then, that methionine exerted a protective action when the protein-free rations containing fat or without fat were fed at full caloric intake.

On the other hand, when the caloric intake of the animals was restricted, striking differences occurred from that described above. The presence of faintly staining areas in which cell outlines were obliterated and nuclei which stained faintly, if at all, suggested that an abnormal protein had been formed (Benbrook*). The change in the percentage of nitrogen in the livers of these animals supports this assumption. It may be possible, according to the hypothesis of Samuels (1946), that since the body adapts to changes in diets by a shift in the proportions of enzymes and other metabolites required to metabolize food, the cellular changes observed may have been influenced, in part, by this factor. It is also quite possible, that since food intake was restricted to one-fourth of the normal ingestion resulting in decreased intakes of protein, fat, and carbohydrate, the faintly straining areas may represent altered or inactive hepatic tissue.

The striking effect of methionine in bringing the appearance of the livers of the animals in this group to

*Dr. E. A. Benbrook, in conference with the author.

that of the normal rat may be due to the fact that it provided a critical moiety necessary for the elaboration of vital hepatic proteins, possibly enzymatic in nature. That the beneficial effects are more evident in the hepatic tissues of animals fed protein free-fat free rations than in the tissues of animals fed rations containing fat suggests that fat may serve in lieu of methionine. This observation is also supported by the fact that sections of liver from the rats fed the low calorie-high fat diet do not show the changes described to as marked a degree as those from the comparable group of rats fed the low fat ration. That methionine exerted a protective effect is corroborated with observations recorded previously in this thesis relating to the ratio of vitamins to nitrogen in hepatic tissue, the concentrations of urea and amino nitrogen in the blood, and the partition of nitrogen in the urine.

TABULAR SUMMARY OF RESULTS

So many data had been accumulated in the present investigation that it is difficult to see the full picture. In order to facilitate comparisons and permit future study and interpretation of the data, findings have been summarized in Table 23.

In order to permit the compilation of the data on one page, the table has been designed to show the influence of the supplementation of the four experimental diets with methionine. In each column the first figure represents the value for a specific determination that was characteristic of the unsupplemented group; the second figure, that of the group which received methionine.

TABLE 23. INFLUENCE OF METHIONINE ON VARIOUS BODY CONSTITUENTS

Determination	Unit	High fat 56 calories	Low fat 56 calories	High fat 14 calories	Low fat 14 calories
Body weight	gm.	248 to 247	246 to 239	223 to 222	223 to 231
Integrity of hepatic tissue		Improved	Improved	Improved in some cases	Improved
Nitrogen balance	mg./5 da.	-288 to -215	-290 to -236	-514 to -528	-1465 to -495
Nitrogen partition					
Total N	mg./5 da.	180 to 188	180 to 164	459 to 450	1389 to 450
Urea N	mg./5 da.	79 to 68	85 to 61	330 to 207	875 to 234
Ammonia N	mg./5 da.	5 to 10	2 to 16	42 to 135	349 to 97
Creatinine N	mg./5 da.	14 to 17	18 to 4	14 to 23	28 to 27
Allantoin N	mg./5 da.	80 to 64	61 to 62	71 to 72	81 to 76
Amino N	mg./5 da.	4 to 11	11 to 8	9 to 9	4 to 14
Urea N to total N	%	44 to 36	47 to 37	72 to 46	63 to 52
NH_3N to total N	%	0.3 to 10	1 to 10	9 to 30	25 to 22
NH_3N to urea N	%	5 to 30	3 to 30	13 to 50	40 to 41
Blood amino N	mg.%	26 to 20	47 to 31	34 to 27	18 to 18
Blood urea	mg.%	6 to 5	2 to 3	6 to 8	31 to 16
Liver					
Moisture	%	68 to 63	70 to 65	71 to 71	73 to 71
Fat	%	13 to 15	11 to 21	5 to 4	3 to 4
N (dry-fat free)	%	13.5 to 10.0	12.2 to 13.4	15.5 to 15.4	15.0 to 15.7
Riboflavin	mcg.	118 to 93	54 to 123	112 to 61	94 to 70
Niacin	mcg.	803 to 718	679 to 955	676 to 391	423 to 732
Glycogen	%	1.8 to 1.7	2.5 to 2.4	2.2 to 1.8	0.7 to 2.5
Weight (dry-fat free)	gm.	1.6 to 1.8	1.5 to 1.4	1.3 to 0.9	1.1 to 1.3
Adrenal weight	mg.	39.7 to 41.2	41.7 to 45.2	41.8 to 41.2	41.7 to 39.9

SUMMARY

In 1945 and 1946, Swanson and coworkers reported the results on the nitrogenous metabolism of a systematic reduction of the number of calories offered to rats maintained on a low protein diet containing 0, 5, 10, 15, and 20 per cent of fat. Most striking observations were made when the diet contained no fat. Omission of this nutrient from the ration when the calories provided were restricted to 25 per cent of the adequate intake resulted in a doubling of the rate of protein catabolism that was characteristic of rats fed a ration containing 20 per cent fat, inadequate for energy needs. In subsequent studies of this body-sparing effect of fat, these investigators observed that the supplementation of the restricted low fat ration with methionine resulted in a reduction in the catabolism of body tissue. For example, the nitrogen balance improved until it approximately equalled that of rats fed the same quantity of a diet containing 20 per cent fat. The mechanism of the action of fat and methionine in the reduction of excretion of urinary nitrogen was not apparent in these studies.

In the present investigation, the effects induced by feeding fat and methionine to rats maintained on a low nitrogen diet have been studied from as many angles as

possible in the hope that further understanding of the interrelations of fat, protein, and carbohydrate in metabolism in this specific experimental situation might be obtained. Male, albino rats were fed low nitrogen diets fortified with adequate amounts of mineral salts and vitamins. One of the rations was deficient in fat, except that supplied in two drops of Wesson oil and cod liver oil daily; the other contained 20 per cent of fat. The rations were fed ad libitum for 14 days, then force-feeding was initiated, 56 calories per day per 300 gm. rat being supplied. After a 4-day period of adjustment to force-feeding, nitrogen balances were determined for a 5-day period.

At the end of 23 days, one-half of the rats receiving each diet was divided into four groups and force-fed either (1) the same diets at the same caloric intake as had been administered previously, or (2) the same diets at 25 per cent of this caloric intake. The remaining animals were divided into four groups and placed on similar experimental regimes; in this instance, however, 44 mg. of methionine was added daily to the high and low fat diets offered at the two planes of energy intake. After a 4-day period of adjustment to these procedures, nitrogen balances were again determined over a 5-day period. The animals were then sacrificed, samples

of blood were taken, and the liver removed. Samples of urine collected in the balance test were prepared for nitrogen partition and for chromatography of urine amino acids. The blood was analyzed for the respective concentrations of urea, amino nitrogen, and alkaline phosphatase; the liver for moisture, fat, nitrogen, riboflavin, niacin, and glycogen. The weights of livers and adrenal glands were determined, and the histology of the liver studied. Comparable groups of animals were used for the determination of glucose tolerance. These data were compared with similar observations made on normal rats fed the stock colony ration.

The marked body sparing actions of fat and methionine that were demonstrated in earlier experiments, were confirmed in these studies using the force-feeding technique. When full calories were fed, the supplementation of the high fat-protein free rations with methionine reduced the negativity of the nitrogen balances from 288 to 215 mg. When calories were restricted, catabolism was increased in both groups, body tissue being used as a source of energy. The surprising observation, however, was the tremendous output of nitrogen in the urines of rats given the low fat diet. The supplementation of the ration of these animals fed restricted calories with methionine resulted in a phenomenal decrease in the quantity of urinary nitrogen excreted, nitrogen balances changing from -1465 to

-495 mg. over the 5-day period. The latter balance approximated that of the rats given the low-calorie diets containing fat.

The partition of urinary nitrogen when fat was omitted from the ration revealed that the increased excretion of nitrogen observed when caloric intakes were restricted could be accounted for by increases in the quantities of urea and ammonia, that of ammonia being disproportionately large. Methionine-supplementation of the low calorie-low fat ration returned the excretion of total urinary nitrogen to a quantity characteric of rats fed the high fat diet in restricted quantities. There were marked decreases in the respective concentrations of urea and ammonia. However, the proportion of these constituents to each other, which had been distorted in the urines of the animals fed the unsupplemented rations, remained abnormal.

It is interesting to note that chromatographic analyses revealed the presence of all amino acids tested in the urine except methionine. In the case of the rats receiving inadequate calories, the quantities of aspartic acid and glutamine were high.

The concentration of amino nitrogen in the blood was high in both experimental groups receiving the unsupplemented diets at full calorie intakes; supplementation of these rations with methionine induced a striking reduction

in this constituent. When the caloric intakes of the animals receiving the high fat ration were restricted, concentrations of blood amino nitrogen rose even higher than had been observed when full calories were fed. Methionine was effective in bringing about a slight reduction in these values. The concentrations of amino nitrogen in the blood of animals fed the low fat ration and restricted in caloric intake were essentially that of the normal animal.

The concentration of blood urea was low when the two test diets were fed at full caloric intake. When the low fat ration was restricted as to calories, however, an increase in the quantity of this constituent in the blood was observed that paralleled the increased quantities of nitrogen excreted by these rats. Here again, methionine was effective in bringing the level of urea to normal, reflecting undoubtedly the concomitant reduction in the quantity of total nitrogen excreted in the urine. In general, the data relating to nitrogen metabolism suggest that a break occurs in the urea-producing mechanisms when the protein-free diets are fed, a disturbance that is accentuated by removal of dietary fat. The high excretion of ammonia by rats fed the restricted low fat ration may reflect a necessary metabolic by-path, since the normal animals excreting equally large quantities of nitrogen seem to be able to effect the synthesis of urea without difficulty.

When caloric intakes were adequate, serum alkaline phosphatase concentrations in animals receiving the low fat ration showed a definite increase as compared to blood concentrations of this constituent in animals fed dietary fat. Supplementary methionine in the ration exerted a slight effect in returning these values to normal. Alkaline phosphatase concentrations in blood serum were also increased with the restriction of caloric intakes to 25 per cent of the normal ingestion. Methionine supplementation, in this instance, induced an increment in the concentration of this blood constituent in the animals fed the low fat ration.

That the ability of the animals to metabolize carbohydrate is disturbed when the protein and/or fat constituents of the ration are omitted was indicated by these experiments. The somewhat distorted glucose tolerance curve characteristic of the animals fed the synthetic diets containing fat at adequate caloric intakes became more diabetic-like in character when fat was omitted from the ration. In all experimental groups except the series fed the low fat diet supplemented with methionine and supplying 14 calories per day, the fasting levels of glycogen were high. It is interesting that this is the group in which nitrogen catabolism was reduced markedly by the administration of methionine. All curves became

more like that of the normal animal when the amino acid was added to the low fat ration, both when full and restricted calories were administered. Whether or not additional dietary thiamin would change this picture needs investigation.

The relative proportion of nitrogen in hepatic tissues was lower when animals were fed either the high or low fat diets at full caloric intake than that in the tissues of the normal animal. When calories were restricted, however, the percentages of nitrogen were increased, and were approximately the same for animals fed the high and low fat rations, animals fed the diets supplemented with methionine, and the normal animal restricted as to food intake.

The concentration of fat in fresh hepatic tissues of the animals fed the high and low fat rations was from 2 to 3 times greater than that in the liver of the normal animal. Methionine when added as a supplement to the diets induced another increment in the fat content of the livers. The value was five times that of the normal animal when rats were fed the low fat rations. When the caloric intakes of these animals were restricted, the fat content of hepatic tissues was approximately that of a normal animal with a restricted food intake. In this instance, methionine supplementation of the rations did not induce an increment in hepatic fat.

When the protein-free rations were fed, decreased quantities of riboflavin were found in the liver. Omission of fat from the diet resulted in further alteration in the concentration of this vitamin, it being reduced by 75 per cent in animals fed full calories. Supplementary dietary methionine brought the quantity of this liver constituent to a level characteristic of animals which received fat in the diet. Likewise, the concentration of niacin fell in the livers of rats fed both test diets at full caloric intake; greatest losses, however, occurred in rats receiving the low fat rations. Here again, methionine exerted a beneficial effect. Ratios of each vitamin to hepatic nitrogen indicated that the decrement in the quantity of each vitamin, except in one instance, paralleled losses in hepatic nitrogen. The exceptions occurred when the low fat rations were fed; in the case of riboflavin at full caloric intake, in the case of niacin when the low fat diet was administered at restricted caloric intake. It seems possible, therefore, that dietary fat defers the disintegration of some important enzyme systems in the liver when protein-free rations are fed.

This concept is supported also by histological studies of hepatic tissue. These observations showed clearly that methionine was responsible for improved cellular structure,

striking improvement in the quality of the cell membrane, and a more even distribution of cellular components. When caloric intakes were restricted, the faintly staining areas in hepatic tissues which were common to both the normal animals and those fed the high and low fat rations disappeared in large measure when methionine supplemented the high fat ration.

Whether the effect of methionine is mediated through maintenance of the status quo of the cell itself or through provision of building blocks for the elaboration of enzyme systems lost in the depletion processes imposed, it seems quite apparent that the body has a need for this amino of the same nature as the need for vitamins or for other essential dietary components. That this effect is not one attributable to the methyl group per se is indicated by the fact that in all experiments adequate quantities of labile methyl groups were provided through dietary choline.

That there too is a vital need for dietary fat, over and above its role in the provision of calories and unsaturated fatty acids, has been indicated by abnormalities in metabolism and nutritional well-being when fat is omitted from the diet. The marked distortion in concentrations of specific body components and metabolites when fat is omitted from the ration support this hypothesis. That methionine and fat may exert similar influences on certain phases of protein metabolism is suggested also by changes induced when each supplements the low fat ration.

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APPENDIX

LIST OF ANALYTICAL PROCEDURES IN THE APPENDIX

Page

LIST OF TABLES IN APPENDIX

Page

Page

Page

Page

Page

LIST OF FIGURES IN THE APPENDIX

ANALYTICAL PROCEDURES

Nitrogen Balance

Method of Analysis for Urine, Feces and Food

The Kjeldahl-Gunning procedure was used in all determinations of total nitrogen.

Urine. Aliquots of urine extract of appropriate size were digested with 20 ml. of concentrated sulfuric acid, 10 gm. of potassium sulfate, and 0.7 gm. of mercuric oxide, for one and one fourth hours, then allowed to cool. The samples were diluted with 200 ml. of cool tap water. A few grains of zinc were added with 50 ml. of concentrated sodium hydroxide-sodium sulfide solution. This amount was a slight excess of that needed to neutralize the acid added. The ammonia released on heating was distilled into a known amount of standard 0.1 normal hydrochloric acid. The acid not neutralized by the ammonia was titrated with standard sodium hydroxide solution, approximately 0.1 normal, with the use of a methylene blue-methyl red indicator. In the appendix are found data illustrating the accuracy of the author in applying this technique to the analysis of a standard creatinine solution.

TABLE 1. CONCENTRATION OF NITROGEN IN A STANDARD CREATININE SOLUTION

Aliquot	Quantity of nitrogen present	Theoretical quantity of nitrogen	Per cent recovery
ml.	mg.	mg.	%
1	9.26	9.28	99.78
2	9.26	9.28	99.78
3	9.26	9.28	99.78
4	9.25	9.28	100.75
		Av.	100.02

TABLE 2. NITROGEN RECOVERED FROM CAGES SPRINKLED WITH ALIQUOTS OF STANDARD AMMONIUM SULFATE SOLUTION

Cage number	Total nitrogen	Theoretical quantity of nitrogen present	Recovery of nitrogen
	mg.	mg.	%
1	10.97	10.94	100.2
	10.99		100.4
	11.00		100.5
2	10.99	10.94	100.4
	11.00		100.5
	11.03		100.8
3	11.01	10.94	100.6
	10.94		100.0
	10.99		100.4
		Av.	100.4

Feces. Twenty-five ml. aliquots of the fecal digests were measured by means of a 25 ml. pipette. These were digested with 35 ml. of concentrated sulfuric acid and 15 grams of a potassium sulfate-mercuric oxide mixture for two hours, and treated similarly to the urine samples.

Dry Diets. Weighed portions of the solid low and high fat diets and of these diets supplemented with methionine were transferred quantitatively to Kjeldahl flasks and were treated as had been the fecal digests.

Food Homogenates. The possible introduction of error in the delivery of the amount of nitrogen desired as a result either of the homogenization or feeding procedures made it desirable to check the quantity of nitrogen actually present in a day's aliquot of the homogenized ration as it had been fed to the animals in this experiment.

Twenty-four ml. of the diet were measured into a 125 ml. Erlenmeyer flask, using the syringe as described earlier. After addition of 50 ml. of 20 per cent hydrochloric acid, this mixture was autoclaved at 15 lbs. pressure for two hours. The samples were cooled and then transferred quantitatively rubbed through a fine sieve, into 250 ml. volumetric flasks. They were made to volume and mixed thoroughly. Twenty-five ml. aliquots were withdrawn and analyzed for nitrogen in the same manner as described for the dry diets. Two aliquots of each diet

were measured from the syringe and digested. Each digest was analyzed for nitrogen in duplicate.

Nitrogen Partition of Urine

General

Urine samples collected for the partition of nitrogenous constituents were analyzed for total nitrogen, urea, ammonia, allantoin, creatinine and amino nitrogen. The samples, which had been previously covered with a layer of toluene and stored in the refrigerator, were brought to room temperature before determinations were made. Pipettes calibrated by the Bureau of Standards were used for all analyses.

The methods used for the analysis of urinary nitrogen constituents are shown in the following table.

