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INFLUENCE OF NON-PROTEIN CALORIES ON THE NITROGEN ECONOMY OF PROTEIN-DEPLETED RATS

by

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INTRODUCTION

The problem of survival when only minimum food and water are available aroused the interest of investigators concerned with basic, applied, and technological aspects of the science of Nutrition during World War II and the immediate post-war period. A person isolated from society by shipwreck, plane crash, or similar disaster must be able to reduce the adverse physiological effects of his environment to a minimum if he is to survive. While ability to protect himself from exposure to unfavorable environmental conditions and to conquer fear so that he approaches the situation intelligently are of primary importance to the survivor, he also must have access to a food supply that partially, at least, will meet his particular needs. The body can withstand water deprivation only for relatively short periods of time; needs for salt too may become critical and are determined to a large extent upon water intake. On the other hand, the physiological need of the body for food is less acute; a castaway rarely dies from starvation. The individual accrues many psychological benefits, however, from the ingestion of food, and its provision becomes an important consideration.

A satisfactory survival ration is one that is palatable, keeps well, and provides the nutrients most essential for the maintenance of physical stamina and the prevention of deterioration of the body or its functions. Calories and protein rank highest among the nutrients in meeting nutritive needs peculiar to these particular stress conditions. Protein, however, is not completely oxidized by the animal body and requires water for elimination of its metabolic end-products, and its use heretofore has not been recommended in survival rations.

Interest in the problem of the formulation of survival rations in the Home Economics Nutrition Laboratory at the Iowa State College was stimulated first by studies in which the nutritive value of egg proteins was investigated. The incorporation of egg proteins into a protein-free diet reduced the quantity of nitrogen excreted in the urine by adult male rats below that occurring when the basal low-nitrogen ration was ingested (Marshall, 1943). This finding indicated, not only that egg proteins were utilized efficiently, but also that their presence in the diet, in amounts commensurate with the maintenance of nitrogen equilibrium, did not impose additional strain upon the organism in respect to the elimination of nitrogenous end-products. That egg proteins or other equally biologically efficient proteins may be used advantageously in survival rations was suggested.

It has been believed that carbohydrate and fat have special merit as components of survival rations. The idea that they are particularly valuable in the conservation of the body water of the castaway is based on the concept that carbohydrate and fat are completely oxidized and give rise to substantial amounts of water in metabolism, thereby reducing dehydration and, at the same time, causing no increase in the amount of obligatory urine.

In starvation, body tissue is broken down to provide energy. This destruction can be averted by the ingestion of either carbohydrate or fat. These nutrients decrease the catabolism characteristic of starvation being oxidized in preference to body tissue and, as a salutary secondary effect, minimize the quantity of water needed for the excretion of protein end-products.

The idea that carbohydrate may possess a greater protein-sparing action than fat has prevailed since the time of Voit (1869). Workers in general have agreed that carbohydrate can exert its sparing effect on either tissue protein or dietary protein (Munro, 1951). For this reason the early survival rations consisted of pure carbohydrate only. The use of carbohydrate instead of fat was favored, also, because metabolism of fat in the absence of adequate carbohydrate is associated with ketosis and disruption of water balance.

Consideration of these ideas suggested that omission of fat from the basal diet used in the studies at the Iowa State College might influence protein metabolism favorably. Experiments were initiated to study the endogenous protein metabolism when varying proportions of carbohydrate and fat served as sources of energy in the diet. The relative efficacies of carbohydrate and fat in reducing body catabolism of adult rats were determined when a low-calorie, nitrogen-free diet was fed that was similar in many respects to the survival ration.

In these experiments, protein-free diets containing either carbohydrate and fat (20 per cent) or carbohydrate only were fed to adult male rats in graded quantities supplying 100, 75, 50, or 25 per cent of the daily energy requirement. When rations were consumed in isocaloric quantities, response to the feeding of the fat-free and fat-containing diets was the same when the ration provided 50 per cent or more of the needed calories. However, when caloric reductions exceeded 50 per cent, a more rapid catabolism of body protein occurred in the rats receiving the fat-free diets than in the animals receiving fat (Stevenson _et_ _al._, 1946). Results, therefore, were exactly the reverse of those that had been expected on the basis of the classic experiments.

Hoover (1950) repeated the same experiment under more precise conditions. Wishing to rule out the influence of faulty appetite and at the same time to administer the fat-containing and fat-free diets in strictly isocaloric quantities, she adopted a force-feeding technique. Again the feeding of a low-fat diet in restricted quantities occasioned a larger increase in nitrogen in the urine than did analogous reduction of the high-fat diet. Increments in urinary nitrogen in response to caloric reduction were even greater when the diets were force-fed than when animals ate of their own accord from a food cup. Hoover found that the augmented excretions of total nitrogen induced by caloric reduction reflected increases in urea and ammonia fractions of the urine. As was true for total nitrogen, the feeding of the low-fat ration caused greater increases in urea and ammonia than did the high-fat diet. The increment in ammonia nitrogen was particularly evident indicating that alterations had occurred in the pathway of nitrogen metabolism.

The results of these experiments raised the question that if fat is needed, how much is needed. Is there a critical level of dietary fat below which nitrogen catabolism is increased? In the next experiment (Willman et al., 1947), rations in which either 20, 15, 10, 5, or zero per cent fat were incorporated into the diet, were fed at the four levels of caloric intake used in the Stevenson et al. study. Body catabolism proceeded at the same rate at each level of caloric intake when the rations provided 20 to 15 per cent of fat. When the fat content was reduced to 10 per cent, however, destruction of body tissue was greatly accelerated when the diet provided only one-fourth of the needed energy. The picture when diets containing 5 per cent of fat were fed was essentially the same at all levels of caloric intake as when the fat-low rations were administered.

These experiments have yielded important basic knowledge regarding the course of protein metabolism in experimental situations uncomplicated by the addition of food protein. However, many questions still remain unanswered. For example,

> Are all fats equally effective in reducing the nitrogen catabolism caused by the feeding of protein-free, low-energy diets?
>
> Does body fat have an influence on the nitrogen metabolism of rats subjected to these dietary conditions?
>
> What property or component of the fat molecule is responsible for its tissue-sparing effect under the specific experimental conditions imposed?
>
> Are the characteristic effects of the inclusion of dietary fat in rations of varying caloric value transient phenomena?
>
> How does the inclusion of fat in a protein-free diet influence time of survival when the

energy value of the ration varies?

The first problem arose because results to date have been obtained from experiments in which a mixture of equal parts butterfat and lard have been used. It seemed desirable to determine whether the favorable influence was peculiar to this particular combination of fats or whether other sources of fat, either singly or in combination, were equally effective in conserving body tissue.

It seemed possible that stores of body fat might function like dietary fat in reducing the rate of tissue catabolism characteristic of underfeeding. It was observed upon necropsy that the quantity of depot fat varied appreciably in rats subjected to caloric restriction. Large body stores of fat were associated with a low incidence of lung infection, small losses in body weight, and, when caloric restriction was imposed, with low excretions of nitrogen in the urine. On the basis of these observations it seemed desirable to study the influence of dietary fat and carbohydrate on protein metabolism in animals reduced to approximately the same degree of fatness before the initiation of the experiment.

One of the fundamental aspects of this problem was to learn why fat favorably influenced the nitrogen metabolism in a protein-starved rat fed a diet providing an inadequate number of calories. It seemed worthwhile to ascertain if the protective effect of fat was related to its essential fatty acid composition or to its degree of saturation.

The beneficial effect of fat in protein-free diets is observed immediately when caloric restriction is imposed. None is evident when the diets are of full caloric value. Whether or not the same phenomena occur when the experiments are continued for the remainder of the life span seemed worthy of investigation.

The respective merits of carbohydrate and fat have not been defined in respect to ability to prolong life in protein-depleted animals receiving rations sufficient and insufficient in energy value. Determination of the rate of catabolism in relationship to the survival time represented another aspect of this interesting problem.

REVIEW OF LITERATURE

Fat has always occupied a prominent place in human dietaries. It provides a concentrated source of energy, has a high satiety value, and imparts a specific appetizing quality to food. Nevertheless, despite its wide acceptance, it generally has been regarded as an optional dietary component replaceable, at least in dietaries of average caloric value, by other sources of energy. Research in the last 25 years, however, has shown that fat has functions in the diet over and beyond the provision of energy.

For instance, it was recognized early, that many natural fats serve as carriers for the fat-soluble vitamins and that they enhance the absorption of these vitamins from the gastrointestinal tract. Interest in fat as a dietary component was stimulated further by the discovery by Burr and Burr that certain unsaturated fatty acids are essential for the normal growth and development of the young rat (1929). Symptoms suggestive of fat deficiency have been observed in infants (Hansen, 1937). Recently, Hansen and Wiese (1954) have presented evidence indicating that human beings may require the essential fatty acids for optimal well being during periods of growth. They showed that the concentrations of dienoic and tetraenoic fatty acids in blood serum were higher in well-nourished than in malnourished children.

That other beneficial effects accrue from the ingestion of substantial amounts of fat as part of the daily dietary has been suggested by recent research. Thus, experiments with animals have shown that rats receiving high-fat diets grow at a faster rate, exhibit better reproductive performance, show increased work capacity, and survive longer following a period of fasting than do animals subjected to a low-fat feeding regimen. The non-caloric functions of fat have been summarized admirably by Deuel (1950).

It has been recognized for many years that fat as an energy-producing component of the diet may profoundly affect the course of protein metabolism in the body. Recently there has been a revival of interest regarding the relative effectiveness of fat and carbohydrate as sparers of dietary and tissue protein. The review that follows is devoted to the general topic of the influence of non-protein calories on protein metabolism. Since only adult animals have been studied in the present investigation, the literature review, for the most part, will be confined to recorded observations on adult animals.

RELATIONSHIP OF ENERGY INTAKE TO PROTEIN METABOLISM

Early Investigations

The concept that either carbohydrate or fat may exert a conserving effect on protein metabolism is one of long standing acceptance. Early investigators demonstrated that protein was metabolized less economically as a sole dietary constituent than it was in the presence of carbohydrate or fat.

In 1881, Carl Voit found that nitrogen equilibrium could not be established in a dog by feeding a quantity of meat providing the amount of protein catabolized during a period of starvation. Equilibrium was attained only when the meat furnished five times the quantity of nitrogen excreted during the fast. Voit and Korkunoff, pupils of Carl Voit, performed a similar experiment and found that in the dog they studied three and one-half times the amount of protein metabolized in starvation was required for equilibrium (1895).

Lusk has recorded (1928), that C. Voit also observed that less protein was burned in starvation when the body was fat than when it was lean. As a result Voit postulated that if protein and fat were ingested together, the amount of protein required for equilibrium would be reduced. Voit and Korkunoff (1895), testing the hypothesis of the master, found that when fat and meat were ingested simultaneously, the quantity of meat necessary to accomplish nitrogen equilibrium was reduced to between 1.5 and 2 times the starvation minimum.

It also has been shown that when the diet provides carbohydrate, it is possible to establish nitrogen equilibrium with less protein than when the diet contains either protein alone or a mixture of protein and fat. Siven (1900) demonstrated for the first time that nitrogen equilibrium may be maintained at even a lower level of protein intake than is represented by the nitrogen excretion during starvation when carbohydrate is present in the diet. He stepwise reduced the protein intake of a healthy man weighing 60 kilograms and consuming a mixed diet containing 16 grams of nitrogen daily. The ration was rich in carbohydrate and provided 2444 Calories daily. Siven found that equilibrium could be established with only 6.26 grams of nitrogen per day. Human subjects characteristically eliminate 10 grams of nitrogen daily during early days of starvation.

Nitrogen Balance And Total Energy Value Of The Diet

The observation that either dietary carbohydrate or fat may influence the protein requirement suggests that the total energy intake may determine the level at which protein metabolism proceeds. Investigations with human subjects and experimental animals have illustrated beautifully the dynamic relationship which exists between protein metabolism and energy intake. Only those studies in which nitrogen excretion has been measured in response to progressive increments in the energy value of the diet will be reported in the following section. Both protein-containing and protein-free diets have been used in these investigations.

When Diets Contain Protein

One of the first experiments in which the quantity of nitrogen appearing in the excreta was measured in response to progressive increments in the energy content of the diet was conducted by Rubner on a man in 1903. Keeping the protein and carbohydrate content of the diet constant, Rubner added fat in increasing quantities and found that each dietary modification was reflected by a lowered output of nitrogen. Similarly, Neumann (1919) studied the elimination of nitrogen by a human subject when carbohydrate was added in increasing quantities to a diet of constant and adequate protein value. The basal diet provided calories inadequate for the attainment of nitrogen equilibrium, but the nitrogen balance improved progressively as the energy value of the food was increased by the addition of carbohydrate. Considerable nitrogen retention occurred at the higher intakes of carbohydrate. In both of these experiments an approximately linear relationship existed between nitrogen balance and energy intake.

Recently Munro and Naismith (1953) confirmed the results of these investigations. Using adult male rats they studied nitrogen balance as they varied the energy intake by altering either the carbohydrate or the fat content of a diet that was adequate in protein. Each increment in energy content, regardless of whether carbohydrate or fat was altered, was accompanied by a corresponding decrease in nitrogen excretion. Again, the relationship between nitrogen balance and energy content of the diet was linear. That the relationship was continuous from points of negative balance through equilibrium to points of positive balance led these authors to postulate that a dynamic relationship exists between these two factors. These results indicate that, providing the protein intake is adequate, the energy content of the diet determines the quantity of nitrogen that is retained.

Results of investigations pertaining to the utilization of protein by a group of 68 women living on their regular self-selected diets bear out this statement (Swanson, 1952). The diets of these women provided 10 grams or more of nitrogen daily. The energy values, however, varied from 1200 to 2400 Calories. When nitrogen retentions were grouped according to the caloric values of the diets, they became progressively less negative with each increment of 400 Calories. The experiments of Rose (1949) with human subjects ingesting amino acid mixtures or acid-hydrolyzed proteins further illustrate the dependence of nitrogen balance upon the energy value of the diet. When nitrogen was provided by amino acids or protein hydrolyzates, more energy was required to establish equilibrium than when an intact protein was fed.

When Diets Contain No Protein

Experiments With Rats. When protein-free diets are administered to adult rats, nitrogen balance no longer serves as a sensitive indicator of the energy content of the diet. Only marked reductions in caloric value of the diet occasion increments in nitrogen output. In this connection, Mitchell reported in 1924 that the amount of a nitrogen-free diet consumed by adult rats bore no direct relationship to the excretion of urinary nitrogen except when its energy value was decreased below the maintenance requirement. Addition of energy-providing nutrients to these submaintenance diets was reflected by improved nitrogen balances. Treichler and Mitchell (1941) also reported that altering the caloric consumption of adult rats in a range close to the maintenance requirement, i.e., from 37.5 to 45.3 Calories per day produced very little change in the quantity of nitrogen excreted in the urine. Willman and associates (1947) in the laboratory of the writer observed that excretion of nitrogen was fairly constant in rats receiving low-nitrogen diets, as long as at least one-half the quantity of energy food ordinarily chosen by the rat was provided. Further decreases in food intake, however, were associated with increases in the negativity of the nitrogen balances.

Experiments With Dogs. Results obtained from experiments on dogs indicate that a close relationship exists between energy intake and nitrogen balance over a wide range of caloric consumption. In 1907, Murlin studied the nitrogen metabolism of dogs fed increasing doses of carbohydrate after a period of starvation. He found that each increment in energy intake was accompanied by a decrease in nitrogen excretion and that this inverse relationship held even after the intake exceeded the energy requirement. Allison and Anderson (1945) added carbohydrate to a protein-free diet fed to dogs and found that as the energy value of the ration was increased from 80 to 100 Calories per kilogram of body weight, a corresponding reduction in nitrogen catabolism occurred. Thus, dogs and rats seem to respond differently to supermaintenance levels of energy intake.

Experiments With Human Subjects. In human subjects nitrogen balance apparently depends upon energy intake to a greater extent than is the case in the rat. Thus, Johnson *et al.* (1947) in Deuel's laboratory demonstrated that nitrogen balance improved as the caloric intake of human subjects consuming a nitrogen-low diet was increased from 600 to 1200 Calories. One person in this study had served as a subject in a similar experiment conducted in 1928 in which the diet provided 2000 Calories (Deuel *et al.*). Comparison of the respective quantities of nitrogen excreted in the two investigations clearly shows the protective influence of energy-producing nutrients on protein metabolism, *i.e.*, 2.74 grams daily when the diet provided 600 Calories *vs.* 1.74 grams when 2000 Calories were furnished.

Basic Energy Value Of Diets And Nitrogen Balance

It has been shown that successive increments in the energy value of the diet progressively decrease catabolism when protein-free diets are fed and improve retention under conditions of protein feeding. The phenomenon also has been demonstrated under various other experimental conditions. In the following section attention has been given to the influence of specific alterations in the energy-producing components of the diet on nitrogen retention.

When Submaintenance Diets Are Fed

Reports in the early literature indicated that when either carbohydrate or fat was added to the diet of dogs receiving insufficient meat to maintain nitrogen equilibrium, nitrogen retention improved (Voit, 1869; Bischoff and Voit, 1860). Munro (1951) in an excellent review article criticizes these reports on the basis that the experiments did not provide for physiological adjustment to the various experimental regimes. Allison, Anderson, and Seeley (1946) have reported an experiment in which dogs were fed a diet providing only 25 per cent of their normal energy intake. When the energy value of the diet was doubled by the addition of either carbohydrate or fat to the diet, improved nitrogen balances resulted in each instance.

When Maintenance Diets Are Fed

Effect Of Decreasing The Energy Value Of The Diet.

One way of measuring the effect of the caloric value of the diet upon the utilization of nitrogen has been to remove carbohydrate or fat from an adequate diet. Lusk (1890), at the suggestion of Voit, tested the hypothesis that the sudden withdrawal of carbohydrate from the food would increase protein metabolism. Lusk first established himself in nitrogen equilibrium. When he then removed 350 grams of carbohydrate from this maintenance diet, protein metabolism increased.

Bosshardt and his co-workers (1948) demonstrated the phenomenon in mice, the removal of either fat or carbohydrate from an adequate diet fed to mice increasing the catabolism of nitrogen to approximately the same extent.

Effect Of Increasing The Energy Value Of The Diet. A type of experiment used by many investigators interested in the topic of energy-nitrogen relationships has been the measurement of the effect of adding extra calories either as carbohydrate, as fat, or as a mixture of the two, to diets capable of supporting nitrogen equilibrium.

Cuthbertson, McGirr, and Munro, in 1937, determined the effect of adding extra calories in the form of either sucrose or glucose to the already adequate diets that were being consumed by two of the authors (Cuthbertson and Munro). The addition of both carbohydrates substantially lowered the quantity of nitrogen excreted in the urine. In a continuation of their studies, Cuthbertson and Munro (1937) also demonstrated that the degree of nitrogen retention was related to the amount of excess energy that was superimposed upon the existing adequate dietary. The quantity of urinary nitrogen excreted daily when 780 additional Calories were provided from a glucose supplement was 2.18 grams less than that excreted on the unsupplemented diet furnishing 2890 Calories. When the supplementary glucose was doubled nitrogen excretion was reduced further, falling to a level 3.72 grams lower than that observed on the original adequate diet.

That carbohydrate and fat exert the same sparing phenomenon on the nitrogen metabolism of dogs has been demonstrated by Larson and Chaikoff in 1937. These authors showed that either glucose or sucrose when added to a diet already adequate in food energy value depressed excretion of nitrogen.

The relative effectiveness of carbohydrate and fat added singly to a diet of adequate energy content has been studied in rats. In 1939 Forbes, Bratzler, Thacker, and Marcy described an experiment in which the dynamic effects of energy-providing nutrients were determined in young and in mature rats. In addition to measurements of energy exchange, the utilization of nitrogen was determined in response to feeding the basal ration and subsequently to feeding the same ration supplemented with either dextrin or lard. In each case, nitrogen retention was improved. In one instance lard spared protein to a greater extent than did dextrin in adult rats. This observation was not confirmed upon repetition of the experiment.

Influence Of Body Reserves Of Carbohydrate And Fat On Nitrogen Excretion During Starvation

The carbohydrate and fat stores of the body represent a potential source of physiological energy, the effect of which can be ascertained by studying nitrogen metabolism during starvation. Early studies (Prausnitz, 1892; Morgulis, 1923) indicated that during a fast the quantity of protein catabolized during the first few days was less than that in subsequent periods. Benedict (1907) in his classical experiments on human fasting attributes this pattern of nitrogen excretion to the sparing effect of reserves of glycogen present in the body during the first few days of a fast. When the glycogen stores are exhausted nitrogen excretion increases.

It is generally believed that in starvation the excretion of nitrogen remains fairly constant as long as the body contains appreciable stores of fat. However, when such reserves are depleted, the quantity of nitrogen excreted in the urine tends to increase. This increment in output of protein end-products during the late stages of prolonged fasting often is referred to as the "premortal rise" in nitrogen excretion.

The administration of fat or carbohydrate to a starving animal with diminished fat stores has been studied by Chambers *et al.* (1939). A marked reduction in nitrogen output occurred when food energy was administered showing that the use of body protein as a source of energy was averted.

DIFFERENCES IN THE PROTEIN-SPARING PROPERTIES OF CARBOHYDRATE AND FAT

In the previous section of the present review, evidence has been presented that indicates that diets of high energy value not only promote retention of nitrogen when protein-containing rations are ingested but also exert protective action against the catabolism of body tissue that occurs when protein-free diets are consumed.

However, the writer believes that part of the interaction between protein and the energy-yielding nutrients may not be inherent in the energy-producing properties of carbohydrate or fat. Results obtained from two types of experiments support this hypothesis. They are:

First: Carbohydrate or fat added to diets of insufficient energy value decreases nitrogen output. This observation may be interpreted to mean that protein from the diet or from the body stores is utilized for energy when the fuel value of the diet is insufficient. This interpretation is invalidated, however, by the observation that when carbohydrate or fat is added to a calorically sufficient diet, nitrogen retention is improved. The protein-protecting power of carbohydrate and fat, therefore, must involve something more than additional calories.

Second: If carbohydrate and fat affect protein metabolism simply by serving as sources of energy, then isocaloric equivalents of the two nutrients should affect nitrogen output in the same manner. However, such is not always the case, and data will be presented that show carbohydrate and fat

vary in their protein-sparing effects
under specific experimental conditions.

Evidence From Feeding Diets Lacking In Carbohydrate

There are few investigations recorded in the literature that report the effect on the human subject of the consumption of diets of adequate energy and protein content but deficient in carbohydrate. In one of the most notable studies of this type (McClellan and DuBois, 1930 and McClellan *et al.*, 1930), two normal men volunteered to live on an exclusive meat diet for a period of one year. Both fat and lean portions of the meat were eaten, between 75 and 85 per cent of the total calories being derived from fat. One subject was able to maintain nitrogen equilibrium for the majority of time that he subsisted on the meat and fat diet. The other subject showed definite negative nitrogen balances in three experimental periods. It was believed that the negative balances could be explained by gastrointestinal disturbances which were experienced by this subject while ingesting the diet.

Evidence From Isocaloric Replacement Studies

Two types of experimental procedures have been used to demonstrate the effect on nitrogen excretion of substituting fat for a calorically equivalent quantity of carbohydrate in diets of adequate energy value. In the first, the subject or experimental animal is provided with a diet supplying normal quantities of carbohydrate, fat, and protein. When a satisfactory adjustment to this diet has been made, carbohydrate is removed and replaced by an isocaloric quantity of fat. The amount of nitrogen excreted in the urine in the two experimental periods is determined. In the second type of experiment individuals do not serve as their own controls. Nitrogen excretion of individuals or of groups is measured following the feeding of diets of constant energy and protein value but varying in their percentage composition of carbohydrate and/or fat.

Complete Replacement Of Carbohydrate With Fat

In 1894, Kayser reported a balance study in which he served as his own subject. He established nitrogen equilibrium while consuming a diet providing 2600 Calories and 21 grams of nitrogen. When the carbohydrate of this diet was replaced by an isocaloric quantity of fat, nitrogen balances became increasingly negative. When carbohydrate was returned to the diet, the protein loss stopped immediately, and positive balance ensued.

Partial Replacement Of Carbohydrate With Fat In Diets Adequate In Energy And Protein

The substitution of fat for part of the dietary carbohydrate has been a favored approach in experiments with human beings as well as with animals. In 1902 Tallqvist, experimenting on himself while working in Rubner's laboratory, studied the effects of partial replacement of carbohydrate in an adequate dietary by an equal number of calories from fat. The diet provided around 2900 Calories daily or 36 Calories per kilogram of body weight, an amount estimated by Rubner as adequate for meeting the energy requirements of the body. Nitrogen balance was determined over a period of eight days, divided into two periods. During the first four-day period, the diet was rich in carbohydrate while in the succeeding four days it was rich in fat.

The replacement of about one-half of the quantity of carbohydrate with fat--an amount representing about one-third of the total fuel value of the diet--shifted the subject from nitrogen equilibrium into negative balance. However, the negative balance persisted only two days. The prompt return to nitrogen equilibrium indicated that partial replacement of carbohydrate with fat may have no influence, or at best only a transitory one, upon the amount of protein metabolized. The short experimental periods and the failure to provide for adjustment precludes deductions of too definite a nature.

Atwater and Benedict studied the protein-sparing properties of fat and carbohydrate incorporated into a diet of high protein value in an athletic young man who performed a considerable amount of work (1903). The experiment lasted for 15 days, divided into four experimental periods, during which diets rich in carbohydrate were alternated with diets rich in fat. Both diets provided around 4500 Calories and the change from one diet to the other involved about 2000 Calories.

Negative nitrogen balance followed the feeding of both diets with a difference in favor of carbohydrate.

Experiments also have been conducted in which dogs have been used as experimental subjects. In general with this species, results have shown that when dietary fat replaces all or part of the carbohydrate, an increase in nitrogen excretion accompanies the increase in fat intake. In 1916, Umeda studied the retention of nitrogen when 30 grams of caseinogen were superimposed upon three types of experimental diets, i.e., one rich in carbohydrate and poor in fat, another intermediate in carbohydrate and fat content, and a third rich in fat and poor in carbohydrate. Before the addition of extra caseinogen the diets all contained 7.2 grams of nitrogen provided by varying proportions of oatflour, dried milk, and caseinogen. The experiments were conducted on a single dog. Significantly better retention of nitrogen from caseinogen was observed on the carbohydrate-rich than on the fat-rich diet, 35 per cent of the added nitrogen being retained from the former as contrasted to 8.5 per cent from the latter. When the diet was intermediate in carbohydrate and fat content, 28.6 per cent of the added nitrogen was retained.

Changes in nitrogen excretion induced by varying the energy components of the diet have been studied in the rat. Lathe and Peters in 1949 maintained rats on a 25 per cent fat diet for five days. Nitrogen excretion was measured during this period and in a subsequent five-day period in which seven animals were transferred to a non-fat diet while eight continued to receive the fat-containing diet. Animals transferred to the non-fat diet excreted slightly less nitrogen than did those receiving fat, but the difference appears too small to be significant.

In the studies noted above, metabolic measurements were made immediately following the substitution of carbohydrate with fat. However, the work of others has shown that if a considerable period elapses between the time that an alteration in the energy components of the diet is made and the time that nitrogen balance is determined, there is no particular benefit in favor of carbohydrate. Indeed, with time allowed for adjustment, improved retention and utilization of nitrogen sometimes is associated with the feeding of diets in which fat replaces carbohydrate as the major energy component. For example, Samuels, Gilmore, and

Reinecke in 1948 administered high-fat and high-carbohydrate diets of equal caloric value to mature male rats for four weeks. Both diets were adequate in protein. The quantity of nitrogen excreted was measured during the last five days of the feeding period and in subsequent intervals of total fasting. While ingesting the two types of diets, rats conditioned to the fat-containing diet excreted less urinary nitrogen than did animals receiving the high-carbohydrate diet (317 mg. vs. 338 mg. per day). During the subsequent period of total fasting the same animals continued to excrete less urinary nitrogen than did animals previously fed a carbohydrate-rich diet (110 mg. vs. 177 mg. per day during the first four days of the fast).

Swift and Black (1949) summarized the results of an extensive series of studies conducted in their laboratory with adult male rats fed diets adequate in protein and varying in fat content from 2 to 30 per cent. Heat production and nitrogen retention were determined in the animals after sufficient time had been allowed for adjustment to the different test rations. Some of the experiments indicated that the presence of a substantial amount of fat in the diet favored nitrogen retention. Careful examination of the results from many experiments, however, led the authors to conclude that the fat content of the diet was without positive influence on the utilization of nitrogen.

Thus, in general, it seems that if the rat is conditioned to fat-feeding, the effect of dietary fat upon the course of nitrogen catabolism is not unfavorable. Adverse nitrogen balances that have been noted in several species following the substitution of fat for carbohydrate in the diet may represent a temporary adjustment that is evident only in the first few days following the dietary modification.

Partial Replacement Of Carbohydrate With Fat In Diets Inadequate In Protein And/Or Energy

The effect of feeding fat or carbohydrate as the major source of calories in submaintenance diets for hogs was reported by Keeton and associates in 1931. These investigators found that the average nitrogen balances accrued in experimental periods of 28 to 41 days duration were not any different in two hogs receiving high-fat diets than they were in two hogs receiving high-carbohydrate diets.

Scheer, Codie, and Deuel (1947) imposed severe caloric restriction on young adult rats. The diets were of equal caloric and protein value but varied in their content of fat. These workers observed slower declines in body weight, fewer deaths, and more rapid gains during recovery from underfeeding in animals receiving liberal amounts of fat than in those receiving fat-free diets. Schwimmer and McGavack (1948) have reported results of a study with human beings receiving isocaloric submaintenance diets. They observed that subjects receiving most of their calories from carbohydrate excreted more nitrogen than subjects receiving a high proportion of calories from fat. The subjects receiving a 30 per cent fat diet in quantities providing 900 Calories and 6 grams of nitrogen per day maintained positive nitrogen balance.

In 1944, Anderson determined nitrogen balances in obese patients receiving diets in which either fat or carbohydrate contributed most of the calories and in which the protein intake was inadequate. Nitrogen output was greater in response to the feeding of the high-fat diet than to the carbohydrate diet. His results were directly opposite to those of Schwimmer and McGavack. However, in Anderson's experiment, nitrogen balances were determined in the immediate interval following the initiation of the experimental diets and hence the results may reflect a transitory adjustment effect rather than a permanent response.

In another study (Zeller, 1914), the effect of substituting fat for part of the carbohydrate was studied when only 3 grams of protein per day were provided in the diets of human subjects. A slight progressive increase in the excretion of nitrogen occurred as the percentage of calories contributed by carbohydrate was decreased from 100 to 50 per cent and the percentage contributed by fat increased correspondingly from 0 to 50 per cent. However, with reduction of carbohydrate to the point where it provided only 25 per cent of the total non-protein calories, the trend was reversed, a drop in the quantity of nitrogen excreted in the urine being observed.

Evidence From Feeding Protein-Free Diets

Protein-free diets also have been employed in studies which have attempted to determine whether or not carbohydrate and fat differ in protein-sparing properties. In these experiments it has been assumed that the urinary nitrogen is of endogenous origin and represents the wear and tear quota plus the quantity of protein catabolized to secure nitrogenous metabolites essential for the maintenance of life. This approach in studies using a protein-depleted rat as the test animal has been used in the laboratory with which the author is associated. Reference has already been made to these studies, and further reference of a detailed nature will be forthcoming in a subsequent section (Stevenson _et al._, 1946; Willman, _et al._, 1947; Swanson _et al._, 1947; Hoover and Swanson, 1950; Swanson, 1951; Edwards and Swanson, 1952). Data in all of these reports show that the inclusion of fat in a protein-free ration retards the rate of catabolism when the nitrogen-low diet is of low caloric value (14 Calories per day). This property of fat is not apparent when the rations meet the energy needs of the protein-depleted rat.

Munro and Naismith (1953) have presented data showing that dietary fat in a protein-free ration is associated with a lesser excretion of nitrogen in the urine than is the case when carbohydrate is the main source of energy. It is interesting that these authors failed to recognize these observations.

A few experiments have been recorded in which the nitrogen excretion of a human subject ingesting a carbohydrate-rich diet has been compared with his nitrogen output while ingesting a fat-rich diet. These studies are of interest despite the fact that inadequate and unbalanced diets were used. For example, Landergren (1903) recorded an experiment in which a human subject ingested low-nitrogen diets of full caloric value in which either carbohydrate or fat provided most of the calories. This subject excreted 12 grams of urinary nitrogen on the day before experimental feeding was begun. After four days of administration of the carbohydrate diets, the excretion fell to less than 4 grams. Replacing the carbohydrate with an equicaloric quantity of fat caused the output of nitrogen to increase promptly. A level of 10 grams was reached by the third day. Landergren found that metabolism also could be reduced to the wear and tear quota of 4 grams of nitrogen by feeding a diet containing one-half of its calories as carbohydrate and one-half as fat.

Cathcart (1922) also studied the metabolism of nitrogen of a human subject fed olive oil alone or olive oil plus varying quantities of dextrose. The diets were approximately equivalent in energy value. Each was fed for a period of three days. The subject excreted a significantly greater quantity of nitrogen when fat was fed singly than when sugar and fat were fed together. As the carbohydrate content of the diet increased, a general tendency for the output of total nitrogen to decrease was observed.

Experiments involving the use of either carbohydrate or fat following a period of starvation also may be considered as falling in the category under discussion. Nitrogen excreted when food energy was provided has been compared with that eliminated during starvation. Several workers have used dogs for experiments of this kind. Voit (1881) found that the feeding of 100, 200, and 300 grams of fat to a fasting dog whose body still contained fat did not alter the quantity of urea excreted. He believed that dietary fat served merely to replace body fat and that the protein catabolism was unaffected by the dietary alteration. Bartmann (1912) confirmed Voit's results reporting that fat when given alone to provide an excess of 50 per cent of the energy requirement reduced nitrogen catabolism of the fasting dog only 7 per cent.

On the other hand, when Wimmer (1912) administered carbohydrate to starving dogs he found that 55 per cent less protein was catabolized than during a previous period of fasting. Richet and Minet also determined the quantity of nitrogen eliminated by dogs during an absolute fast and after alimentation with sugar or fat (1925). In the case of sugar-feeding the quantity of nitrogen excreted was less than during the absolute fast. When fat was given, excretions for the most part were higher than during the fasting period. Eight animals were studied and the excretions varied widely from dog to dog in response to the feeding of fat. The authors believed that the variation could be attributed to differences in the stores of glycogen.

In an experiment with a human subject, Grafe (1910) reported that the nitrogen output was reduced significantly below the fasting level by the administration of sugar.

Rats have also been used in investigations in which the respective abilities of energy-producing nutrients to reduce the nitrogen catabolism characteristic of starvation has been tested. Attempting to ascertain whether or not the animal organism can convert fat to carbohydrate, Gregg (1931) fed to young rats a diet consisting of butterfat and salts. In connection with his studies, he determined the quantity of nitrogen excreted in the urine daily and found that this was lower in all cases for rats receiving butterfat than for a single rat to whom no food was offered. In 1934, Kriss, Forbes, and Miller reported the results of a study in which the nitrogen excretion of fasting rats was compared with that of rats receiving either 3.92 grams of starch or 1.56 grams of olive oil daily. The daily excretions of urinary nitrogen were 122 milligrams for the fasting rats, 30 milligrams for rats receiving starch, and 70 milligrams for rats receiving olive oil.

Evidence From "Time Factor" Studies

Larson and Chaikoff (1937), in supermaintenance feeding experiments with adult dogs receiving adequate diets, observed that nitrogen retention could be improved by the addition of sugar to the diet if the supplement were provided within a four-hour interval before or after the ingestion of the protein-containing meal. The authors emphasized that the sparing effect was not permanent, the elimination of nitrogen beginning several hours after storage had occurred.

Cuthbertson and Munro believe that proximity of carbohydrate to protein ingestion may be required for maximal utilization of dietary protein also under ordinary feeding conditions. In 1939, these investigators carried out experiments with four human subjects using diets which could be separated into two portions, one containing essentially all of the carbohydrate and some of the fat, and the other containing all of the protein and the remainder of the fat. Each subject lost an average of 2 grams more nitrogen per day when the carbohydrate-fat and the protein-fat portions of the diet were consumed separately than when the portions were combined for ingestion. These investigators also have used adult rats as subjects in this type of experiment (Cuthbertson, McCutcheon, Munro, 1940). Again, an increased output of urinary nitrogen occurred when the diet was separated into carbohydrate-fat and protein-fat meals.

More recently, in a strictly controlled experiment using adult male rats, Munro (1949) demonstrated that carbohydrate only was responsible for the alterations in nitrogen balance observed in his earlier experiments induced by variations in the time of ingestion of nutrients. Nitrogen balance remained unchanged when the time of fat ingestion was the only experimental variable. Of special interest is the fact that results of this experiment indicated that the beneficial effect associated with simultaneous ingestion of carbohydrate and protein was transitory. The improved nitrogen balance observed when carbohydrate and protein were fed simultaneously lasted only a few days, after which nitrogen balance returned to its original level.