Table 3. Methods for the Partition of Nitrogen

Urinary component	Method	Reference
Total nitrogen	Kjeldahl-Gunning	Kjeldahl, 1893 Gunning, 1889
Urea	Colorimetric	Ormsby, 1942
Ammonia	Distillation-Nesslerization	Parnas and Heller, 1924 du Vigneaud, 1933
Allantoin	Colorimetric	Young and Conway, 1942
Creatinine	Colorimetric	Folin, 1914
Amino nitrogen	Van Slyke-gasometric	Van Slyke et al., 1943

Total Nitrogen

Twenty-five ml. aliquots of urine samples were analyzed by the Kjeldahl-Gunning procedure as described earlier.

Urea

The method developed by Ormsby (1942) depends on a reaction first described by Fearon (1939). When urea is heated with diacetylmonoxime in acid solution, a yellow color develops, deepening with subsequent oxidation with potassium persulfate. Many substituted ureas give a red color, but only urea yields a yellow pigment, as measured

under the conditions of this method.

Appropriate samples of urine, estimated so as to contain from 0.10 to 0.20 mg. of urea were placed in test tubes with an amount of distilled water to make the total volume to 3.0 ml. One ml. and 2 ml. aliquots of urea standard containing 0.1 mg. urea per ml. were added to similar tubes, with additional water to make the total volume to 3.0 ml.

To each tube was added 5.0 ml. of accurately measured concentrated hydrochloric acid and 0.5 ml. of diacetyl-monoxime. The contents of the tubes were mixed by rotation and placed in a vigorously boiling water bath for exactly 10 minutes. To prevent evaporation during this period, hollow glass bulbs were placed on the tops of these tubes. The tubes were removed from the water bath simultaneously and cooled for 2 minutes in a running water bath. A round wire test tube rack which held approximately fifty test tubes, facilitated this procedure.

To the cooled tubes, 0.25 ml. of 1 per cent potassium sulfate solution was added with shaking. An interval timer was used to accurately measure the 10 second intervals between addition of the reagent to these samples. To insure thorough mixing, the tubes were inverted two or three times in this step of the procedure.

After the contents of the tubes had been poured into standard calibrated Klett-Summerson tubes, the intensity of color was read at intervals in a Klett-Summerson photoelectric colorimeter, using a blue No. 42 filter. Since the time required for development of maximum color depended on the concentration of urea, tubes were read at 5, 10 and 15 minute intervals. The maximal reading, usually obtained at the 10 minute interval, was taken for calculation of results.

Standard curves run earlier with a pure urea sample had indicated that the color concentration maintained a linear relation to the quantity of urea present in the range used in this experiment. Therefore, results were calculated by reference to the standard. Since the slope of the curve depended on the diacetylmonoxime reagent, the same solution was used for all analyses.

This method provided a simple, accurate procedure which required no distillation or aeration as commonly found in other procedures for the determination or urea. Ammonia does not interfere and no preliminary preparation of the sample was necessary. With the size of aliquots used in these determinations, no significant interference from allantoin was noted, as measured from readings of standard urea samples to which known amounts of allantoin

had been added. The use of the blue filter further minimized the small amount of red color which would have been given by allantoin.

Ammonia

The method used for the determination of ammonia involved essentially the distillation of the ammonia into a volumetric flask and determination of the quantity present by nesslerization.

Appropriate samples of urine were placed in the distilling chamber of a micro-distillation unit. These were washed down with small amounts of distilled water. One drop of 1 per cent phenolphthalein and 1.25 ml. of 0.1 normal sodium hydroxide, or enough to develop a faint pink color, were added with 10 ml. of the borax buffer. After attaching the receiver, a 25 ml. volumetric flask into which had been placed 2 ml. of 1:1 sulfuric acid, steam distillation was commenced, continuing until approximately 10 ml. had been distilled. The contents of the flask were diluted to volume and aliquots were taken for analysis.

At intervals, a standard solution of ammonium sulfate containing 1 mg. of nitrogen per 10 ml., or 0.4716 gm. of ammonium sulfate, was distilled as above into 2 ml. of 0.1 normal sodium hydroxide. This mixture was read after

nesslerization against an undistilled standard.

Ten ml. of the diluted distillate were placed in a test tube and one drop of gum ghatti and 3 ml. of Nessler's solution were added. The contents were mixed and transferred to a colorimeter tube. Using filter No. 54, these solutions were read between 10 and 30 minutes after addition of the Nessler's solution. After deducting the value of the reagent blank from all readings, comparison was made against the 10 ml. standard.

The blank contained 10 ml. of a 1 to 50 ml. dilution of 1:1 sulfuric acid. Occasionally 1 ml. of 1 per cent phosphoric acid was distilled through the apparatus and analyzed similarly.

Using this method, 0.005 mg. of ammonia could be determined with less than 5 per cent error.

Allantoin

Appropriate aliquots of urine were added to test tubes graduated at 25 ml. and made up to a total volume of 5 ml. One ml. of 0.5 normal sodium hydroxide was added and the tubes were placed in a vigorously boiling water for exactly 7 minutes. At the end of this time, the tubes were placed in a water bath maintained at 20° C.

After adjustment of the acidity of the solutions to

approximately 0.2 normal, usually requiring very little acid, 1 ml. of 0.5 normal hydrochloric acid was added. Five ml. of a standard allantoin solution, containing 0.02 mg. of allantoin per ml. of 0.01 normal sodium hydroxide, were analyzed simultaneously with the urine samples, after the pH had been adjusted by the addition of 5 drops of 0.5 normal hydrochloric acid. Following the addition of 1 ml. of 0.33 per cent phenylhydrazine hydrochloride solution, the tubes were shaken and placed in a boiling water bath for exactly two minutes. They were immediately immersed in a dry ice bath and chilled to incipient freezing in about 3 minutes.

On removal from the ice bath, 3 ml. of concentrated hydrochloric acid, also chilled to -10° C., were added to each tube with 1 ml. of 1.67 per cent potassium ferricyanide solution. The contents of the tubes were well mixed. After 30 minutes, the tubes were diluted to volume with distilled water. Samples of these solutions were poured into Klett-Summerson colorimeter tubes and read immediately, using a green filter.

Calculation of the allantoin present in the samples was made by comparison to the standard solution.

Amino Nitrogen

The procedure used for the determination of amino nitrogen in urine involves first the removal of urea with urease, then reaction of the amino groups with ninhydrin, triketohydrinedenehydrate, and finally determination of the carbon dioxide evolved therefrom in the Van Slyke manometric chamber.

Appropriate samples of urine were placed in a Thunberg tube and one drop of 0.04 per cent brom-thymol blue was added. One normal sodium hydroxide was added until the color was just blue. When the reaction was thus adjusted, 175 mg. of phosphate buffer of pH 6.2 were added with 0.2 ml. of 1 per cent urease solution and a crystal of thymol. The Thunberg tubes were stopped loosely to retard evaporation of water, but not the escape of carbon dioxide, and incubated overnight at 37° F.

After incubation, one drop of 0.04 per cent brom cresol green and one drop of capryl alcohol were added. Five normal of sulfuric acid was cautiously added until the solutions were just yellow. Two glass beads were placed in the tubes to prevent bumping, and they were boiled for exactly 50 seconds over a micro burner to expel the carbon dioxide.

After the samples had been cooled in tap water below 25° C., 100 mg. of accurately measured ninhydrin were added. The vessels were closed at once with the ground glass stoppers, and evacuated by use of water suction apparatus. It was important to use heavy grease in preparing the Thunberg tubes for use so that the stoppers would not be blown out in the subsequent boiling process. For this purpose, regular Van Slyke grease was used.

After evacuation of the tubes, a heavy piece of rubber tubing, approximately two and one-half inches in length was slipped over the side arm. The side arm was evacuated and immediately closed with a piece of solid glass rod, approximately one and one-half inches long, which had been previously fire polished and covered with a thin film of glycerol. This procedure was necessary to prevent exposure of the sample to the air during the subsequent determination of carbon dioxide in the manometric chamber. The tubes were boiled for exactly 8 minutes in a boiling water bath.

Carbon dioxide evolved from the amino acids in the urine sample was measured in the Van Slyke-Neil Manometric chamber. The carbon dioxide was transferred and measured according to the procedure of Van Slyke et al., (194).

A blank analysis was performed simultaneously with

the samples, substituting 2 ml. of water in the place of urine. The pressure of the carbon dioxide evolved from the carboxyl groups of the amino acids, p_{co_2}, was determined by subtracting the p_2 reading, after absorption of the carbon dioxide with sodium hydroxide, from p_1 the reading after liberation of carbon dioxide from the basic solution with lactic acid. A correction, the reading observed in a blank analysis was subtracted from all readings. Conversion factors were obtained from tables found in the publication of Van Slyke et al., (1943).

Creatinine

The method for the determination of creatinine was one first published by Folin, (1914).

Appropriate samples of urine were placed in 100 ml. volumetric flasks. In similar flasks were placed 1 and 2 ml. of a standard creatinine solution, containing 1 mg. of creatinine per ml. To each flask, 20 ml. of picric acid solution and 1.5 ml. of 10 per cent sodium hydroxide were added. The solutions were allowed to stand 10 minutes. At the end of that time they were diluted to volume with distilled water, mixed, and compared with the standard solution using a Klett-Summerson photoelectric colorimeter.

Calculation of the creatinine in the urine sample was made by comparison to the standard solutions.

Chromatographic Analysis of Urine Amino Acids

General

The method used for chromatographic analysis depends on the differences in partition coefficients between the mobile phase and water saturated filter paper. Within a homologous series of solvents, the amino acids, by virtue of differences in physical properties, migrate on filter paper at different rates. The relative position of the amino acids on the developed chromatogram, however, depends on the solvent used. Hence, by development first in one direction with one solvent, followed by development in a direction at right angles with another solvent, amino acids in a drop of protein hydrolysate placed near the corner of a sheet of paper will give a two dimensional chromatogram characteristic of the solvents used. Advantage is taken of the color reaction of these compounds with ninhydrin to reveal the positions of the amino acids.

This method can be made roughly quantitative by careful preparation of solutions of the solvent, amino acid solutions and hydrolysate, and by accurate measurement and

delivery of samples on to the filter paper.

The samples used for chromatographic analysis were collected over two day intervals. Pairs of rats were placed in metabolism cages at the end of the 18 day depletion period. A wire screen, placed between the cage and pyrex plate served to catch the feces and particles of hair and thus to prevent contamination of the urine samples. At the end of the two day collection period, the screens were removed and brushed free of feces and hair. They were washed several times with a stream of lukewarm water applied under pressure. The washings were added to the original urine sample in the pyrex plate. The contents of the plates were filtered with suction through Whatman filter paper and added to pyrex 600 ml. Erlenmeyer flasks. After the addition of 17 ml. of concentrated hydrochloric acid, they were immediately autoclaved for 15 minutes at 15 lbs. pressure. This procedure served to hydrolyze peptide linkages which may have been present and to preserve the samples from bacterial spoilage.

The solutions were transferred quantitatively to volumetric flasks, and made up to appropriate volumes. Samples were transferred to 12 oz. pharmacy bottles and stored until the time of analysis. The procedure for collection of urine was repeated in the second period after

the dietary modification had been introduced.

Chambers used for the preparation of chromatograms were circular pyrex glass cylinders of diameter. These were fitted with heavy lids and further reinforced with lead bricks, so that no air could enter. Whatman No. 1 filter paper, 18.5 x 22.5 inches, was used for development of the chromatograms.

Standard solutions of nineteen pure amino acids and glutamine were prepared in concentrations of 1 mg. per ml. These were stored in a refrigerator under toluene. In some cases, amino acids with widely differing Rf values were prepared together. In all cases when this was done the spots produced by both amino acids when sprayed with ninhydrin were easily separable.

Thirty lambdas of the hydrolysate and 10 lambdas of the pure amino acid solutions were used because it had been found in preliminary work that this amount gave a color which could be easily compared.

One Dimensional Chromatogram

A horizontal line, approximately two inches from the bottom of the paper, was drawn with an ordinary lead pencil. Thirty lambdas of the hydrolyzed urine sample, adjusted to the same pH as the pure amino acid solutions,

was delivered from a specially calibrated micro pipette in the center of this line and allowed to dry. On either side, spaced approximately 3 inches apart, 10 lambdas of the amino acid solutions and glutamine were placed. The amino acids used were:

phenylalanine	leucine
tyrosine	threonine
glutamic acid	glutamine
histidine	cysteine
norleucine	leucine
arginine	arginine
methionine	norvaline
serine	cystine
histidine	lysine
hydroxyproline	aspartic acid

These solutions were allowed to dry, care being taken that the paper at no time came in contact with an object. The drying process was facilitated by the use of an apparatus which blew a stream of air on to the paper at moderate speed.

The chamber was covered with a one and one-half inch layer of a solution of phenol saturated with water. The ends of the filter paper, vertical to the line on which the amino acid solutions had been placed, were attached to each other securely by paper clips, after small slits had been made in one edge with a razor blade.

The paper, now circular, was carefully lowered into the phenol solution and the top was placed securely on the

chromatographic chamber. The chromatograms were allowed to develop overnight. On the following morning the papers were removed with care and hung in a vertical position to day.

After the papers were completely dry, they were sprayed with a solution of ninhydrin in butyl alcohol, 5 mg. per ml. The instrument used for this procedure had been designed by Dr. Arnoff in the Physics Department at the Iowa State College and is described in the appendix.

The color resulting from the reaction of ninhydrin with the amino acids was developed in a specially built steam chamber designed to give even heating (See appendix). After the paper had been allowed to heat for approximately 20 minutes, it was removed and allowed to cool. Interpretation of the chromatograms was made immediately by comparing the position of migration of the amino acids in the hydrolyzed urine sample to those of the pure amino acids. Over extended periods of time the ninhydrin color fades; therefore, the areas which marked the amino acids were encircled with ink.

Two Dimensional Chromatograms

In order to determine accurately which amino acids were present it was desirable to run a two dimensional

chromatogram of the amino acids which were thought to be present. The migration of the amino acids, as mentioned earlier, in different solvents is characteristic. Therefore a measurement of the movement of the pure amino acid conducted simultaneously with the hydrolysate in two different solvents provided a check as to the specific presence of any one acid.

The filter paper was prepared as before for the one dimensional chromatogram except that after 30 lambdas of hydrolysate had been placed on the paper and dried, 10 lambdas of the pure amino acid was placed directly over it. After the second application had dried, the paper was placed in the phenol solution as described earlier, allowed to stand overnight, and taken out to dry the following morning.

The paper was turned at right angles and placed in a solution of normal butanol with water and pyridine, 1:1:1. Separate papers were prepared for each amino acid which had been indicated to be present in the one dimensional chromatograms. The chromatograms were allowed to develop overnight again and removed the next morning to dry. They were sprayed with ninhydrin as before and heated in the steam chamber.

Thus, earlier findings were confirmed, and a rough

quantitative estimate of the concentration of amino acids present was obtained.

Organ Analyses

Moisture in Liver

After extirpation of the liver by severing the hepatic vessels and ligaments, the gland was removed, trimmed free of connective tissue and blood vessels, and blotted to remove surface blood.

Approximately one gram samples were placed in weighing bottles for the moisture determinations. The color and general appearance were noted at this time. Samples were weighed immediately and then dried to constant weight in an air oven at 105° F. After seven days, the samples were removed from the oven, weighed again, and the per cent moisture calculated.

Fat in Liver

The determination of fat was based on the method of Bloor (1929). In this method, fat is estimated as total alcohol-ether soluble substances. Moisture-free samples of liver were ground in a mortar with one-half teaspoon of acid washed sand and transferred quantitatively to a 125 ml.

Erlenmeyer flask. The weighing bottle and pestle were rinsed with three portions of alcohol-ether mixture. Approximately 25 ml. of a 3 to 1 mixture of absolute alcohol and anhydrous ethyl ether were added and the mixture was boiled on a steam bath with shaking for 5 minutes.

After being cooled to room temperature, the solution was filtered quantitatively through Whatman No. 45 fat-free paper into a 200 ml. volumetric flask. The filter paper holding the ground tissue was washed with several small portions of the alcohol-ether mixture and it, with its contents, was extracted for five hours in a Goldfisch extraction apparatus with anhydrous ethyl ether.

The ether extract, plus rinsings of the extraction cup, was added to the contents of the 200 ml. volumetric flask, and the solution made up to volume with ether. Fifty ml. aliquots of this solution were measured into large weighing bottles, evaporated to dryness on a steam bath, and brought to constant weight in an air oven at 80° F.

The total amount and the per cent fat in the sample were calculated from the data thus obtained.

Total Nitrogen in Liver

Liver samples for the determination of nitrogen were placed in stoppered 125 ml. Erlenmeyer flasks which had been previously weighed. After sample weights had been obtained, the tissue was covered with 50 ml. of 20 per cent hydrochloric acid and autoclaved at 15 lbs. pressure for two hours. These solutions were cooled and pressed through a medium sieve into 100 ml. volumetric flasks. The samples were diluted to volume with distilled water and 10 ml. aliquots taken for analysis of nitrogen by the Kjeldahl-Gunning procedure.

Vitamins in Liver

Nicotinic acid and riboflavin determinations were made on the livers of the animals in this experiment using the microbiological method of Landy and Dickens (1942).

After removal of the liver, it was trimmed free of adhering fat and connective tissue and blotted to remove excess blood. Samples were then transferred to 125 ml. Erlenmeyer flasks and weighed.

Tissues were homogenized in a Waring Blendor with 100 ml. of sodium acetate-acetic acid buffer of pH 4.5, and made up to a total weight of 200 grams. The solutions were

transferred to half pint jars, covered with tightly fitting metal lids, and frozen immediately in a deep freeze unit. By the use of this method, tissues could be kept in the frozen state for long periods of time without loss of vitamin activity.

Riboflavin. After thawing of the liver samples at room temperature, portions were removed which contained approximately 8 to 10 micrograms of riboflavin. Ten grams were weighed directly into a 125 ml. Erlenmeyer flask. Fifty ml. of 0.1 normal sulfuric acid were added and the contents of the flasks autoclaved for twenty minutes at 15 lbs. pressure.