Geiger _et al._ (1950) express the opinion that the favorable effect on nitrogen balance observed by the above investigators may have been only transitory because the adult well-fed animal has a limited capacity to store nitrogen. Working with growing rats, Geiger (1948) found that better growth resulted from feeding carbohydrate and protein together than from feeding the two nutrients separately and that the improved growth persisted for the duration of the 21-day experiment. In this case, undoubtedly, retained nitrogen is converted into new body tissue.

Subsequently Geiger and collaborators (1950) tested the ability of protein-depleted adult rats to store nitrogen when the time of feeding of protein was separated from that of carbohydrate. They found that nitrogen retention and weight gains of animals receiving carbohydrate and protein together were in all cases superior to those in rats fed the two nutrients separately. The effect was not restricted to a period of only two or three days but lasted for extended periods of at least 14 days, i.e., until repletion of the protein-depleted rats was complete.

Another illustration of the importance of time considerations is seen in Leverton's study in which negative nitrogen balances were induced in human subjects (college women) when an adequate supply of protein originally distributed over the three meals of the day was redistributed so that it occurred in lunch and dinner only (Leverton and Gram, 1949).

The effect of feeding extra carbohydrate apart from the protein of the diet has been studied recently in man, the dog, and the rat (Munro and Wikranmanayake, 1954). These investigators found that extra carbohydrate can reduce nitrogen excretion even when carbohydrate and protein are fed separately, the maximum periods of separation being 5-1/2 hours in man and 12 hours in the rat and the dog. Isocaloric quantities of fat were also effective in improving nitrogen retention in the rat when administered apart from the protein component of the diet. These investigators believe that carbohydrate can affect protein metabolism in two ways. The first effect is illustrated by the favorable influence on protein utilization observed when carbohydrate and protein are ingested simultaneously. This influence is specific for carbohydrate. The second effect, supported by the data in their report, is independent of time and is exhibited by both carbohydrate and fat. Presumably the latter is related to the energy-producing capacities of the two nutrients.

Summary Of The Effects Of Carbohydrate And Fat On Protein Metabolism

In the review of literature herein presented, an attempt has been made to clarify the position of non-protein calories in the metabolism of protein. The studies described show that the problem has been investigated under a wide variety of experimental situations. The complexity thus introduced makes it extremely difficult to summarize facts that seem to have been established about the energy-protein relationship.

Many variables influence the relationship. Some of these are: (1) species of animal, (2) length of experiment, (3) general nutritional state of the experimental animal, (4) existing body stores of protein, fat, and carbohydrate, (5) method of feeding, (6) time allowed for adjustment of animal to specific dietary treatment, (7) presence or absence of protein from the diet, (8) time of ingestion of protein in relation to carbohydrate, (9) quantity of dietary protein, (10) source of non-protein calories, and (11) energy value of diet.

The experimental evidence supports the view that part of the protein-sparing effect of fat and carbohydrate is attributable to their energy-producing properties. The addition of calories, either as fat or as carbohydrate, to diets of adequate protein value occasions an improvement in nitrogen balance than can be demonstrated in many experimental animals over a wide range of caloric intakes varying from submaintenance to supermaintenance levels. The progressive improvement in nitrogen balance that occurs with increments in the caloric value of the diet suggests that a dynamic relationship exists between energy value of the diet and utilization of protein. That carbohydrate or fat added as supplements to diets of adequate protein and energy value can improve retention of nitrogen is noteworthy.

In certain species, the addition of energy-producing nutrients to a protein-free diet usually improves nitrogen balance. The situation in respect to the mature rat, however, needs definition. While nitrogen balance can be improved in rats maintained on protein-free or low-protein diets of low energy value, increments in the caloric value of the ration beyond 50 per cent of daily needs have no effect on the nitrogen metabolism.

The relative efficiency of fat and carbohydrate in sparing protein seems to depend upon the experimental conditions employed. Generally, initial nitrogen balance is more favorable when diets containing adequate protein provide a high proportion of carbohydrate than when a large part of the calories are furnished by fat. Nitrogen excretions of animals transferred from a high carbohydrate to a high-fat diet usually increase. However, "conditioning" to fat feeding, particularly in the rat, may reverse the process so that nitrogen metabolism proceeds more efficiently when fat rather than carbohydrate provides a high proportion of calories.

When protein-free rations are ingested nitrogen balances are of the same order for rats receiving either a mixture of carbohydrate and fat or carbohydrate alone as long as the energy needs of the animal are met. Reduction of calories below 50 per cent of the energy requirement is accompanied by an increased rate of catabolism. However, when fat is present in the calorically restricted ration, the destruction of body tissue is decidedly less marked than when the ration is devoid of fat. On the other hand, limited evidence from human experiments in which protein-free diets of adequate energy value have been used indicates that nitrogen metabolism proceeds more efficiently when high-carbohydrate rather than high-fat diets are ingested.

Comparison of nitrogen excretions of animals incurred during a period of starvation with subsequent excretions when either carbohydrate or fat is administered generally has indicated a greater sparing action for carbohydrate than for fat. The administration of fat alone during starvation produces different effects in various species. In the rat, the feeding of fat has been associated with lower excretions of nitrogen than observed during starvation, whereas in man and in the dog, the metabolism characteristic of starvation has been maintained when fat has been used as the principal source of energy.

The protein-sparing effect of carbohydrate is enhanced when the protein and carbohydrate portions of the diet are ingested simultaneously. The favorable effect is temporary in adult well-nourished rats and dogs but persists for a longer period of time in growing animals and in animals depleted of their protein reserves. Excess carbohydrate and fat, on the other hand, improve nitrogen retention irrespective of the time at which they are fed.

Throughout the present review attention has been directed to the influence of specific experimental conditions in determining the effect of non-protein calories on the course of protein metabolism. The advisability of interpreting results in relation to the experimental conditions employed is obvious. In this connection the writer believes that experimenters working in this field should make sure that the results of their experimentations are defined in terms of the experimental conditions under which the researches were conducted.

SUGGESTED MECHANISMS BY WHICH FAT EXERTS ITS PROTEIN-SPARING ACTION IN A PROTEIN-DEPLETED ANIMAL

In the Home Economics Nutrition Laboratory of the Iowa State College it has been demonstrated consistently that fat, when fed as a part of a protein-free ration of limited energy value, has the unique ability of conserving body protein. It is interesting to speculate regarding the nature of the mechanism by which fat exerts this sparing action. A number of separate possibilities will be presented in this section of the review.

Pathway Of Energy Production When Carbohydrate Metabolism Is Impaired

The energy aspects of metabolism have received much attention. The body is often compared with a heat-producing machine with protein, fat, and carbohydrate identified as the energy-yielding nutrients. The actual production of energy for work and the maintenance of body activities is secondary to the direction of energy into important metabolic channels. This requires highly specialized metabolic machinery, and materials must be available for construction of these working parts.

Metabolism has been classified into two categories, energy-producing and operative (Peters, 1951). While the three major foodstuffs can all provide fuel for the body machine, each has exclusive metabolic functions. No one of these can replace another entirely in providing certain of the operative components of the body in metabolism.

Peters has designated some of the operative aspects involved in the metabolism of each of the three major foodstuffs. Thus, carbohydrate is needed for the formation of pyruvic acid which, in turn, by combining with carbon dioxide is converted to oxaloacetic acid. Both substances are required for the functioning of the Krebs' cycle. Pyruvic acid is the principal source of fuel for the production of energy, but, in case of emergency, the two-carbon derivatives of fatty acids can substitute for pyruvic acid. This, however, does not eliminate completely the need for pyruvic acid since it serves as a precursor for oxaloacetic acid, an operative metabolite, that is needed for the initiation of the reaction sequence of the Krebs' cycle. Some of the oxaloacetic acid is used up as energy production proceeds and it must then be replaced in order to keep the cycle intact.

The essential fatty acids, in line with Peters' postulation, are needed for the formation of phospholipids. These are important constituents of tissue cells which presumably serve as vehicles for the transport of the fatty acids and as instruments that facilitate their oxidation. The phospholipids thus form essential parts of the body machinery operating in metabolism.

The structural functions of protein distinguish it from the other major foodstuffs. It is well recognized that certain amino acids must be supplied in the food.

These together with non-essential amino acids are synthesized into tissue protein. But proteins also have operative functions in the body. For example, they are the building stones for the elaboration of other proteins, such as enzymes and hormones, needed for the differentiation and specialization of vital activities. Protein also in serving as a source of energy for the body provides the essential pyruvic acid. But in addition it has other specific operative functions. The difference between the operative and energy-producing functions of protein is illustrated by the type of nitrogenous end-products that are excreted. The production of urea and ammonia describes the energy-producing aspect; the quantity of each varies directly with the amount of protein that is oxidized. On the other hand, uric acid and creatinine are formed as a result of operative functions and their excretion remains essentially unchanged in response to variations in protein intake.

A mechanism based on the operative aspect of metabolism has been postulated to explain the protein-sparing effect of fat in the protein-depleted animal. Hoover, working in the same laboratory as the writer, demonstrated that the glucose tolerance curves of animals conditioned to a protein-free diet containing no fat except a source of the essential fatty acids were mildly diabetic in character. Apparently these animals were not metabolizing carbohydrate efficiently. It may be assumed that when carbohydrate can no longer be used effectively for the production of pyruvic and oxaloacetic acids, the metabolites needed for energy production in the Krebs' cycle, the catabolism of protein increases to provide pyruvic acid. In the animal receiving no energy-producing food except carbohydrate, the degradation of protein serves as the last resort for the maintenance of life. The excessive quantities of urinary nitrogen excreted by Hoover's rats (1389 mg. per 5 days) suggest strongly that body protein was serving as a principal source of energy.

In the protein-depleted animal receiving a 20 per cent fat diet the metabolic processes function more efficiently than in the animal receiving no fat. Not only is the expenditure of tissue nitrogen less lavish (459 mg. per 5 days) but also the glucose tolerance curve approaches that of the normal animal. In accord with the modern concept of metabolism, it is postulated that in the fat-fed animal, the two-carbon derivatives of fatty acid metabolism serve as the source of fuel for the Krebs' machinery. These two-carbon fragments cannot, however, be used for the manufacture of oxaloacetic acid, the operative component needed for keeping the Krebs' energy-producing machine intact. When the mechanism is impaired by which carbohydrate is oxidized to pyruvic acid and the pyruvate in turn is converted to oxaloacetate, an alternative pathway may operate in the animal receiving part of its calories as fat. Peters suggests that, in this case, fatty acids may be conveyed to the liver by a less used metabolic route. Here they are degraded to the conventional two-carbon groups and these are immediately coupled to form the four-carbon intermediates, acetoacetic and beta-hydroxybutyric acids. These keto acids now can be used to replace oxaloacetic acid and hence may explain the mechanism by which the energy-producing scheme continues to function in the animal that utilizes fat in preference to carbohydrate.

The replacement of oxaloacetic acid by the keto acids formed during the metabolism of fatty acids is carried out less efficiently than the formation of oxaloacetate from pyruvate. However, these metabolites may contribute significantly to the machinery of energy production in this particular type of experimental animal. Diminished protein catabolism on the part of animals receiving an appreciable portion of their calories as fat suggests that less demands are placed on body protein for the provision of intermediates in the Krebs' cycle.

In Hoover's experiments (1950), analyses of the glycogen contents of livers of protein-depleted animals receiving high- and low-fat diets in restricted quantities revealed that animals receiving fat in the ration stored glycogen than did animals ingesting high-carbohydrate diets, i.e., 2.0 per cent vs. 0.7 per cent in the carbohydrate-fed group. Apparently rats fed the restricted low-fat ration were unable to convert the non-nitrogenous portions of the degraded protein molecule into glycogen. It should be noted that the ability to retain glycogen was associated with a glucose tolerance curve of close similarity to that exhibited by the normal animal; on the other hand, a decreased ability to store glycogen and abnormal glucose tolerance in an animal deprived of fat indicated some impairment of the body to utilize carbohydrate.

The work of Samuels, Reinicke, and Ball (1942) agrees with Hoover's findings. They reported that glycogen disappeared from the liver less rapidly when diets of high-fat content were fed than when a high-carbohydrate feeding regime was employed.

That ketosis develops as a consequence of replacing dietary carbohydrate with fat has been recognized for a long time. An observation in the early literature (Zeller, 1914) suggests that the appearance of ketosis in a human subject receiving a low-protein diet in which carbohydrate was progressively replaced by fat, was associated with a lowered output of nitrogen in the urine. An excretion of 5.75 grams of nitrogen attained when the subjects received 75 per cent of their calories as carbohydrate was reduced to 5.04 grams when the percentage of calories contributed by carbohydrate was decreased to 10 per cent. At this juncture, the provision of only 10 per cent calories as carbohydrate and the other 90 per cent as fat, ketosis was apparent.

Even less nitrogen was excreted when 25 per cent of the calories were provided by carbohydrate and 75 per cent by fat. Since ketosis did not appear on this feeding regime it may mean that keto acids were being used efficiently in the provision of intermediates for the Krebs' cycle. Further substitution of fat for carbohydrate increased the production of ketone bodies where they could not be utilized completely and ketosis was evidenced.

Alterations In Pathways Of Metabolism

Hoover's studies shed some light on the course of protein metabolism in animals receiving limited energy either from carbohydrate alone or from a combination of carbohydrate and fat. Urea production increased in both groups of animals, the animals excreting 330 mg. per five days on the high-fat diet and 875 mg. per five days on the low-fat diet. Urea accounted for a large share of the augmented nitrogen output that accompanied caloric restriction. These data show that protein was being broken down for energy purposes. The quantity of ammonia nitrogen excreted by both groups of animals also increased, the output being appreciably greater for animals deprived of fat than for animals receiving a fat-containing ration (349 mg. *vs.* 42 mg. per five days). The ratio of ammonia nitrogen to urea nitrogen was 13 per cent for animals receiving the high-fat diet and 40 per cent for animals not given fat. The high proportion of ammonia nitrogen to urea nitrogen in the urine of the semistarved animal may indicate that when fat is absent from the diet the animal is unable to handle the acidic intermediates released during the degradation of protein and, hence, the kidney attempts to compensate by manufacturing ammonia for neutralizing the acidic products.

In these experiments the rations were force-fed, the high- and low-fat rations being administered at both full and restricted levels of caloric intake. When the amino acid composition of the urines of the animals subjected to the various feeding regimes was studied by one dimensional chromatography, certain differences were apparent. Animals receiving sufficient calories to meet their requirements from the diet of low-fat content excreted considerably more of the amino acids tyrosine, arginine, cysteine, cystine, glycine, lysine, aspartic acid, isoleucine, and norleucine than did animals receiving restricted amounts of the low-fat ration or any of the animals to which fat was fed. Methionine was not identified as an excretory product in any of the animals studied. This observation stimulates further speculation because earlier observations from this laboratory (Swanson et al., 1947 and confirmed by Hoover, 1950) have shown that methionine incorporated into the low-fat, low-calorie diet is as effective as fat in preventing the destruction of body tissue. It has been postulated that methionine may serve as an essential component of important metabolite(s) in animals deprived of a dietary source of protein and that they raid their own tissues to secure it for this purpose.

Other workers have observed that when rats were fed diets of adequate protein value in which calories were provided by either fat or carbohydrate, nitrogen retention was better in animals receiving the fat-containing diet (Pearson and Panzer, 1949). A reduced fecal output of phenylalanine, valine, lysine, and methionine and a lower urinary output of valine and methionine was associated with the feeding of fat. Again a high priority for methionine is indicated.

Role Of Methionine

Reference has been made to the series of investigations conducted in the laboratory of the author in which the favorable influence of fat in preventing excess catabolism of tissue protein during a period of severe caloric restriction has been demonstrated. Reports from this laboratory (Swanson et al., 1947 and Hoover, 1950) have shown that methionine incorporated into the low-fat diet was equally as effective as fat in preventing untoward losses in body nitrogen observed in connection with feeding an unsupplemented low-fat ration in restricted quantities.

Hoover observed that the nitrogen losses in the urine brought about by restricting the intake of the fat-free ration were associated with increases in both its urea and ammonia nitrogen contents. The increase in ammonia was particularly evident so that the usual proportion of urea to ammonia nitrogen was distorted markedly. The addition of methionine to the low-fat ration was successful in reducing outputs of both urea and ammonia. The ratio between the two components, however, remained distorted. Although methionine is capable of decreasing the total nitrogen excretion of rats receiving low-fat diets, the observation that it does not alter the ratio of urea to ammonia nitrogen from that observed on the unsupplemented ration suggests that methionine functions in regulating the speed of the process by which body tissue is catabolized.

That no methionine was excreted by any of the animals receiving protein-free rations regardless of whether or not fat was provided further illustrates the vital importance of this amino acid to the animal organism. Apparently the body is very conservative in eliminating the methionine obtained from raiding its own tissues.

The mildly diabetic glucose tolerance curves exhibited by animals deprived of fat and receiving restricted diets became more nearly like those of the normal animal when methionine was fed in conjunction with the low-fat ration. This finding may explain in part the "methionine effect". Researches of Griffiths (1950) and Lazarow (1946) have suggested that sulfhydryl compounds play a significant role in the alleviation of alloxan and uric acid induced diabetes. Griffiths further demonstrated that uric acid was diabetogenic only when glutathione in the blood was depleted by the feeding of a methionine- and cystine-deficient diet. Methionine then may exert a favorable effect upon carbohydrate utilization by furnishing appropriate sulfhydryl moieties for the manufacture of certain enzyme systems.

If it is assumed that an impaired ability to utilize carbohydrate is one of the factors operating in the protein-depleted animal receiving all of its food energy from carbohydrate, the ability of methionine to avert excessive tissue catabolism in these animals suggests that this amino acid may be a component of an enzyme system required for the utilization of carbohydrate. It has been established that acetyl coenzyme A arising from the union of decarboxylated pyruvic acid and coenzyme A is a vital intermediate in carbohydrate as well as in fat metabolism. Coenzyme A contains a sulfhydryl component. The beneficial effect of methionine suggests that in rats fed a low-calorie, low-fat diet deficient in protein, carbohydrate metabolism may be affected in a way other than that postulated in a preceding section. Instead, the metabolism of carbohydrate by the normally operating route may be disrupted because the synthesis of coenzyme A is retarded in the absence of dietary protein. The provision of methionine to these animals then stimulates the manufacture of coenzyme A and carbohydrate metabolism proceeds at the regular rate.

The common ability of fat and methionine to reduce tissue destruction in the particular type of experimental animal used in this laboratory does not mean necessarily that they are performing an identical function. The ability of the animal body to adjust metabolically to a wide variety of conditions is well recognized. Possibly the apparent identity of these substances in this particular aspect of metabolism represents just such an adjustment phenomenon. It may be that dietary fat opens up a new metabolic route whereas dietary methionine permits metabolism to proceed along usual pathways.

Specific Dynamic Effect

Ingestion of food is followed by increments in heat production, the increments varying in magnitude according to the type of foodstuff fed. This stimulating effect of food on energy production is known as specific dynamic action, and is greater for protein than for either of the other energy-yielding nutrients. Forbes and Swift (1944) preface the report of their work on the associative dynamic effects of protein, carbohydrate, and fat by a statement to the effect that a nutrient fed alone is never metabolized alone. These workers tested the dynamic effects of protein, carbohydrate, and fat singly and in four combinations fed as supplements to a nutritively complete, basal, maintenance diet. This procedure, they felt, was more representative of nutritive practice than the usual method of measuring the dynamic effect of single test meals fed to animals in a post-absorptive state. Forbes and Swift observed dynamic effects of 32 per cent, 20 per cent, and 16 per cent for beef protein, cerelose, and lard respectively. The dynamic effects of the mixed supplements containing fat were lower than the effect of fat alone. They concluded that the fat content is the most important factor in determining the specific dynamic effect of a diet.

In the same laboratory Forbes, Swift, Elliott, and James (1946) conducted respiration experiments, with mature albino rats as subjects, to determine the extent to which the heat increments produced by the feeding of complete diets was influenced by their content of fat. Heat increments were measured as the difference in heat production from maintenance to supermaintenance diets, containing 2, 5, 10, 30 per cent fat and equal quantities of protein and calories. Heat increments decreased as the fat content of the diet increased and, at the same time, both the digestibility and retention of nitrogen improved. The authors attributed the inverse relationship that existed between energy expense of utilization and the fat content of the ration to a decreased catabolism of carbohydrate and a decreased synthesis of fat from carbohydrate in animals receiving significant amounts of fat.

It seems logical to assume that the dynamic effect associated with the utilization of protein is still operating when body protein is metabolized for the production of energy. In the protein-depleted rat receiving a limited number of calories from carbohydrate alone or from a mixture of carbohydrate and fat, energy production in one instance is influenced by a mixture of protein and carbohydrate and in the other case by a mixture of the three energy-producing nutrients. It may be recalled that Forbes and Swift (1944) found a significantly higher dynamic effect associated with the utilization of a mixture of carbohydrate and protein than with a mixture of the three energy-providing nutrients. It is conceivable that a lower specific dynamic action associated with the utilization of the three-nutrient mixture may conserve energy for the operation of body processes and may explain, at least partially, the lowered catabolism of body protein that has been observed in connection with the feeding of high-fat, low-calorie rations.

Changes In Metabolic Rate

Another possible mechanism by which fat may spare body protein when the diet contains insufficient energy and protein is by influencing the metabolic rate. Wesson and Burr (1931) and Burr and Beber (1937) studied the respiratory quotients and metabolic rates of animals conditioned to diets containing no fat as compared with those of rats receiving a mixed diet. They found that a high metabolic rate was associated with fat deficiency and that it was present in a pronounced degree in rats just entering the condition recognizable as characteristic of the deficiency disease. Also they were able to demonstrate that the animals maintained on fat-deficient diets exhibited respiratory quotients above one indicating that they had not lost their ability to form fat from carbohydrate. In neither experiment, were the investigators able to account for the difference in metabolic rate by a difference in the activity of the animals receiving the two types of diets.

Alterations In Enzyme Activity

Evidence is accumulating which indicates that liver enzyme activity may be altered markedly in response to dietary conditions. Thus, it was demonstrated by Miller (1948) that catalase, alkaline phosphatase, xanthine dehydrogenase, and cathepsin from rat liver all decreased in activity as the result of a seven-day fast. Loss of these vitally important functional proteins accompanies general loss of protein from the body.

Since all of the rats used in the present studies have been conditioned to the feeding of protein-free diets, one might expect that they would exhibit diminished enzyme activity. Other evidence suggests, however, that the activity of certain enzyme systems may be greater in animals conditioned to a fat-free diet than to diets containing fat. Thus, Swanson and Artom (1950) found that the mitochondria of the cells in the liver have a high lipide content. Many of the essential oxidation processes occur in the mitochondria, including those represented in the succinic oxidase and cytochrome oxidase and cytochrome oxidase systems. On the basis of these observations, Kunkel and Williams (1951) postulated that the activity of these enzymes might be affected by fat deficiency. They proceeded to study the relation of essential fatty acid deficiency to enzyme activity. They found that hepatic tissue of a fat-deficient rat exhibited a marked increase in cytochrome oxidase activity, and a marked decrease in endogenous respiration. On the other hand, the activity of succinic oxidase remained unchanged. The results suggest that the body has the ability to retain certain enzyme systems during fat deficiency but that the balance between the various respiratory enzyme systems may be altered during fat deprivation. The observation that an increase in cytochrome oxidase activity occurs concomitantly with an overall decrease in endogenous respiration suggests that the increased activity of this enzyme may occur at the expense of a decrease in another respiratory enzyme system. As already indicated, the basal metabolic rate of animals deprived of fat tends to increase. A simultaneous increase in metabolic rate and in the activity of the cytochrome oxidase system in fat deficiency may indicate that the regulation of the speed of metabolism is mediated, at least partially, by this particular enzyme.

Unpublished data collected in a preliminary experiment in this laboratory are in accord with the results of Kunkel and Williams. The cytochrome oxidase activity of hepatic tissues of rats subjected to protein-free diets providing limited calories from either carbohydrate alone or from a mixture of carbohydrate and fat was measured. All of the animals fed the fat-free diets showed greater activity of cytochrome oxidase than did the animals receiving fat. These animals also excreted greater quantities of nitrogen in the urine in response to a reduction in caloric intake than did animals receiving fat. The destruction of body tissue observed when the low-fat, low-calorie diet was fed may reflect an increased rate of metabolism controlled, in part at least, by the cytochrome oxidase enzyme system. Since caloric needs are not met by the food, it follows that a high metabolic rate would stimulate catabolism of tissue protein for energy purposes.

EXPERIMENTAL PROCEDURE

PLAN OF THE EXPERIMENT

Experiments from the Nutrition Laboratory of the Home Economics Research Department at the Iowa State College have shown repeatedly that dietary fat incorporated into a protein-free ration for adult male rats is effective in averting excessive destruction of tissue protein occasioned by the imposition of severe caloric restriction. That the presence of fat in the diet exerts a more favorable effect upon nitrogen metabolism than is observed when carbohydrate serves as the principal source of energy is a surprising observation. It has been the tendency of investigators ever since the time of Voit to attribute a greater nitrogen-sparing action to carbohydrate than to fat.

The research to be presented is divided into four units, each of which is described below.

Experiment I. Relative efficacy of different fats in sparing body protein of rats subjected to severe caloric restriction

The source of fat in the original experiments was a mixture composed of equal parts of butterfat and lard. The first step in studying the influence of dietary fat on metabolism under the experimental conditions imposed was to ascertain whether or not all fats were equally effective in retarding catabolism caused by the caloric reduction of a protein-free ration. Several common fats and different combinations of these fats were chosen for the study. In the first part of this experiment, animals were fed from a food cup. The response of these animals to calorically inadequate diets was compared to that of another group of animals similarly treated that received diets of adequate energy value.

In the second phase of Experiment I, the fats and fat mixtures associated with the greatest differences in performance were retested using a force-feeding technique.

Experiment II. Dependence of the protein-sparing action of fat on the essential fatty acid content or on the degree of hydrogenation of the fat molecule

The second study was planned to identify, if possible, the essential component or property of the fat proved to be most potent in alleviating the destructive course of catabolism in protein-deficient animals maintained on restricted caloric rations. The fat of demonstrated superior performance in Experiment I had an essential fatty acid content different than that of other fats tested. It also was saturated to a different degree.

In view of these variables, the influence of feeding an essential fatty acid and a sample of fully hydrogenated coconut oil was tested.

Experiment III. Effect of body stores of fat on the metabolism of protein-depleted animals receiving rations of inadequate energy value

It seems logical that body fat may spare tissue protein in the same manner as dietary fat. If body fat is capable of averting excess destruction of tissue protein, then the previous fat stores of the animal may determine the level at which protein metabolism proceeds. It was believed that reducing animals to the same original body weight through starvation before the initiation of the experimental regime might explain, at least partially, the role of body fat in this particular experimental situation.

Experiment IV. Relationship of the rate of nitrogen catabolism to survival time in rats fed a protein-free diet in restricted or unrestricted quantities as influenced by the distribution of energy-producing nutrients in the ration

The beneficial effect of fat reported in previous experiments was observed during relatively short periods of caloric restriction. The permanent nature of this effect was questioned. Accordingly, Experiment IV was designed to determine the rate of nitrogen catabolism in animals fed fat-free or fat-containing diets when the period of caloric restriction was extended until death intervened. The survival times of animals subjected to the various experimental regimes were observed.

This study was enlarged to include animals upon which no caloric restrictions were imposed. It seemed desirable to trace the course of protein metabolism as affected by the prolonged ingestion of two types of protein-free diets in the latter group of animals. The effect of the level of nitrogen metabolism upon the survival of animals was determined in rats subjected to the two experimental regimes.

EXPERIMENTAL ANIMALS

Adult male rats of Wistar stock, strain A, were used. Studies were conducted over a three-year period so that the animals represented the 101st to the 110th generations of rats inbred by brother and sister matings in the Nutrition Laboratory. They ranged in age from six to eight months at the time of the initiation of each experiment.

The rats at weaning (28 days) were housed either singly or in pairs in round wire-meshed cages until the beginning of the experimental period. Body weights were recorded once each week during this interval. During this time they were fed the laboratory stock ration designated as Steenbock XVII. This ration has been used for the maintenance of male animals in the stock colony of the Nutrition Laboratory since June, 1949. It differs from the Steenbock V diet used prior to this time in that dried whole milk is incorporated directly into the ration. No milk was included in the Steenbock V ration and reconstituted dried milk was offered daily as a supplement.

Rats used in these experiments ranged in weight from 280 to 440 grams. The animals were examined carefully before being assigned to the experiments. Rats with erratic growth performance or with obvious signs of respiratory infection were discarded.

In each main experiment, the rats were distributed throughout the groups on the basis of body weight on the day of assignment to the various groups. Each group initially was comprised of from six to ten animals, the weights of the individual animals in each group representing the range of weights characteristic of the entire group of animals selected for each particular experiment. Average weights of the animals in all groups in a particular experiment were almost identical. Litter mates were not assigned to the same experimental group.

EXPERIMENTAL DIETS

The synthetic low-nitrogen ration that has been used in this laboratory for a number of years was used in all of the investigations. This diet is considered adequate in all respects except for protein. The diets differed only in regard to their fat content. It was necessary to provide a source of essential fatty acids to animals receiving the low-fat rations. Fifty mg. of Wesson oil per day were used as a carrier for alpha tocopherol which was offered to animals receiving both types of diets. In the fat-containing rations, either a single fat or a mixture containing equal parts of two fats comprised 20 per cent of the diet. In diets from which fat was omitted dextrin substituted for an equivalent quantity of fat. The composition of the basal diets is indicated in Table 1.

Table 1. Composition of the low-nitrogen basal diets

Ingredients	High-fat diet	Low-fat diet
	%	%
Dextrin[a]	73	93
Fat	20	-
Osborne and Mendel salts[b]	4	4
Ruffex[c]	2	2
Sodium chloride[d]	1	1
Total	100	100

[a] Fisher Scientific Co.

[b] Osborne, T. B. and Mendel, L. B., J. Biol. Chem. 37, 557-601 (1919).

[c] Eimer and Amend, New York.

[d] Baker Chemical Co.

Certain fats used in the diets needed special preparation. Butter was purchased from the Iowa State College Dairy in a large enough quantity to cover the needs of one experiment. It was prepared in the following manner. The butter was melted in the top of a double-boiler and the milk solids removed from the top by careful skimming. The melted fat was then filtered through a hot-water funnel, using several thicknesses of cheesecloth. Margarine fat was prepared in the same manner. It was noted that the margarine contained appreciably larger quantities of milk solids or other non-fatty material than did the butter. Lard used in the experiments was Cudahay's "Pure Leaf" lard purchased on the local market. The hydrogenated coconut oil was a preparation provided by Dr. H. J. Deuel, Jr., of the University of Southern California. The source of hydrogenated vegetable fat was Crisco; the vegetable oil, Wesson oil; and the margarine, Allsweet.

All solid fats were liquefied before they were combined with the other dietary ingredients. The salt mixture and Ruffex were passed through a fine sieve; this procedure insured more uniform mixing of the dietary ingredients and disposed of small lumps which may block the needle or catheter used in force-feeding. Dry ingredients were blended thoroughly and transferred to the Hobart mixer after which the liquid fats were added. Diets were mixed at medium speed for thirty minutes, material adhering to the paddle and to the sides of the bowl being scraped into the mixture at intervals. Diets were refrigerated immediately after preparation.

The low-fat ration was prepared in the same manner. Dextrin was substituted for the fat component of the diet.

Two vitamin preparations were used. The composition of these preparations is given in Table 2. The one used in the first part of Experiment I (Fall, 1950) was the standard vitamin preparation that has been fed in previous experiments. It contained all known pure vitamins except B_{12} and folic acid with rice bran polish serving as a source of unidentified factors. All of the vitamins with the exception of tocopherol and the fat-solubles furnished by cod liver oil were mixed with dextrin so that 500 mg. of the mixture provided the daily allowance of each vitamin. Vitamins were prepared in 500-gram lots, a quantity which provided 1000 daily doses. Before each experiment began a sufficient number of lots was prepared to last for the duration of the experiment; the lots were combined and carefully blended. The preparation was stored in brown bottles in the deep-freeze to prevent deterioration from light and heat.

Table 2. Composition of the vitamin supplements

Vitamin	Allowance per rat per day		Gm. per 1000 doses	
	Prep. I	Prep. II	Prep. I	Prep. II
Choline chloride	5.0 mg.	5.0 mg.	5.000	5.000
Biotin	1.0 mcg.	1.0 mcg.	0.001	0.001
Rice Bran Polish Factor II	100.0 mg.	---	100.000	---
Thiamine	40.0 mcg.	40.0 mcg.	0.040	0.040
Riboflavin	60.0 mcg.	60.0 mcg.	0.060	0.060
Pyridoxine	40.0 mcg.	40.0 mcg.	0.040	0.040
Inositol	10.0 mg.	10.0 mg.	10.000	10.000
Para-amino benzoic acid	10.0 mg.	10.0 mg.	10.000	10.000
Calcium pantothenate	0.1 mg.	0.1 mg.	0.100	0.100
Nicotinic acid	0.5 mg.	0.5 mg.	0.500	0.500
Ascorbic acid	1.0 mg.	1.0 mg.	1.000	1.000
Folic acid	---	8.0 mcg.	---	0.008
Alpha tocopherol	0.75 mg.	0.75 mg.	---	---
Cod liver oil	50.0 mg.	50.0 mg.	---	---
B_{12}	---	0.5 mcg.	---	---

Small amounts were removed and kept refrigerated during the course of the experiment.

The laboratory supply of rice bran polish was exhausted by the time the 1950 experiment was completed, and a new supply could not be procured. By this time, folic acid and vitamin B_{12} were available and were used in the mixture in lieu of the rice bran polish extract. Eight mcg. of folic acid and 1.5 mcg. of vitamin B_{12} were provided per rat per day. The quantity of vitamin B_{12} was later reduced to 0.5 mcg.

Detailed descriptions of the methods used in the preparation of the dry portions of the two vitamin mixtures are on file in the Nutrition Laboratory.

Dry vitamins for all animals were measured in a calibrated spoon during the preliminary conditioning period. This method was continued for the entire experiment when rats were allowed to eat from food cups; when force-feeding was used, vitamins were mixed with the rations. The only exception to this practice was made in Experiment II in which vitamins were force-fed apart from the regular rations.

Vitamin B_{12}, alpha tocopherol dissolved in Wesson oil, and cod liver oil were measured from calibrated droppers. These liquids were added to the supplement cup containing the dry vitamins or, in case of force-feeding, they were homogenized with the diet in appropriate amounts.

Nitrogen balance tests with animals receiving adequate food energy from protein-free rations containing 20 per cent fat supplemented with each of the two vitamin mixtures have been conducted. The results of these tests indicate that nitrogen metabolism is unaffected by the alteration that was made in the composition of the vitamin supplement.

Administration Of The Diets

In the experiments reported by Stevenson _et_ _al._ (1946) and by Willman _et_ _al._ (1947) in which a favorable influence of fat in diets of low-protein, low-energy value was demonstrated, food was offered _ad_ _libitum_ during the preliminary depletion period and in the subsequent balance test during which response to full caloric feeding was measured. When calories were reduced, the animals were given the appropriate fraction of the actual quantity of food they had consumed during the first metabolic period. Faulty appetite is commonly observed in animals ingesting protein-free diets. Hoover (1950) introduced a force-feeding technique in order to insure a specific energy value of the administered diets and to equalize the caloric intakes of animals receiving the different diets. Both methods of feeding have been employed in the present investigation.

Feeding From Food Cup

All animals fed from a food cup were offered the rations *ad libitum* during the preliminary depletion period (18 days) and the subsequent five-day test period in which metabolic measurements were made. Animals which were maintained on diets of unrestricted energy value continued to receive the diet in the same manner throughout the experiment. When calories were reduced, two procedures were used in determining the quantity of food to be offered. In Experiment I, the quantity of food offered during the period of caloric restriction was assigned arbitrarily. In this experiment *ad libitum* consumption in the first period of the metabolic test was somewhat below the quantity that the rats were expected to eat, the rats fed the low-fat ration consuming an average of 35.7 Calories per day and rats receiving the various fat-containing diets ingesting on the average 38.4 to 45.1 Calories daily during the unrestricted feeding period. It was believed that reducing calories to one-fourth of the actual ad libitum intake would impose too great a strain upon the organism. It was decided to offer slightly more than one-fourth of the quantity of food consumed in the first metabolic test period. Each rat was offered a quantity of diet that would provide 12 Calories.

In subsequent experiments, adjustment of food intake during the period of caloric restriction was based on body weight. The average number of grams of food consumed daily per gram of body weight was calculated from data collected in the first metabolic test (calories unrestricted), the average body weights of animals during this period being used. Each rat received one-fourth of this average calculated value times its actual average body weight during caloric restriction. For Experiments III and IV, the average number of calories provided by high- and low-fat rations on the basis of the above calculations was 12.4. The low-calorie rations when fed from a food cup were offered in two portions daily.