After being allowed to cool to room temperature, the pH of the samples was adjusted to 4.5 with 2.5 molar sodium acetate. Congo red paper was added directly to the sample so that the end point could be determined precisely. The samples were filtered through Whatman filter paper into a 100 ml. volumetric flask. These were diluted then to volume, and the solutions were transferred to dark green bottles, covered with a layer of toluene, and refrigerated.

Appropriate aliquots of the solutions were added to 100 ml. volumetric flasks and the pH adjusted with brom thymol blue (pH 6.6-6.8) with sodium hydroxide. A dark greenish-blue color was chosen as the end point. The

flasks were made up to volume, giving a final dilution of approximately 0.02 micrograms per ml. Lactobacillus casei was used as the assay organism.

Nicotinic Acid. Ten grams of the thawed liver samples were weighed into 125 ml. Erlenmeyer flasks; this quantity contained approximately 30 to 40 micrograms of nicotinic acid. After the addition of 50 ml. of 1 normal sulfuric acid, the samples were autoclaved at 15 lbs. pressure for 25 minutes. They were cooled to room temperature, filtered into 100 ml. volumetric flasks, and diluted to the maniscus. The samples were transferred to green bottles, covered with a layer of toluene, and stored in the refrigerator.

At the time of assay, appropriate aliquots of the solutions were delivered into 100 ml. volumetric flasks and the pH adjusted to 6.6 using concentrated sodium hydroxide with brom thymol blue. They were then made up to volume, thus containing between 0.3 to 0.4 micrograms of nicotinic acid per ml. Lactobacillus arabinosus served as the test organism.

Assay Procedures. The basal medium used for both the riboflavin and nicotinic acid assays is shown in Table 4. This medium differed for the two determinations only in that riboflavin was omitted in the assay of riboflavin,

Table 4. Complete Basal Medium Used in the Analysis of Riboflavin and Niacin

Ingredients for 100 tubes	Quantity
Casein hydrolysate	5 gm.
Sodium acetate	6 gm.
Glucose	10 gm.
Asparagine	250 mg.
Tryptophane	100 mg.
Cystine	100 mg.
Salts A	5 ml.
Salts B	5 ml.
Guanine.HCl	5 mg.
Adenine sulfate	5 mg.
Uracil	5 mg.
Xanthine	5 mg.
Bring this portion of basal medium to pH 6.6-6.8	
Thiamine	200 mcg.
Pyridoxine	600 mcg.
p-amino-benzoic acid	200 mcg.
Biotin	1 mcg.
Folic acid	1 mcg.
Riboflavin	400 mcg.
Niacin	400 mcg.
Pantothenic Acid	400 mcg.
Bring basal medium to pH 6.6 with brom-thymol blue to 500 ml. volume.	

and nicotinic acid was not added to the basal medium for the niacin assay.

The standard solution for the riboflavin assay contained 0.05 micrograms riboflavin per ml. The niacin standard solution contained 0.2 micrograms per ml. of nicotinic acid. Forty tubes were used for determining the standard curve, of which 6 were blanks; 5 tubes were used at the 0.5 ml., 1.0, 1.5, 2.0, and 2.5 ml. levels. Three tubes were assayed on the 3.0, 4.0, and 5.0 ml. levels.

Aliquots of the final dilution of the liver hydrolysate contained 1, 2, 3, 4, and 5.0 ml. of solution. These were placed in test tubes, contained in rectangular wire racks. Five ml. of the basal medium were added to each tube and an additional amount of distilled water to make a final volume of 10 ml. The tubes were covered with metal caps, sterilized for 15 minutes at 15 lbs. pressure in the autoclave, and allowed to cool.

The inoculum of the appropriate organism was prepared the day before the assay was set up. Stabs of Lactobacillus casei and Lactobacillus arabinosus were transferred asceptically to tubes of nutrient broth and incubated for 18 to 24 hours in an oven at 37° F. These tubes were centrifuged for 10 minutes at 10 RPM. The supernatant fluid was decanted and 10 ml. of sterile distilled

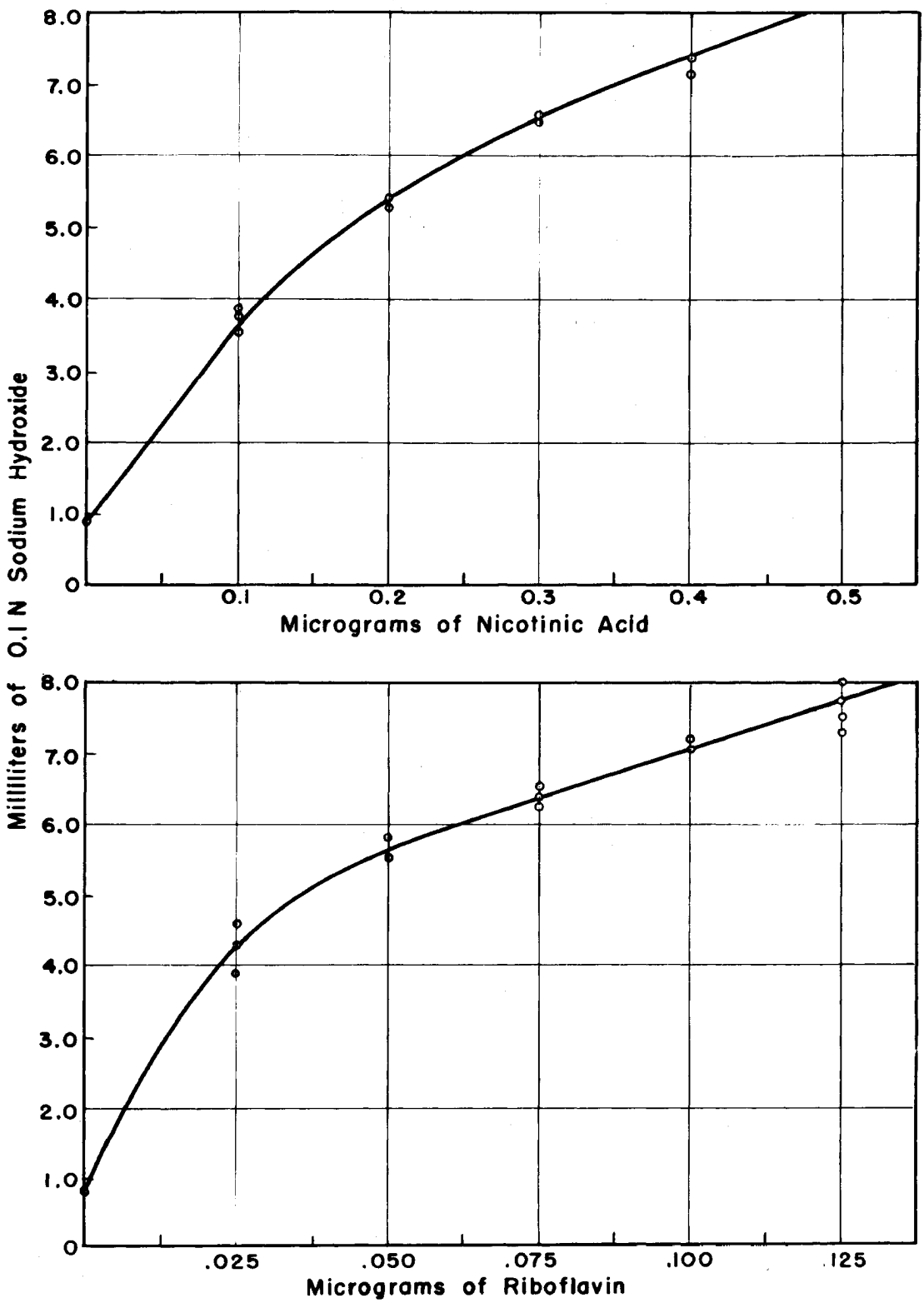

FIGURE 3. STANDARD CURVES FOR RIBOFLAVIN AND NICOTINIC ACID ASSAYS

water was added with gentle agitation of the tube. The contents of the tube were transferred asceptically into a 125 ml. Erlenmeyer flask containing 50 ml. of sterile distilled water. After being twirled a few times, the solution was ready for use.

Cooled tubes containing the hydrolyzed liver tissue and basal medium or the standard solutions for the vitamin assay curves were inoculated asceptically with the appropriate assay organism, and placed immediately in an incubator at 37° C. The tubes were allowed to incubate for 72 hours. At the end of this time, growth was stopped by placing the tubes in a refrigerator, where they remained until the time of analysis. All tubes were titrated with 0.1 normal NaOH.

Concentrations of riboflavin and nicotinic acid were calculated from standard curves which had been run simultaneously with the liver samples.

Weights of Adrenal Glands

The adrenal glands from each animal were removed, and carefully trimmed free of adhering fat and connective tissue. They were placed in small weighing bottles and weighed immediately.

Extreme care was exercised in the removal of these

tissues, and in their subsequent handling to prevent damage. The gross appearance was noted and recorded.

Blood Analyses

General

Immediately after the animal was stunned, an incision was made in the linea alba. The chest cavity was opened by making a "V" shaped incision through the ribs, care being taken so as not to sever the diaphragm. In all cases, the animal was still breathing, and the heart beating.

Using a glass syringe fitted with a No. 21 stainless steel needle, 3 to 5 ml. of blood were withdrawn from the right ventricle of the heart. The blood was delivered into a 15 ml. centrifuge tube into which two drops of a solution containing 10 mg. of sodium fluoride and 1 mg. of thymol per ml. had been dispersed and dried. A few grains of sodium oxalate were added to prevent coagulation. According to Peters and Van Slyke (1932) this solution prevents any change in blood constituents over a period of 10 days. The samples were stirred with a fine glass rod, immediately placed in the freezing unit of the refrigerator and chilled to 0° C.

At the time of analysis, the samples were thawed at room temperature and appropriate aliquots taken for the determination of blood constituents.

Methods of Analysis

Urea. One ml. aliquots of blood were blown into a tungstic acid solution, contained in centrifuge tubes and prepared according to the method of Folin and Wu They were inverted with shaking and centrifuged immediately for 15 minutes. The supernatant liquid was transferred to a 50 ml. Erlenmeyer flask and one drop of 0.5 per cent brom thymol blue added. The flasks were shaken with a horizontal, whirling motion for 45 seconds unstoppered. A tube attached to an air jet was inserted, and air was allowed to pass through the flasks for a few seconds. This process was repeated two times. A blank containing acidified 0.9 per cent sodium chloride solution was treated in the same manner.

With a 5 ml. stopcock pipette, provided with a rubber tip and calibrated to deliver between two marks, 5 ml. of the filtrate were transferred to the chamber of the Van Slyke manometric apparatus. A drop of caprylic alcohol had been previously drawn into the capillary beneath the mercury, and 3 to 4 ml. of water added. The tip of the

pipette was immersed in the mercury, so that the solution was delivered directly into the chamber of the gas apparatus.

Five tenths of a ml. of 10 per cent urease solution, measured from a burette, was run into the chamber under the mercury seal in the same manner. The urease and filtrate were mixed, by lowering and raising the mercury in the chamber a few centimeters, and permitted to react for one and one-half minutes.

Two tenths of a ml. of 1 normal lactic acid were run in under the mercury seal in the same manner as the sample and urease. The chamber was evacuated and shaken for one and one-half minutes. The gas volume was reduced to 0.5 ml. and readings were taken as described by Van Slyke (1932).

The urea content of the blood samples was calculated from factors given by Van Slyke after the carbon dioxide had been absorbed in sodium hydroxide.

Amino Nitrogen. Appropriate aliquots of the blood samples were pipetted into centrifuge tubes containing 10 ml. of a 1 per cent picric acid solution and 1 ml. of distilled water. The tubes were centrifuged for thirty minutes, and the centrifugate was transferred into a clean centrifuge cone. Five ml. aliquots were pipetted into

Thunberg tubes and 2 glass beads added with one drop of capryl alcohol. The tubes were heated over a microburner for exactly 50 seconds to release amino nitrogen from sources other than the amino acids.

After the tubes had been cooled, 100 mg. of ninhydrin which had been accurately measured, were added. Immediate evacuation, closing of the tube, attachment of the rubber side arm, and sealing with solid glass rods followed as described under urine amino nitrogen.

The procedure subsequently followed was essentially the same as described for urine amino nitrogen, with the exception that 2 normal lactic acid was added (in a 25 per cent sodium chloride solution). The gas chamber was shaken 3 minutes to extract the carbon dioxide.

Blanks were also analyzed which contained distilled water instead of the blood filtrates. Ninhydrin was not added in this case.

Serum Alkaline Phosphatase. The method used was that of Bessey, Lowry and Brook (1946). Ten centimeters of serum were transferred to the bottom of a 3/8 x 3 inch culture tube in a wire rack. The tubes were immersed in ice water and the ice cold disodium-p-nitrophenyl phosphate reagent added. The contents were mixed by tapping the tubes. Blanks and standards containing 10 ml. of

water and graded amounts of a standard solution containing 1, 2, and 4 millimoles per liter of p-nitrophenol, were analyzed simultaneously with blood samples.

All tubes were immersed simultaneously in a water bath at 38° C. at a sufficient depth to cover the bottom half of the tubes. After exactly 30 minutes, the rack was again immersed in ice water and 1.0 ml. of 0.02 normal sodium hydroxide was added to each tube using sufficient force to mix the sample. The outside of the tubes were dried and they were read in a Coleman Spectrophotometer at 415 mu. The machine was set with distilled water as a blank.

After the initial reading, R_1, 0.02 ml. of 1:3 hydrochloric acid were added and a second reading taken, R_2.

The calculation of serum alkaline phosphatase present in the samples was made by subtracting R_2 from R_1, and referring to a table of factors given by the authors of the method. A millimole unit is defined as the phosphatase activity which will liberate one millimole of nitrophenol per liter of serum per hour.

Specially built pipettes designed to deliver the microquantities required in this method were made by a worker in the Nutrition Laboratory. The method of construction of this instrument is reported in the appendix.

Carbohydrate Metabolism

Glucose Tolerance Test

Abnormalities in carbohydrate metabolism are indicated when distorted glucose tolerance curves, as compared to those of normal animals, are obtained after the feeding of a known quantity of glucose. In some instances, for example diabetes mellitus, the concentration of glucose in the blood remains high for a longer period of time than it does in the normal state, and in certain cases, not even returning to the fasting level.

The glucose tolerance test, thus is based on the principle that any increase in the sugar concentration of the blood due to the ingestion of a glucose solution is rapidly taken care of by the normal animal. Samples of blood are taken at the fasting level, and at intervals after the injection of a known amount of glucose. The mg. per cent of sugar in each sample is determined during these periods, and follows a definite curve, the peak occurring shortly after the feeding of the glucose solution. A rapid decrease occurs in the normal animal with return of the glucose concentration in the blood to the fasting level.

In the present experiment, the animal was judged to be in a fasting state after a 10 hour starvation period.

At this time, 0.1 ml. of tail blood was taken for the analysis of glucose. A sugar solution containing 8.75 per cent glucose was then fed by stomach tube, and samples of blood were withdrawn for analysis one-half hour, one, two, three, and four hours following.

Drawing of Blood. The blood was obtained by making a horizontal slit in the tail of the animal with the use of a sharp Bard-Parker blade. The animal had been first wrapped in a soft towel, and securely fastened with a safety pin. The tail was placed in warm water for a second to dilate the veins and then covered with a small amount of vaseline at the point of incision. Blood thus was obtained in large drops which could be easily transferred to an enameled spot plate disc. Sodium oxalate was added in small amounts to prevent coagulation while aliquots were pipetted for the glucose determinations.

After the blood had been taken from the animal, the incision was closed with collodion, and a cotton bandage was applied if necessary. The rat was gently unwrapped and placed into its cage. Blood pipettes were rinsed temporarily by drawing ammonium hydroxide solution through them, and at the end of a series of determinations, they were washed with distilled water and ether, and dried with suction.

Feeding of Glucose Solution. In the present study, the 8.75 per cent solution of pure glucose was fed by stomach tube. The amount of glucose given each rat was calculated on the basis of 0.35 gms. of sugar per one hundred grams of body weight (Treadwell, 1942). The glucose was weighed accurately on an analytical balance and made to volume in a 100 ml. volumetric flask with distilled water. The solution was kept in the refrigerator until the time of use. Before feeding, it was warmed to room temperature thus reducing the shock which might be induced by the introduction of a cold liquid to the animal, and insuring accurate delivery of the desired amount from the syringe.

The technique used to feed the glucose solutions was the same as that described earlier, except that a glass syringe calibrated to hold 5 ml. was used. Any air bubbles present were carefully removed before the solution was fed.