Force-Feeding

When force-feeding was used in Experiments I and II, the diets were prepared in the manner described by Hoover (1950). Twenty-four ml. of diet containing 12 gm. of high-fat diet or 15 gm. of low-fat diet and 0.5 gm. of vitamin mixture were fed to a 300 gm. rat daily during the period of unrestricted feeding. The weight of dry diet was increased or decreased by 0.8 gm. for each 10 gm. increase or decrease in body weight. When calories were reduced, 24 ml. of the diet contained 3 gm. of high-fat diet or 3.75 gm. of low-fat diet and 0.5 gm. of vitamin mixture. The volume to be fed to each rat was calculated from the body weight at the beginning of force-feeding. The same amount was fed throughout the remainder of the experiment.

Force-fed diets were administered three times daily, slightly smaller quantities being fed in the afternoon than in the morning and evening because of the shorter time between feedings. Animals were fed at 9 A. M., 4 P. M., and 9 P. M. Rats selected for force-feeding experiments were offered the rations _ad libitum_ for the first 15 days of the test. Force-feeding was initiated on the 16th day. In order to accustom the animals to the technique, one-third of the total quantity of diet was force-fed on the 16th day, two thirds on the 17th day, and the full amount on the 18th day. On days when only part of the food was force-fed, an additional quantity of food was provided in a food cup.

The force-feeding technique as developed for use in this laboratory is described in detail by Hoover (1950) and by Clark (1950).

EVALUATION OF NITROGEN METABOLISM

Plan Of The Test

When protein-free diets are fed to rats, the lavish expenditure of nitrogen observed in the first few days following the ingestion of the diet gradually decreases until finally a more or less steady state of catabolism is reached. The plan of the present investigation demanded that a constant level of nitrogen excretion be attained before metabolic measurements could be initiated. Hoover (1950) determined the quantities of urinary nitrogen eliminated at successive intervals following force-feeding of low-nitrogen diets. Results indicated that nitrogen excretion did not plateau until 18 days had passed. This work was repeated during the present investigation using ad libitum feeding of the high-fat, protein-free diet as well as force-feeding twice daily and force-feeding three times daily. In the present experiment the plateau was attained at approximately the same time as in the corresponding test conducted by Hoover, and it was decided to retain the experimental periods suggested by her. It is interesting that in animals force-fed the diet three times daily, urinary nitrogen decreased more regularly and a more steady plateau was maintained than was observed for animals fed ad libitum or force-fed twice daily. Partly because of this observation, the procedure of force-feeding three times daily was adopted in this investigation.

In general, each experiment extended over 32 days, divided into the following periods:

Full calorie feeding period	
Preliminary adjustment period	18 days
Collection period (Period I)	5 days
Reduced calorie feeding period	
Adjustment period	4 days
Collection period (Period II)	5 days

When caloric restriction was not imposed, the animals were maintained on the rations for the same length of time as the restricted animals, collections during Period II being made in response to the ingestion of a diet of adequate energy value.

Stock diet was removed from the cage at 10 P. M. on the day before animals were offered the experimental diets. This procedure was also followed on the day preceding each balance period for animals fed from a food cup. In the case of animals force-fed the rations, the last feeding was administered at 4 P. M. on the day prior to the initiation of a balance period. On the first day of the balance period, the animals were transferred to wide-meshed metabolism cages at 8 A. M. The feeding schedule for the last day of the balance period corresponded to that on the day preceding the test. Animals were removed from the metabolism cages the following morning at the same hour as they had been placed on balance.

Collection Of Urine And Feces

Urine was collected on acid-treated filter paper*. Seven of these papers were placed on Pyrex plates under each metabolism cage for each five-day collection period. One was removed each day and placed in a 1-liter, wide-mouthed, Erlenmeyer flask which contained 200 ml. of 20 per cent hydrochloric acid.

Feces were collected daily, brushed free from hair, and placed in an Erlenmeyer flask containing 50 ml. of 20 per cent hydrochloric acid. Ferric oxide was employed as a fecal marker, 100 mg. per 100 gm. representing the proportion of marking material to dry diet. Red diets were fed on the first day of each balance period and on the day following the completion of each period. On the first day on which feces were collected, only red ones were saved. Subsequently all were collected until red feces appeared again marking the end of the balance period.

At the end of each test interval, rats were transferred to ordinary cages and the metabolism cages were

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*500 9-inch filter papers (Schleicher and Schull, No. 597) were left overnight in a solution of 900 ml. of 95 per cent alcohol and 100 ml. of glacial acetic acid and dried.

washed quantitatively with hot distilled water and the washings added to the flask for urine collection. The papers were washed with hot distilled water using suction. The volume of the acid solution of urine was adjusted to 2 liters, and a suitable aliquot was saved for analysis.

In Experiment IV urine collections were extended beyond the regular balance periods. Urine was collected daily during this interval. In this case the flasks containing filter papers and urine in hydrochloric acid, representing a one-day collection, were autoclaved for one hour. The contents of the flask were transferred quantitatively to a 1-liter volumetric flask, and the volume was adjusted accordingly.

Determination Of Nitrogen

Concentration of nitrogen in food, urine, and feces was determined in Experiment I using the Kjeldahl-Gunning procedure. Ammonia was collected in approximately 0.1 N hydrochloric acid containing a mixed indicator of methylene blue and methyl red. In the remaining experiments only urinary nitrogen was determined.

The size of sample, volume of concentrated sulfuric acid used in digestion, quantity of catalyst, and the length of the digestion period were adjusted in relation to the type of material being analyzed.

The ability of the author to recover nitrogen from a standard solution was demonstrated. Recovery of nitrogen from the metabolism cage sprinkled with a standard solution of $(NH_4)_2SO_4$ was also satisfactory. (See Appendix, Tables A and B.)

RESULTS AND DISCUSSION

EXPERIMENT I

The first part of this experiment, conducted in Fall, 1950, was designed to determine the effects of a number of single dietary fats and various combinations of these fats on the protein catabolism of protein-depleted animals receiving a ration of limited energy value.

Since a butterfat and lard mixture was the fat component of the original diet with which a favorable influence was associated, the protein-sparing properties of each of these fats fed alone were investigated. It was not known whether the influence was specific for one of the fats or whether it represented an additive or supplementary effect of two single fats. It seemed possible that single fats might act differently than the same fats in combination. In addition to butterfat and lard, three other common sources of fat were selected for study, namely, cottonseed oil, hydrogenated vegetable fat, and margarine fat. The effect of combining butterfat or lard with each of the other fats was determined to ascertain whether any other mixture was as effective as the butterfat-lard mixture in decreasing catabolism under the specific conditions of this experiment. Three butterfat-containing and three lard-containing mixtures were formulated, *i.e.*, butterfat and lard each were combined with cottonseed oil, hydrogenated vegetable fat, or margarine fat.

The various fats and fat combinations were incorporated into the basal protein-free diet in quantities to provide 20 per cent of the diet. In all of the mixtures of fat equal parts of the two fats were used. The response of rats to the feeding of these diets was compared to the response of animals receiving butterfat and lard as the source of fat and to that of animals receiving no dietary fat. In the low-fat diet additional dextrin was added at the expense of fat.

Thirteen experimental diets were used, each fed to a group of 12 rats. Animals in each group were fed the respective rations *ad libitum* for the first 23 days of the experiment. During the last five days of this feeding regimen, nitrogen balances were determined. On the 24th day, the dietary modification was introduced, *i.e.*, restriction of calories to approximately one-fourth of the quantity voluntarily eaten by the rats. At this juncture, calories were decreased for six of the animals, while the remaining six continued to consume the rations *ad libitum*.

This method of procedure permitted two types of comparisons:

(1) Comparison of the nitrogen balances accrued by the same animals while ingesting full or restricted quantities of one diet; in this way each animal served as his own control, *i.e.*, a longitudinal control;

(2) Comparison of the nitrogen balances of different animals receiving full and restricted quantities of one diet during the second interval of the test period, *i.e.*, a cross-sectional control.

The nitrogen metabolism of animals fed the various diets in unrestricted quantities (Period I) and in restricted quantities (Period II) is indicated in Table 3 (the longitudinal control study). The mean nitrogen balance of animals fed the low-fat, unrestricted ration in Period I for five days was -213 mg. Corresponding mean balances in the same interval for animals receiving the different fat-containing diets ranged from -238 to -296 mg. The latter difference is of the order of the standard

Table 3. Mean nitrogen metabolism of rats ingesting diets containing different sources of fat and various combinations of these fats during intervals of <u>ad libitum</u> feeding (Pd. I) and of restricted feeding (Pd. II)

Source of dietary fat	Number of rats	Daily energy value of diet		Nitrogen balance per 5 days		
		Periods		Periods		Increase in catabolism
		I	II	I	II	
		Cal.	Cal.	mg.	mg.	mg.
Low-fat (50 mg. oil/day)	5	35.7	9.9	-213	-739	526
Cottonseed oil	6	41.7	10.7	-296	-348	52
Butterfat and lard	6	40.0	11.4	-239	-343	104
Butterfat and margarine fat	6	42.3	11.4	-259	-455	196
Lard and margarine fat	6	42.9	11.4	-246	-449	203
Lard and hydrogenated fat	6	40.2	11.4	-239	-456	217
Lard	5	45.1	11.5	-250	-472	222
Hydrogenated vegetable fat	5	41.8	11.0	-238	-475	237
Butterfat and hydrogenated fat	6	42.0	11.5	-255	-502	247
Lard and cottonseed oil	6	40.5	11.4	-245	-494	249
Butterfat and cottonseed oil	6	40.2	11.4	-246	-553	307
Margarine fat	6	38.4	11.4	-242	-572	330
Butterfat	6	39.3	11.4	-244	-657	413

deviation from the mean balance of some 300 nitrogen balances of rats fed the nitrogen-low, 20 per cent fat diet in this laboratory. The significance of the lower balance accrued by rats ingesting unrestricted quantities of the low-fat ration will be discussed in relation to Experiment IV.

When the energy value of the diet was decreased (Period II), nitrogen catabolism increased in all of the animals studied. Nitrogen balances in the five-day period of reduced calorie feeding ranged from -343 mg. to -739 mg. Feeding the restricted low-fat diet brought about the greatest increase in the negativity of the nitrogen balance. Considerable variation was noted in response to the feeding of the low-calorie, fat-containing diets. In animals receiving butterfat, the rate of nitrogen expenditure increased to about the same extent as it did when animals were provided the low-fat ration. On the other hand, the feeding of a diet containing cottonseed oil or a diet containing a mixture of butterfat and lard was accompanied by very small increments in the rate of nitrogen catabolism.

It may be noted that the caloric values of the daily diets consumed by the different groups of animals were not identical in Period I. In this experiment, rats were allowed free access to the respective rations during the first period of metabolic measurement, and ate differently. The average energy values of the food chosen by the various groups of rats were between 35.7 and 45.1 Calories per day. Although isocaloric quantities of the restricted rations were provided the animals in Period II, failure in some cases to consume the full quota of rations was reflected by slight differences in the caloric value of the food eaten. It is believed, however, that the differences in energy value of the consumed rations occasioned by the method of feeding were without effect upon the results observed, except perhaps in the case of the rats fed the low-fat diet. Animals receiving cottonseed oil as the source of fat demonstrated less evidence of catabolism than did animals receiving any of the other rations, yet the former animals actually consumed fewer calories than animals fed any of the other fat-containing rations (Period II).

An interesting observation was made in connection with the nitrogen balance findings. It has been suggested that the size of the standard deviation in a value measuring the response of animals to experimental diets varying in respect to one component may measure the general nutritional adequacy of the diets. The greater the variation within a group the less adequate is the diet. Individual animals, in general, within the groups exhibiting a less favorable nitrogen balance following the reduction in caloric intake showed greater variation from the mean values for their respective groups than did animals from groups in which the response to caloric restriction was more favorable. The standard deviation characteristic of each mean nitrogen balance was calculated. These data were then plotted against the mean nitrogen balances (Figure 1). The relationship is clear, and indicates differences in the nutritive value of the fats fed.

Although results of this analysis are suggestive, whether or not the large standard deviations observed in connection with the feeding of certain of the experimental diets were entirely of nutritional origin was questioned. It was believed also that the physical condition of the animal at the end of the experiment might provide some explanation for the variations in nitrogen excretions observed among the animals.

Findings at necropsy revealed the presence of infection in the lungs in a small proportion of animals.

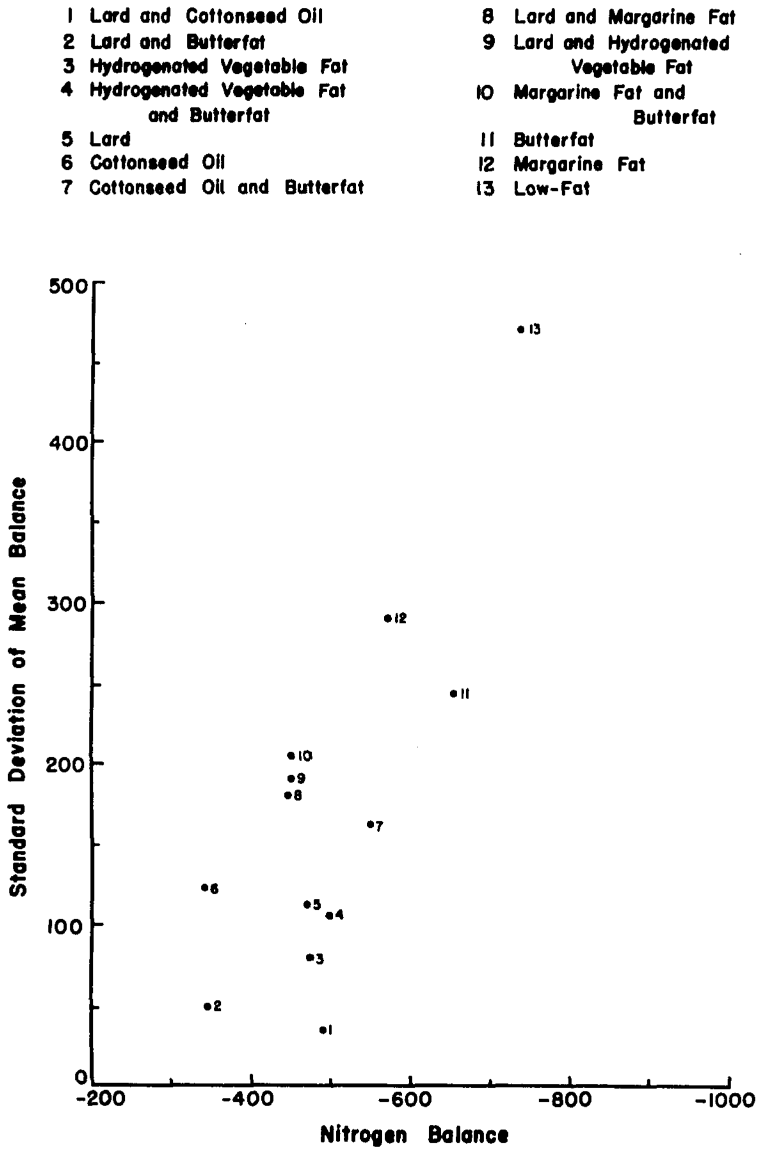

Figure 1. Scattergram showing relation of standard deviation of mean nitrogen balance to degree of negativity of nitrogen balance

There also was a wide variation in the stores of body fat among the animals; some animals were completely devoid of visible body fat. It was noted that weight losses varied appreciably during the period of caloric restriction. Some of the animals exhibited precipitous drops in body weight in response to caloric restriction and may have been approaching death. Increased catabolism often appeared in individual rats in which the following symptoms appeared: large losses in body weight, presence of lung infection, and absence of body fat. Nitrogen metabolism data obtained from animals who met two out of three of the following criteria were eliminated: presence of moderate lung infection, a 40-gram or greater loss in body weight during the nine days of caloric restriction (four days adjustment; five days, Period II), absence of visible body fat. The results obtained from animals exhibiting a severe degree of lung infection or from animals who were moribund at the end of the experiment were similarly omitted. The remaining data represent results uncomplicated by the effects of pathological conditions that might affect the performance of the rats in the nitrogen balance test. The data were reanalyzed and results compared with those obtained in the analyses just presented. This procedure has been followed in presenting results from all of the experiments that follow.

The screened nitrogen balance data of animals free from pathological abnormalities and receiving single fats first in unrestricted and subsequently in restricted quantities are shown in Table 4. Results still show that the nitrogen balances occasioned by caloric restriction, in general, were of the same order but of lower magnitude than those reported in Table 3. As before, the use of butterfat in the rations was associated with a greater catabolism than that induced by feeding the low-fat diet and again cottonseed oil rated the highest of all of the fats fed. Data from animals free from abnormalities indicated that other fats fell into the following order: margarine fat, hydrogenated vegetable fat,and lard. The observation that butterfat and lard, the components of the original fat mixture with which a sparing effect was demonstrated, were both ineffective as single fat components of the protein-free rations is of particular interest.

A two-way control was used in the present experiment. The results obtained when the cross-sectional type of

Table 4. Longitudinal control study of mean nitrogen metabolism of rats ingesting diets containing single fats during intervals of <u>ad libitum</u> feeding (Pd. II)

Source of dietary fat	No. of rats	Daily energy value of diet		Nitrogen balance per 5 days			Urinary nitrogen per 5 days		
		Periods		Periods		Increase in catabolism in Period II	Periods		Increase in urinary nitrogen in Period II
		I	II	I	II		I	II	
		Cal.	Cal.	mg.	mg.	mg.	mg.	mg.	mg.
Low-fat	3	37.5	10.4	-241	-434	193	151	395	244
Cottonseed oil	5	40.9	10.6	-280	-303	23	193	288	95
Margarine fat	3	42.1	11.5	-258	-363	105	173	336	163
Hydrogenated vegetable fat	3	41.4	11.0	-285	-419	134	175	383	208
Lard	4	44.7	11.5	-283	-463	180	188	422	234
Butterfat	3	39.8	11.4	-257	-493	236	176	454	278

control was used are of interest also. Results are presented in Table 5. The extent of nitrogen catabolism indicated by the comparisons of these two types of measurements is of slightly higher magnitude but of exactly the same order as was shown when each animal served as his own control.

In Table 6, nitrogen balance data for animals receiving the rations containing butterfat alone or butterfat combined with other sources of fat are presented. While the presence of butterfat as the only source of fat in the ration was associated with a marked increment in the rate of tissue destruction, the feeding of butterfat in combination with certain other fats evoked a more favorable response. The butterfat-lard combination was most effective in averting excessive losses of body nitrogen, then in order came butterfat-margarine fat, butterfat-hydrogenated vegetable fat, and butterfat-cottonseed oil. It should be noted that the combination of butterfat and cottonseed oil had no protective influence.