Determination of Glucose. From the porcelain dish, 0.1 ml. aliquots of blood were pipetted with a clean, dry, blood pipette calibrated to hold that amount. The blood was immediately blown into centrifuge tubes containing 10.0 ml.of dilute tungstic acid; the fluid was pulled into the pipette several times to insure quantitative

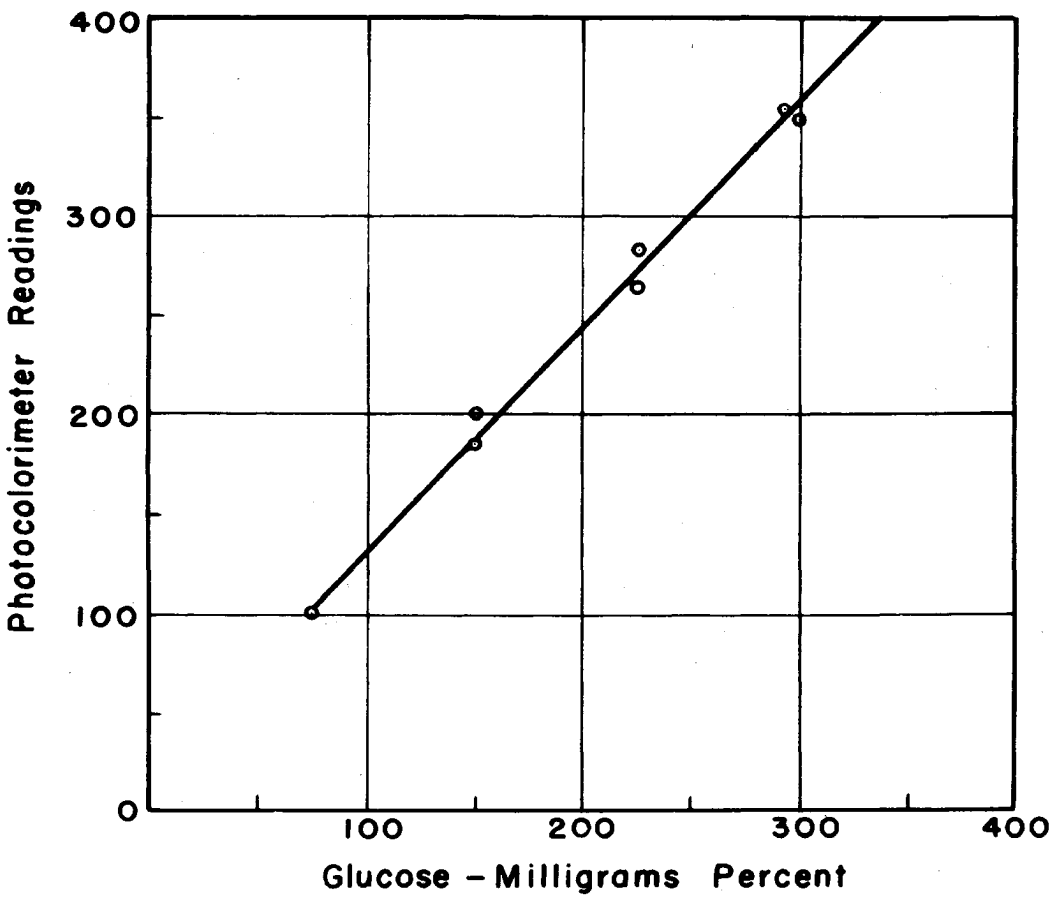

FIGURE 5. STANDARD CURVE FOR GLUCOSE DETERMINATIONS

removal. Samples were centrifuged immediately, covered with tin foil, and placed in the refrigerator.

Two aliquots of 4 ml. each of the tungstic acid filtrates were taken for analysis. These were pipetted into calibrated 25 ml. test tubes, and to them were added 2 ml. of potassium ferricyanide and 1 ml. of sodium carbonate-cyanide solutions. The tubes, in a wire basket, were heated in a boiling water bath for 20 minutes, then placed in cold water. At the end of the two minutes allowed for cooling, the tubes were removed from the water, and 5 ml. of ferric iron solution were added from a pipette. After a five minutes, the solutions were made to volume with distilled water. The intensity of color was measured in a Klett-Summerson colorimeter, using a blue No. 42 filter. Standard glucose solutions and blanks were analyzed simultaneously with the samples of blood filtrate.

Glycogen in Liver

Glycogen was determined essentially as described by Good, Kramer, and Somogyi (1933). This method is a modification and simplification of the classic method of Pfluger (1904), making it possible to perform the method in a few hours.

The process was carried on in 50 ml. pyrex centrifuge

tubes with round bottoms and pourout spouts. The rounded bottoms made it possible to mix the contents by gentle agitation without the use of a stirring rod. These tubes were pierced by two small openings on opposite sides near the upper edge and fitted with handles made of fine non-corrosive piano wire, so that they could be hung from the beam of an analytical balance.

On the day that the animal was to be killed, two tubes were charged with 8 ml. of 30 per cent potassium hydroxide, approximately 2 ml. per gram of tissue. After the introduction of the liver, the centrifuge tubes were tightly stoppered with corks covered with tin foil, weighed on an analytical balance, and then immersed immediately in a boiling water bath. Usually about 10 or 15 minutes elapsed between the time of stunning the rat and the placing of the tubes into hot water. Only one to two minutes, however, were required to open the animal, remove the liver, and drop it into the potassium hydroxide.

Hydrolysis of Liver Glycogen. The tubes were kept in the boiling water bath one-half hour or until a homogenous solution had formed. Occasional agitation facilitated the solution of the liver and prevented small bits of tissue from adhering to the sides of the tubes.

After removal from the water bath, the tubes were

cooled in a water bath at 20° C. and the glycogen was precipitated by the addition of 9.6 ml. of 95 per cent ethyl alcohol. This quantity of alcohol provided 1.2 volumes in relation to the original volume of potassium hydroxide. The tubes were carefully shaken to mix the alcohol and alkaline liver solution and then replaced in the boiling water bath until the mixture began to boil. During this time the precipitate became more flocculant and began to settle out.

The tubes were removed, cooled to room temperature and centrifuged for 10 minutes with the rheostat set at 14. The mother liquor was easily decanted as a result of this procedure. After the tubes had been drained, excess alcohol was removed by heating in the water bath for a few minutes.

Four ml. of hot distilled water were added to the precipitate to dissolve it; a small glass rod facilitated this process. The dissolved glycogen was reprecipitated by the addition of 9.6 ml. of 95 per cent ethyl alcohol, added in such a manner as to wash off the stirring rod and the sides of the tubes. After the mixture had been heated to boiling, it was cooled to room temperature and recentrifuged. The precipitated glycogen was now fairly white in color, and the supernatant liquid, a clear amber fluid,

was poured off. The excess alcohol was again removed by heating and 10 ml. of normal sulfuric acid were added. The tubes were covered loosely with tin foil, replaced in the hot water bath, and hydrolyzed for three hours.

After hydrolysis, the tubes were cooled and analyzed for glucose.

Determination of Glucose in Liver Samples. The contents of the tubes were transferred quantitatively to 50 ml. volumetric flasks with small amounts of warm distilled water. A few drops of phenol red were added as an indicator and the acid contents neutralized with normal sodium hydroxide. When the color of the solution changed to red, a drop or two of normal sulfuric acid was added until the yellow color returned. The contents of the flasks were diluted to volume and mixed. Appropriate aliquots were taken for analysis of glucose.

The method of Schaffer and Somogyi (1933) was used for the glucose determinations. With an accurate pipette, 0.5 ml. of the sugar solutions were transferred into pytex test tubes, 25 x 200 ml. Five ml. of the copper reagent were added in a manner so as to rinse the sides of the test tube. Blanks were treated similarly using 5 ml. of distilled water. The solutions were mixed with gentle shaking and covered with small marbles. They were placed

in a metal test tube rack and heated in a vigorously boiling water bath for exactly 15 minutes. At the end of this time, the tubes were removed.

After the tubes had been cooled to room temperature, 2 ml. of a solution containing 2.5 per cent potassium iodide and potassium oxalate and 5 ml. of normal sulfuric acid were added. The bulbs were replaced and the solutions mixed well to dissolve the cuprous iodide which had precipitated. The solutions were allowed to stand, covered, from 5 to 10 minutes with occasional agitation. At the end of this time the bulbs and walls of the tubes were rinsed with water and poured into 50 ml. Erlenmeyer flasks. They were titrated with 0.005 normal sodium thiosulfate. When the straw color of the iodine liberated faded to a yellow color, one ml. of starch indicator was added. The end point of the titration was reached when the dark blue of the starch indicator changed sharply to the green-blue of copper sulfate.

Calculation of the glucose was made by subtracting the titration volume of the sample from the value of the heated blank. These values were then referred to the standard curve obtained by similar treatment of pure glucose solutions of known concentration.

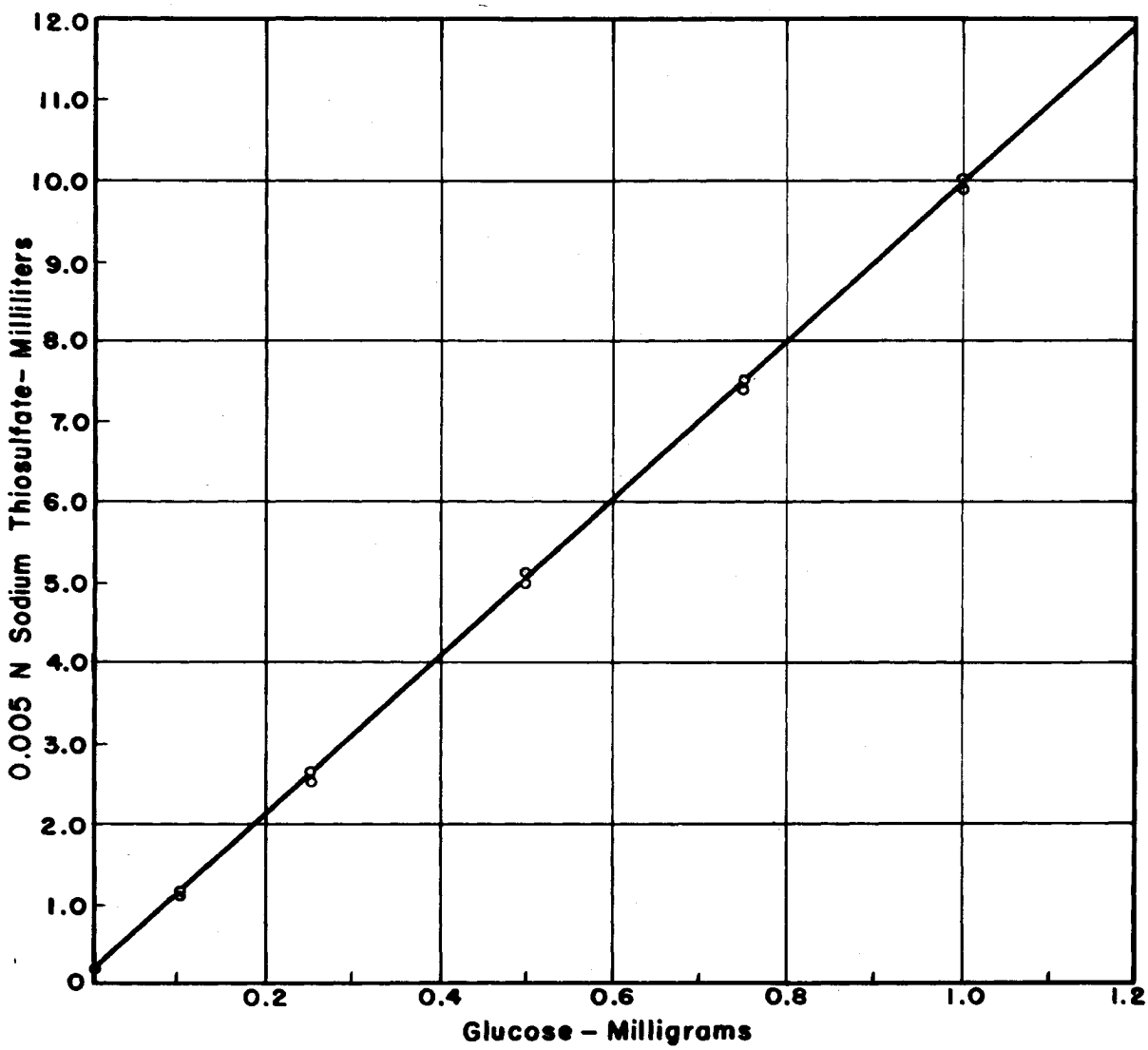

FIGURE 4. STANDARD CURVE FOR GLUCOSE DETERMINATIONS (COPPER REAGENT)

Standard Curve. Glucose solutions were prepared containing quantities of glucose which were similar to those contained in the liver samples. Five ml. aliquots of these solutions were analyzed according to the method previously described. Analyses were run in duplicate or triplicate. The volumes of sodium thiosulfate, 0.005 normal, required for the titration of these samples were plotted against the concentration of glucose present in each aliquot.

Calculation of Glycogen. The weight of glucose, as calculated from the standard curve, was converted to glycogen by use of the factor, 0.927. (Pfluger, 1904).

Histological Analyses

Muscle, bone, skin, and fibrous tissue may remain alive for some time, but the cells of the alimentary tract and the glandular organs disintegrate immediately; therefore it is important that the latter be obtained within a few seconds after death. In these experiments, in all cases, the animal was breathing and the heart still beating when the initial incision was made. Usually only 50 to 60 seconds elapsed before the liver was placed in the fixative and within a comparable time, the kidneys and adrenal glands were also removed and fixed.

Histological examinations of the liver, kidneys, and

adrenal glands were made in a series of rats especially fed for that purpose. After the animals had been starved for 10 hours, their physical condition was noted and recorded. The procedures used in removing tissues and sacrificing the animal are the same as those described earlier.

In the autopsy procedure, a definite routine was followed. The color and consistency of each organ was noted as it was removed. The esophagus was examined for signs of excessive irritation. The stomach and intestines were examined for ulcers and signs of hemorrhage. The relative amounts of fat in the subcutaneous, abdominal, perirenal, pericardial, and intramuscular depots were also noted. Any abnormal condition which could be noted by gross observation in the lungs, liver, kidneys, adrenal glands, or spleen was recorded.

Sections from the lobes of the liver and from the kidney were removed by the use of a carefully sharpened Bard-Parker blade. These samples and the adrenal glands were placed immediately into the fixative.

Usually only 30 to 60 seconds elapsed between the time of stunning of the animal and the removal of the liver. No attempt was made to remove the adhering connective tissue and fat. Small sections from each lobe were placed immediately into bottles containing (1) 10 per cent formalin

for hematoxylin and eosin staining, (2) a mixture of 90 per cent absolute alcohol and 10 per cent glacial acetic acid for glycogen staining, and (3) 10 per cent formalin for fat staining.

No attempt was made to keep the lobes of the liver separate. However, all tissues from one rat were kept together. One container was used for the liver, kidneys, and adrenals to be stained with hematoxylin and eosin, another for glycogen staining of the liver, and a third container for sections to be used for the staining of fat. Three to five serial sections of each organ were mounted on each slide. Five such slides were made of each organ, the sections on each slide being taken from different parts of the organ.

In analyzing the histological sections from any one organ, a composide description first was made of all the sections mounted on the five slides. Component parts were always examined in the same order. For example, in studying the sections taken from the kidney, the cortex was examined first, then the medulla, and lastly, the pelvis. In so far as it was practical, all sections from one organ, for example the liver, from every rat in the experimental groups were described before a different organ was studied.

TABLE 4. CHANGES IN BODY WEIGHT OF RATS FED THE STEENBOCK XVII DIET

Rat number	Initial weight	Collection period I		
		2nd day	5th day	Change in weight
	gm.	gm.	gm.	gm.
		Normal food intake		
48474	274	290	286	-4
48475	298	316	324	+8
48404	260	276	278	+2
48261	296	323	318	-5
48449	274	307	312	+5
48488	266	284	282	-2
Average				+1
		25 per cent of the normal food intake		
50104	312	286	280	-6
50105	324	294	290	-4
50106	337	308	290	-18
50096	330	294	282	-12
50090	326	308	296	-12
50097		283	278	-5
Average				-10

TABLE 5. CHANGES IN BODY WEIGHT OF RATS FED LOW NITROGEN DIETS CONTAINING 20 PER CENT FAT

Rat number	Original weight	Collection period I			Adjustment period			Collection period II		
		2nd day	5th day	Change in weight	2nd day	4th day	Change in weight	2nd day	5th day	Change in weight
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
		56 Calories per day per 300 gm. rat in period II								
47918	272	231	230	-1	228	222	-6	222	209	-13
47933	299	254	260	+6	246	244	-2	240	231	- 9
48024	302	271	265	-6	260	258	-2	252	252	- 0
48017	321	274	266	-8	264	258	-2	256	248	- 8
47984	329	289	284	-5	280	278	-2	274	272	- 2
47834	349	290	294	+4	290	288	-2	283	274	- 9
Average				-2			-3			- 7
		14 Calories per day per 300 gm. rat in period II								
47961	292	256	248	-8	238	230	-8	216	182	-34
47886	303	261	256	-5	255	236	-19	228	219	- 9
47963	313	276	270	-6	253	246	-7	237	222	-15
47864	316	280	271	-9	260	254	-6	244	233	-11
47954	360	314	310	-4	306	286	-20	276	262	-14
47808	302	258	256	-2	243	233	-10	224	218	-6
Average				-6			-12			-15

TABLE 6. CHANGES IN BODY WEIGHT OF RATS FED A LOW NITROGEN DIET CONTAINING NO FAT

Rat number	Initial weight	Collection period I			Adjustment period			Collection period II		
		2nd day	5th day	Change in weight	2nd day	4th day	Change in weight	2nd day	5th day	Change in weight
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
		56 Calories per 300 gm. rat per day in period II								
48023	278	238	232	-6	235	226	-9	230	216	-14
47915	294	252	248	-4	248	242	-6	240	231	- 9
47789	324	278	270	-8	260	265	+5	258	256	- 2
47951	328	288	287	-1	275	270	-5	268	255	-13
49869	342	301	296	-5	295	294	-1	290	286	- 4
47813	308	256	255	-1	253	251	-2	248	241	- 7
Average				-4			-3			- 8
		14 Calories per 300 gm. rat per day in period II								
48172	277	245	251	+6	243	244	+1	235	215	-20
48138	288	258	258	-0	250	233	-17	222	193	-29
48060	296	252	256	+4	250	231	-19	223	200	-23
48207	306	243	250	-7	244	224	-20	215	184	-31
48650	320	260	266	+6	261	242	-19	220	200	-20
48115	304	246	252	+6	250	225	-25	217	186	-31
Average				+3			-17			-26

TABLE 7. CHANGES IN BODY WEIGHT OF RATS FED A LOW NITROGEN DIET CONTAINING 20 PER CENT FAT AND SUPPLEMENTED WITH METHIONINE IN PERIOD II

Rat number	Initial weight	Collection period I			Adjustment period			Collection period II		
		2nd day	5th day	Change in weight	2nd day	5th day	Change in weight	2nd day	5th day	Change in weight
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
		56 Calories per day per 300 gm. rat in period II								
47996	282	244	238	- 6	232	230	-2	232	232	0
48170	292	252	240	-12	246	242	-4	244	238	-6
48208	296	292	253	-39	248	242	-6	253	242	-11
48076	324	284	282	- 2	272	274	+2	273	268	-5
48194	320	280	274	- 6	267	273	+6	274	274	0
48055	276	238	236	- 2	230	229	-1	234	228	-6
Average				-10			-1			-5
		14 Calories per day per 300 gm. rat in period II								
48092	305	274	276	+ 2	264	248	-16	242	218	-24
48171	282	250	253	+ 3	243	226	-17	224	209	-15
48109	298	260	247	-13	248	234	-14	230	200	-30
47963	320	262	276	+14	268	258	-10	252	222	-30
48025	310	274	266	- 8	264	250	-14	246	232	-14
48069	321	275	276	+ 1	270	252	-18	253	230	-23
Average				0			-15			-23

TABLE 8. CHANGES IN BODY WEIGHT OF RATS FED A LOW NITROGEN DIET CONTAINING NO FAT AND SUPPLEMENTED WITH METHIONINE IN PERIOD II

Rat number	Initial weight	Collection period I			Adjustment period			Collection period II		
		2nd day	5th day	Change in weight	2nd day	4th day	Change in weight	2nd day	5th day	Change in weight
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
56 Calories per day per 300 gm. rat in period II										
48091	280	240	230	-10	248	232	-16	238	232	- 6
48061	284	250	248	- 2	250	244	- 6	246	240	- 6
48108	304	270	271	+ 1	266	260	- 6	263	258	- 5
48202	292	254	244	-10	244	242	- 2	243	234	- 9
48054	310	272	263	- 9	262	256	- 6	262	250	-12
48178	308	264	250	-14	246	242	- 4	250	238	-12
Average				- 7			- 7			- 8
14 Calories per day per 300 gm. rat in period II										
4871	323	279	262	-17	250	239	-11	228	216	-12
47916	313	269	252	-17	240	229	-11	218	206	-12
47960	336	286	278	- 8	290	256	-34	250	243	- 7
47962	332	286	283	- 3	293	261	-32	254	240	-14
47985	297	253	248	- 5	240	229	-11	214	180	-34
47788	360	313	311	- 2	300	286	-14	276	266	-10
Average				- 9			-19			-15

TABLE 9. NITROGEN BALANCES OF RATS FED THE STEENBOCK XVII DIET

Rat number	Average body weight at the end of period I	Nitrogen balance Period I
	gm.	mg.
	Normal food intake	
48474	289	+458
48475	320	+674
48404	278	+293
48361	318	+463
48449	308	+772
48488	284	+340
Average	300	+499
	25 per cent of the normal food intake	
50104	284	-268
50105	294	-209
50106	302	-490
50096	291	-514
50090	304	-451
50097	284	-298
Average	293	-372

TABLE 10. NITROGEN BALANCES OF RATS FED A LOW NITROGEN DIET CONTAINING 20 PER CENT FAT

Rat number	Average body weight		Nitrogen balance		Difference
	Period I	Period II	Period I	Period II	
	gm.		mg.		mg.
	56 Calories per day per 300 gm. rat				
47918	231	214	-261	-246	-15
47933	258	234	-281	-257	-24
48024	270	248	-259	-245	-14
48017	272	251	-264	-329	+65
47984	287	265	-288	-317	+29
47834	295	277	-349	-336	-13
Average	269	248	-284	-288	
	14 Calories per day per 300 gm. rat				
47961	254	202	-282	-1276	+994
47886	258	223	-331	-316	-15
47963	274	231	-321	-525	+204
47864	277	239	-316	-338	+22
47954	314	271	-376	-304	-72
47808	259	221	-238	-327	+89
Average	273	231	-310	-514	

TABLE 11. NITROGEN BALANCES OF RATS FED A LOW NITROGEN DIET CONTAINING NO FAT

Rat number	Average body weight		Nitrogen balance		Difference
	Period I	Period II	Period I	Period II	
	gm.		mg.		mg.
	56 Calories per 300 gm. rat per day				
48023	239	221	-209	-280	+71
47915	265	244	-259	-293	+34
47789	274	258	-268	-280	+12
47951	288	263	-311	-271	-40
49869	265	250	-259	-287	+28
47813	258	245	-249	-327	+78
Average	265	247	-259	-290	
	14 Calories per 300 gm. rat per day				
48172	254	205	-280	-1460	+1180
48138	255	211	-281	-1216	+ 935
48060	255	211	-281	-1470	+1190
48207	247	202	-263	-1427	+1164
48650	262	209	-294	-1285	+ 991
48115	250	204	-283	-1931	+1648
Average	254	207	-280	-1465	

TABLE 12. NITROGEN BALANCES OF RATS FED A LOW NITROGEN DIET CONTAINING 20 PER CENT FAT AND SUPPLEMENTED WITH METHIONINE IN PERIOD II

Rat number	Average body weight		Nitrogen balance		Difference
	Period I	Period II	Period I	Period II	
	gm.	gm.	mg.	mg.	mg.
	56 Calories per 300 gm. rat per day				
47996	239	230	-326	-179	-147
48170	248	238	-364	-196	-168
48208	261	242	-305	-245	- 60
48076	264	270	-341	-272	- 69
48194	280	273	-327	-192	-135
48055	237	228	-303	-203	-100
Average	257	247	-328	-215	
	14 Calories per 300 gm. rat per day				
48092	264	232	-313	-524	+211
48171	250	217	-195	-314	+119
48109	254	217	-263	-724	+461
47863	273	238	-240	-759	+519
48025	269	238	-244	-314	+ 70
48067	275	241	-243	-530	+287
Average	264	231	250	-528	

TABLE 13. NITROGEN BALANCES OF RATS FED A LOW NITROGEN DIET CONTAINING NO FAT AND SUPPLEMENTED WITH METHIONINE IN PERIOD II

Rat number	Average body weight		Nitrogen balance		Difference
	Period I	Period II	Period I	Period II	
	gm.	gm.	mg.	mg.	mg.
	56 Calories per 300 gm. rat per day				
48091	237	233	-264	-217	-47
48061	252	237	-254	-236	-18
48108	258	237	-250	-240	-10
48202	251	240	-310	-233	-77
48054	268	256	-281	-257	-24
48178	246	238	-252	-232	-20
Average	252	239	-251	-236	
	14 Calories per 300 gm. rat per day				
47871	280	238	-262	-496	+234
47916	261	214	-314	-401	+ 85
47960	283	248	-230	-398	+168
47962	288	249	-269	-551	+282
47985	252	201	-269	-732	+463
47788	315	280	-226	-394	+168
Average	280	238	-261	-495	

TABLE 14. PARTITION OF NITROGEN IN URINES OF RATS IN THE CONTROL GROUP FED THE STEENBOCK XVII DIET (PART I)

Rat number	Total nitrogen	Allantoin nitrogen	Creatinine nitrogen	Amino nitrogen
		Period I	Period I	Period I
	mg.	mg.	mg.	mg.
		Adequate food intake		
48474	2197	66	21	8.1
48475	2243	67	21	11.5
48404	1828	56	22	15.3
48261	2036	64	22	6.1
48449	2003	64	21	16.7
48488	1998	61	21	11.2
Average	2050	64	21	10.9
		25 per cent of the adequate food intake		
50104	1170	65	25	0.2
50105	1091	58	25	8.9
50106	1394	65	24	2.4
50096	1408	65	22	10.2
50090	1317	65	25	8.4
50097	1189	60	25	6.2
Average	1261	63	25	6.0

TABLE 15. PARTITION OF NITROGEN IN URINES OF RATS FED A LOW NITROGEN DIET CONTAINING 20 PER CENT FAT (PART I)

Rat number	Total nitrogen		Allantoin nitrogen		Creatinine nitrogen		Amino nitrogen	
	Period I	Period II	Period I	Period II	Period I	Period II	Period I	Period II
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
				56 Calories per day per 300 gm. rat				
47918	178	154	68	65	22	13	8.1	4.7
47933	189	157	72	71	25	16	11.4	4.5
48024	177	159	71	73	21	12	7.6	4.5
48017	176	198	45	93	20	11	5.4	3.2
47984	211	206	77	91	20	11	4.7	4.2
47834	239	202	80	89	23	14	4.3	4.4
Average	195	180	70	80	23	14	12.8	4.2
				14 Calories per day per 300 gm. rat				
47961	178	1232	66	88	21	14	8.0	10.7
47886	222	258	70	72	22	15	8.4	7.2
47963	205	495	71	81	21	14	2.2	6.8
47864	207	276	71	63	22	15	1.8	6.1
47954	221	233	70	62	21	14	5.3	7.4
47808	124	270	56	60	16	9	2.3	9.5
Average	193	459	67	71	21	14	4.6	8.5

TABLE 16. PARTITION OF NITROGEN IN URINES OF RATS FED A LOW NITROGEN DIET CONTAINING NO FAT (PART I)

Rat number	Total nitrogen		Allantoin nitrogen		Creatinine nitrogen		Amino nitrogen	
	Period I	Period II	Period I	Period II	Period I	Period II	Period I	Period II
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
			56 Calories per day per 300 gm. rat					
48023	171	162	77	60	24	20	3.4	11.5
47915	155	172	64	70	21	16	12.5	11.9
47789	181	236	64	54	22	18	13.2	20.4
47951	141	147	48	59	22	18	11.8	4.3
49869	169	180	64	61	21	16	10.1	8.9
47813	169	179	63	60	22	18	10.3	9.0
Average	169	180	63	61	22	18	10.2	11
			14 Calories per day per 300 gm. rat					
48172	182	1148	66	85	18	24	10.3	2.4
48138	179	1341	59	81	17	28	7.3	2.9
48060	206	1848	65	75	19	26	6.8	5.5
48207	181	1150	64	84	18	28	4.6	3.6
48650	179	1350	64	82	18	27	7.0	5.3
48115	183	1840	62	75	18	28	7.4	5.3
Average	196	1389	63	81	18	28	7.2	4

TABLE 17. PARTITION OF NITROGEN IN URINES OF RATS FED A LOW NITROGEN DIET CONTAINING 20 PER CENT FAT AND SUPPLEMENTED WITH METHIONINE (PART I)

Rat number	Total nitrogen		Allantoin nitrogen		Creatinine nitrogen		Amino nitrogen	
	Period I	Period II	Period I	Period II	Period I	Period II	Period I	Period II
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
				56 Calories per day per 300 gm. rat				
47996	178	176	60	53	17	16	10.2	13.2
48170	225	181	72	67	15	16	7.1	13.8
48208	176	211	65	74	15	17	6.4	12.1
48076	186	198	55	71	17	19	10.3	9.0
48194	206	179	66	60	19	16	12.7	10.0
48055	198	184	67	59	17	15	9.3	10.3
Average	195	188	64	64	17	17	9.2	11.4
				14 Calories per day per 300 gm. rat				
48092	221	471	28	89	20	21	2.9	9.0
48171	167	292	37	59	16	19	5.0	9.7
48109	196	705	37	59	17	24	2.8	10.9
47863	206	723	38	86	21	25	6.4	11.2
48025	192	295	34	61	18	19	1.9	6.3
48067	174	507	37	71	18	23	4.7	7.1
Average	193	450	35	72	18	23	3.9	9.0

TABLE 18. PARTITION OF NITROGEN IN URINES OF RATS FED A LOW NITROGEN DIET CONTAINING NO FAT AND SUPPLEMENTED WITH METHIONINE IN PERIOD II (PART I)

Rat number	Total nitrogen		Allantoin nitrogen		Creatinine nitrogen		Amino nitrogen	
	Period I	Period II	Period I	Period II	Period I	Period II	Period I	Period II
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
				56 Calories per day per 300 gm. rat				
48091	159	154	60	54	16	5	4.0	5.0
48061	178	168	60	70	17	4	5.0	9.7
48108	161	171	58	61	15	3	7.2	10.3
48202	161	155	60	55	16	3	5.0	8.0
48054	177	167	59	69	17	4	5.2	7.5
48178	160	171	59	61	15	3	5.6	9.3
Average	166	164	59	62	17	4	5.4	8.3
				14 Calories per day per 300 gm. rat				
47871	194	337	64	78	28	27	13.8	7.1
47916	164	344	52	63	24	27	31.4	14.0
47960	178	485	72	69	27	29	11.6	15.6
47962	166	730	70	103	21	23	9.4	15.3
47985	175	352	63	65	25	27	10.4	14.6
Average	175	450	64	76	25	27	15.3	13.6

TABLE 19. PARTITION OF NITROGEN IN URINES OF RATS FED THE STEENBOCK XVII DIET (PART II)

Rat number	Total nitrogen	Urea nitrogen Period I	Ammonia nitrogen Period I	Total nitrogen in constituents
	mg.	mg.	mg.	mg.
		Adequate food intake		
48474	2197	2087	15	2197.1
48475	2243	2108	19	2226.5
48404	1828	1700	18	1811.3
48261	2036	1913	10	2015.1
48449	2003	1882	32	2015.7
48488	1998	1837	31	1962.2
Average	2050	1927	21	2037.9
		25 per cent of the adequate food intake		
50104	1170	1006	61	1157.2
50105	1091	949	87	1127.9
50106	1394	1235	106	1432.4
50096	1408	1210	86	1393.2
50090	1317	1118	68	1284.4
50097	1189	986	43	1120.2
Average	1261	1084	76	1252.5

TABLE 20. PARTITION OF NITROGEN IN THE URINES OF RATS FED A LOW NITROGEN DIET CONTAINING 20 PER CENT FAT (PART II)

Rat number	Total nitrogen		Urea nitrogen		Ammonia nitrogen		Total nitrogen in constituents	
	Period I	Period II	Period I	Period II	Period I	Period II	Pd. I	Pd. II
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
			56 Calories per day per 300 gm. rat					
47918	178	154	82	73	8	5	188.1	160.7
47933	189	158	126	65	10	3	244.5	159.5
48024	177	159	84	65	11	6	194.6	160.5
48017	176	198	86	94	12	4	167.5	205.2
47984	211	206	86	76	8	6	195.7	188.2
47834	239	203	114	95	6	5	227.3	207.4
Average	195	180	96	79	9	5	202.3	180.4
			14 Calories per day per 300 gm. rat					
47961	178	1232	81	936	4	111	180.0	1159.7
47886	222	258	107	185	2	21	209.4	300.2
47963	205	485	94	325	2	39	190.2	465.8
47864	207	276	83	196	1	19	178.8	299.1
47954	221	233	101	170	5	19	202.3	272.4
47808	124	270	54	194	5	32	133.3	304.5
Average	193	459	83	330	3	42	182.3	466.9

TABLE 21. PARTITION OF NITROGEN IN THE URINES OF RATS FED A LOW NITROGEN DIET CONTAINING NO FAT (PART II)

Rat number	Total nitrogen		Urea nitrogen		Ammonia nitrogen		Total nitrogen in constituents	
	Period I	Period II	Period I	Period II	Period I	Period II	Pd. I	Pd. II
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
			56 Calories per day per 300 gm. rat					
48023	171	162	81	72	0.9	1.6	186.3	165.1
47915	155	172	83	72	0.6	1.6	181.1	171.5
47789	181	236	81	114	0.7	3.6	180.9	210.0
49951	141	147	63	79	1.0	1.4	145.8	165.7
49869	169	180	77	85	0.7	1.7	169.8	172.6
47813	169	179	76	84	0.9	3.4	172.1	174.2
Average	169	180	77	85	0.8	2.2	172.7	176.5
			14 Calories per 300 gm. rat per day					
48172	182	1148	83	700	0.7	280	178.0	1091.4
48138	179	1341	84	764	0.7	362	178.0	1238
48060	206	1848	101	1257	0.8	416	193	1779
48207	181	1150	98	770	0.5	263	185	1149
48650	179	1350	90	754	0.7	364	180	1232
48115	183	1840	91	1251	0.8	460	179	1819
Average	196	1369	91	875	0.7	349	182	1384

TABLE 22. PARTITION OF NITROGEN IN THE URINES OF RATS FED A LOW NITROGEN DIET CONTAINING 20 PER CENT FAT AND SUPPLEMENTED WITH METHIONINE (PART II)

Rat number	Total nitrogen		Urea nitrogen		Ammonia nitrogen		Total nitrogen in constituents	
	Period I	Period II	Period I	Period II	Period I	Period II	Pd. I	Pd. II
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
				56 Calories per 300 gm. rat per day				
47996	178	176	86	56	0.9	16	174	154
48170	225	181	98	56	0.4	14	193	167
48208	176	211	80	104	0.9	21	167	218
48076	186	198	87	75	1.3	23	171	197
48194	206	179	105	59	1.0	20	204	165
48055	198	184	97	61	1.0	18	191	163
Average	195	188	92	68	0.9	20	183	178
				14 Calories per 300 gm. rat per day				
48092	221	471	108	278	0.9	85	160	382
48171	167	292	76	166	0.7	44	135	297
48109	196	705	83	408	0.6	141	140	643
47863	206	723	82	296	0.8	188	148	606
48025	192	295	89	120	0.6	59	144	265
48067	174	507	83	243	0.7	101	143	445
Average	193	450	87	207	0.7	103	145	489

TABLE 23. PARTITION OF NITROGEN IN THE URINES OF RATS FED A LOW NITROGEN DIET CONTAINING NO FAT AND SUPPLEMENTED WITH METHIONINE (PART II)