The feeding of lard as the only source of fat was accompanied by an increase in the rate of nitrogen expenditure; an increment of 180 mg. was observed for the

Table 5. Cross-sectional control study of mean nitrogen metabolism of rats ingesting diets containing single fats during intervals of *ad libitum* feeding (Pd. II) and of restricted feeding (Pd. II)

Source of dietary fat	Daily energy value of diet		Nitrogen balance per 5 days			Urinary nitrogen per 5 days		
	Periods		Periods		Increase in catabolism	Periods		Increase in urinary nitrogen
	II	II	II 100% Cal.	II 25% Cal.		II 100% Cal.	II 25% Cal.	
	Cal.	Cal.	mg.	mg.	mg.	mg.	mg.	mg.
Low-fat	35.2	10.4	-213 (6)[a]	-434 (3)	221	141 (6)	395 (3)	254
Cottonseed oil	44.8	10.6	-248 (5)	-303 (5)	55	177 (5)	288 (5)	111
Margarine fat	40.5	11.5	-242 (6)	-363 (3)	121	172 (6)	336 (3)	164
Hydrogenated vegetable fat	43.1	11.0	-238 (6)	-419 (3)	181	162 (6)	383 (3)	221
Lard	45.3	11.5	-250 (6)	-463 (4)	213	169 (6)	422 (4)	253
Butterfat	45.2	11.4	-244 (6)	-493 (3)	249	172 (6)	454 (3)	282

[a] Number in parenthesis indicates number of animals.

Table 6. Mean nitrogen metabolism of rats ingesting diets containing butterfat or butterfat combined with other fats during intervals of *ad libitum* feeding (Pd. I) and of restricted feeding (Pd. II)

Source of dietary fat	No. of rats	Daily energy value of diet		Nitrogen balance per 5 days			Urinary nitrogen per 5 days		
		Periods		Periods		Increase in catabolism in Period II	Periods		Increase in urinary nitrogen in Period II
		I	II	I	II		I	II	
		Cal.	Cal.	mg.	mg.	mg.	mg.	mg.	mg.
Butterfat	3	39.8	11.4	-257	-493	236	176	454	278
Butterfat and lard	6	40.0	11.4	-244	-343	99	173	312	139
Butterfat and margarine fat	5	42.5	11.4	-260	-382	122	175	349	174
Butterfat and hydrogenated vegetable fat	4	43.0	11.5	-299	-444	145	203	403	200
Butterfat and cottonseed oil	4	40.3	11.4	-275	-483	208	185	452	267

five-day period (Table 7). As was the case for butterfat, diets containing combinations of lard with margarine fat or hydrogenated vegetable fat retarded excessive destruction of tissue protein while the diet containing a combination of lard with cottonseed oil did not. It may be noted that the mixture of lard and margarine fat was as effective as the mixture of lard and butterfat in preventing losses of nitrogen from the body. Comparison of the data presented in Tables 6 and 7 may indicate that the influence of the butterfat-lard mixture upon the course of nitrogen metabolism is more dependent upon its content of lard than its content of butterfat.

In all of the results presented, when the quantities of nitrogen excreted in the urine by these animals are compared with the nitrogen balances for these same animals, it is seen readily that the order of the two estimates is alike. The quantity of nitrogen excreted in the feces varies with the total quantity of diet fed but is approximately constant in response to the different dietary modifications used. In view of this observation, the quantity of nitrogen excreted in the urine has been used as the index of measuring differences in the nutritional values of the diets.

Table 7. Mean nitrogen metabolism of rats ingesting diets containing lard or lard combined with other fats during intervals of *ad libitum* feeding (Pd. I) and of restricted feeding (Pd. II)

Source of dietary fat	No. of rats	Daily energy value of diet		Nitrogen balance per 5 days			Urinary nitrogen per 5 days		
		Periods		Periods		Increase in catabolism in Period II	Periods		Increase in urinary nitrogen in Period II
		I	II	I	II		I	II	
		Cal.	Cal.	mg.	mg.	mg.	mg.	mg.	mg.
Lard	4	44.7	11.5	-283	-463	180	188	422	234
Lard and butterfat	6	40.0	11.4	-244	-343	99	173	312	139
Lard and margarine fat	4	43.7	11.4	-271	-343	72	179	309	130
Lard and hydrogenated vegetable fat	5	40.3	11.4	-260	-383	123	178	350	172
Lard and cottonseed oil	3	40.4	11.5	-267	-494	227	197	455	258

The results of this experiment have been summarized in Table 8. Single fats as judged by their ability to decrease the rate of catabolism when the caloric value of the protein-free diet was reduced rated in efficiency as follows: cottonseed oil, margarine fat, hydrogenated vegetable fat, lard, and butterfat. With cottonseed oil in the diet, the quantity of nitrogen in the urine was increased by only 95 mg. in the five-day period; with butterfat, 278 mg. Results of arranging the various fat mixtures tested in order of their effectiveness disclosed an interesting trend. While cottonseed oil far exceeded the other single fats in retarding catabolism, the combinations containing cottonseed oil and either butterfat or lard were less effective than any of the other fat combinations.

It is interesting that butterfat acquired beneficial properties when combined with lard or margarine fat. When fed alone it ranked at the bottom of the list. Combining butterfat with either of these fats brought about approximately one-half as great an increase in catabolism in response to caloric restriction as did the feeding of a diet containing only butterfat.

Table 8. Increase in quantities of nitrogen excreted in the urine in response to reduction of calories when rats receive single fats or fat mixtures

Urinary nitrogen per 5 days			
	Difference between Pd. I and Pd. II		Difference between Pd. I and Pd. II
	mg.		mg.
Fats		Fat mixtures	
Cottonseed oil	95	Lard and margarine fat	130
Margarine fat	163	Lard and butterfat	139
Hydrogenated vegetable fat	208	Lard and hydrogenated fat	172
Lard	234	Butterfat and margarine fat	174
Butterfat	278	Butterfat and hydrogenated fat	200
		Lard and cottonseed oil	258
		Butterfat and cottonseed oil	267

In the spring of 1952, a confirmatory experiment was conducted. It seemed desirable to repeat the experiment with the rigid control of the energy value of the daily food consumed that force-feeding permitted. Four of the experimental diets used in the original investigation were reevaluated, _i.e._, the diet low in fat and diets containing a mixture of butterfat and lard, butterfat only, and cottonseed oil only. Two methods of feeding were employed in the 1952 study, feeding from a food cup and force-feeding. The quantity of nitrogen excreted in the urine was the index used for measuring the relative efficiencies of the various diets. Results obtained in the food-cup feeding test are presented in Table 9. Data from the 1950 test are included for comparison. Results from the 1952 experiment are of the same order as they were in the original experiment. Differences between the groups were statistically significant in the 1950 experiment but not in the 1952 test.

When the same diets were force-fed, increases in the rate of catabolism following the restriction of the diet were not as large as in previous experiments ranging now from 70 to 120 mg. per five days (Table 10). Again

Table 9. Mean quantities of nitrogen in the urine of rats ingesting diets containing different sources of fat during intervals of *ad libitum* feeding (Pd. I) and of restricted feeding (Pd. II) in two experiments

Source of dietary fat	Urinary nitrogen per 5 days				Increase in urinary nitrogen	
	Period I		Period II			
	1950	1952	1950	1952	1950	1952
	mg.	mg.	mg.	mg.	mg.	mg.
Low-fat	151	151	395	386	244	235
Cottonseed oil	193	195	288	318	95	123
Butterfat and lard	173	193	312	377	139	184
Butterfat	176	175	454	386	278	211

Table 10. Mean quantities of nitrogen in the urine of rats force-fed diets containing different sources of fat during intervals of full (Pd. I) and restricted (Pd. II) feeding

Source of dietary fat	No. of rats	Urinary nitrogen per 5 days		
		Period I	Period II	Increase in urinary nitrogen
		mg.	mg.	mg.
Low-fat	6	193	313	120
Cottonseed oil-20% low-fat	6	221	294	73
Butterfat-10% lard-10%	4	209	279	70
Butterfat-20%	6	186	297	111

the use of butterfat in the diet was without benefit (increases in urinary nitrogen: low-fat diet, 120 mg. per 5 days; butterfat diet, 111 mg. per 5 days). Cottonseed oil and the butterfat-lard mixture both decreased the increment in excretion of nitrogen following caloric restriction to the same extent (50 mg. per 5 days).

The relatively small increases in urinary nitrogen occurring in this test suggest that when the restricted diet was force-fed it was utilized more efficiently than when it was eaten from a food cup. The difference is hard to explain. However, force-feeding distributed the limited quantities of the diet over three intervals of the day. These rats were quiescent and did not seem to be hungry. When allowed to eat from a food cup the rats consumed the rations immediately and exhibited symptoms of hunger between intervals of feeding. They were constantly searching for food and expended a great deal of energy pawing the cage and attempting to escape. The greater activity of animals fed from a food cup may have increased their requirement for food energy and may explain, at least partially, why they excreted more nitrogen in response to caloric reduction than animals force-fed isocaloric quantities of the same diets.

Periods between feedings approximating starvation may also have been an influencing agent.

EXPERIMENT II

The observation that cottonseed oil incorporated into a protein-free ration was particularly effective in preventing destruction of tissue protein caused by caloric restriction suggested that the protective influence might be attributed to some particular property or component of the cottonseed oil. The highly unsaturated nature of cottonseed oil and its content of linoleic acid, an essential fatty acid, distinguished it from the other fats tested.

It was believed that the favorable influence of cottonseed oil might reflect either its high degree of unsaturation or its content of linoleic acid. Two test procedures were adopted. A negative approach was used to study the effect of the degree of saturation upon the protein-sparing effect. A laboratory prepared, purified sample of hydrogenated coconut oil which was almost entirely free of unsaturated fatty acids was procured. It was believed that if the protective influence of cottonseed oil was dependent upon its high degree of unsaturation then the feeding of a completely saturated fat should evoke the same response as feeding a diet devoid of fat. The effect of adding the methyl ester of linoleic acid to the low-fat ration was also tested.

The response of rats to the feeding of a low-fat diet supplemented with 100 mg. methyl linoleate daily or a diet containing 20 per cent hydrogenated coconut oil was compared to that of rats fed the low-fat diet or diets containing either 20 per cent cottonseed oil or 20 per cent butterfat.

All animals in this experiment were force-fed the experimental rations in three portions daily. In order to provide a vehicle for the methyl linoleate, the vitamin preparation was homogenized with a small quantity of water and force-fed apart from the regular ration. The vitamin mixture, either with or without methyl linoleate, was administered daily immediately preceding the feeding of the first portion of the appropriate diet. The supplements were force-fed during the entire 32-day experimental period. Animals who became accustomed to the force-feeding of the vitamin mixture from the beginning of the experiment offered little resistance to the force-feeding of the entire ration. In this experiment, animals adjusted to caloric restriction for three instead of four days before metabolic measurements were made.

Results are presented in Table 11. Animals in this experiment responded in much the same manner as already was noted for animals force-fed rations in Experiment I. Again caloric restriction was accompanied by a relatively small increase in the rate of catabolism. It should be noted that the rats in this experiment were in unusually good physical condition at the beginning of the experiment and came through in excellent condition. Animals receiving the ration containing the hydrogenated coconut oil or the diet supplemented with an unsaturated essential fatty acid showed essentially the same response as animals fed the low-fat diet, the respective increments in excretion induced by caloric restriction being 36, 64, and 46 mg. for the five-day period. When the diet containing 20 per cent cottonseed oil was fed the level of excretion remained constant during periods of full and restricted feeding. Once again, the ineffectiveness of butterfat was demonstrated. The incorporation

Table 11. Mean nitrogen metabolism of animals force-fed the basal low-fat or high-fat rations during intervals of full (Pd. I) and restricted (Pd. II) feeding

Source of dietary fat	No. of rats	Urinary nitrogen per 5 days		
		Period I	Period II	Increase in urinary nitrogen
		mg.	mg.	mg.
Low-fat 100 mg. methyl linoleate	7	168	232	64
Hydrogenated coconut oil (20%)	7	193	229	36
Low-fat	7	173	219	46
Cottonseed oil	7	233	240	7
Butterfat	6	187	238	51

of equal amounts of either butterfat or hydrogenated coconut oil into the ration brought about increments in excretion of 51 and 36 mg. respectively.

When fed as a supplement to a low-fat diet already adequate in essential fatty acids, linoleic acid exerts no protective influence. Perhaps it might be effective at a higher dosage. Also, it is entirely possible that other unsaturated fatty acids may possess a protein-sparing effect. Since oleic acid and linoleic acid occur in approximately the same proportions in cottonseed oil and account for most of its unsaturated fatty acid content, the possibility still exists that oleic acid may be effective in preventing increments in the output of nitrogen in the urine in response to caloric restriction.

At this point it may be well to call attention to a phenomenon that has appeared before and appears even more clearly in this experiment. Rats receiving cottonseed oil in the diet excreted more nitrogen when the ration was administered in a quantity estimated to meet the energy requirements of the animal (Period I) than did animals force-fed an isocaloric quantity of low-fat ration. This problem will be treated further in Experiment IV.

Although the results of the present experiment are inconclusive, nevertheless, this experiment has stimulated considerable thought regarding other approaches that might be used in studying the protein-sparing properties of fat. One might ascertain, for example, whether the protective property characterizes the saponifiable or the non-saponifiable portion of the fat molecule. Since cottonseed oil contains large quantities of tocopherols, it is conceivable that these may be functioning in a special capacity under the particular stress conditions that have been described, *i.e.*, over and beyond their recognized functions as vitamin E and as antioxidants. The quantity of tocopherol furnished by the carbohydrate diet is significantly less than by the diet containing cottonseed oil. It is also possible that some other fraction of the non-saponifiable portion of cottonseed oil may be responsible for its protective action. It would also be desirable to test the effectiveness of the saponifiable portion or specific fractions thereof of cottonseed oil. The potency of progressively hydrogenated fractions of a pure sample of cottonseed oil might be one approach. The value of the glycerol fraction of fat should not be overlooked.

EXPERIMENT III

A beneficial effect of dietary fat in preventing excess catabolism of tissue protein under the stress of caloric restriction has been demonstrated when protein-free rations are fed to rats depleted of their reserves of body protein. It seems reasonable that under this stress condition stores of fat in the body may act in the same manner as dietary fat. Findings at necropsy in previous experiments had indicated that considerable variation existed among animals in regard to the size of fat stores. Animals with little or no visible fat often catabolized body protein faster than animals with appreciable reserves of fat.

The rate of nitrogen catabolism has been determined in a group of animals in which an attempt was made to equalize fat stores. Two groups of animals were used, one designated to receive the low-fat basal ration and the other to receive a 20 per cent fat ration containing cottonseed oil. Stores of fat were equalized by reducing all animals to the same body weight through starvation before initiating the 32-day test. The starting weight was set at 325 gm. In previous experiments, rats of this starting weight have withstood caloric restriction without complete exhaustion of body fat. Animals whose initial weight was 325 gm. were assigned immediately to the experimental rations. Animals whose weight exceeded 325 gm. received no food except for the daily vitamin supplement until they reached the designated weight. They were allowed free access to water.

Data, screened in respect to incidence to lung infection and unduly large losses in body weight as described in Experiment I, are reported for 13 animals, seven receiving the low-fat ration and six, the high-fat ration (Table 12). At the time of selection the body weights ranged from 323 to 440 gm. From zero to ten days of starvation were required by the individual animals to reduce body weight the desired amount. The animals remained unusually calm and exhibited no unusual symptoms during the starvation period. The rats

Table 12. Individual and mean nitrogen metabolism of animals reduced in weight to 325 gm. before initiation of experiment in response to full (Pd. I) and restricted (Pd. II) feeding

Rat no.	Original body wt.	Starvation period	Urinary nitrogen per 5 days		
			Pd. I	Pd. II	Increase in urinary nitrogen in Pd. II
	gm.	days	mg.	mg.	mg.
		Low-fat diet			
3	327	0	170	259	89
4	333	0	202	280	78
6	337	1	175	278	103
7	370	4	132	296	164
8	376	5	133	235	102
9	408	9	114	272	158
10	423	10	111	214	103
Av.			148	262	114
		High-fat diet			
11	323	0	213	256	43
12	334	1	224	248	24
13	345	1	193	310	120
14	348	1	210	256	40
17	379	5	160	217	57
19	440	10	216	223	7
Av.			203	252	49

were permitted to eat ad libitum between the time that feeding was initiated and the end of Period I of the metabolism test so that body weights varied somewhat during the period that nitrogen excretion was measured. Even so, the range in actual body weights was less (270 gm. to 319 gm.) than that observed in experiments of this type when no control over body weight was exercised.

Individual and mean excretions of nitrogen are presented in Table 12. The constant nature of the individual data, particularly in response to the feeding of low-calorie diets is notable. This observation coupled with the fact that the present animals were of greater body weight than many of those used formerly may indicate that uniform stores of body fat are influencing the response of the rats given the low-fat diet.

Increments in excretion were small in comparison to other results reported herein when the low-fat ration was fed in restricted quantities. Values for the rats fed the high-fat diet, however, were of approximately the same order as those previously noted. The analyses are particularly difficult to interpret because the long period of starvation affected the response of certain rats (note rats 7, 8, 9, and 10 and rat 17). The excretions of nitrogen of these animals were very low in Period I. Starvation apparently had induced the protective adjustment in nitrogen excretion that occurs in protein deprivation.

As in previous experiments, the increase in catabolism demonstrated by animals receiving low-fat diets was double that of the increase in animals fed fat. The excretion of nitrogen in Period I was maintained at a high level by the fat-fed rats, and emphasized the difference in response of the two groups. However, if the rats that had been starved from four to ten days in the low-fat group are omitted, the effect of reducing caloric intake is about the same as that observed previously. It is of interest that the administration of the fat diet kept the excretion of the rats that had been starved either five or ten days at a high level in Period I. In general, the experiments to date have shown that animals receiving fat-containing diets *ad libitum* maintained nitrogen catabolism on a higher plane than animals receiving all of their calories from carbohydrate. In view of this fact, a long-time study of nitrogen metabolism in animals receiving fat-free or fat-containing diets in full or restricted quantities was indicated. Such an experiment is reported in the following section.

EXPERIMENT IV

Results to date have indicated that dietary fat has the unique ability of conserving tissue protein when incorporated into a protein-free diet supplying approximately one-fourth of the calories needed daily by the rat. In these experiments, results were evaluated in terms of the degree of tissue catabolism induced by feeding high- and low-fat diets deficient in protein and of varying caloric value. The nitrogen metabolism data that were reported reflect the immediate reaction to caloric restriction of rats fed either fat-free or fat-containing diets. It was recognized that the more favorable nitrogen metabolism associated with the feeding of the fat-containing diets under conditions of caloric restriction might represent a temporary adjustment. Whether or not the effect of the inclusion or fat in low-calorie, protein-free diets continues to be manifested over a long period of time was studied in this phase of the investigation. In addition, the influence of fat when present in diets providing the full quota of required energy was investigated in a similar long-time experiment.

Also time of survival of the animals fed the various test diets provided another measurement of the relative effectiveness of fat-free and fat-containing diets in protein metabolism. The course of protein metabolism in the two groups of animals was followed, therefore, day by day until all of the rats in each group had died. Four main groups of animals were studied in this experiment:

(1) Animals fed the low-fat diet in restricted quantities until death(10 rats),

(2) Animals fed the 20 per cent fat diet in restricted quantities until death(10 rats).

(3) Animals fed the low-fat diet <u>ad libitum</u> until death(3 rats), and

(4) Animals fed the 20 per cent fat diet <u>ad libitum</u> until death (3 rats).

In order to provide a basis for initial comparison, the regular five-day metabolism test first was conducted under the conditions of full and restricted feeding. At the completion of this test, the rats were fed the same diet fed in Period II until they died. The quantity of nitrogen excreted in the urine daily by some of the rats in each group was determined. Four rats receiving restricted quantities of each diet and two animals receiving each of the rations *ad libitum* were studied.

Cottonseed oil was selected as the source of fat in this experiment because of the demonstrated superiority of this fat over other single fats in retarding the nitrogen catabolism caused by the ingestion of calorically inadequate diets containing no protein. The heat of combustion of cottonseed oil as determined by bomb calorimeter analysis was 9.3 Calories per gram. With the use of this value the caloric value of the cottonseed oil-containing ration was calculated to be 4.8 Calories per gram in contrast to 3.7 Calories per gram for the low-fat ration. Mean quantities of diets consumed daily were isocaloric (Table 13).

Rate Of Catabolism In Initial Five-Day Test And Length Of Life

It should be noted that animals fed fat again excreted more nitrogen in Period I than animals receiving the low-fat ration. The differences in response induced

Table 13. Catabolism of animals fed protein-free rations, either fat-free or fat-containing, at initiation of long-time metabolic test

Rations fed	No. of rats	Daily energy value of diet		Urinary nitrogen per 5 days		
		Pd. I	Pd. II	Pd. I	Pd. II	Increase in catabolism in Period II
		Cal.	Cal.	mg.	mg.	mg.
When rations are inadequate in energy value						
Low-fat	10	49.6	12.4	161	398	237
20% Cotton-seed oil	10	49.1	12.4	225	308	83
When rations are adequate in energy value						
Low-fat	3	36.6	44.6	170	162	8
20% Cotton-seed oil	3	37.6	43.5	199	199	0

by the feeding of the two types of diets in Period I magnified the difference between the excretions of Period I and Period II for rats receiving the carbohydrate diet. The small increment in excretion occasioned by caloric restriction in animals receiving the fat-containing diet partly reflected the higher nitrogen output of these animals in Period I of the metabolism test. However, a comparison of the Period II excretions (398 mg. per five days, low-fat diet; 308 mg. per five days, high-fat diet) showed that the low excretion of animals fed the low-fat diet during Period I did not account for all of the unfavorable increment in excretion that accompanied the feeding of this diet in restricted quantities.

Animals that continued to receive ad libitum quantities of the two diets maintained a constant level of excretion in the two periods of the test. These data demonstrate clearly that the premise on which these experiments have been based is valid, i.e., the maintenance of a steady state of catabolism during the two metabolic test periods when a diet of adequate energy value is fed.

When the catabolism in Period II was associated with time of survival, interesting information regarding the influence of fat in response to different dietary conditions was obtained (Table 14). Thus animals fed a fat-containing diet withstood caloric restriction for 39 days as compared to 27 days for rats ingesting a low-fat diet. A low excretion of urinary nitrogen in Period II seems to be associated with longer survival. On the other hand, rats allowed to consume the protein-free ration *ad libitum* lived longer when no fat was included in the diet than when fat comprised 20 per cent of the ration. Again survival time apparently could be predicted by the nitrogen excretion. Animals receiving fat-containing diets *ad libitum* excreted more nitrogen during each of the two five-day metabolism tests than animals deprived of fat (Table 13). The favorable influence of fat in low-calorie diets and the unfavorable influence in diets of adequate energy value were further emphasized by the study of the daily nitrogen excretions.

Table 14. Output of nitrogen during Period II and survival times of animals receiving fat-free or fat-containing diets in restricted or unrestricted quantities

Ration fed	No. of rats	Daily energy value of diet fed in survival test	Urinary nitrogen per 5 days prior to initiation of survival test	Survival
		Cal.	mg.	days
Low-fat	10	12.4	398	27
20% Cottonseed oil	10	12.4	308	39
Low-fat	3	44.6	162	174
20% Cottonseed oil	3	43.5	199	121

Day By Day Metabolism Of Rats Fed Calorically Restricted Protein-Free Diets

The mean daily outputs of nitrogen in the urine for four rats ingesting restricted low-fat rations and four rats ingesting restricted high-fat rations are shown in Figure 2. These measurements were made between the tenth day of caloric restriction and the time that death occurred for each animal*. It is immediately obvious that nitrogen catabolism continued at a higher plane over the entire remaining life span in the absence of dietary fat than when fat was present. Although rats in each group excreted increasing quantities of nitrogen as caloric restriction was prolonged, rats ingesting fat showed a more gradual increase in excretion than did those receiving none. They never attained as high a level of excretion as did rats deprived of fat (180 vs. 266 mg. nitrogen per day). The mean peak in urinary nitrogen came on the eighth day for rats receiving restricted low-fat rations. The peak

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*The determination of the amount of nitrogen excreted daily was initiated at the completion of Period II. Day 1 in this series, therefore, actually represents the tenth day of caloric restriction. This fact should be kept in mind in regard to statements that follow.

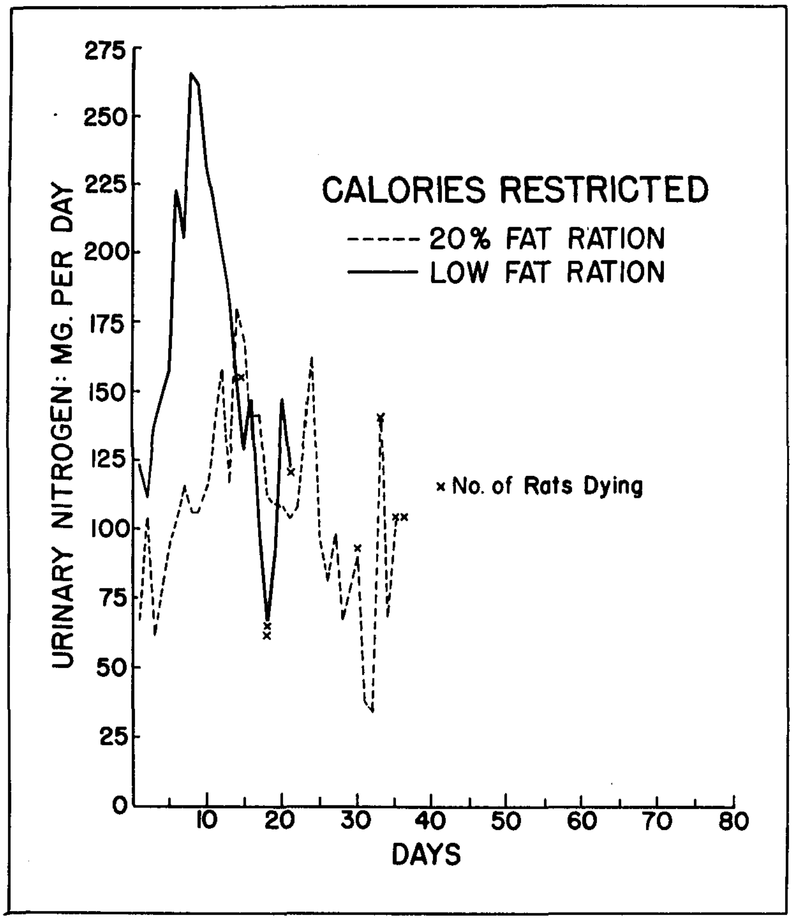

Figure 2. Mean quantities of nitrogen in urine excreted day by day by groups of rats fed different protein-free rations providing one-fourth of the needed calories

excretions for individual rats in this group all came between the sixth and ninth days, the individual excretions ranging from 269 to 296 mg. per day. The highest individual daily excretions in this group were observed eight to fifteen days prior to death. The first death in this group occurred on the 14th day.

For animals ingesting fat, several peaks in excretion occurred. The first and highest came on the 14th day. Again, the rats reached their highest excretions over a very narrow time range, i.e., between the 12th and 15th days of the daily balance studies. The peak excretion of each rat in this group was lower than that in any animals deprived of fat and ranged from 178 to 243 mg. per day. The first animal died on the 30th day of the test.

The trends depicted in Figure 2 are emphasized when the data are pooled and arranged to show excretions during successive five-day intervals. The rearranged data for rats receiving restricted rations are presented in Figure 3. Successive five-day weight losses and nitrogen outputs of individual animals ingesting restricted low-fat and restricted high-fat rations are presented in Tables 15 and 16. Inspection of the

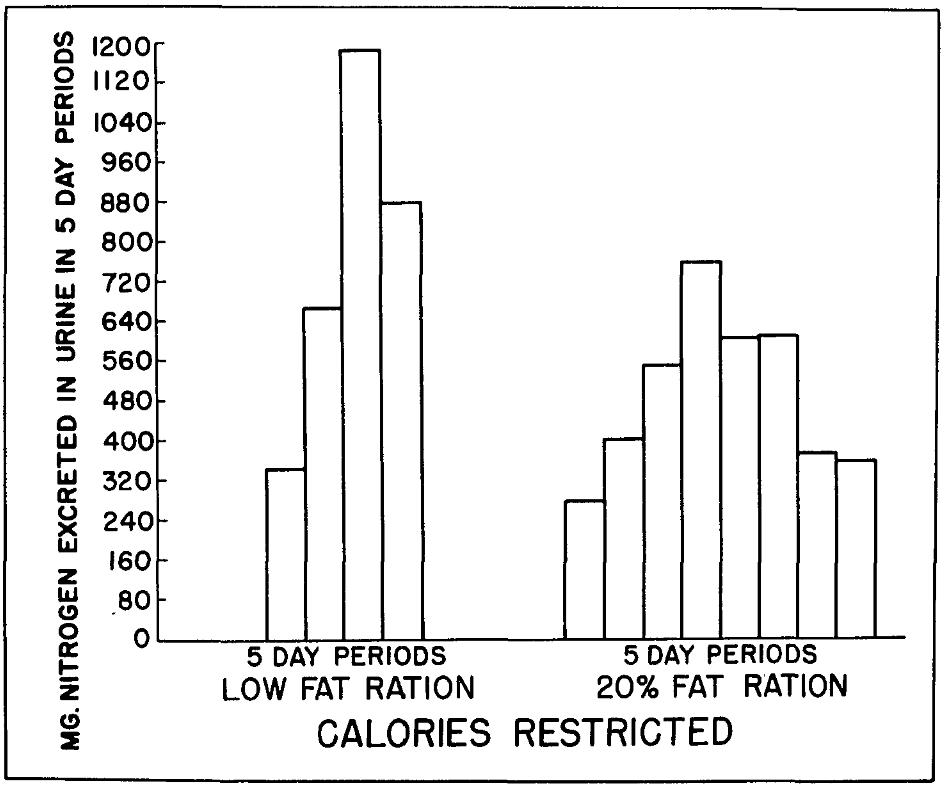

Figure 3. Mean quantities of nitrogen in urine excreted in successive five-day intervals by groups of rats fed different protein-free rations providing one-fourth of the needed calories

Table 15. Nitrogen metabolism and loss in body weight during successive 5-day intervals of individual rats fed low-fat rations in restricted quantities (after 28 days on protein-free, low-fat ration)

Interval of test	1st five days[a]		2nd five days		3rd five days		4th five days		5th five days	
Rat no.	N in urine	Loss in wt.	N in urine	Loss in wt.	N in urine	Loss in wt.	N in urine	Loss in wt.	N in urine	Loss in wt.
	mg.	gm.	mg.	gm.	mg.	gm.	mg.	gm.	mg.	gm.
4	355	14	835	30	1149	38	710	17	---	--
5	330	10	623	31	1076	32	875	28	---	--
6	315	15	505	29	1273	38	945	22	542	14
8	405	16	735	27	1265	35	996	25	---	--
Av.	351	14	674	29	1191	38	881	23	---	--

[a] Period II of metabolism test.

Table 16. Nitrogen metabolism and loss in body weight during successive 5-day intervals of individual rats fed high-fat rations in restricted quantities (after 28 days on protein-free, high-fat ration)

Rat no.	N in urine	Loss in wt.	N in urine	Loss in wt.	N in urine	Loss in wt.	N in urine	Loss in wt.
	mg.	gm.	mg.	gm.	mg.	gm.	mg.	gm.
	1st five days		2nd five days		3rd five days		4th five days	
35	295	14	455	19	755	23	869	31
36	275	14	420	20	540	15	695	24
37	295	13	390	18	470	10	783	31
38	255	15	354	19	445	12	705	18
Av.	280	14	405	19	552	15	763	26
	5th five days		6th five days		7th five days		8th five days	
35	603	16	655	11	340	3		
36	665	18	657	21	366	4	420	19
37	622	16	560	20	453	6		
38	565	18	597	17	350	7	300	9
Av.	614	17	617	17	377	2	360	14

figure and tables indicates clearly that the feeding of a low-calorie, fat-free ration was punctuated by an immediate and severe breakdown of body tissue that resulted in early death. In rats similarly fed fat-containing rations, catabolism, as evidenced by the amount of urinary nitrogen excreted, proceeded at a slower rate and life was prolonged considerably.

Losses in body weight paralleled losses in body nitrogen. The largest losses in weight occurred between the 15th and 19th days of caloric restriction (Interval 3, Table 15) for animals fed fat-free diets, between the 20th and 24th days (Interval 4, Table 16) for animals receiving fat. These intervals corresponded to the periods in which the highest excretion of nitrogen was observed. The greatest individual weight losses in this five-day period ranged from 32 to 38 grams for animals ingesting carbohydrate diets, and from 18 to 31 grams for animals receiving fat.

In both groups of animals the peak excretions were followed by periods of lower output. Animals ingesting fat lived 15 to 20 days after they had attained the peak excretion. In this interval, they showed regular decreases in output of nitrogen and a lessened tendency to lose body weight. Animals deprived of fat exhibited the same tendency but apparently they were unable to decrease the rate of catabolism soon enough, and, hence, the decline in nitrogen output was interrupted by death (approximately 10 days after the peak excretion). An immediate premortal rise in the excretion of nitrogen was not observed in either group.

Day By Day Metabolism Of Rats Fed Protein-Free Diets Adequate In Energy

When fat was included in a protein-free ration fed in <u>ad libitum</u> quantities it no longer exerted a protective influence upon the rate of tissue catabolism. The average daily excretions of nitrogen of two rats receiving fat in the diet and two rats receiving only carbohydrate during the interval corresponding to the tenth day of caloric restriction until death are shown in Figure 4. It was noted previously that rats ingesting fat-containing diets excreted more nitrogen in Period I of the regular metabolic test than animals deprived of fat and that length of life was shorter for rats fed fat than for those ingesting carbohydrate only in unrestricted quantities. Apparently in animals ingesting fat-containing

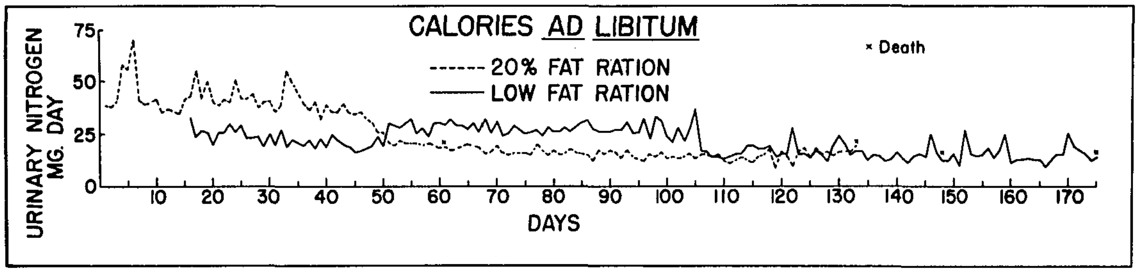

Figure 4. Mean quantities of nitrogen in urine excreted day by day by groups of rats fed different protein-free rations of adequate caloric value

diets *ad libitum*, the high level of nitrogen catabolism characteristic of the metabolism test was maintained for approximately 50 days. During this time animals fed fat-free diets continued to decrease their excretion below that observed during the regular metabolic test intervals conducted before studies of day by day metabolism were initiated.

Tables 17 and 18 present data pooled in five-day intervals and arranged to show individual nitrogen excretions and losses in body weight for animals fed the two rations *ad libitum*. The bar graph (Figure 5) presents average excretions during successive five-day intervals of the day by day metabolism experiment. The data demonstrate that under these experimental conditions the ameliorative influence of fat is lost and that, therefore, rats receiving the high-fat diet cannot do as good a job in extending survival as the rats fed carbohydrate only. In the latter group, catabolism proceeded at a low and fairly uniform plane for approximately 100 days. Then daily excretion of urinary nitrogen dropped by one-half and continued at this point until death. These low excretions probably represent the "wear and tear" quota of nitrogen or the "nitrogen minimum".

Table 17. Nitrogen metabolism and loss in body weight during successive 5-day intervals of individual rats fed low-fat rations *ad libitum* (after 28 days on protein-free, low-fat ration)

Rat no.	N in urine	Loss in wt.	N in urine	Loss in wt.	N in urine	Loss in wt.	N in urine	Loss in wt.	N in urine	Loss in wt.
	mg.	gm.	mg.	gm.	mg.	gm.	mg.	gm.	mg.	gm.
	1st five days[a]		3rd five days		4th five days		5th five days		6th five days	
65	178.3	8	108.3	5	102.7	9	124.2	10	120.6	11
68	-	4	152.9	4	169.5	5	103.6	11	96.4	6
Av.		6	130.7	4.5	136.1	7	114.0	10.5	108.6	8.5
	7th five days		8th five days		9th five days		10th five days		11th five days	
65	112.3	7	107.5	14	102.8	4	155.4	3	145.2	7
68	89.5	5	93.1	8	90.4	4	141.3	3	129.7	8
Av.	101.0	6	100.5	11	97.0	0	148.4	0	137.6	7.5
	12th five days		13th five days		14th five days		15th five days		16th five days	
65	148.9	2	148.5	6	131.1	5	131.8	3	150.3	7
68	142.6	10	138.9	1	127.5	5	131.0	3	126.2	6
Av.	145.4	6	143.7	3.5	129.3	5	131.4	3	138.3	6.5
	17th five days		18th five days		19th five days		20th five days		21st five days	
65	129.5	1	144.7	3	156.3	11	159.1	1	74.0	5
68	141.2	0	128.6	9	132.5	5	128.3	1	70.6	3
Av.	135.5	0.5	136.7	6	144.5	8	143.8	1	72.4	4

	12th five days		13th five days		14th five days		15th five days		16th five days	
65	148.9	2	148.5	6	131.1	5	131.8	3	150.3	7
68	142.6	10	138.9	1	127.5	5	131.0	3	126.2	6
Av.	145.4	6	143.7	3.5	129.3	5	131.4	3	138.3	6.5

	17th five days		18th five days		19th five days		20th five days		21st five days	
65	129.5	1	144.7	3	156.3	11	159.1	1	74.0	5
68	141.2	0	128.6	9	132.5	5	128.3	1	70.6	3
Av.	135.5	0.5	136.7	6	144.5	8	143.8	1	72.4	4

	22nd five days		23rd five days		24th five days		25th five days		26th five days	
65	95.7	6	84.4	3	71.1	5	96.9	8	77.3	2
68	69.6	10	83.1	2	79.9	9	76.3	0	83.6	6
Av.	82.7	8	83.7	0.5	85.5	7	86.7	4	80.6	4

	27th five days		28th five days		29th five days		30th five days		31st five days	
65	68.6	1	64.5	0	-	-	-	-	-	-
68	71.7	1	70.2	0	81.4	5	81.2	5	83.2	5
Av.	70.3	0	67.5	0						

	32nd five days		33rd five days		34th five days	
65	-	-	-	-	-	-
68	60.6	3	76.5	11	77.0	6
Av.						

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[a]Intervals.

Table 18. Nitrogen metabolism and loss in body weight during successive 5-day intervals of individual rats fed high-fat ration *ad libitum* (after 28 days on protein-free, low-fat ration)

Rat no.	N in urine	Loss in wt.	N in urine	Loss in wt.	N in urine	Loss in wt.	N in urine	Loss in wt.	N in urine	Loss in wt.
	mg.	gm.	mg.	gm.	mg.	gm.	mg.	gm.	mg.	gm.
	1st five days[a]		2nd five days		3rd five days		4th five days		5th five days	
22B	222.9	13	233.1	11	229.9	13	182.0	5	232.4	10
16B	210.6	18	-	1	-	8	-	8	-	10
Av.		15.5		6		10.5		6.5		10
	6th five days		7th five days		8th five days		9th five days		10th five days	
22B	217.4	5	209.7	5	222.8	3	189.0	9	179.3	8
16B	-	7	-	4	-	9	-	7	-	2
Av.		6		4.5		6		8		5
	11th five days		12th five days		13th five days		14th five days		15th five days	
22B	152.5	3	104.0	5	-	11	-	-	-	-
16B	89.2	8	82.9	3	84.2	6	81.8	2	76.6	6
Av.		5.5		4		8.5				
	16th five days		17th five days		18th five days		19th five days		20th five days	
22B	-	-	-	-	-	-	-	-	-	-
16B	82.0	6		4	67.1	4	70.9	3	63.9	3
Av.										

	mg.	gm.	mg.	gm.	mg.	gm.	mg.	gm.	mg.	gm.
	1st five days[a]		2nd five days		3rd five days		4th five days		5th five days	
22B	222.9	13	233.1	11	229.9	13	182.0	5	232.4	10
16B	210.6	18	-	1	-	8	-	8	-	10
Av.		15.5		6		10.5		6.5		10
	6th five days		7th five days		8th five days		9th five days		10th five days	
22B	217.4	5	209.7	5	222.8	3	189.0	9	179.3	8
16B	-	7	-	4	-	9	-	7	-	2
Av.		6		4.5		6		8		5
	11th five days		12th five days		13th five days		14th five days		15th five days	
22B	152.5	3	104.0	5	-	11	-	-	-	-
16B	89.2	8	82.9	3	84.2	6	81.8	2	76.6	6
Av.		5.5		4		8.5				
	16th five days		17th five days		18th five days		19th five days		20th five days	
22B	-	-	-	-	-	-	-	-	-	-
16B	82.0	6		4	67.1	4	70.9	3	63.9	3
Av.										
	21st five days		22nd five days		23rd five days		24th five days			
22B	-	-	-	-	-	-	-	-		
16B	59.4	5	60.4	1	80.0	5	79.8	3		
Av.										

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[a] Intervals.

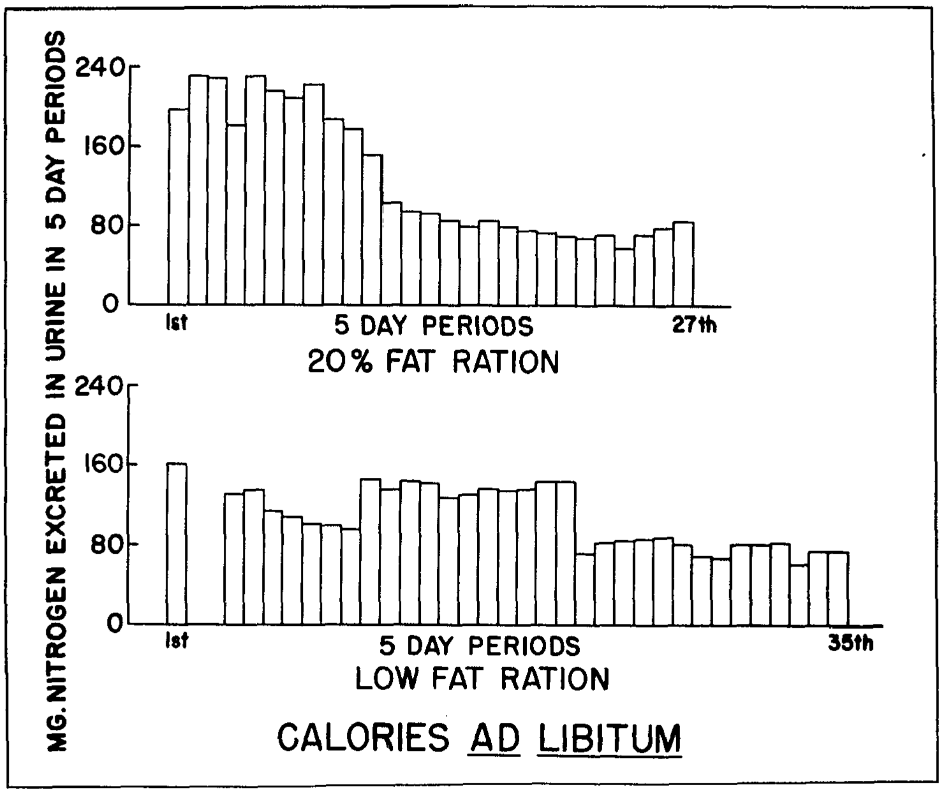

Figure 5. Mean quantities of nitrogen in urine excreted in successive five-day intervals by groups of rats fed different protein-free rations of adequate caloric value

In contrast to the rats fed the low-fat diet, very high and sustained excretions of nitrogen by animals receiving fat were apparent for the first 50 days. Then the excretions drop to about the same level as the excretions of the carbohydrate-fed rats. The augmented excretion in the early part of the period was reflected in a shorter life. During the interval in which nitrogen outputs were large, losses in body weight also exceeded those in animals fed low-fat diets. No premortal rise in nitrogen output was observed. It may be recalled that animals fed in restricted quantities showed no tendency to excrete more nitrogen than usual in the immediate interval prior to death.

In Table 19 the survival times and total weight losses incurred from the beginning of the total experiment until the time that death occurred are presented for individual animals used for the long-time nitrogen study. Length of life when diets free of protein and low in energy were fed was related to the average daily loss in weight incurred. Rats fed the low-fat diet were able to tolerate average losses of 7.2 gm. per day over a 27-day period. On the other hand, mean daily losses in body

Table 19. Total weight losses from beginning of experiment until death and survival times of individual animals fed fat-free or fat-containing diets

Low-fat diet			Fat-containing diet		
Rat no.	Total loss in wt. during experiment	Survival after calories reduced in some groups	Rat no.	Total loss in wt.	Survival after calories reduced in some groups
	gm.	days		gm.	days
When diets are inadequate in energy value					
1	163	20	31	179	28
2	163	14	32	214	39
3	199	30	33	192	32
4	192	24	34	182	30
5	196	27	35	209	42
6	157	31	36	219	46
7	213	32	37	216	39
8	223	29	38	229	46
9	189	17	39	230	46
10	249	46	40	248	46
Av. values	195	27	--	232	39
Av. wt. loss/day	7.2	--	--	6.2	--
When diets are adequate in energy value					
65	232	156	16B	167	156
68	205	183	22B	168	70
11B	224	182	13B	180	137
Av. values	220	173	--	172	121
Av. wt. loss/day	0.8	---	--	1.4	--

weight of 6.2 gm. per day extended life on the average to 39 days in the fat-fed group.

Exactly the reverse is true when the protein-free diets were fed ad libitum. Now mean losses in body weight of 0.8 gm. per day were associated with a life span of 173 days in the carbohydrate-fed group. Rats given the fat diet lost, on the average, 1.4 gm. per day and lived for only 121 days. Thus, it is seen that the dietary conditions employed have exerted a profound influence upon the rate at which body weight was lost.

Time of survival, losses in body nitrogen and losses in body weight may be correlated from data that are presented in Table 20. Rats fed fat lost weight at the rate of 3 gm. per day and body nitrogen at the rate of 97 mg. per day during the interval of the daily metabolism tests. Comparable daily weight and nitrogen losses for animals receiving restricted quantities of low-fat ration were 4.1 gm. and 120 mg., respectively. Thus, fat in the diet retarded daily losses, and as a result rats maintained on this diet lived longer and were able to withstand a greater total output of body nitrogen than rats fed the carbohydrate diet inadequate in energy.

Table 20. Cumulated weight losses, cumulated losses in body nitrogen and survival times of rats fed various diets from time that specific variations in caloric feeding were initiated until death

Diet	Rat no.	Survival on exp't'l. regime	Loss of body nitrogen	Loss of body weight
Restricted low fat	4	24	3049	99
	5	27	2904	105
	6	31	3580	125
	8	29	3401	121
	Av. values	27	3244	112
	Av. loss body N/day	--	120	---
	Av. loss body wt./day	--	----	4.1
Restricted high fat	35	42	3972	100
	36	46	4038	128
	37	39	3573	114
	38	46	3571	120
	Av. values	39	3788	115
	Av. loss body N/day	--	97	---
	Av. loss body wt./day	--	----	3.0
<u>Ad libitum</u> low fat	65	156	3336	146
	68	183	3678	156
	Av. values	173	3507	151
	Av. loss body N/day	--	20	---
	Av. loss body wt./day	--	----	0.88
<u>Ad libitum</u> high fat	16B	156	3000[a]	131
	22B	70	2459	101
	Av. values	121	2729	116
	Av. loss body N-day	--	22	---
	Av. loss body wt./day	--	----	0.96

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[a] Values for first 10 5-day periods estimated on basis of performance of other rats.

Rats allowed to eat low-fat rations <u>ad libitum</u> lived longer (173 <u>vs.</u> 121 days) and excreted a greater quantity of nitrogen (3507 <u>vs.</u> 2729 mg. N) than animals consuming high-fat rations <u>ad libitum</u>. Animals fed fat now lost 2 mg. more nitrogen daily and 0.1 gm. more body weight daily than animals deprived of fat. The protective value of the energy components of the diet is clearly illustrated in this table.

SUMMARY

Four questions have been raised in the present investigation regarding the influence of non-protein calories on the rate of catabolism characteristic of rats depleted of bodily reserves of protein and maintained on nitrogen-low rations of varying caloric value. Answers to these questions were sought within a specific experimental framework.

A protein-free ration providing energy from either carbohydrate alone or from a mixture of carbohydrate and fat was fed to adult male rats. The quantity of nitrogen excreted in the urine under the various experimental regimes imposed was used to measure rate of body catabolism. The respective rations were fed *ad libitum* for 18 days after which the nitrogen metabolism of the animals was measured for a five-day interval. The energy content of the diet then was reduced to approximately one-fourth of its original value, and after a four-day adjustment period another five-day metabolism test was conducted. Animals in a control group received the same rations in amounts that met their energy needs in the second metabolic interval. In one experiment, the rations

administered in the metabolism test were fed until death intervened and urinary nitrogen was determined daily.

The four questions raised and the experimental work designed to answer the questions are presented below.

Are all fats equally effective in preventing excess catabolism of body protein induced by caloric restriction of protein-free diets? The effect of butterfat, lard, margarine fat, hydrogenated vegetable fat, and cottonseed oil, as well as of certain fat mixtures, was determined. Reactions of animals to the feeding of diets containing the different sources of fat at two caloric levels were compared to those of animals ingesting a low-fat diet. Fats apparently vary appreciably in their ability to spare body protein under the stress of caloric restriction. Of the single fats tested cottonseed oil was the most effective; butterfat, the least effective. Margarine fat ranked second, while hydrogenated vegetable fat and lard were intermediate in effect. The lard-butterfat and the lard-margarine fat combinations were the most effective fat mixtures, while the mixtures containing cottonseed oil were the least effective. The margarine fat-butterfat mixture and the two mixtures containing hydrogenated vegetable fat were of intermediate value. Force-feeding of the test rations was associated with smaller increments in excretion of nitrogen than was feeding from a food cup. Effects produced by force-feeding some of the diets described were of the same order as produced by cup-feeding.

What properties of cottonseed oil are responsible for its protein-sparing effect? It was thought that the superiority of diets containing cottonseed oil in reducing catabolism caused by caloric restriction might reflect the high degree of unsaturation of the oil or its content of essential fatty acids. If the unsaturated fatty acids exert a protective influence, the feeding of a completely saturated fat should induce a negative response. A saturated preparation of coconut oil did prove ineffective when fed as the source of dietary fat, and in the next test, therefore, the low-fat ration was supplemented with a pure preparation of methyl linoleate. No decrease in the excretion of urinary nitrogen occurred. Thus, the favorable influence of cottonseed oil cannot be attributed to linoleic acid. Other unsaturated fatty acids present in cottonseed oil may be active, however. Or, its potency may be associated with the non-saponifiable fraction of the fat molecule.

Does body fat act in the same manner as dietary fat in preventing excess catabolism of tissue protein in response to caloric restriction? Animals were starved until they reached a weight of 325 gm. after which they were subjected to the regular experimental metabolic regime. Individual rats in this test exhibited very uniform performance, especially in response to caloric restriction, suggesting that body reserves of fat may play a part in the body-sparing phenomenon.

What is the long-time effect upon the nitrogen metabolism of protein-depleted rats of feeding nitrogen-low rations of adequate and inadequate caloric value? Whether or not the apparent protein-sparing effect of cottonseed oil in diets of low energy value was a permanent or a transient phenomenon was studied as well as the extent to which the rate of nitrogen catabolism affected the time of survival of animals receiving the various test rations. Animals were fed in full and restricted quantities either the basal low-fat ration or the ration containing 20 per cent of cottonseed oil until death occurred. Nitrogen excreted in the urine by the rats was determined daily. As the period of caloric restriction lengthened, animals receiving low-fat, low-calorie diets excreted increasingly large quantities of urinary nitrogen daily over an interval of about 20 days. Then excretion of the constituent dropped from 240 to 180 mg. per day. The animals survived caloric restriction for an average of 27 days. Animals receiving fat, in comparison, metabolized protein at a slower rate and life was prolonged considerably, i.e., 39 days after the initiation of caloric restriction. At no time did these rats excrete more than 150 mg. of nitrogen per day. They exhibited a markedly low catabolism in the latter days of life (70 mg. of urinary nitrogen per day).

Dietary fat behaved in a different manner when the same diets were fed in amounts that met the energy requirement of the rats. Rats receiving the fat diet in unrestricted amounts maintained the high excretion of nitrogen observed in the first period of the regular metabolism test for an average of 50 days when feeding the diet was continued. Then, in the remaining days of life, they decreased their excretion of nitrogen by approximately one-half. On the other hand, the rats receiving the carbohydrate ration of full caloric value excreted considerably less nitrogen in the urine than did the fat-fed group in the first intervals of the experiment. As a result they survived on the average 174 days instead of 121 days.

CONCLUSIONS

Unequivocal substantiation has been obtained of data previously reported from this laboratory that under specific experimental conditions dietary fat plays a role in the maintenance of nutrition over and beyond the provision of energy and essential fatty acids. It so acts only when a ration low in nitrogen is fed to protein-depleted rats in quantities inadequate to meet energy needs. Under these circumstances a ration containing fat reduces the rate at which body tissue is catabolized, so that ability to survive under the stress conditions imposed is extended. On the other hand, fat in a protein-free diet of adequate energy value is associated with a shortened life span and an increased rate of catabolism.

Dietary fats vary in efficacy in decreasing the rate of nitrogen catabolism in animals in response to caloric restriction of protein-free diets. Of the fats tested, cottonseed oil consistently has retarded breakdown of body tissue to a greater extent than any other fat.

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APPENDIX

Table A. Concentration of nitrogen in 15 ml. of a standard creatinine solution (15.01 mg. per cent N)

0.1 N HCl neutralized	Nitrogen in 25 ml. aliquot	Theoretical quantity of nitrogen present	Recovery of nitrogen
ml.	mg.	mg.	per cent
10.57	14.80	15.01	98.60
10.65	14.91		99.33
10.55	14.77		98.40
10.59	14.83		98.80
10.74	15.04		100.20
10.62	14.87		99.07
10.66	14.92		99.40
		Average	99.11

Table B. Recovery of nitrogen from a standard solution of ammonium sulfate distributed at intervals over metabolism cages

Cage no.	Quantity of nitrogen theoretically present	Quantity of nitrogen recovered	Recovery of nitrogen
	mg.	mg.	per cent
1	401.15	399.74	99.65
		400.27	99.78
		397.66	99.13
2	401.15	401.18	100.00
		398.54	99.35
		399.42	99.57
3	401.15	400.79	99.91
		396.18	98.76
		397.58	99.11
4	401.15	400.55	99.85
		398.42	99.32
		399.74	99.65