Rat number	Total nitrogen		Urea nitrogen		Ammonia nitrogen		Total nitrogen in constituents	
	Period I	Period II	Period I	Period II	Period I	Period II	Pd. I	Pd. II
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
			56 Calories per 300 gm. rat per day					
48091	159	154	82	62	0.6	14	162	140
48061	178	168	76	62	0.7	13	149	159
48108	161	171	69	61	1.0	19	150	154
48202	161	155	73	61	0.8	16	155	143
48054	177	167	76	60	0.9	17	158	159
48178	169	171	76	61	1.0	17	158	150
Average	166	164	76	61	0.8	16	156	151
			14 Calories per 300 gm. rat per day					
47871	194	337	92	179	0.8	74	199	365
47916	164	344	67	192	0.7	62	200	358
47960	178	485	52	293	0.7	121	157	528
47962	166	730	68	394	0.7	168	169	703
47985	175	352	66	162	0.5	73	165	342
Average	175	450	70	234	0.7	97	178	459

TABLE 24. RELATIVE PROPORTIONS OF ALLANTOIN NITROGEN, CREATININE NITROGEN, AND AMINO NITROGEN IN THE URINES OF RATS FED THE STEENBOCK XVII DIET (PART I)

Rat number	Allantoin N / Total N	Creatinine N / Total N	Amino N / Total N
	Period I	Period I	Period I
	%	%	%
	Adequate food intake		
48474	3	1.0	0.1
48475	3	0.5	1.0
48404	4	0.8	1.1
48261	3	0.7	0.3
48449	3	0.8	0.1
48488	4	1.0	0.2
Average	3	1	0.5
	25 per cent of the adequate food intake		
50104	4	1.8	0.2
50105	5	2.0	0.8
50106	4	1.6	0.2
50096	4	1.5	0.1
50090	5	1.7	0.6
50097	5	1.8	0.5
Average	5	2	0.4

TABLE 25. RELATIVE PROPORTIONS OF ALLANTOIN NITROGEN, CREATININE NITROGEN, AND AMINO NITROGEN IN THE URINES OF RATS FED A LOW NITROGEN DIET CONTAINING 20 PER CENT FAT (PART I)

Rat number	Allantoin N / Total N		Creatinine N / Total N		Amino N / Total N	
	Period I	Period II	Period I	Period II	Period I	Period II
	%	%	%	%	%	%
56 Calories per day per 300 gm. rat						
47918	38	42	13	10	4.5	4.7
47933	38	45	13	10	6.0	4.5
48024	40	46	12	9	4.3	4.5
48017	26	47	11	8	3.1	1.5
47984	36	44	9	6	2.2	2.0
47834	34	44	9	6	1.8	2.1
Average	35	45	11	8	3.7	2.9
14 Calories per day per 300 gm. rat						
47961	37	7.1	12	5	4.5	0.9
47886	32	28	10	3	3.8	2.8
47963	35	17	10	3	1.1	1.4
47864	34	23	10	3	0.9	2.2
47954	32	27	9	2	2.4	3.2
47808	45	22	13	6	1.8	3.5
Average	36	15	10	3	2.4	2

TABLE 26. RELATIVE PROPORTIONS OF ALLANTOIN NITROGEN, CREATININE NITROGEN, AND AMINO NITROGEN IN THE URINES OF RATS FED A LOW NITROGEN DIET CONTAINING NO FAT (PART I)

Rat number	Allantoin N / Total N		Creatinine N / Total N		Amino N / Total N	
	Period I	Period II	Period I	Period II	Period I	Period II
	%	%	%	%	%	%
56 Calories per day per 300 gm. rat						
48023	45	37	14	10	2.1	7.1
47915	42	41	13	9	8.0	6.9
47789	36	23	12	8	7.3	8.6
47951	34	40	15	11	8.4	2.9
49869	39	35	14	10	6.4	6.4
47813	38	36	14	10	6.5	6.4
Average	39	35	14	10	6.4	6.4
14 Calories per day per 300 gm. rat						
48172	36	7	10	2	5.7	0.2
48138	33	6	9	2	4.1	0.2
48060	32	4	9	2	3.2	0.5
48207	35	7	9	1	7.2	0.2
48650	34	5	10	2	3.8	0.2
48115	32	5	9	1	3.7	0.3
Average	34	6	9	2	3.8	0.27

TABLE 27. RELATIVE PROPORTIONS OF ALLANTOIN NITROGEN, CREATININE NITROGEN, AND AMINO NITROGEN IN THE URINES OF RATS FED A LOW NITROGEN DIET CONTAINING 20 PER CENT FAT AND SUPPLEMENTED WITH METHIONINE (PART I)

Rat number	Allantoin N / Total N		Creatinine N / Total N		Amino N / Total N	
	Period I	Period II	Period I	Period II	Period I	Period II
	%	%	%	%	%	%
56 Calories per day per 300 gm. rat						
47996	33	30	10	9	5.7	7.5
48170	32	37	7	9	3.2	7.6
48208	37	35	8	8	3.6	5.7
48076	30	36	8	10	5.5	4.6
48194	32	33	10	8	6.2	5.6
48055	34	32	8	8	4.9	6.2
Average	33	34	9	9	4.9	6.2
14 Calories per day per 300 gm. rat						
48092	28	19	9	4	1.3	1.9
48171	37	20	10	7	3.0	1.6
48109	37	10	9	3	1.4	1.6
47863	38	10	10	3	3.1	1.6
48025	34	21	9	6	1.0	2.2
48067	37	14	10	5	2.7	1.4
Average	35	16	9	5	2.1	1.7

TABLE 28. RELATIVE PROPORTIONS OF ALLANTOIN NITROGEN, CREATININE NITROGEN, AND AMINO NITROGEN IN THE URINES OF RATS FED A LOW NITROGEN DIET CONTAINING NO FAT AND SUPPLEMENTED WITH METHIONINE (PART I)

Rat number	Allantoin N / Total N		Creatinine N / Total N		Amino N / Total N	
	Period I	Period II	Period I	Period II	Period I	Period II
	%	%	%	%	%	%
	56 Calories per day per 300 gm. rat					
48091	38	35	14	2	2.5	3.3
48061	34	42	15	3	2.8	5.8
48108	36	36	15	3	4.5	5.9
48202	38	35	12	1	3.0	5.0
48054	35	41	14	2	3.2	5.5
48178	35	37	14	2	3.5	5.1
Average	36	37	14	2	3.3	5.1
	14 Calories per day per 300 gm. rat					
47916	33	23	14	6	7.1	7.1
47960	32	18	15	7	19.0	14.0
47962	40	14	15	7	6.5	15.6
47985	42	14	12	4	5.7	15.4
47788	36	18	14	6	6.0	4.6
Average	37	18	14	6	8.9	2.6

TABLE 29. RELATIVE PROPORTIONS OF UREA NITROGEN AND AMMONIA NITROGEN IN THE URINES OF RATS FED THE STEENBOCK XVII DIET (PART II)

Rat number	Urea N / Total N	Ammonia N / Total N	Ammonia N / Urea N
	Period I	Period I	Period I
	%	%	%
	56 Calories per 300 gm. rat per day		
48474	95	0.7	
48475	94	0.9	
48404	93	1.0	
48261	94	0.5	
48449	94	1.6	
48488	92	1.6	
Average	94	1.0	.01
	14 Calories per 300 gm. rat per day		
50104	86	5.2	
50105	87	8.0	
50106	86	7.6	
50096	86	6.1	
50090	85	5.2	
50097	86	4.5	
Average	86	6.1	.07

TABLE 30. RELATIVE PROPORTIONS OF UREA NITROGEN AND AMMONIA NITROGEN IN THE URINES OF RATS FED A LOW NITROGEN DIET CONTAINING 20 PER CENT FAT (PART II)

Rat number	Urea N / Total N		Ammonia N / Total N		Ammonia N / Urea N	
	Period I	Period II	Period I	Period II	Period I	Period II
	%	%	%	%	%	%
	56 Calories per 300 gm. rat per day					
47918	46	47	5	3		
47933	67	41	5	2		
48024	47	41	7	4		
48017	49	48	6	2		
47984	41	37	4	1		
47834	46	47	3	3		
Average	50	44	5	3	.05	.05
	14 Calories per 300 gm. rat per day					
47961	47	76	0.2	9		
47886	48	71	0.8	8		
47963	46	67	0.1	8		
47864	40	71	0.6	7		
47954	46	73	0.3	8		
47808	44	72	0.3	11		
Average	45	72	0.3	9	.04	.13

TABLE 31. RELATIVE PROPORTIONS OF UREA NITROGEN AND AMMONIA NITROGEN IN THE URINES OF RATS FED A LOW NITROGEN DIET CONTAINING NO FAT (PART II)

Rat number	Urea N / Total N		Ammonia N / Total N		Ammonia N / Urea N	
	Period I	Period II	Period I	Period II	Period I	Period II
	%	%	%	%	%	%
56 Clories per 300 gm. rat per day						
48023	48	44	0.5	1		
47915	54	42	0.4	1		
47789	45	47	0.4	2		
47951	45	54	0.5	1		
49869	47	47	0.5	1		
47813	46	47	0.5	2		
Average	47	47	0.5	1	.01	.03
14 Calories per 300 gm. rat per day						
48172	45	61	0.4	25		
48138	47	57	0.4	27		
48060	47	68	0.4	22		
48207	47	67	0.3	23		
48650	46	57	0.4	27		
48115	47	68	0.5	25		
Average	47	63	0.4	25	.01	.40

TABLE 32. RELATIVE PROPORTIONS OF UREA NITROGEN AND AMMONIA NITROGEN IN THE URINES OF RATS FED A LOW NITROGEN DIET CONTAINING 20 PER CENT FAT AND SUPPLEMENTED WITH METHIONINE (PART II)

Rat number	Urea N / Total N		Ammonia N / Total N		Ammonia N / Urea N	
	Period I	Period II	Period I	Period II	Period I	Period II
	%	%	%	%	%	%
56 Calories per day per 300 gm. rat						
47996	48	32	0.5	9		
48170	44	31	0.2	8		
48208	45	49	0.5	10		
48076	47	38	0.7	12		
48194	51	33	0.5	11		
48055	49	33	0.5	10		
Average	47	36	0.5	10	.08	.30
14 Calories per day per 300 gm. rat						
48092	49	59	0.4	18		
48171	45	57	0.4	15		
48109	43	58	0.3	20		
47863	40	41	0.4	26		
48025	46	41	0.3	20		
48067	47	48	0.4	20		
Average	45	46	0.4	20	.08	.50

TABLE 33. RELATIVE PROPORTIONS OF UREA NITROGEN AND AMMONIA NITROGEN IN THE URINES OF RATS FED A LOW NITROGEN DIET CONTAINING NO FAT AND SUPPLEMENTED WITH METHIONINE (PART II)

Rat number	Urea N / Total N		Ammonia N / Total N		Ammonia N / Urea N	
	Period I	Period II	Period I	Period II	Period I	Period II
	%	%	%	%	%	%
56 Calories per day per 300 gm. rat						
48091	52	40	0.4	9		
48061	43	37	0.4	8		
48108	43	36	0.6	11		
48202	46	35	0.5	10		
48054	45	34	0.5	10		
48178	45	37	0.6	11		
Average	46	37	0.5	10	.01	.30
14 Calories per 300 gm. rat per day						
47871	48	53	0.4	22		
47916	47	56	0.4	18		
47960	48	50	0.4	25		
47962	47	54	0.4	23		
47985	48	46	0.3	21		
Average	48	52	0.4	22	.01	.41

TABLE 34. CONCENTRATIONS OF AMINO NITROGEN AND UREA IN THE BLOOD OF RATS FED THE STEENBOCK XVII DIET

Rat number	Urea		Amino nitrogen
	Urea	Urea nitrogen	
	mg./100 ml.		mg./100 ml.
	Normal food intake		
48606	22.8	10.6	15.66
48605	21.5	10.1	14.7
48607	40.9	19.1	18.0
48657	35.0	16.4	17.0
48643	38.3	17.9	17.7
48651	38.3	17.9	17.3
Average	32.8	15.3	16.7
	25 per cent of the normal food intake		
50083	41.6	19.4	13.7
50090	33.3	15.5	13.5
50031	32.5	15.2	13.5
50097	41.4	19.4	13.7
Average	37.2	17.4	13.6

TABLE 35. CONCENTRATIONS OF AMINO NITROGEN AND UREA IN THE BLOOD OF RATS FED A LOW NITROGEN DIET FOR 23 DAYS

Rat number	Urea		Amino nitrogen
	Urea	Urea nitrogen	
	mg./100 ml.		mg./100 ml.
20 per cent fat - 56 Calories per 300 gm. rat per day			
49255	7.5	3.5	19.6
49157	9.4	4.4	17.3
49154	3.9	1.8	13.3
49153	10.4	4.8	14.4
49281	7.6	3.6	15.8
Average	7.8	3.6	16.1
No fat - 56 Calories per 300 gm. rat per day			
49166	27.6	12.9	8.7
49246	15.1	7.1	11.8
49245	14.0	6.5	9.5
49263	12.5	5.8	9.7
Average	17.3	8.1	9.9

TABLE 36. CONCENTRATIONS OF AMINO NITROGEN AND UREA NITROGEN IN THE BLOOD OF RATS FED LOW NITROGEN DIET CONTAINING 20 PER CENT FAT

Rat number	Urea		Amino nitrogen
	Urea	Urea nitrogen	
	mg./100 ml.		mg./100 ml.
56 Calories per day per 300 gm. rat			
47918	9.2	4.3	25.5
47933	11.2	5.2	28.1
48024	11.1	5.1	26.8
48017	17.3	8.1	23.7
47984	9.1	4.3	23.4
47834	19.0	8.9	28.0
Average	12.8	6.0	25.9
14 Calories per day per 300 gm. rat			
47961	12.9	6.0	37.2
47886	12.4	5.8	33.4
47963	21.3	9.2	31.0
47864	11.4	5.5	31.0
47954	12.4	5.8	32.0
47808	12.4	5.8	44.1
Average	12.2	5.8	34.1

TABLE 37. CONCENTRATIONS OF AMINO NITROGEN AND UREA IN THE BLOOD OF RATS FED A LOW NITROGEN DIET CONTAINING NO FAT

Rat number	Urea		Amino nitrogen
	Urea	Urea nitrogen	
	mg./100 ml.		mg./100 ml.
56 Calories per 300 gm. rat per day			
48023	5.9	2.7	46.8
47915	5.9	2.7	46.4
47789	3.5	1.6	50.5
47951	5.5	2.5	43.6
Average	4.9	2.3	46.8
14 Calories per 300 gm. rat per day			
48172	64.7	30.2	15.8
48138	68.6	32.0	20.7
48060	67.4	31.4	20.7
48207	65.6	31.2	16.4
Average	66.6	31.2	18.4

TABLE 38. CONCENTRATIONS OF AMINO NITROGEN AND UREA IN THE BLOOD OF RATS FED A LOW NITROGEN DIET CONTAINING 20 PER CENT FAT AND SUPPLEMENTED WITH METHIONINE IN PERIOD II

Rat number	Urea		Amino nitrogen
	Urea	Urea nitrogen	
	mg./100 ml.		mg./100 ml.
56 Calories per 300 gm. rat per day			
47996	14.3	6.7	21.8
48170	6.6	3.1	18.0
48208	5.8	2.7	23.1
48076	9.6	4.5	20.1
48194	14.6	6.8	19.2
48055	6.8	3.2	18.4
Average	9.6	4.5	20.1
14 Calories per 300 gm. rat per day			
48092	16.4	7.5	22.8
48171	18.3	8.3	33.8
48109	17.1	7.8	24.2
47863	17.3	7.9	26.9
Average	17.3	7.9	26.9

TABLE 39. CONCENTRATIONS OF AMINO NITROGEN AND UREA IN THE BLOOD OF RATS FED A LOW NITROGEN DIET CONTAINING NO FAT AND SUPPLEMENTED WITH METHIONINE IN PERIOD II

Rat number	Urea		Amino nitrogen
	Urea	Urea nitrogen	
	mg./100 ml.		mg./100 ml.
56 Calories per 300 gm. rat per day			
48091	4.4	2.1	21.4
48061	7.7	3.6	38.4
48108	6.9	3.2	30.5
48202	10.2	4.8	31.7
Average	7.3	3.4	31.0
14 Calories per 300 gm. rat per day			
47871	32.3	15.0	16.9
47916	29.0	13.5	16.7
47960	24.5	11.4	16.4
47962	41.7	19.4	20.9
47738	39.1	18.2	20.8
Average	33.3	15.5	18.3

TABLE 40. ABSORPTION CURVE FOR P-NITROPHENOL

Wave length	Slit width	E reading
325	.28	.042
335	.24	.064
345	.16	.096
355	.16	.142
365	.11	.196
375	.11	.262
385	.11	.323
395	.13	.360
405	.05	.361
410	.05	.352
415	.05	.326
425	.05	.252
450	.04	.072
475	.04	.004

TABLE 41. CONCENTRATION OF SERUM ALKALINE PHOSPHATASE IN THE BLOOD OF RATS FED A LOW NITROGEN DIET CONTAINING EITHER 20 PER CENT OR NO FAT AND THE STEENBOCK XVII DIET

Rat number	E	Serum alkaline phospha-tase	Rat number	E	Serum alkaline phospha-tase	Rat number	E	SAP
	units	units*		units	units		units	units
	56 Calories per 300 gm. rat per day					Normal food intake		
	20 per cent fat			No fat		Steenbock XVII		
49681	0.257	10.8	49808	0.218	8.9	50168	0.396	17.3
49828	0.305	12.2	49843	0.701	30.6	50154	0.560	24.4
49622	0.251	9.9	49634	0.397	16.8	49462	0.394	16.6
Average		11.0			18.8			19.5
	14 Calories per 300 gm. rat per day					25% of normal intake		
49782	0.330	14.0	49785	0.600	26.7	50083	0.490	21.6
50050	0.484	20.8	50047	0.345	14.4	50031	0.898	39.3
49703	0.330	13.7	49620	0.391	16.4	50202	0.766	33.3
Average		16.2			19.2			31.4

*p-nitrophenylphosphate units

TABLE 42. CONCENTRATION OF SERUM ALKALINE PHOSPHATASE IN THE BLOOD OF RATS FED A LOW NITROGEN DIET CONTAINING EITHER 20 PER CENT OR NO FAT AND SUPPLEMENTED WITH METHIONINE IN PERIOD II

Rat number	E	Serum alkaline phosphatase	Rat number	E	Serum alkaline phosphatase
	units	units*		units	units
	56 Calories per 300 gm. rat per day				
	20 per cent fat			No fat	
49607	0.207	8.5	49979	0.323	13.8
			49643	0.658	28.6
Average		8.5			21.2
	14 Calories per 300 gm. rat per day				
49768	0.220	9.0	50068	0.554	24.5
49666	0.292	12.0	49807	0.710	31.3
49784	0.550	24.1	49777	0.486	21.0
Average		15.0			25.6

*p-nitrophenylphosphate units

TABLE 43. DETERMINATION OF GLUCOSE IN THE BLOOD OF RATS FED A LOW NITROGEN DIET CONTAINING 20 PERCENT FAT IN PERIOD II: PHOTOCOLORIMETER READINGS

Rat number	Minutes after glucose injection					
	0	30	60	120	180	240
	56 Calories per 300 gm. rat per day					
47782	130	370	334	265	214	146
47787	134	415	350	326	239	144
47810	128	412	340	279	207	140
47850	116	391	302	262	201	129
47857	110	340	277	234	182	146
Average	124	386	321	274	209	141
	14 Calories per 300 gm. rat per day					
47870	209	412	350	294	266	227
47885	199	379	370	326	299	242
47919	217	427	392	309	411	217
47941	186	414	380	322	283	249
47959	189	396	376	319	292	235
Average	190	406	374	303	278	234

TABLE 44. DETERMINATION OF GLUCOSE IN THE BLOOD OF RATS FED A LOW NITROGEN DIET CONTAINING 20 PER CENT FAT AND SUPPLEMENTED WITH METHIONINE IN PERIOD II: PHOTOELECTRIC COLORIMETER READINGS

Rat number	Minutes after glucose injection					
	0	30	60	120	180	240
	56 Calories per 300 gm. rat per day					
48099	108	359	313	246	181	140
48085	110	386	297	243	173	146
48106	106	347	314	234	189	136
48026	100	352	293	239	181	167
48135	114	364	297	233	181	141
Average	108	359	313	239	181	140
	14 Calories per 300 gm. rat per day					
48150	128	394	346	290	214	163
48201	136	383	348	289	215	188
48101	138	402	344	290	215	168
48077	130	395	347	286	206	148
48157	129	397	318	266	219	166
48203	136	382	394	333	225	173
Average	133	394	347	290	215	168

TABLE 45. DETERMINATION OF GLUCOSE IN THE BLOOD OF RATS FED A LOW NITROGEN DIET CONTAINING NO FAT: PHOTOELECTRIC COLORIMETER READINGS

Rat number	Minutes after glucose feeding					
	0	30	60	120	180	240
			56 Calories per 300 gm. rat per day			
47833	197	423	372	347	306	227
47856	166	399	379	339	235	159
47952	156	399	374	315	252	222
47887	156	420	379	348	259	226
47865	168	401	374	339	242	157
47809	196	399	372	315	306	223
Average	173	402	375	334	269	215
			14 Calories per 300 gm. rat per day			
47866	190	408	374	315	246	209
47869	184	379	352	326	238	---
47953	169	342	335	321	266	239
47976	202	414	386	331	263	230
47855	197	387	362	323	254	226
Average	197	387	362	323	254	226

TABLE 46. DETERMINATION OF GLUCOSE IN THE BLOOD OF RATS FED A LOW NITROGEN DIET CONTAINING NO FAT AND SUPPLEMENTED WITH METHIONINE: PHOTOELECTRIC COLORIMETER READINGS

Rat number	Minutes after glucose feeding					
	0	30	60	120	180	240
	56 Calories per 300 gm. rat per day					
48065	173	394	334	226	229	192
48116	168	412	329	279	215	173
48193	142	408	355	273	227	186
48155	128	287	333	293	246	198
48068	186	360	313	253	226	202
Average	160	392	333	265	229	190
	14 Calories per 300 gm. rat per day					
48075	184	347	313	247	207	181
48597	168	352	305	255	214	193
48643	146	396	326	266	215	186
48100	207	394	321	251	293	193
48179	166	306	301	237	210	180
Average	164	359	313	251	214	187

TABLE 47. DETERMINATION OF GLUCOSE IN THE BLOOD OF RATS FED THE STEENBOCK DIET AT NORMAL FOOD INTAKE

Rat number	Minutes after glucose injection					
	0	30	60	120	180	240
			Photocolorimeter readings			
48458	122	335	307	226	184	146
48388	133	350	321	241	176	152
48461	112	268	303	227	186	144
48406	120	303	291	238	176	153
48450	126	326	311	234	178	141
48487	117	334	306	233	180	157
Average	120	335	306	234	180	149
			Milligrams per cent glucose			
48458	92	252	231	170	138	110
48388	100	263	241	181	132	114
48461	85	277	228	171	140	108
48406	90	228	219	179	132	115
48450	95	242	234	176	134	106
48487	88	251	230	175	135	118
Average	90	252	230	176	135	112

TABLE 48. MILLIGRAMS PER CENT GLUCOSE IN THE BLOOD OF RATS FED A LOW NITROGEN DIET CONTAINING 20 PER CENT FAT IN PERIOD II

Rat number	Minutes after glucose injection					
	0	30	60	120	180	240
	56 Calories per 300 gm. rat per day					
47782	98	278	251	199	161	110
47787	101	312	263	245	180	108
47810	96	310	256	210	156	105
47850	87	294	227	197	151	97
47857	83	256	208	176	137	110
Average	93	290	241	206	157	106
	14 Calories per 300 gm. rat per day					
47870	157	310	263	221	200	171
47885	149	285	278	245	225	182
47919	163	321	295	232	187	163
47941	140	311	286	242	213	187
47959	141	298	283	240	220	177
Average	150	305	281	236	209	176

TABLE 49. MILLIGRAMS PER CENT GLUCOSE IN THE BLOOD OF RATS FED A LOW NITROGEN DIET CONTAINING NO FAT

Rat number	Minutes after glucose feeding					
	0	30	60	120	180	240
	56 Calories per 300 gm. rat per day					
47833	148	318	280	261	230	171
47856	125	300	285	255	179	148
47952	117	300	281	237	197	167
47887	117	316	285	262	195	170
47865	126	302	281	255	181	148
47809	147	300	280	237	230	168
Average	130	306	282	251	202	162
	14 Calories per 300 gm. rat per day					
47866	143	307	281	237	185	157
47869	138	285	265	245	179	---
47953	127	257	252	241	200	180
47976	152	311	290	249	196	173
47855	140	291	272	243	191	170
Average	140	290	272	243	190	170

TABLE 50. MILLIGRAMS PER CENT GLUCOSE IN THE BLOOD OF RATS FED A LOW NITROGEN DIET CONTAINING 20 PER CENT FAT AND SUPPLEMENTED WITH METHIONINE IN PERIOD II

Rat number	Minutes after glucose feeding					
	0	30	60	120	180	240
	56 Calories per 300 gm. rat per day					
48099	81	270	235	185	136	105
48085	83	290	240	183	130	110
48106	80	261	236	176	142	98
48026	75	265	220	180	136	103
48735	86	274	240	175	136	106
Average	81	270	235	180	136	105
	14 Calories per 300 gm. rat per day					
48150	96	296	260	218	161	124
48201	102	288	262	217	162	141
48101	104	302	259	218	162	126
48077	98	297	261	215	155	111
48157	97	298	237	200	165	125
48023	102	295	296	250	169	130
Average	100	296	261	218	162	126

TABLE 51. MILLIGRAMS PER CENT GLUCOSE IN THE BLOOD OF RATS FED A LOW NITROGEN DIET CONTAINING NO FAT AND SUPPLEMENTED WITH METHIONINE IN PERIOD II

Rat number	Minutes after glucose feeding					
	0	30	60	120	180	240
	56 Calories per 300 gm. rat per day					
48065	130	296	251	170	172	144
48116	126	310	247	210	162	130
48193	107	307	267	205	171	140
48155	97	291	250	220	185	149
48068	140	271	235	190	170	152
Average	120	295	250	199	172	143
	14 Calories per 300 gm. rat per day					
48075	138	261	235	186	156	136
48597	126	265	229	192	161	145
48643	110	298	245	200	162	140
48100	156	296	241	189	168	145
48179	125	230	226	178	158	135
Average	131	270	235	189	161	141

TABLE 52. CONCENTRATIONS OF GLYCOGEN IN THE LIVERS OF RATS FED THE STEENBOCK XVII DIET

Rat number	Weight of rat	Weight of liver sample	Weight of glycogen in sample	Per cent glycogen in sample	Total weight of liver	Total glycogen in liver	Per cent glycogen in liver
	gm.	gm.	mg.	%	gm.	mg.	%
			Normal food intake				
50168	336	3.171	9.6	0.3	7.333	23.5	0.3
		4.162	13.9	0.3			
50154	336	4.681	13.9	0.3	8.218	26.0	0.3
		3.537	12.1	0.3			
49462	336	3.259	15.6	0.4	8.241	35.9	0.4
		4.982	20.3	0.4			
Average							0.33
			25 Per cent of the normal food intake				
50083	276	2.931	37.4	1.3	5.705	84.7	1.8
		2.774	47.3	1.7			
50031	307	2.287	67.3	2.9	5.747	153.4	2.7
		3.460	86.1	2.5			
50202	303	2.675	31.4	1.2	6.616	81.4	1.3
		3.941	50.0	1.3			
Average							1.90

TABLE 53. CONCENTRATIONS OF GLYCOGEN IN THE LIVERS OF RATS FED A LOW NITROGEN DIET CONTAINING 20 PER CENT FAT

Rat number	Weight of rat	Weight of liver sample	Weight of glycogen in sample	Per cent glycogen in sample	Total weight of liver	Total glycogen in liver	Per cent glycogen in liver
	gm.	gm.	mg.	%	gm.	mg.	%
			56 Calories per 300 gm. rat per day				
49681	278	3.870	67.8	1.8	7.443	164.3	2.2
		3.573	96.5	2.7			
49828	292	4.801	67.8	1.4	8.601	121.1	1.4
		3.800	53.2	1.4			
Average							1.8
			14 Calories per 300 gm. rat per day				
49782	286	2.938	73.5	2.5	5.522	137.5	2.5
		2.584	64.1	2.5			
50040	300	3.312	63.1	1.9	6.103	106.1	1.9
		2.791	53.0	1.9			
Average							2.2

TABLE 54. CONCENTRATIONS OF GLYCOGEN IN THE LIVERS OF RATS FED A LOW NITROGEN DIET CONTAINING NO FAT

Rat number	Weight of rat	Weight of liver sample	Weight of glycogen in sample	Per cent glycogen in sample	Total weight of liver	Total glycogen in liver	Per cent glycogen in liver
	gm.	gm.	gm.	mg.	%	mg.	%
			56 Calories per 300 gm. rat per day				
49808	285	3.629 4.227	7.857	120.0 135.3	3.3 3.2	255.3	3.3
49843	277	4.221 4.336	8.557	94.2 95.4	2.2 2.2	189.6	2.2
49634	280	3.867 4.495	8.362	75.8 90.0	2.0 2.0	165.8	2.0
Average							2.5
			14 Calories per 300 gm. rat per day				
49785	292	3.0969 2.5346	5.632	56.5 45.6	1.8 1.8	102.1	1.8
50047	282	2.023 3.084	5.107	2.3 2.1	0.1 0.1	4.4	0.09
49620	276	2.637 2.320	4.957	2.9 2.9	0.1 0.1	5.8	0.12
Average							0.67

TABLE 55. CONCENTRATIONS OF GLYCOGEN IN THE LIVERS OF RATS FED A LOW NITROGEN DIET CONTAINING 20 PER CENT FAT AND SUPPLEMENTED WITH METHIONINE IN PERIOD II

Rat number	Weight of rat	Weight of liver sample	Weight of glycogen in sample	Per cent glycogen in sample	Total weight of liver	Total glycogen in liver	Per cent glycogen in liver
	gm.	gm.	gm.	mg.	%	mg.	%
			56 Calories per 300 gm. rat per day				
49607	282	5.465	10.477	88.3	1.6	176.9	1.7
		5.012		88.6	1.8		
			14 Calories per 300 gm. rat per day				
49768	256	2.726	5.857	62.7	2.3	135.9	2.3
		3.131		73.2	2.3		
49666	265	2.717	4.875	32.0	1.2	57.9	1.2
		2.159		25.9	1.2		
Average							1.8

TABLE 56. CONCENTRATIONS OF GLYCOGEN IN THE LIVERS OF RATS FED A LOW NITROGEN DIET CONTAINING NO FAT AND SUPPLEMENTED WITH METHIONINE IN PERIOD II

Rat number	Weight of rat	Weight of liver sample	Weight of glycogen in sample	Per cent glycogen in sample	Total weight of liver	Total glycogen in liver	Per cent glycogen in liver
	gm.	gm.	mg.	%	gm.	mg.	%
			56 Calories per 300 gm. rat per day				
49979	283	6.0317	144.8	2.4	11.689	277.9	2.4
		5.657	133.1	2.4			
			14 Calories per 300 gm. rat per day				
50068	268	4.676	102.9	2.2	9.326	205.0	2.2
		4.650	102.1	2.2			
49807	287	2.661	103.3	3.9	7.349	201.2	2.7
		4.688	97.9	2.1			
Average							2.5

TABLE 57. WEIGHTS OF LIVERS OF RATS FED THE STEENBOCK XVII DIET AND THEIR RESPECTIVE CONCENTRATIONS OF MOISTURE AND FAT

Rat number	Body weight	Weight of liver			Moisture		Fat		
		Fresh basis	Dry basis	Fat-free basis	Total weight	Per-cent	Total weight	Wet basis	Dry basis
	gm.	gm.	gm.	gm.	gm.	%	gm.	%	%
				Normal food intake					
48474	286	9.061	2.872	2.482	6.189	68.3	0.390	4.3	13.6
48475	324	10.019	3.116	2.715	6.903	68.9	0.401	4.0	12.9
48404	278	8.291	2.823	2.483	5.468	68.1	0.340	4.1	12.0
48261	318	9.176	2.991	2.633	6.185	67.4	0.358	3.9	12.0
48449	312	8.619	2.723	2.370	5.896	68.4	0.353	4.1	13.0
48488	282	8.071	2.534	2.235	5.537	68.6	0.299	3.7	11.8
Average	300	8.873	2.843	2.486	6.029	68.3	0.357	4.0	12.6
				25 per cent of the normal food intake					
50096	282	5.693	1.805	1.441	3.888	68.3	0.364	6.4	20.2
50090	296	5.789	1.835	1.499	3.954	68.3	0.336	5.8	18.3
50097	278	5.823	1.817	1.520	4.006	68.8	0.297	5.1	16.3
Average	285	5.768	1.819	1.487	3.950	68.4	0.332	5.8	18.3

TABLE 58. WEIGHTS OF LIVERS OF RATS FED A LOW NITROGEN DIET FOR 23 DAYS AND THEIR RESPECTIVE CONCENTRATIONS OF MOISTURE AND FAT

Rat number	Body weight	Weight of liver			Moisture		Fat		
		Fresh basis	Dry basis	Fat-free basis	Total weight	Percent	Total weight	Wet basis	Dry basis
	gm.	gm.	gm.	gm.	gm.	%	gm.	%	%
20 per cent fat - 56 Calories per 300 gm. rat per day									
49153	254	7.495	2.242	1.613	5.253	70.1	0.629	8.4	28.1
49282	272	7.592	2.278	1.655	5.314	70.0	0.623	8.2	27.3
49281	266	7.327	2.213	1.598	5.114	69.8	0.615	8.4	27.8
Average	264	7.471	2.244	1.622	5.227	70.0	0.622	8.3	27.7
No fat - 56 Calories per 300 gm. rat per day									
49165	272	7.615	2.216	1.645	5.399	70.9	0.571	7.5	25.8
49166	285	9.601	2.880	1.846	6.721	70.0	1.034	10.8	35.9
49169	272	8.283	2.427	1.682	5.856	70.7	0.745	9.0	30.7
Average	276	8.500	2.508	1.724	5.992	70.5	0.783	9.1	30.8

TABLE 59. WEIGHTS OF LIVERS OF RATS FED A LOW NITROGEN DIET CONTAINING 20 PER CENT FAT IN PERIOD II AND THEIR RESPECTIVE CONCENTRATIONS OF MOISTURE AND FAT

Rat number	Body weight	Weight of liver			Moisture		Fat		
		Fresh basis	Dry basis	Fat-free basis	Total weight	Percent	Total weight	Wet basis	Dry basis
	gm.	gm.	gm.	gm.	gm.	%	gm.	%	%
			56 Calories per 300 gm. rat per day						
47918	209	8.986	3.019	1.572	5.967	66.4	1.447	16.1	47.9
47933	231	8.118	2.476	1.023	5.642	69.5	1.453	17.9	58.7
48024	252	8.551	2.805	1.657	5.746	67.2	1.148	14.6	40.9
48017	248	8.689	2.850	1.781	5.839	67.2	1.069	12.3	37.5
47984	272	8.431	2.689	1.930	5.742	68.1	0.759	9.0	28.2
47834	274	8.555	2.592	1.856	5.963	69.7	0.736	8.6	28.4
Average	248	8.555	2.739	1.637	5.816	68.0	1.102	13.1	40.3
			14 Calories per 300 gm. rat per day						
47961	182	5.779	1.485	1.127	4.294	74.3	0.358	6.2	24.1
47886	219	5.411	1.694	1.402	3.717	68.7	0.292	5.4	17.2
47963	222	5.590	1.420	1.174	4.170	74.6	0.246	4.4	17.3
47864	233	5.601	1.708	1.411	3.893	69.5	0.297	5.3	17.4
47954	262	5.732	1.708	1.370	4.024	70.2	0.338	5.9	19.8
47808	218	5.457	1.615	1.320	3.842	70.4	0.295	5.4	18.3
Average	223	5.595	1.605	1.301	3.987	71.3	0.304	5.4	19.0

TABLE 60. WEIGHTS OF LIVERS OF RATS FED A LOW NITROGEN DIET CONTAINING NO FAT AND THEIR RESPECTIVE CONCENTRATIONS OF MOISTURE AND FAT

Rat number	Body weight	Weight of liver			Moisture		Fat		
		Fresh basis	Dry basis	Fat-free basis	Total weight	Percent	Total weight	Wet basis	Dry basis
	gm.	gm.	gm.	gm.	gm.	%	gm.	gm.	gm.
				56 Calories per 300 gm. rat per day					
48023	216	7.639	2.261	1.650	5.378	70.4	0.611	8.0	27.0
47915	256	7.495	2.706	1.477	4.789	63.9	1.229	16.4	45.4
47789	255	6.888	2.080	1.570	4.808	69.8	0.510	7.4	24.5
47951	241	7.356	2.163	1.575	5.193	70.6	0.588	8.0	27.2
49869	250	7.842	2.290	1.239	5.552	70.8	1.051	13.4	45.9
47813	255	6.825	1.986	1.276	4.839	70.9	0.710	10.4	35.8
Average	246	7.341	2.248	1.465	5.093	69.6	0.783	10.6	34.3
				14 Calories per 300 gm. rat per day					
48172	253	5.517	1.490	1.302	4.027	73.0	0.188	3.4	12.6
48138	193	3.529	0.978	0.840	2.551	72.3	0.138	2.9	14.1
48060	254	4.807	1.303	1.165	3.504	72.9	0.139	2.9	10.7
48207	184	4.679	1.259	1.128	3.420	73.1	0.131	2.8	10.4
48650	253	4.922	1.324	1.112	3.598	73.1	0.212	4.3	16.0
48115	200	4.704	1.303	1.181	3.401	72.3	0.122	2.6	9.4
Average	223	4.869	1.276	1.121	3.417	72.8	0.155	3.3	12.2

TABLE 61. WEIGHTS OF LIVERS OF RATS FED A LOW NITROGEN DIET CONTAINING NO FAT AND SUPPLEMENTED WITH METHIONINE IN PERIOD II AND THEIR RESPECTIVE CONCENTRATIONS OF MOISTURE AND FAT

Rat number	Body weight	Weight of liver			Moisture		Fat		
		Fresh basis	Dry basis	Fat-free basis	Total weight	Percent	Total weight	Wet basis	Dry basis
	gm.	gm.	gm.	gm.	gm.	%	gm.	gm.	gm.
				56 Calories per 300 gm. rat per day					
48091	232	9.257	3.545	1.823	5.712	61.7	1.722	18.6	48.6
48061	247	10.293	3.757	1.173	6.536	63.5	2.584	25.1	68.8
48108	220	8.531	2.952	1.306	5.579	65.4	1.646	19.3	55.8
48202	234	8.271	2.729	1.877	5.542	67.0	0.852	10.3	31.2
48054	250	10.436	3.465	1.221	6.971	66.8	2.244	21.5	64.8
48178	226	9.138	3.143	1.160	5.995	65.6	1.983	21.7	63.1
Average	239	9.321	3.265	1.427	6.055	65.0	1.833	21.2	55.4
				14 Calories per 300 gm. rat per day					
47871	236	5.517	1.600	1.335	3.917	71.0	0.265	4.8	19.9
47916	206	4.922	1.575	1.294	3.347	68.0	0.281	5.7	21.7
47960	246	5.187	1.535	1.234	3.652	70.4	0.301	5.8	24.4
47962	238	5.236	1.534	1.277	3.702	70.7	0.257	4.9	20.1
47985	228	4.892	1.487	1.262	3.405	69.6	0.225	4.6	17.8
47788	236	5.705	1.683	1.381	4.022	70.5	0.302	5.3	21.9
Average	231	5.243	1.569	1.297	3.674	70.5	0.272	5.2	21.0

TABLE 62. WEIGHTS OF LIVERS OF RATS FED A LOW NITROGEN DIET CONTAINING 20 PER CENT FAT AND SUPPLEMENTED WITH METHIONINE IN PERIOD II AND THEIR RESPECTIVE CONCENTRATIONS OF MOISTURE AND FAT

Rat number	Body weight	Weight of liver			Moisture		Fat		
		Fresh basis	Dry basis	Fat-free basis	Total weight	Per-cent	Total weight	Wet basis	Dry basis
	gm.	gm.	gm.	gm.	gm.	%	gm.	%	%
				56 Calories per 300 gm. rat per day					
47996	232	8.101	3.313	2.349	4.788	59.1	0.964	11.9	29.1
48170	238	8.086	2.862	1.667	5.224	64.6	1.195	14.8	41.8
48208	242	8.900	3.115	1.856	5.785	65.0	1.259	14.1	40.4
48076	268	9.478	3.526	1.861	5.952	62.8	1.665	17.6	47.2
48194	274	7.427	2.711	1.497	4.716	63.5	1.214	16.3	44.8
48055	228	8.141	2.857	1.710	5.284	64.9	1.147	14.1	40.1
Average	247	8.356	3.1	1.8	5.291	63.3	1.241	14.8	40.6
				14 Calories per 300 gm. rat per day					
48092	218	2.919	0.750	0.610	2.169	74.3	0.140	4.8	18.7
48171	209	3.970	1.270	1.099	2.700	67.9	0.171	4.3	13.4
48109	200	2.586	0.685	0.593	1.901	73.5	0.092	3.4	13.5
47863	222	2.623	0.687	0.620	1.936	73.8	0.067	2.6	9.8
48025	232	4.644	1.625	1.391	3.019	65.0	0.234	5.0	14.4
48067	230	3.064	0.861	0.780	2.203	71.9	0.081	2.6	9.4
Average	222	3.301	1.0	0.85	2.320	71.1	0.131	3.8	13.2

TABLE 63. TITRATION VALUES FOR STANDARD CURVE FOR NICOTINIC ACID ASSAY

Aliquot of the standard solution	Nicotinic acid content of the aliquot	Titration value
ml.	mcg.	ml.
0	---	0.9 0.8 0.9 0.8 0.9
0.5	0.1	3.5 3.5 3.6 3.8 3.9
1.0	0.2	5.3 5.4 5.5 5.4 5.4
1.5	0.3	6.5 6.6 6.4 6.5 6.5
2.0	0.4	7.40 7.40 7.3 7.4 7.4
2.5	0.5	8.2 8.2 8.3 8.2 8.2

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TABLE 63 (CONT'D)

Aliquot of the standard solution	Nicotinic acid content of the aliquot	Titration value
ml.	mcg.	ml.
3.0	0.6	8.8 8.7 8.8
4.0	0.8	9.2 9.3 9.3
5.0	1.0	10.0 10.0 9.9

TABLE 64. TITRATION VALUES FOR STANDARD CURVE FOR RIBOFLAVIN ASSAY

Aliquot of standard solution	Riboflavin content of aliquot	Titration value
ml.	mcg.	ml.
0	--	0.9
		0.8
		0.9
		0.9
		0.8
0.5	0.025	3.9
		4.3
		4.3
		4.5
		4.3
1.0	0.050	5.7
		5.6
		5.7
		5.8
		5.6
1.5	0.075	6.2
		6.3
		6.4
		6.5
		6.3
2.0	0.100	7.0
		7.2
		7.3
		7.0
		7.2
2.5	0.125	7.7
		7.9
		8.0
		7.7
		7.7

(Continued on next page)

TABLE 64 (CONT'D)

Aliquot of standard solution	Riboflavin content of aliquot	Titration value
ml.	mcg.	ml.
3.0	0.150	8.5
		8.5
		8.5
4.0	0.200	9.9
		9.9
		9.8
5.0	0.250	11.0
		10.9
		11.0

TABLE 65. CONCENTRATIONS OF TOTAL NITROGEN, RIBOFLAVIN, AND NICOTINIC ACID IN THE LIVERS OF RATS FED THE STEENBOCK XVII DIET

Rat number	Total nitrogen	Riboflavin		Nicotinic acid	
		Total	Ratio: riboflavin to nitrogen x 10^{-5}	Total	Ratio: nicotinic acid to nitrogen x 10^{-4}
	mg.	mcg.	units	mcg.	units
		Normal food intake			
49486	318	201	63	1130	36
49474	318	201	63	1135	36
49515	318	201	63	1131	36
Average	318	201	63	1131	36
		25 per cent of the normal food intake			
50097	220	101	46	785	36
50083	224	109	49	770	34
50031	224	108	48	769	34
Average	223	106	48	771	35

TABLE 66. CONCENTRATIONS OF TOTAL NITROGEN, RIBOFLAVIN, AND NICOTINIC ACID IN THE LIVERS OF RATS FED A LOW NITROGEN DIET CONTAINING 20 PER CENT FAT

Rat number	Total nitrogen	Riboflavin		Nicotinic acid	
		Total	Ratio: riboflavin to nitrogen x 10^{-5}	Total	Ratio: nicotinic acid to nitrogen x 10^{-4}
	mg.	mcg.	units	mcg.	units
		56 Calories per 300 gm. rat per day			
49308	216	116	54	800	37
49465	218	118	54	803	37
48614	219	119	54	805	37
Average	218	118	54	803	37
		14 Calories per 300 gm. rat per day			
49445	199	110	55	680	34
49353	204	116	55	671	33
49354	202	112	55	676	33
Average	202	112	55	676	33

TABLE 67. CONCENTRATIONS OF TOTAL NITROGEN, RIBOFLAVIN, AND NICOTINIC ACID IN THE LIVERS OF RATS FED A LOW NITROGEN DIET CONTAINING NO FAT

Rat number	Total nitrogen	Riboflavin		Nicotinic acid	
		Total	Ratio: riboflavin to nitrogen $x10^{-5}$	Total	Ratio: nicotinic acid to nitrogen $x10^{-4}$
	mg.	mcg.	units	mcg.	units
		56 Calories per 300 gm. rat per day			
49434	180	53	30	670	37
49402	181	54	30	690	38
49147	181	54	30	679	37
Average	181	54	30	679	37
		14 Calories per 300 gm. rat per day			
49383	186	94	51	423	23
49338	186	94	51	423	23
49170	184	91	49	422	23
Average	186	94	51	423	23

TABLE 68. CONCENTRATIONS OF TOTAL NITROGEN, RIBOFLAVIN, AND NICOTINIC ACID IN THE LIVERS OF RATS FED A LOW NITROGEN DIET CONTAINING 20 PER CENT AND SUPPLEMENTED WITH METHIONINE IN PERIOD II

Rat number	Total nitrogen	Riboflavin		Nicotinic acid	
		Total	Ratio: riboflavin to nitrogen x 10^{-5}	Total	Ratio: nicotinic acid to nitrogen x 10^{-4}
	mg.	mcg.	units	mcg.	units
		56 Calories per 300 gm. rat per day			
49427	181	92	51	718	39
49426	183	95	51	718	39
49306	182	93	51	717	39
Average	182	93	51	718	39
		14 Calories per 300 gm. rat per day			
49400	126	61	48	391	31
49424	126	60	48	391	31
49396	125	63	50	391	31
Average	126	61	48	391	31

TABLE 69. CONCENTRATIONS OF TOTAL NITROGEN, RIBOFLAVIN, AND NICOTINIC ACID IN THE LIVERS OF RATS FED A LOW NITROGEN DIET CONTAINING NO FAT AND SUPPLEMENTED WITH METHIONINE IN PERIOD II

Rat number	Total nitrogen	Riboflavin		Nicotinic acid	
		Total	Ratio: riboflavin to nitrogen x 10^{-5}	Total	Ratio: nicotinic acid to nitrogen x 10^{-4}
	mg.	mcg.	units	mcg.	units
		56 Calories per 300 gm. rat per day			
49443	223	121	55	955	43
49385	224	123	55	950	42
49444	224	123	55	959	43
Average	224	123	55	955	43
		14 Calories per 300 gm. rat per day			
49411	189	116	58	731	39
49412	185	110	59	730	39
49462	185	107	59	735	39
Average	186	111	59	732	39

TABLE 70. WEIGHTS OF ADRENAL GLANDS OF RATS FED THE STEENBOCK XVII DIET

Rat number	Body weight	Weight of adrenal glands	Ratio: adrenal wt. / body wt.
	gm.	mg.	x10-5
	Normal food intake		
48474	286	40.1	14
48475	312	39.9	13
48404	278	33.8	12
Average	292	37.9	13
	25 per cent of the normal food intake		
50096	278	54.5	20
50090	284	51.9	18
50097	276	52.6	19
Average	279	52.9	19

TABLE 71. WEIGHTS OF ADRENAL GLANDS OF RATS FED A LOW NITROGEN DIET FOR 23 DAYS

Rat number	Body weight	Weight of adrenal glands	Ratio: adrenal wt. / body wt.
	gm.	mg.	x10^{-5}
20 per cent fat - 56 Calories per 300 gm. rat per day			
49153	254	46.9	18
49282	272	36.5	13
49281	266	40.4	15
Average	264	41.3	15
No fat - 56 Calories per 300 gm. rat per day			
49165	272	43.9	16
49166	285	47.6	17
49169	272	51.6	19
Average	276	47.7	17

TABLE 72. WEIGHTS OF ADRENAL GLANDS OF RATS FED A LOW NITROGEN DIET CONTAINING 20 PER CENT FAT

Rat number	Body weight	Weight of adrenal glands	Ratio: adrenal wt. / body wt.
	gm.	mg.	x10^{-5}
56 Calories per 300 gm. rat per day			
47918	209	30.0	14
48024	252	38.0	15
48017	248	43.1	17
47984	272	41.4	15
47834	274	45.8	17
Average	251	39.7	16
14 Calories per 300 gm. rat per day			
47961	182	41.8	23
47886	219	50.7	23
47963	222	51.0	23
Average	208	41.8	23

TABLE 73. WEIGHTS OF ADRENAL GLANDS OF RATS FED A LOW NITROGEN DIET CONTAINING NO FAT

Rat number	Body weight	Weight of adrenal glands	Ratio: adrenal wt. / body wt.
	gm.	mg.	x10^{-5}
56 Calories per 300 gm. rat per day			
47951	255	46.1	18
47813	241	37.6	16
48023	223	40.5	18
47915	242	42.4	18
Average	240	41.7	18
14 Calories per 300 gm. rat per day			
47871	253	39.3	16
47916	250	39.0	16
47960	245	38.1	16
Average	249	38.8	16

TABLE 74. WEIGHTS OF ADRENAL GLANDS OF RATS FED A LOW NITROGEN DIET CONTAINING 20 PER CENT FAT AND SUPPLEMENTED WITH METHIONINE IN PERIOD II

Rat number	Body weight	Weight of adrenal glands	Ratio: adrenal wt. / body wt.
	gm.	mg.	$\times 10^{-5}$
56 Calories per 300 gm. rat per day			
48055	218	36.3	17
48208	242	44.1	18
48076	268	51.1	19
Average	242	43.8	18
14 Calories per 300 gm. rat per day			
48109	200	41.5	21
48025	231	41.0	18
48067	230	41.0	18
Average	220	41.2	19

TABLE 75. WEIGHTS OF ADRENAL GLANDS OF RATS FED A LOW NITROGEN DIET CONTAINING NO FAT AND SUPPLEMENTED WITH METHIONINE IN PERIOD II

Rat number	Body weight	Weight of adrenal glands	Ratio: adrenal wt. / body wt.
	gm.	mg.	$\times 10^{-5}$
56 Calories per 300 gm. rat per day			
48091	232	42.2	18
48202	234	52.6	22
48054	220	39.6	18
48178	247	46.4	19
Average	233	45.2	19
14 Calories per 300 gm. rat per day			
47960	240	39.1	16
47962	180	50.0	28
47788	286	40.4	14
47871	246	35.7	15
47916	236	40.7	17
47985	228	33.5	15
Average	236	39.9	18

TABLE 76. CONDITION OF MALE RATS AT NECROPSY

Rat No._____ Diet_____ Weight_____ Age_____

General Description:
Appearance_____ Alert_____ Paws Pink_____ Ears Pink_____
Fat_____ Gaunt_____ Lice_____ Muscle Tone_____

Gait: Dragging_____ Sprawling_____ Awkward_____ Elevated_____

Skin: Sores_____ Scaly_____ Color_____ Eyelids: Inflamed_____ Infected____

Hair: Clean_____ Smooth_____ Thick_____ Colored_____ Brown patches_____

Tail: Clean_____ Smooth_____ Discolored_____ Sores_____

Respiration: Sniffles_____ Wheeziness_____ Palpitation_____

Exudates: Nasal_____ Genital_____ Oral_____ Genitals: Development of tests_____
Priapism_____

Fat Depots: Subcutaneous_____ Peritoneal_____ Omental_____ Perirenal_____
Genital_____ Intramuscular_____

Liver: Yellow_____ Mottled_____ Friable_____ Spongy_____

Kidneys: Color_____ Friability_____ Hemorrhage_____ Stomach Ulcers: Number_____ Severity_____

Lungs: (lobes) 1 2 3 4 5 Pus Pockets:
Infections _____ _____ _____ _____ _____ Ear_____ Rt._____ Left_____
Atelectasis _____ _____ _____ _____ _____ Base of tongue_____
Emphysema _____ _____ _____ _____ _____

Remarks: