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(1875–1955)

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(October 16, 1875–October 7, 1955)

Chemist, nutritionist, teacher, humanitarian

Henry Clapp Sherman was born on a farm near Ash Grove, Virginia on October 16, 1875, the son of Franklin and Caroline Alvord Sherman, and died October 7, 1955. He thus lived just 9 days short of 80 years. During the span of his scientific career most of modern nutritional science as we know it developed. The concept of protein quality and the essentiality of certain amino acids was firmly established, and man's requirement for protein measured. The vitamins were discovered, their biological and chemical functions elucidated, their chemical structure determined, and their syntheses accomplished. The bases of our present knowledge concerning the functions and quantitative requirements for mineral elements were established. Important enzymes of digestion, food utilization, and respiration were studied and many enzymes were purified and crystalized. Henry Sherman made important contributions to all of these aspects of nutrition research. His range of interest was probably broader than that of any other authority of his day, yet his work was never careless or superficial. He came to look upon nutrition, not negatively as simply the prevention of deficiency diseases, but positively as a means of improving the health of the individual and the enrichment of the life of a nation. Starting as an analytical chemist he progressed successively to nutritionist, experimental biologist, and thence to great humanitarian.

In 1903 he married Cora Aldrich Bowen. Their three living children have all distinguished themselves in their respective professions. The older son, Henry Alvord Sherman, was graduated from the engineering school of Columbia University and is now a chemical engineer. William Bowen Sherman is

a distinguished physician of New York City and is currently Associate Clinical Professor of Medicine at the College of Physicians and Surgeons of Columbia University. Professor Sherman's daughter, Caroline — now Mrs. Oscar Lanford, is an authority in nutrition in her own right. After receiving the Doctor of Philosophy degree from Yale University she collaborated with her father in a number of studies dealing with calcium retention. She is co-author with him of two of his later books.

It is rare for a man to have had continuous association with one institution for 60 years, but that was true for Henry C. Sherman and Columbia University. Following his graduation at the age of 18 from Maryland Agricultural College (now University of Maryland) in 1893 with the Bachelor of Science degree, he was an assistant in chemistry at that school until 1895. From 1895 until his death in 1955 he was either a student or a faculty member at Columbia University. He was a fellow in chemistry at Columbia from 1895 until 1897, receiving the Doctor of Philosophy degree in that year. From 1897 until 1899 he was associated with Dr. W. O. Atwater in nutrition investigations at Wesleyan University. Returning to Columbia in 1899 he was successively a Lecturer, Instructor, Adjunct Professor, Professor, and finally Mitchell Professor of Chemistry. For 20 years, from 1919 to 1939, he was Executive Officer of the Department. At the time of his death he was Emeritus Mitchell Professor of Chemistry.

For several short periods of time he was granted leaves of absence from his duties at Columbia. He served as a member of a Red Cross team to Russia in 1917, and from 1943 to 1944 he was Chief of the Bureau of Human Nutrition and Home Economics of the U. S. Department of Agriculture.

He was the recipient of a number of medals, honors, and honorary degrees. These include honorary doctoral degrees from Maryland and Columbia, membership in the National Academy of Sciences, and the presidency of the American Society of Biological Chemists and of the American Institute

of Nutrition. He was awarded medals by the Franklin Institute, the American Institute of Nutrition, the American Chemical Society, and the American Institute of Chemists. He was the author of 10 books, several of which went through a number of editions.

His early training in analytical chemistry left a profound influence upon his approach to any research problem, whether it involved the determination of calcium or the bioassay for vitamin A. He was one of the first — if not the first — to use statistical methods to evaluate data on animal growth. His "Chemistry of Food and Nutrition," through many of its revisions, carried an Appendix containing the "Student" method for computing "standard deviation" and "probable error," this latter being approximately two-thirds of the now more commonly used "standard error." These statistical values have in recent years been to a large extent replaced by the use of the "t" test which is felt by some to be more valuable as a measure of reliability of data. In any event, Sherman must be regarded as a pioneer in the use of statistical methods in evaluating biological data. His doctoral students were expected to use such methods in reporting their results.

His laboratory was largely responsible for placing the bioassay for many of the vitamins on a quantitative basis. Actually these quantitative methods laid the groundwork for later successes of a more spectacular nature by others in identifying and synthesizing the vitamins as pure compounds. No one else had the patience, humility, and insight to do this fundamental work.

He was never statisfied with a single experiment. He would repeat the experiment over and over, until he was completely satisfied that the results were valid. Thus he never found it necessary to publish a retraction.

As a result of his venture into the realm of the essential amino acids, Sherman (with Alice T. Merrill) believed that cystine was an essential amino acid for the rat. Using whole milk as the sole source of protein, "diluting" it with starch, they found that ". . . cystine is the first limiting amino acid

of the protein of cow's milk for the growth of young rats'' (1925). This was of course before the importance of methionine as an essential amino acid was recognized. In view of our present knowledge, and in complete agreement with the findings of Rose, cystine promoted growth by reason of its ability to ''spare'' methionine. Using the rat-growth response, Sherman and Woods developed a bioassay technic for cystine.

Henry Sherman sometimes appeared to be a lonely man, professionally. When he attended scientific meetings, and that seemed to be as infrequently as possible, he would not be found in a boisterous group of men swinging along the Atlantic City boardwalk or sitting late in bars. More likely he would be seen walking hastily through a hotel lobby with serious intent showing on his face. And a day or two before the end of the meeting he would leave inconspicuously carrying his well-worn valise, back to unfinished work at his office and laboratory. His deep religious faith together with a sympathy for people as individuals combined to provide the motivation for his studies of human nutrition. Rather early in his career he was a member of the Red Cross team which studied the post-revolution food situation in Russia. Even earlier he had studied the economics of food habits of families in New York under supervision of the Association for Improving the Condition of the Poor. He recognized that better nutrition knowledge, education, and practice could do much to eliminate malnutrition and alleviate the effects of poverty. With these as his goals he found no time for petty arguments over such matters as credit for priority of publication or for disputes over minor differences of interpretation of scientific data. He was self-effacing because he believed his *task* transcended *self*. His frequent periods of isolation from people did not indicate indifference to human companionship, but rather a willingness to sacrifice temporary pleasure for the more important serious study and writing.

Although Professor Sherman is most widely known for his research and teaching in nutrition, his contributions to en-

zymology were numerous and significant.[1] Between 1911 and 1934 he and his collaborators published nearly 50 papers in this field, most of them dealing with the properties and purification of amylases. Professor Mary L. Caldwell was associated with these studies over most of that time and was his right-hand "man" for many years. The brilliance of their work was temporarily outshone by that of others who first succeeded in crystallizing enzymes. After the crystallization of trypsin by Northrop and Kunitz, Caldwell, Booher and Sherman reported the crystallization of pancreatic amylase. Reviewers sometimes overlook the importance of the studies by Sherman, Caldwell and their students in clearly showing the protein nature of enzymes. He pointed out the errors in Willstätter's claim that certain enzyme preparations were protein-free—that the test for an enzyme is much more sensitive than the color test for a protein. Thus, as in so many other scientific areas, Sherman made the basic discoveries and developed the technics which other groups extended and exploited.

We now know that most of the water-soluble vitamins are constituents of specific enzyme systems. The association is so common that the word "enzyme" brings to mind the word "vitamin," and *vice versa*. It is ironic that Henry Sherman, who did outstanding work in *both* enzymes and vitamins, failed to bring the two together in his own research. Had he chosen to study an oxidative rather than a hydrolytic enzyme, it is just possible that he would have made many of the fundamental discoveries of the respiratory enzyme systems.

Doctor Sherman's interest in nutrition encompassed all aspects of the science — energy metabolism, food composition, inorganic elements, protein quality and requirement, vitamins, and the practical problems of food selection and distribution. He had no pet hobbies to the exclusion of others, but if there

[1] A complete list of his publications together with reprints of the more significant of his papers in nutrition are contained in a volume "Selected Works of Henry Clapp Sherman," The Macmillan Company, 1948, New York. The Preface contains a list of his many honors and awards. A few copies of this book are still available.

was one nutritional essential to which he gave the most attention it was undoubtedly calcium. In the 10-year period from 1934 to 1944 he published 16 papers on the various facets of calcium utilization and requirement. It was therefore inevitable that he should become interested in the availability of calcium from oxalate-containing foods. Experiments published in 1922 (with Edith Hawley) indicated that vegetable calcium was less readily utilized than milk calcium. In 1935 he and Fincke published experiments designed to determine accurately the effect of oxalate on calcium absorption. They found that the calcium from spinach was poorly utilized and that spinach rendered the calcium of concurrently-fed milk partially unavailable. In contrast, the calcium of kale (which is oxalate-free) was almost as well utilized as that of milk. In his book "The Science of Nutrition" (1934) he states: "...but science *does not* specifically seek the sanctification of spinach! In fact spinach is now known to be an unfortunate choice among the green-leaf vegetables because it contains a relatively large amount of oxalic acid, which is not a desirable substance for human consumption in any case, and which renders practically unavailable and useless the calcium which spinach, chard and other leaves of the Goosefoot family contain." It is indeed unfortunate that the presumed nutritional value of spinach has become so firmly imbedded in American folk-lore that the scientific facts have not yet caught up with comic strip fiction!

Although Sherman (with Harriet Edgeworth) once ventured into the microbiological assay field, with a study of the yeast-growth method for thiamine and comparing it with the rat-growth response, he did not further pursue this line of investigation. One can only speculate on the reasons for his lack of interest in a method which later proved to be so useful and time-saving in the hands of other people. Bacteria and yeasts were foreign to his knowledge, experience, and interest. And at the time he was 48 years of age — possibly too old to undertake a completely new investigative approach. Furthermore, he was never one to seek an easy way of doing things!

The most significant of Sherman's nutrition studies were long-term breeding experiments designed to compare two (or more) diets differing quantitatively with regard to one or more food constituents. The longest of these experiments, performed with the able assistance of Dr. H. Louise Campbell, extended over two decades and more than 44 generations of rats, and is in fact being continued at the present time. It was courageously planned, meticulously executed, the data critically evaluated, and the results boldly applied to human nutrition. Comparable groups of litter-mate rats were given two diets which differed only in the relative amounts of whole milk powder and ground whole wheat. Diet A (16) contained one-sixth milk powder and five-sixths wheat. Diet B (13) contained one-third milk powder and two-thirds wheat. Salt was added to each as 2% of the weight of the wheat. The animals received these diets and distilled water ad libitum. Both diets were adequate in that they supported growth and reproduction. However, rats receiving the better diet (B) grew faster, attained somewhat larger size at all ages, reached sexual maturity earlier, were more successful in rearing young, and lived longer. The reproductive success of rats receiving the better diet B was characterized by more numerous young per litter and a greater number of young reaching weanling age. The duration of the reproductive span was longer on this diet, and food utilization was better. To quote from the author:

"Diet A, then is adequate in the usual meaning of the word, but is not optimal; Diet B is better and probably capable of still further improvement. We have here a nutritional improvement upon a dietary which was already adequate for the support of normal nutrition. In the averages of sufficiently large numbers of cases the evidences of nutritional improvement of an already adequate diet (1) expedited growth and development, (2) resulted in a higher level of adult vitality as shown by several criteria, and (3) extended the average length of adult life, or improved the life expectation of the adult.

"Special interest has attached to the influence of improvement of food supply upon the adult life-expectation. This is partly because in the great advance made during the past two or three generations in the life-expectation at birth there had been practically no advance in the life-expectation of the adult. The diminution of death rates had been practically confined to the early ages. And, moreover, studies on longevity had succeeded only in correlating it with heredity. Hence the present experimental correlation of length of adult life with an improvement in an already adequate food supply was a finding unexpected to those to whom we had previously owed our chief knowledge in this field; and it is optimistic and constructive where the previous view had been pessimistic and fatalistic."

"The increase in average length of adult life here found would correspond to an extension of the longstanding human-adult life expectation of 70 years to 77 years instead Yet inasmuch as previous improvements in the average length of life have (or had) been so closely confined to the lowering of *early* death rates as to leave the average length of *adult* life unchanged, the possibility of extending this adult average by a better use of food is of interest from several points of view." *(Chemistry of Food and Nutrition,* seventh edition, pp. 529–532).

No doubt his informal associations with Mary Swartz Rose of Teachers College, Columbia University had more influence upon American life than either of them realized. As Professor of Nutrition, Doctor Rose was more concerned with practical applications of nutrition knowledge. Her "Feeding the Family" was long a classic among home economics textbooks. Although her laboratories were only a stone's throw across 120th Street beyond the Columbia "green," they did not collaborate in a single published piece of research. Yet there is no doubt that each influenced the thinking and teaching of the other. Contrary to the mathematical principle, the whole of their influence was greater than the sum of its parts.

Professor Sherman's research, teaching, and writing have had great and continuing influence upon the food habits of the American people and the people of the entire world. Although his nutrition research was as fundamental as such work can be, he nevertheless attempted to make practical applications of his results. His influence upon food practices was largely indirect; few of the "average man" personally heard him speak. But his graduate students often went back to university laboratories where they taught potential high school teachers such of his precepts as "a quart of milk a day for every person." And the high school teachers in turn carried the gospel of good nutrition to every city and town in the land. His books, also, have had an immeasurable impact upon American food habits. Never flamboyant, his books were written in the impersonal style of the scientist, yet they were understandable to the layman and his words carried conviction. His fluency has the studied precision of the reflective thinker.

One of his distinguished students, Edward C. Kendall, said of him in 1954: His "... scientific papers do not reflect his genial capacity for friendship, his deep understanding of human nature, his lack of malice and intrigue, his sense of humor, his modesty and generosity; in short, his kindly spirit which endeared him to his students and associates."

Professor Sherman was retiring in disposition and seemingly timid in interpersonal relations. Although quick of wit he did not engage in breezy repartee; neither did he ever raise his voice in argument or anger. In contemporary jargon, he was an introvert. Had he been given a modern battery of intelligence tests as a child he possibly would not have scored in the genius class. But he had a genius — for long hours and long years of hard work, a dogged determination, and an unfailing faith in the importance of the work he was doing. He was dedicated to a Cause — *the nutritional improvement of life*. Henry Clapp Sherman was one of the world's great crusaders.

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PANTOTHENIC ACID REQUIREMENT OF THE GROWING AND ADULT RAT [1]

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INTRODUCTION

The pantothenic acid requirement of the rat is reported to decrease with age. Weanling rats consuming 5.3 gm food daily required 80 to 100 μg of pantothenic acid for optimal growth as compared with a food consumption of 15.5 gm and a daily requirement of about 25 μg of pantothenic acid in 10-week old rats (Unna and Richards, '42). Other investigators noticed that, in rats maintained on suboptimal amounts of pantothenic acid, the deficiency symptoms tend to disappear with progressing age (Henderson et al., '42). However, mature rats, put on a diet totally deficient in pantothenic acid, stop growing in about one month and die later showing typical signs of deficiency (Miller and Baumann, '44). In view of the multiplicity and importance of metabolic functions in which the metabolically active form of pantothenic acid, coenzyme A takes part (Novelli, '55), this would be expected.

On the other hand, the diets used by some of the above investigators were lacking in several factors, which, according to some reports, can influence the utilization of pantothenic acid. Folic acid and biotin are thought to be involved in enzyme systems to which pantothenic acid is related (Wright and Welch, '44). The biotin deficiency, produced by feeding succinylsulfathiazole, was reportedly influenced by the panto-

[1] This study was supported by a grant in aid from the Nutrition Foundation, Inc.

thenic acid content of the diet (Emerson and Wurtz, '44), and *p*-aminobenzoic acid was also implicated as necessary for certain biological functions (Ansbacher, '44). The possible relationship between these factors and pantothenic acid and the tentative mechanism of their action were reviewed by Frost ('48), and by Ralli and Dumm ('53).

In an attempt to learn more about the pantothenic acid requirement of the rat during the whole span of life, long term experiments which involved the feeding of diets fortified with the factors thought to be of influence in the utilization of pantothenic acid, were carried out.

EXPERIMENTAL

Male weanling rats of the Sprague-Dawley strain with initial weights of about 50 gm were used in the experiment. The animals were divided into 6 groups, each consisting of 9 animals. The rats were housed separately in metal, screen-bottom cages in an air-conditioned room maintained at 70°F. and 45% relative humidity. Food and water were supplied ad libitum, and the animals were weighed twice weekly. The composition of the basal diet in percent was as follows: vitamin-free casein, 25[2]; salts IV,[3] 4; corn oil, 5; choline chloride, 0.2; cystine, 0.2 and sucrose 65.6. Vitamins were supplied in milligrams per 100 gm of diet as follows: thiamine, 1.0; riboflavin, 1.0; pyridoxine, 1.0; nicotinic acid, 10.0; *l*-inositol, 20; *p*-aminobenzoic acid, 20.0; folic acid, 0.1; biotin, 0.1; menadione, 1.0; and α-tocopherol acetate, 10.0. Vitamins A and D were supplied in the form of halibut oil and Drisdol.[4] The following concentrations of calcium pantothenate in milligrams per 100 gm of diet were used: 0.0, 0.2, 0.4, 0.8, 1.0, and 10.0. When each group of animals had been on its particular diet for about one year, an acetylation study was made using the method described by Riggs and Hegsted ('48). The rats were injected with 3 mg of sulfanilamide intraperitoneally.

[2] Labco, Borden Company.
[3] Hegsted et al., '41.
[4] Winthrop-Stearns, Inc.

The urine for the following 24 hours was collected and analyzed for total and free sulfanilamide by the method of Bratton and Marshall ('39). After about two years, the animals started to show signs of senile debility, loss of appetite and decrease in weight. It was decided, therefore, to terminate the experiment after 680 days. The animals which died during

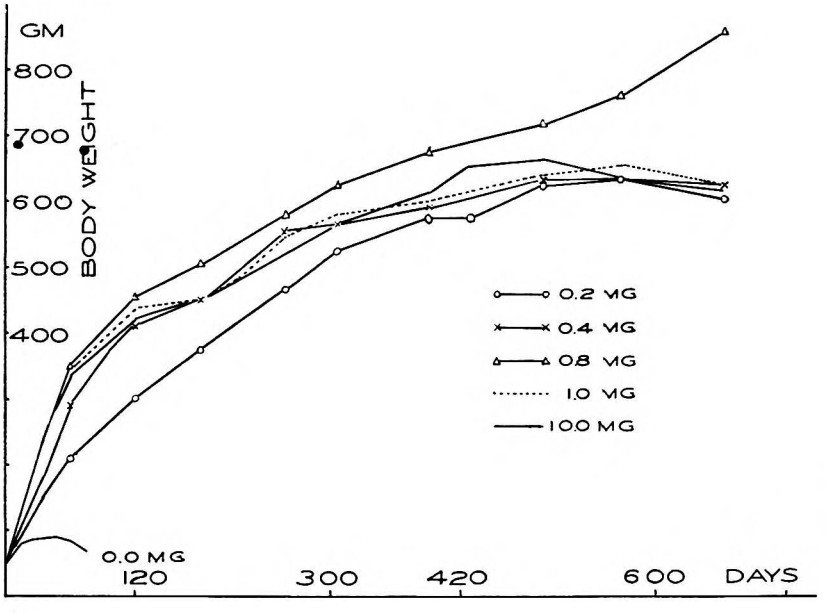

Fig. 1 Growth curves of rats fed various concentrations of calcium pantothenate. Concentrations in milligrams per 100 gm of diet.

the experiment as well as those sacrificed at the end were autopsied and the adrenals, kidneys, liver and testes preserved in buffered 10% formalin for subsequent histological study.

RESULTS

The data on weight gain and acetylation are summarized in table 1; to illustrate better the group differences during the initial period of rapid growth and the subsequent stage of slower growth, the average growth curves are represented in figure 1.

The animals on the diet without any pantothenic acid supplement ceased to grow after 14 to 20 days. Later, external signs of deficiency, such as porphyrin whiskers, rough, reddish fur and bald patches appeared in most of these animals. After 4 weeks, they started to succumb and all animals in this group were dead before the 75th day of the experiment. Most of these rats, upon autopsy, were found to have an atrophied thymus, little or no fat deposition around the kidneys and adrenals, and the intestines were distended with gas. The adrenal glands were dark brown in color: a few of them were enlarged and hemorrhagic. In 4 animals hemorrhagic lungs were observed. Histological examination of the adrenals revealed capillary congestion of the zona reticularis and very low fat content in the zona fasciculata. With one exception, the zona glomerulosa seemed to be unaffected. Most of the glands had some accessory cortical tissue.

In the groups that received supplements of calcium pantothenate, the animals receiving 0.2 mg per 100 gm of diet grew first at a significantly lower rate than those in the other groups. During the first 162 days of the experiment this group gained an average of 310 gm per animal as compared with 403 gm for the group receiving 0.4 mg calcium pantothenate per 100 gm diet and 457 gm for the animals getting 0.8 mg per 100 gm diet. After about 10 months of the experiment, the animals on the higher levels of pantothenic acid supplementation exhibited a decrease in their growth rate with the result that the group on the lowest pantothenic acid level was able to catch up with them. The animals receiving 0.8 mg of calcium pantothenate per 100 gm of diet showed slightly higher weight gains over almost the whole span of the experiment. However, the difference was not statistically significant ($P = 0.1$, Snedecor, '46). No gross external deficiency signs were observed in animals receiving the pantothenic acid supplements; there were no effects on the life span of the administration of the vitamin in the different amounts used here: 6 animals out of 9 died in each group during the first two years of the experiment, in most cases

toward the end of this period. At autopsy, the vital organs did not show any appreciable pathological changes which could be attributed to pantothenic acid deficiency. Histological examination of different tissues revealed slight capillary congestion of the adrenal zona reticularis in a few animals without any relation to the diet fed. A few cases of liver congestion, low liver fat and swollen kidney tubules were also observed, but again there was no correlation with the diet fed. In most cases, the testes of the animals on the two lowest levels of pantothenic

TABLE 1

Weight gain of rats on experimental diets and percentage of sulphonamide acetylated

CALCIUM PANTOTHENATE PER 100 GM DIET	NUMBER OF ANIMALS	INITIAL AVERAGE WEIGHT	WEIGHT GAIN [1]		FINAL AVERAGE WEIGHT [2]	SULPHONAMIDE ACETYLATED [1,3]
			on 162nd day	on 425th day		
mg		*gm*	*gm*	*gm*	*gm*	*%*
0.0	9	47.7				
0.2	9	48.0	310.6 ± 24.8	525.1 ± 43.5	606	33.6 ± 5.4
0.4	9	49.7	403.2 ± 15.5	588.4 ± 44.8	624	41.4 ± 3.3
0.8	9	48.4	457.3 ± 17.2	639.8 ± 49.3	858	76.2 ± 2.0
1.0	9	46.5	416.8 ± 18.6	576.8 ± 33.3	630	70.9 ± 3.0
10.0	9	46.8	416.4 ± 13.5	606.9 ± 36.4	618	67.0 ± 1.5

[1] Average and its standard error.
[2] Average of three animals.
[3] Average of 5 animals.

acid evidenced a moderate to severe depletion of connective tissue and a lower number of sperm. A similar condition was observed, however, in a few animals on higher vitamin levels. Because of the advanced age of the animals at autopsy, it is rather difficult to decide whether the pathological changes observed in some animals were definitely caused by the diet or whether they were to be interpreted as signs of senile debility. On the basis of our other experiments, we favor the view that the changes observed in the testes were, at least partially, the result of an insufficient supply of pantothenic acid.

The acetylation study was carried out on 5 animals in each group after they had been on their respective diets for about one year. The results are summarized in table 1. The animals on the lowest vitamin level showed the lowest acetylation value, 33.6%. The animals receiving 0.8 mg of calcium pantothenate per 100 gm of diet had the highest acetylation percentage, 76.1%. The difference is very great and also proves to be statistically significant.

DISCUSSION

Brown and Sturtevant ('49) reviewed the literature concerning the pantothenic acid requirement of growing rats and came to the conclusion that the optimal concentration lies between 0.8 to 1.0 mg per 100 gm of diet. This figure agrees very well with our findings. A lower level of vitamin supply tended to slow down the growth, while higher vitamin concentration did not result in greater weight gains.

In mature, slow growing animals, the weight does not seem to be a sensitive enough criterion for the measurement of vitamin requirement, certainly for pantothenic acid. Riggs and Hegsted ('48) and Shils et al. ('49) noticed a decrease in acetylation of sulfonamides in growing pantothenic acid-deficient rats. Zucker et al. ('55) confirmed this finding and showed that it is valid also for mature animals. In our experiments the acetylation of sulfonamide was used as a measure for the degree of pantothenic acid deficiency. A decreased acetylation was observed on lower pantothenic acid levels without any concomitant external signs of deficiency. These results seem to indicate that the lower pantothenic acid requirement of adult rats as observed by Unna and Richards ('42) relates only to the growth requirement. For normal metabolic functions, as represented by acetylating ability, adult rats probably require higher pantothenic acid concentrations. The optimum level seems to be the same as for young, growing animals, namely 0.8 mg of calcium pantothenate per 100 gm of diet. Emerson and Evans ('41) in an experiment concerning the graying of hair in "filtrate factor"-

deficient rats also noticed that the requirement of pantothenic acid for growth was less than for prevention of graying.

The amount of pantothenic acid necessary to prevent external signs of deficiency seems to be quite low in adult rats. Miller and Baumann ('44) noticed the development of deficiency signs in adult rats kept on pantothenic acid-deficient diets for 4 months. In our experiment 0.2 mg of calcium pantothenate per 100 gm of diet was sufficient to prevent their appearance. It is felt that, in adult rats, a criterion other than growth rate should be used to measure the degree of pantothenic acid deficiency.

During the course of these experiments, reports appeared about the possible influence of vitamin B_{12} deficiency on coenzyme A metabolism (Boxer et al., '55; Wong and Schweigert, '56). Two to three times higher levels of coenzyme A were found in the livers and kidneys of vitamin B_{12}-deficient rats than in the controls. This finding could indicate some metabolic relationship between pantothenic acid and vitamin B_{12}. However, the relatively high casein content, 25% employed in the present study is believed adequate to prevent any vitamin B_{12} deficiency. Certainly the control animals that received pantothenic acid grew at a rate considered to be normal.

SUMMARY AND CONCLUSIONS

Six groups of male albino rats, each group consisting of 9 animals, were maintained on diets differing only in pantothenic acid concentrations. The pantothenic acid levels used were: 0.0, 0.2, 0.4, 0.8, 1.0, and 10.0 mg of calcium pantothenate per 100 gm of diet.

All animals on the diet without any pantothenic acid supplement developed some signs of deficiency and none of those animals survived 75 days of the experiment. In the supplemented groups, the animals receiving 0.2 mg of calcium pantothenate per 100 gm of diet showed significantly lower weight gains during the first half year of the experiment. Later, the difference gradually diminished until after one year the weight gains of the various groups were practically equal. Starting

with the second month of the experiment, the animals receiving 0.8 mg of calcium pantothenate per 100 gm of diet showed slightly higher weight gains than the other supplemented groups. They maintained this lead during the whole experiment; however, this difference was not statistically significant.

The animals on lower levels of pantothenic acid supply acetylated significantly less of the injected sulfonamide than did the animals on higher vitamin levels. It is believed that the optimal level of pantothenic acid needed for acetylation in adult rats lies between 0.8 to 1.0 mg of calcium pantothenate per 100 gm of diet.

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PANTOTHENIC ACID DEFICIENCY AND HYPOPHYSECTOMY [1]

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INTRODUCTION

One of the main symptoms of pantothenic acid deficiency in the young growing rat is a fairly rapid retardation and eventual cessation of growth. The administration of growth hormone reportedly does not induce further a growth response (Beare et al., '54). If growth hormone is administered to adult animals deficient in pantothenic acid, the initial increased growth rate levels off after a short time and signs of pantothenic acid deficiency appear (Lotspeich, '50). Normally, it is rather difficult to produce signs of pantothenic acid deficiency in adult rats (Unna and Richards, '42).

It seems probable, therefore, that a lack of pantothenic acid interferes with the growth process. It is not clear whether this interference is a direct one, or, the result of impaired adrenal function (Cowgill et al., '52) which further reflects a disturbed function of the adrenal-pituitary axis. To gain further insight into this problem and to limit the variables involved, experiments were done with groups of hypophysectomized rats, which were either given or denied pantothenic acid and either treated or not treated with growth hormone.

EXPERIMENTAL

Following hypophysectomy, 30 male rats of the Sprague-Dawley strain with an initial weight of about 80 gm were

[1] This work was supported by a grant in aid from the Nutrition Foundation.

divided into two groups. Twenty animals were fed the diet deficient [2] in pantothenic acid, while the other 10 rats received the same diet supplemented with 10.0 mg of Ca-pantothenate per 100 gm of diet. The animals were housed in individual, metal, screen-bottom cages in an air-conditioned room, weighed twice a week and closely observed for the development of deficiency signs. Food and water were supplied ad libitum. Autopsies were made on all animals which died during the experiment, as well as on those which were sacrificed at the end. All animals were carefully examined for any possible pituitary remnants. The adrenal glands were removed for subsequent histological study.

Four weeks after hypophysectomy, the animals were considered to have recovered from the side-effects of the operation and those in the deficient group to be sufficiently depleted of their stores of pantothenic acid as well. One half of the rats in each of the main groups then received daily intraperitoneal injections of 3 mg of growth hormone.[3] Beginning with the 13th week, the injected animals on the deficient diet were fed the diet supplemented with 1.0 mg of Ca-pantothenate per 100 gm. On the 112th day of the experiment, all surviving animals were sacrificed and the experiment terminated. The growth preparation was assayed for its pantothenic acid content using *Lactobacillus arabinosus* as the test organism (Association of Vitamin Chemists, '51). No measurable pantothenic acid activity was found.

RESULTS

Growth curves are presented in figure 1. For the first 4 weeks none of the animals showed any appreciable growth.

[2] The composition of the basal, pantothenic acid-deficient diet was as follows: vitaminized casein, 10% (provided an adequate supply of menadione and vitamin B-complex except for pantothenic acid); Labco casein, 15%; vitaminized corn oil, 5% (adequate supply of vitamins A, D, and tocopherol); mineral salt mixture IV, 4%; choline Cl, 0.2%; cystine Cl, 0.2%; and sucrose, 65.6%.

[3] Armour, Lot GH-3, showing about 25% of the activity of the 22KR2 Standard. We are indebted to Dr. I. Bunding, Armour Laboratories for the supply of growth hormone.

When the injections were started, both the deficient and control groups responded with growth. The growth rate of the deficient group (GH-D, fig. 1) was about one half of that of the control (GH-PA). Toward the end of the experiment, a plateau appeared in the growth curve of the injected, control group. Similar "leveling off" of the growth rate has been reported by other investigators and attributed to the formation of antibodies after prolonged administration of the

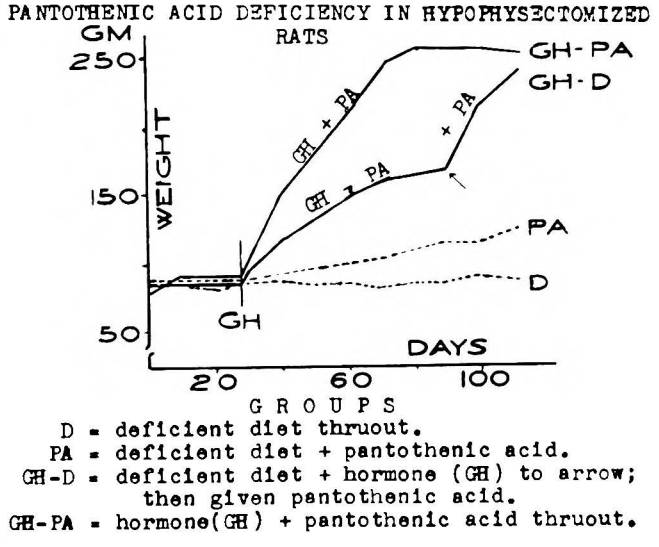

Fig. 1 Growth curves of hypophysectomized rats receiving or not receiving pantothenic acid and treated or not treated with growth hormone.

growth hormone (Emerson, '55). The addition of Ca-pantothenate to the deficient diet of the surviving growth hormone-injected rats (GH-D) resulted in renewed growth which enabled these animals by the end of the experiment to reach the weight of the injected control group which had received the pantothenic acid from the beginning.

In the groups not receiving growth hormone, the control animals (PA) which were given pantothenic acid, showed only a slight increase in weight, while the pantothenic acid-deficient rats (D) retained an almost constant weight.

No animals died in the control pantothenic acid-supplemented groups, but mortality was rather high in both the injected and non-injected deficient groups: 6 animals died during the first 4 weeks; after the administration of growth hormone was begun, 5 animals in both hormone-injected and non-injected groups were lost. The animals in the injected group died earlier in the experiment: their average survival time was 31 days as compared with 55 days for the non-injected group. Neither of these groups showed gross external pathology or signs of pantothenic acid deficiency. The adrenal glands in all 4 groups were smaller than in normal rats of comparable size, especially of the non-injected animals. The adrenal cortices of the deficient but injected animals were congested and hemorrhagic, especially the zona glomerulosa and inner portion of the zona fasciculata.

DISCUSSION

Contrary to the experience with intact, deficient animals in which the administration of growth hormone apparently did not promote further growth (Beare et al., '54), hypophysectomized deficient rats showed quite remarkable growth in response to growth hormone. A possible explanation might involve two factors: (a) some of the original tissue supply of pantothenic acid is retained in spite of the depletion period which was imposed before beginning the administration of hormone, and, (b) the demands of the hypophysectomized rats for pantothenic acid are reduced because of the more quiescent metabolism in the hormone-deficient animals. The presence of a retained store of pantothenic acid is indicated by the fact that it usually takes from 14 to 20 days to depress the growth of rapidly growing weanling rats when they are given a pantothenic acid-deficient diet.

It has also been reported (Everson et al., '54) that the tissues of young rats born to deficient mothers have a much lower content of pantothenic acid than the tissues of young born to mothers receiving a supplementation of this vitamin.

The hypophysectomized animals did not grow during the depletion period, and as the pantothenic acid requirement of non-growing animals is rather low (Unna and Richards, '42), it is possible that the vitamin need was met by amounts synthetized by the intestinal microflora and that the tissue stores were left intact. Furthermore, it has been reported (De Caro et al., '54) that in hypophysectomized thiamine-deficient rats, the thiamine content of brain, muscles and liver falls more slowly than in intact thiamine-deficient animals. Such a mechanism might be assumed to operate also in pantothenic acid deficiency. The rapid growth response of the pantothenic acid-deficient hormone-injected animals when they were finally given pantothenic acid, as well as the shorter survival time show that the lack of pantothenic acid was the limiting factor in these experiments.

Recently, Selye and Bois ('55) reported an antagonism between glucocorticoids and growth hormone as well as a synergism between mineralocorticoids and growth hormone. In their experiments, adrenalectomized rats maintained on hydrocortisone did not grow after receiving growth hormone; the animals maintained on desoxycorticosterone reacted to growth hormone with rapid growth. In pantothenic acid deficiency the zona glomerulosa, which is the suggested site of mineralocorticoid synthesis, is said to be either unaffected (McQueeny et al., '47) or affected only in the very last stage of the deficiency (Ashburn, '47), and therefore it could be assumed that mineralocorticoid synthesis is not affected. In spite of the above-mentioned possible synergism between mineralocorticoid and growth hormone, intact deficient animals, according to Beare et al. ('54), do not react to the hormone. The interference caused by the deficiency would seem, therefore, to involve other mechanisms. The site of the interference could be in protein synthesis, since it was reported (Hazelwood et al., '55) that pantothenic acid-deficient animals retained significantly less nitrogen under the influence of the growth hormone than did the controls which received pantothenic acid.

The occurrence of adrenal lesions in hypophysectomized pantothenic acid-deficient rats again raises the question of the etiology of these lesions. It was suggested (Dean and McKibbin, '46) that pantothenic acid deficiency acts as an "alarming agent," which triggers the adaption syndrome in the sense of Selye's adaptation theory ('37). It has been suggested (Cowgill et al., '52) that this causes an increased production of adrenocorticotropic hormone, which, because of lack of corticoid precursors would lead first to exhaustion and hypertrophy, and later to lesions in the adrenals. Recent investigations by Butler and Morgan ('55) however, have failed to find an increased level of ACTH in the blood of animals deficient in pantothenic acid; the occurrence of adrenal lesions in hypophysectomized animals also indicates that stress mediated through the pituitary is not necessary for the development of adrenal lesions. It could, however, be a contributory factor.

SUMMARY

The influence of pantothenic acid and growth hormone on the growth process has been investigated in young hypophysectomized rats. With hypophysectomized rats, whether or not they received pantothenic acid, growth hormone induced a marked growth response; the growth rate of the deficient group was about one half of that of the control. With rats not receiving growth hormone, pantothenic acid-fed animals demonstrated only a slight weight increase, while pantothenic acid-deficient animals failed to gain weight.

Pantothenic acid-deficient, growth hormone-injected rats frequently had congested and hemorrhagic adrenal cortices. The histological study of the adrenals of the three other groups revealed only the adrenal glands that might be expected after hypophysectomy. The relation of growth hormone and pantothenic acid under these experimental conditions is discussed.

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RELIABILITY OF THE CHROMIC OXIDE INDICATOR METHOD FOR THE DETERMINATION OF DIGESTIBILITY WITH GROWING CHICKENS

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In feeding experiments and balance studies with chicks, the determination of the digestibility of the nutrients involves some unique difficulties. The calculation of the digestion coefficient in the growing chick by the conventional method of collecting all the droppings for several days is very laborious and time-consuming. Some feed will be scattered by the chicks and mixed with the droppings, making the exact measurement of feed intake and the quantitative collection of excreta very difficult. Further, a special apparatus and careful attention are required.

The chromic oxide index method advocated by Edin et al. ('44) and developed by Olsson ('50), Dansky and Hill ('52), Eriksson ('53) and Mueller ('53, '56) helped greatly to simplify the procedure. Dansky and Hill ('52) studied the application of this procedure to growing chicks and they found that changes in the level of feed intake were reflected quickly in the level of chromic oxide in the excreta and that two or more successive 24-hour periods were necessary to get reliable data. Their discussion was based on the percentage of chromic oxide in the dry matter of the excreta and the daily variation of the solids of the urine was neglected.

In this report, the reliability of a simplified method, using chromic oxide as an indicator and collecting about half of

the daily excrement, is discussed on the basis of the digestion coefficient of starch. Since our data, using the birds with artificial anus, indicate a large daily variation in the dry matter of the urine, the discussion is based not on the percentage of chromic oxide in the excreta but on the digestibility of starch. A study has been made of the differences between (a) coefficients determined by the conventional time-collection method and by the index method; (b) those determined with individual chicks and with the same chicks as a group; (c) those obtained from both day and night feces and, finally (d) those with droppings collected from different halves of the pans.

In addition, the daily excretion of reducible matter in the urine of birds having an artificial anus has been determined and the error caused by disregarding this factor, as in the case of this study, has been discussed.

EXPERIMENTAL

Five White Leghorn cockerels, 6 months of age, were reared in individual cages with raised screen floor and fed 70 gm daily of a corn diet containing chromic oxide. Excreta were collected quantitatively into a polyethylene beaker attached with rubber band to a polyvinyl tube which was stitched on the surface of the anus. Excreta were spread on a china dish, dried, weighed and analyzed every day for 11 days of the experimental period. On the 4th and 5th days from the beginning of the trial, the excreta were collected in two parts, one voided in the daytime (from 8:30 A.M. to 5:30 P.M) and the other during the night (from 5:30 P.M. to 8:30 A.M.), and analyzed separately. Then the birds, as a group, were transferred to a large cage battery with a raised screen floor and fed the same quantity of the same diet. After a three-day preliminary period, the excreta for the 4th and 5th days were collected on the zinc tray placed under the floor. In this trial the excreta were also divided into two parts (right and left halves). Since the chicks slept at the rear part of the battery, each part of the sample contained the day and night feces in the proportion occurring naturally. Thereafter the chicks were

returned to the individual cages and a sweet potato diet was fed for 5 days. The excreta for day time and for night time of the 4th and 5th days were collected separately. Again chicks were transferred to the group cage and treated in the same manner as before. This procedure was repeated with the cooked sweet potato diet.

The corn diet used was composed of ground yellow corn, fish meal, yeast, salt mixture, cellulose flour and vitamin B supplements. The sweet potato diet contained sun-dried sweet potato instead of corn. The diet was given mashed with water, or cooked for 15 minutes.

The chromic oxide content was estimated colorimetrically by the method of Schürch et al. ('50) modified by Dansky and Hill ('52); the amount of reducing substances was determined with Somogyi's phosphate reagent ('45) after hydrolysis with 0.7 N hydrochloric acid.

At the end of the trial, three of 5 chicks were operated on so as to place an artificial anus on the surface of the abdomen. One week after the operation, urine was collected daily into a polyethylene sack through a small funnel of glass and polyvinyl stitched on the surface of original anus. The volume of urine was recorded and its content of reducing sugar was determined with Somogyi's reagent ('45).

In experiments 21 and 22, 10 starting chicks were kept in a cage with a raised screen floor and fed a chick starter containing corn, fish meal, soybean meal, wheat bran, defatted rice bran, alfalfa meal, calcium carbonate and salt. Feed and water were supplied ad libitum. At 18 days of age, chromic oxide was mixed in the diet at a level of 0.1%. Thereafter feed intake was recorded daily. The excreta were collected daily, for a 9-day experimental period on a tray of plastic resin placed under the floor. The excreta were divided into two parts (right and left halves of the tray), weighed and the parts were analyzed separately.

In experiments 23, 24 and 25, a part of the excreta of 15 starting chicks, only slightly contaminated with scattered feed, was collected and analyzed daily. No feed intake was recorded.

RESULTS AND DISCUSSION

The digestibility coefficients determined by the usual method of 8-day collection and by the chromic oxide index method, and the recovery of chromic oxide are summarized in table 1. Although the weight of air-dried excreta voided daily ranged from 26.0 to 15.0 gm, the daily variation of digestibility was very small. The agreement of the results obtained by the two methods is satisfactory.

In table 2 mean values of the coefficients of digestibility obtained on 5 chicks for the 4th and 5th days from the beginning of feeding are compared with the coefficients determined

TABLE 1

Comparison of digestibility coefficients determined by total collection method and by chromic oxide method

CHICK NO.	TOTAL COLLECTION METHOD	Cr_2O_3 [1] INDEX METHOD	RECOVERY OF Cr_2O_3
	%	%	%
501	91	92 ± 1.1 [2]	103.9
503	92	91 ± 0.3	92.8
505	91	91 ± 0.8	103.5
507	91	91 ± 0.8	106.9
509	91	91 ± 0.9	98.4
Mean	91	91	101.1

[1] Determined on the excreta of successive 24-hour periods.

[2] Mean value ± standard deviation.

with the same chicks as a group. Statistical analysis shows that the differences between the two procedures and between sampling places in group feeding are insignificant. The standard deviation of the coefficients shown in tables 1 and 2 was not large, compared with that of Eriksson's report ('53).

Results of the trials with starting chicks are given in table 3. Digestion coefficients by the time-collection method and recovery of chromic oxide are calculated on the data for feed intake, fecal output and the content of starch and chromic oxide for 7 days of the experimental period. The unexpected agreement of the chromic oxide intake and output in trial 21 shows such factors as the small loss of feed and excreta which

cannot be recovered, and the difference between the intestinal volume of the chick at the beginning and at the end of the trial counterbalance one another. Variance analysis shows that the error due to the place of sampling statistically is insignificant. The correlation coefficients between feed intake and output, and between feed intake and daily digestion coeffi-

TABLE 2

Digestion coefficient [1] determined with individual chicks and with the same chicks as a group and the difference between two samples of excreta

DIET	DIGESTION COEFFICIENTS		
	Individual sampling	Group sampling	
		Right half	Left half
	%	%	%
Corn	91	91	91
Sweet potato	89	90	89
Sweet potato (cooked)	92	93	93

[1] Mean of two samples of every 24-hour excreta.

TABLE 3

Feed intake and digestion coefficient of starting chicks

TIME [1]	EXP. 21			EXP. 22			EXPERIMENTS 23	24	25
	DAILY FEED INTAKE	DIGESTION COEFF.		DAILY FEED INTAKE	DIGESTION COEFF.		DIGESTION COEFF.	DIGESTION COEFF.	DIGESTION COEFF.
		Right half	Left half		Right half	Left half			
days	*gm*	%	%	*gm*	%	%	%	%	%
3	317	82	82	189	81	79	80	80	80
4	219	80	81	236	80	79	81	80	80
5	356	81	80	261	80	79	81	81	81
6	334	80	80	226	81	80	80	81	78
7	326	81	83	257	81	82	80	80	79
8	346	80	79	257	80	79			80
9	276	81	81	283	81	80			79
Mean		81 ± 1.0 [2]			80 ± 0.84 [2]		81	80	80
Coeff. calculated by total intake and output for 7 days		81			80			80 ± 0.87 [2]	
Recovery of Cr_2O_3		100.0			102.3				

[1] Days after mixing of Cr_2O_3 in the feed.

[2] Mean value ± standard deviation.

cient are 0.97 and 0.22 respectively. Regression analysis shows the former is significant at the 1% level. This may be further support for the view that, in the chick, the feed is passed rapidly through the digestive canal and that nearly all of the diet eaten on a given day is excreted during the same day. The correlation between feed intake and digestion coefficient was

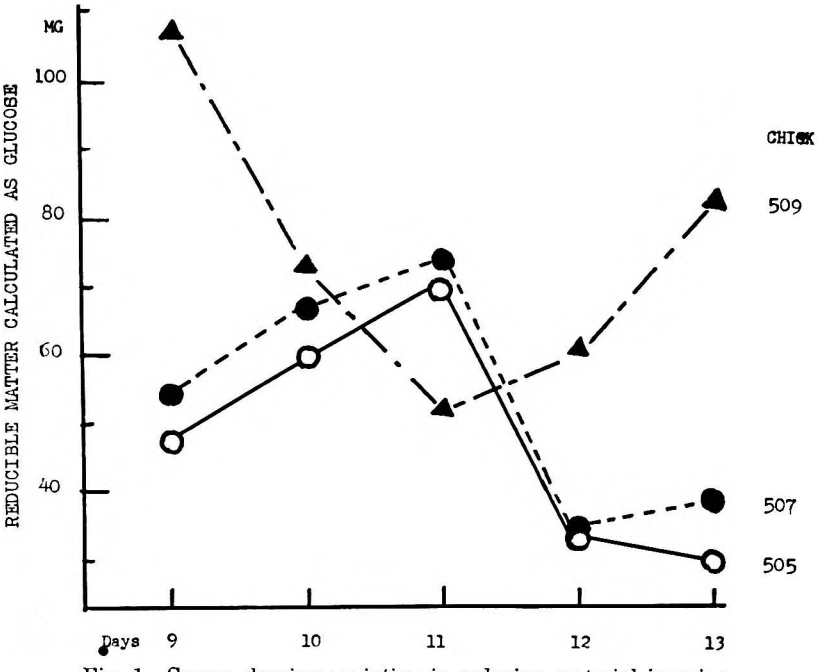

Fig. 1 Curves showing variation in reducing material in urine.

insignificant. Therefore, contrary to the supposition of Dansky and Hill ('52), the level of feed intake has little influence on the efficiency of the utlization of the diet.

As shown in figure 1, the daily variation in urinary excretion of reducible matter, calculated as glucose, was very large. The largest value was 107 mg and this corresponds to about 0.3% in terms of the digestion coefficient when chicks were fed the diet containing nearly 50% of starchy materials. Therefore the relation of urinary reducing substances to the diges-

tion of starchy material in the alimentary tract, may be negligible in this case.

When birds are reared in the usual cage battery, the droppings are more or less stained with scattered feed. In extreme cases, the droppings are completely covered with feed. It is very difficult and troublesome to avoid this and to get clean samples for digestion tests. If a suitable feeder is placed at one side of the cage and the droppings of the opposite half are collected, it is possible to get samples only slightly contaminated with feed. The results shown in tables 2 and 3 clearly indicate that samples thus obtained can be representative ones for the digestion test. The standard deviation of digestibility coefficient obtained with the procedure above mentioned was less than 1% at the digestibility of 80%. According to Mueller's critical study ('53, '56), this may be in agreement with the limit of the accuracy of the chromic oxide index method. Therefore, approximately half of the excreta voided for one day seems to be satisfactory as a representative sample for the determination of the digestion coefficient with an error of $\pm$ 2%.

In data not recorded here, the difference in digestibility between day and night feces was found to be significantly large. This is in agreement with the work of Mueller ('53, '56) and of Dansky and Hill ('52). Care must be taken to secure samples which contain day and night feces in natural proportions.

SUMMARY

The reliability of a simplified method for digestion trials with groups of growing chicks is discussed on the basis of the digestion coefficient of starch. Samples of feces were collected from suitable half areas of the cage floors for 24-hour periods and analyzed, using chromic oxide as an index substance.

Not only in the case of restricted feeding, using pullets, but also in the case of ad libitum feeding with starting chicks, the sample thus collected is found to be a representative one. It is necessary to collect excreta containing day and night feces in natural proportions.

The largest value of reducible material in urine, calculated as glucose is 107 mg per day per 7-month-old chick with an artificial anus. This is of negligible effect on the measurement of the digestion coefficient.

The correlation between feed intake and digestion coefficient is no greater than 0.22. A change in the level of feed intake is not reflected directly in digestibility.

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DIET-AGE PATTERN FOR HEPATIC ENZYME ACTIVITY

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The demonstrated relationship between the activity of certain hepatic enzymes and the age of the rat (Ross and Ely, '54a) and between the enzymes and diet (Ross and Ely, '54b; Ross and Batt, '56) indicated that it might be possible to alter the level of hepatic enzyme activity of any one age to that of another age. The activity of most of the hepatic enzymes or enzyme systems previously reported (Ross and Ely, '54a) showed an increase with age; a few showed a decrease. By maintaining adult rats on protein-free diets high in carbohydrate the activity of these enzymes was altered to that of the young rat (Ross and Ely, '54b). The activity of one of the enzymes, alkaline phosphatase, was shown to be dependent not only upon the ratio of protein to carbohydrate but also upon the absolute amounts of both in the diet (Ross and Batt, '56).

The results reported in the present paper indicate that it is possible to evoke in a young or a mature rat a hepatic enzyme activity identical to that of a rat of a different chronological age by altering the proportion of protein and of carbohydrate in the diet. A strict relationship has been found between the age of the rat, the protein-carbohydrate composition of the diet and the hepatic enzyme activity.

EXPERIMENTAL

Male rats of the Wistar strain, 494 in all, were maintained on a commercial food [1] from weaning age until the age at which they were placed on a specific diet. The initial ages of the rats with their corresponding average weights were: 4 weeks, 45 gm; 6 weeks, 110 gm; 7½ weeks, 160 gm; 10 weeks, 250 gm; 12 weeks, 300 gm; 60 weeks, 450 gm.

Washed casein [2] was used as the only source of protein (86% crude protein). Dextrose was the only source of carbohydrate. The amounts of these two constituents were varied to obtain the several diets used but the combined weight of the two constituents was always 89% of the total diet. The remaining 11% consisted of salt mixtures USP XII, 4%; corn oil, 5%; rice bran extract,[3] 2% supplemented with 0.02% riboflavin. All diets were essentially isocaloric.

Rats of the same age were placed in groups of not less than 10. All had free access to water and food at all times during the experiment; the amount of food consumed has been found not to have an effect on hepatic enzyme activity (Ross and Batt, '56). A feeding period of 21 to 23 days was chosen because it had been determined previously that the greatest effect of any diet upon enzyme activity was produced during the first 5 days of the feeding and that after this period of time enzyme activity exhibited relatively little change.

At the conclusion of the feeding period the rats were decapitated. Weighed pieces of liver were homogenized immediately in cold distilled water or in buffer solution for 30 seconds in a Waring Blendor at low speeds.

Alkaline phosphatase activity determinations were made on the water homogenates at pH 9.1. Sodium β-glycerophosphate was used as the substrate at a final concentration of 0.050 M. The homogenate, which represented approximately 25 mg of fresh liver, was added to the substrate-buffer mixture, prewarmed to 37°C. Incubation was allowed to proceed at

[1] Purina Fox Food Blox (23% protein).
[2] Fisher Scientific Company.
[3] Vitab.

37°C. for one hour. At the end of the incubation period, trichloroacetic acid was added to the mixture and the inorganic phosphorus content was determined on an aliquot portion of the filtrate by the method of King ('32). Alkaline phosphatase activity was measured as micrograms of phosphorus liberated per milligram of fresh liver.

Histidase activity determinations were made by the method of Walker and Schmidt ('44), on samples of the phosphate-buffered liver homogenate at pH 8.0 containing approximately 0.3 gm of liver. Histidase activity was measured on the basis of fresh liver weight.

For testing of the diet-age-enzyme activity pattern, cathepsin, which decreases in activity with age, and D-amino acid oxidase, which increases in activity with age, were investigated. Cathepsin activity determinations were made on the acetate buffered homogenate, pH 3.5, according to the method of Anson ('39). The homogenate contained approximately 25 mg of liver. Cathepsin activity was measured on the basis of fresh liver weight. D-Amino acid oxidase activity determinations of the phosphate buffered homogenate at pH 7.8 were made according to the method of Bernheim and Bernheim ('34). The liver homogenate containing approximately 200 mg of fresh liver, was added to the DL-alanine substrate and phosphate buffer containing NaF in Warburg flasks at 37°C. D-Amino acid oxidase activity was measured as the microliters of oxygen consumed per hour per gram of fresh liver.

RESULTS

Alkaline phosphatase. The results of the assay of hepatic alkaline phosphatase activity show that at every age the activity varies inversely with the casein content and directly with the dextrose content of the diet. It was found, when rats 4, 5, 7½ and 60 weeks old were maintained on diets which differed in their proportions of casein and dextrose, that the level of activity differed at different ages (fig. 1) and the

older the rat the lower the alkaline phosphatase activity. In the young rat, when most active growth was occurring, the level of hepatic alkaline phosphatase activity changed most rapidly from one time period to another, and in the older rat, when relatively little growth was occurring, the activity changed little from one time period to another. Therefore,

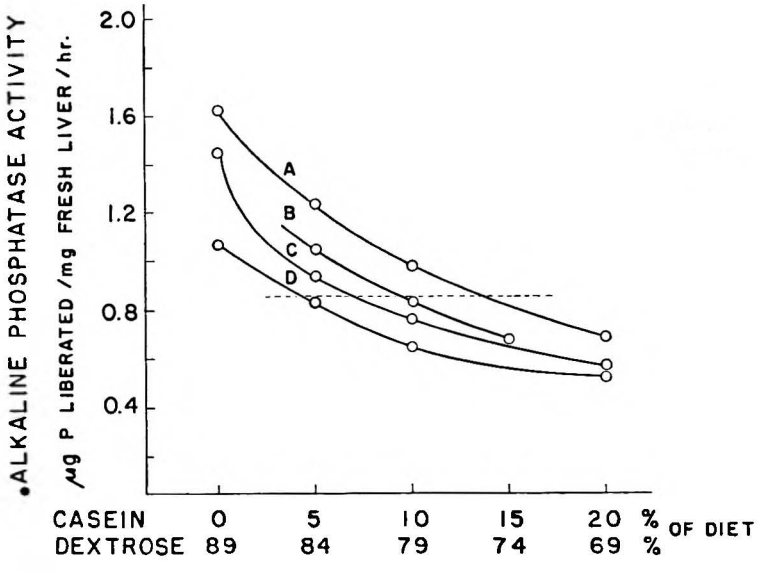

Fig. 1 Effect of diet on hepatic alkaline phosphatase activity. Initial age and weight of rats — A, 4 weeks old, 45 gm; B, 5 weeks old, 110 gm; C, 7½ weeks old, 175 gm; D, 60 weeks old, 450 gm; time period on diet, 21 to 23 days.

The intersection of the dotted line with the curve indicates the proportion of casein and of dextrose in the diet which should be allowed to a rat regardless of age in order to obtain 0.86 units of alkaline phosphatase activity.

the level of activity appears to be dependent upon the diet and the age or growth of the rat. This conclusion suggests that a single value for hepatic alkaline phosphatase activity could be obtained for a rat of any age by placing it on the proper diet.

The level of activity which was obtained in a rat with an initial weight of 175 gm at 7½ to 8 weeks of age, when fed

7% casein and 82% dextrose was calculated to be 0.86 units (see fig. 1, C). This level was obtained in rats of each of the other ages that were fed the diet indicated by the abscissa at which the dotted line in figure 1 (A, B and D) intercepts the corresponding curve. Inasmuch as the weight of a normal growing rat can be used as a measure of its age, an estimate of the casein-dextrose requirement at each age was made on the basis of the initial rat weight. It was found that smaller amounts of dietary casein were required to produce

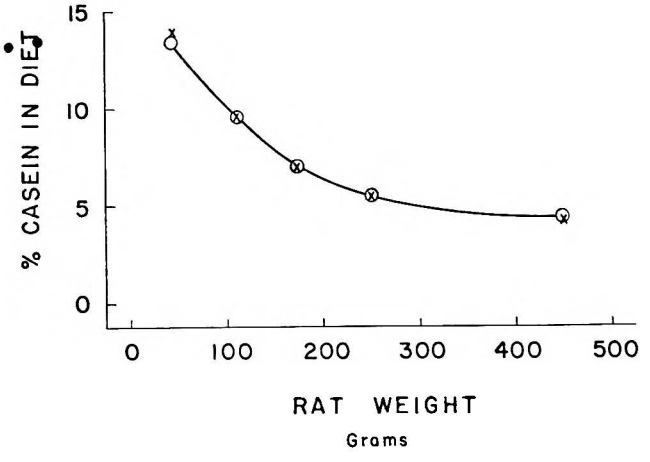

Fig. 2 Diet-age-hepatic enzyme pattern for obtaining a constant enzyme level. Initial rat weight has been used as the measure of rat age.
Alkaline phosphatase = ○
Histidase = ×

a certain level of activity in heavier, that is, older animals (fig. 2).

To ascertain whether the calculated amount of dietary casein, as estimated in figure 2, would indeed produce a level of activity of 0.86 units, rats 6 weeks old weighing 110 gm were allowed to feed on a diet containing 9.5% casein. Rats 10 weeks old, weighing 250 gm, were allowed to feed on a diet containing 5.5% casein. The results, given in table 1, indicate that the expected level of alkaline phosphatase activity was obtained.

Histidase. To determine the effect of diet on histidase activity which increases with age, as contrasted with alkaline phosphatase, which decreases with age, rats of three ages, 4, 7½ and 60 weeks old were maintained on diets which differed in their proportion of casein and dextrose. Histidase activity at every age was found to vary directly with the casein content and inversely with the dextrose content of the diet. The level of activity on any one diet varied with age (fig. 3); the older the rat the higher the hepatic histidase activity. The levels of both alkaline phosphatase and histidase are dependent upon the diet and the age of the rat. The greatest change in activity of both enzymes from one time period to another occurs in the young, rapidly growing rat. The level of histidase activity,

TABLE 1

Dietary effect on hepatic alkaline phosphatase at different ages

CASEIN IN DIET	NUMBER OF RATS	AGE	WEIGHT	ALKALINE PHOSPHATASE ACTIVITY	
				Calculated	Observed
%		*weeks*	*gm*	*μg P/mg liver*	
9.5	13	6	110	0.86	0.87 ± 0.05 [1]
5.5	11	10	250	0.86	0.88 ± 0.05

[1] ± S.E.$_{mean}$.

like that of alkaline phosphatase, can be altered at any given age by a change in the diet. This should make it possible to obtain a given level of histidase activity for any age by maintaining the rat on a diet containing a given proportion of casein and dextrose.

A comparison was made of the dietary requirements which would produce a given effect on histidase activity with those requirements necessary to produce a similar effect on alkaline phosphatase. The same basis used for alkaline phosphatase activity was used for histidase activity, namely, the level of histidase activity that would be obtained in a rat with an initial weight of 175 gm at 7½ to 8 weeks of age if fed 7% casein. This level was found to be 0.91 units. The amount of dietary casein which will result in this level of histidase

activity for any age varies inversely with age. This amount of casein is identical with that which will produce, for any age, a level of 0.86 units of alkaline phosphatase activity (see fig. 2).

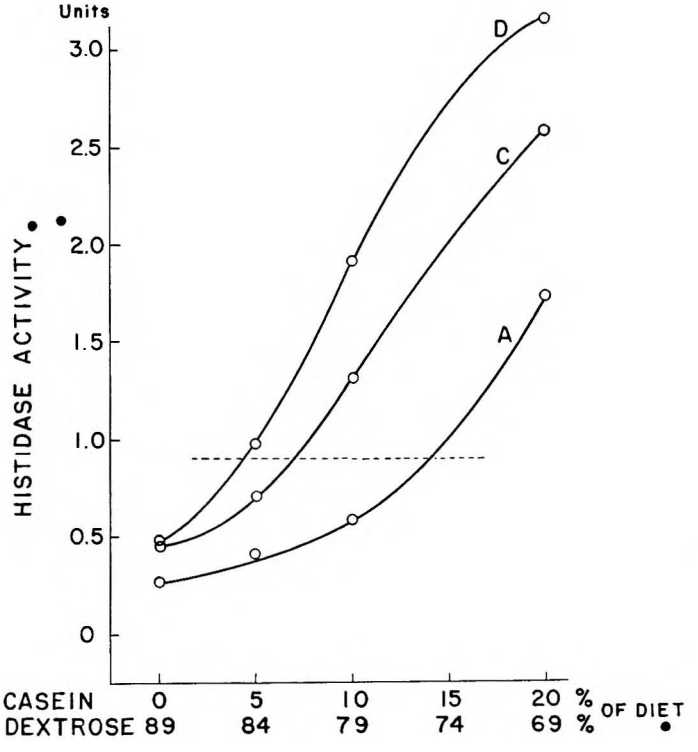

Fig. 3 Effect of diet on hepatic histidase activity. Initial age and weight of rats — A, 4 weeks old, 45 gm; C, 7½ weeks old, 175 gm; D, 60 weeks old, 450 gm; time period on diet, 21–23 days.

The intersection of the dotted line with the curve indicates the proportion of casein and of dextrose in the diet which should be allowed to a rat regardless of age in order to obtain 0.91 units of histidase activity.

To prove that the amount of dietary casein estimated from figure 2 will produce a level of histidase activity of 0.91 units for other ages, rats 6 weeks old, weighing 110 gm, were allowed to feed on a diet containing 9.5% casein. Rats 10 weeks old, weighing 250 gm, were allowed to feed on a diet

containing 5.5% casein. The expected level of hepatic histidase activity was obtained in both groups (table 2). A given diet therefore produces the same degree of effect on the levels of activity of both histidase and alkaline phosphatase even though these enzymes differ in respect to the direction of the changes that occur with age and diet. A diet containing small amounts of casein changes the levels of activity of both enzymes in older animals to the level found in younger animals while a diet containing large amounts of casein changes the levels of activity of both enzymes in younger animals to those found in older animals.

TABLE 2

Dietary effect on hepatic histidase at different ages

CASEIN IN DIET	NUMBER OF RATS	AGE	WEIGHT	HISTIDASE ACTIVITY	
				Calculated	Observed
%		*weeks*	*gm*	*units*	
9.5	12	6	110	0.91	0.88 ± 0.078 [1]
5.5	10	10	250	0.91	0.93 ± 0.079

[1] ± $S.E._{mean}$.

Diet-age-enzyme activity pattern. In order to confirm the predictability of changes in enzyme activity from one age to that of another age by an alteration of the diet, the activities of D-amino acid oxidase and cathepsin were measured. Activities of D-amino acid oxidase and of cathepsin are like those of histidase and alkaline phosphatase, respectively, in direction of changes with age and in changes with reduction of the protein content of the diet. The determinations of D-amino acid oxidase and cathepsin were made on rats of 4 ages which had been given diets containing the same percentages of casein and dextrose as had been used for adjusting (fig. 2) the alkaline phosphatase and histidase activity levels. The same level of hepatic D-amino acid oxidase was obtained for each age (table 3).

The same level of cathepsin activity was obtained (table 4) for each age when rats of different ages and weights were given diets corresponding to their age. The amounts of casein and of dextrose (fig. 2) which should be given to rats of any age to produce a designated level of enzyme activity regardless of the age of the rat have been shown to be identical for all 4 enzymes.

TABLE 3

Dietary effect on hepatic D-*amino acid oxidase at different ages*

CASEIN IN DIET	NUMBER OF RATS	AGE	WEIGHT	D-AMINO ACID OXIDASE ACTIVITY
%		*weeks*	*gm*	*μl O_2/mg liver/hr.*
13.5	10	4	45	212 ± 16 [1]
8.0	10	7	150	222 ± 28
5.0	10	12	300	213 ± 15
4.5	10	60	460	241 ± 22

[1] ± $S.E._{mean}$.

TABLE 4

Dietary effect on hepatic cathepsin at different ages

CASEIN IN DIET	NUMBER OF RATS	AGE	WEIGHT	CATHEPSIN ACTIVITY
%		*weeks*	*gm*	*units*
13.5	16	4	50	10.40 ± 0.37 [1]
8.0	16	7	160	10.51 ± 0.39
5.0	16	12	300	9.72 ± 0.48
4.5	16	60	440	10.34 ± 0.60

[1] ± $S.E._{mean}$.

DISCUSSION

Regardless of the direction of change with age in the level of activity of any of the enzymes investigated, a diet containing a particular proportion of casein and of dextrose always evoked a predictable response in the level of enzyme activity. When mature rats were given a diet in which the proportion of casein in the mixture was small, the level of enzyme activity was always changed to that level ordinarily found in younger rats. When young rats were given a diet in which the proportion of casein was large, the level of enzyme activity was always changed to a level ordinarily found in

older rats. This quantitative enzymatic adaptation evidently enabled an arbitrary adjustment by dietary means of the activities of any of the enzymes studied from one age level to another, without, however, analogous weight change. It is reasonable to assume from these developments and from earlier conclusions (Ross and Ely, '54b) that any one diet will evoke the same degree of response in all hepatic enzymes. The dependence of the level of hepatic enzyme activity of a rat of any age on the composition of the diet indicates that a definite diet-age pattern exists for hepatic enzymes.

Biological aging may be a manifestation of the naturally occurring enzymatic changes. If so, by an alteration of the enzymological activities through diet, it may be possible to influence biological age.

SUMMARY

The relationship has been investigated between activity of hepatic enzymes and age of the rat and between hepatic enzymes and diet. The activity levels of alkaline phosphatase, histidase, D-amino acid oxidase and cathepsin have been adjusted from that of one age to that of another age by means of diet. Diets containing large proportions of casein altered the levels of activity from those of young rats to those of older rats while diets containing small proportions of casein altered the levels of activity of older rats to those of more youthful rats. At each age, these hepatic enzymes responded to diet to the same degree. It is suggested that a diet-age pattern exists for all hepatic enzyme activity.

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STUDIES ON THE KIDNEY IN VITAMIN E DEFICIENCY [1]

I. POST-MORTEM AUTOLYSIS IN KIDNEYS OF RATS FED A VITAMIN E-DEFICIENT DIET RICH IN LONG-CHAIN UNSATURATED FATTY ACIDS

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Despite numerous studies of the manifestations of vitamin E deficiency in a wide variety of organs and various animal species, comparatively little attention has been directed to the kidney. The first indication of a renal abnormality associated with vitamin E deficiency is the report by Martin and Moore ('36) of hyalin tubular degeneration in the kidneys of rats maintained for periods up to 15 months on a vitamin E-deficient diet containing 20% lard. Subsequent reports (Martin and Moore, '38, '39; Moore, '39) described extensive degenerative changes and sloughing of epithelium involving practically all of the convoluted tubules and unaccompanied by inflammatory reaction. In more recent studies of the effects of unsaturated fats and fatty acids in the diet of vitamin E-deficient rats (Mason and Emmel, '45; Filer, Rumery and Mason, '46; Rumery, '47) similar observations have been recorded, but their nature and significance have not been investigated.

The descriptions of the above findings are strongly reminiscent of the changes observed during certain phases of post-mortem autolysis in the normal rat kidney (Emmel, '40).

[1] Supported by grant no. A-903, U. S. Public Health Service.

The fact that the lesions described in kidneys from vitamin E-deficient rats were not found in controls suggested the possibility that if post-mortem autolysis were a factor in producing these changes, it might have progressed more rapidly in the deficient than in the control animals. In some preliminary studies undertaken with this question in mind (Emmel, '55a, b), it was found that the kidneys of vitamin E-deficient rats on a diet containing large amounts of unsaturated fat do indeed undergo post-mortem changes much more rapidly than do the kidneys of normal or vitamin E-treated animals. The present report provides data to establish this fact further, and to indicate that continued investigation of this matter should lead to new insight into the interrelationships between vitamin E and lipids in renal structure and function and into the general role of vitamin E in vital processes.

MATERIALS AND METHODS

Albino rats of both sexes were weaned at 21 to 28 days of age and transferred to a synthetic diet [2] in which 30% of the calories were supplied by the mixed free fatty acids of linseed oil.[3] The latter mixture had an iodine number (Wijs) of about 175 and contained about 40% of linolenic acid. Its tocopherol content was negligible.[4] The diet was prepared in small batches once or twice weekly and stored in a refrigerator. Food and water were given ad libitum, but any food uneaten after 24 hours was replaced with a fresh supply. Twice weekly all animals received oral supplements of 400 units of vitamin A and 40 units of vitamin D. In addition to this the controls

[2] Composition of the diet is as follows:

Crude casein	238 gm	Salt mixture (H.M.W.)	30 gm
Cerelose (Dextrose)	489 gm	Linseed fatty acids	155 gm
Dried brewers' yeast	89 gm		

Subsequent experiments have shown that the same results are obtained with a diet in which the fat is stabilized against oxidation by adding 0.1% by weight of citric acid and propylgallate to the fat before mixing into the diet.

[3] Woburn Chemical Corp., Harrison, N. J. (Product no. 34-A.)

[4] Chemical analyses and vitamin supplements were obtained through the courtesy of Dr. P. L. Harris, of Distillation Products Industries, Rochester, N. Y.

received 25 mg of α-tocopheryl acetate [4] twice weekly. Animals were killed at appropriate intervals by decapitation.

Kidneys for the study of autolysis were promptly removed, decapsulated and gently sliced into a suitable number of pieces. One piece was immediately fixed in Bouin's fluid, and the others were placed in a small sealed dish in which the atmosphere was in equilibrium with Ringer's solution, to prevent drying of the specimens. Temperature was maintained at 37°C. Specimens removed for fixation at intervals up to 6 hours were handled gently to avoid mechanical injury to tissues softened by autolysis. All were subsequently combined in a single paraffin block for sectioning, then processed simultaneously through staining with hematoxylin and eosin. Specimens for the determination of tissue non-protein nitrogen were similarly maintained at 37°C. for various intervals before chemical analysis.

OBSERVATIONS

Microscopic. The histologic changes during the course of autolysis in the kidneys of a vitamin E-deficient and a vitamin E-treated animal are compared in figures 3 to 8 (plate 1). In freshly fixed specimens (figs. 3, 6) there are no conspicuous differences between the kidneys of these two groups of animals. However, after incubation for one hour and 6 hours it is apparent that much greater change has occurred in the kidney of the vitamin E-deficient animal (figs. 7, 8) than in that of the vitamin E-treated animal (figs. 4, 5). In the convoluted portions of the proximal tubules of the former there is loss of nuclear staining and extensive general disorganization of the cytoplasm; whereas in those of the vitamin E-treated animal, although there are obvious post-mortem changes including nuclear pyknosis, the changes are much less extensive than in the vitamin E-deficient specimens.

In a former study of mitochondrial changes during renal ischemia (Emmel, '40) it was noticed that these begin later in the distal than in the proximal tubules. However, when changes start in the distal tubules they proceed rapidly and

soon become even more extensive than those in the proximal tubules. This finding is again borne out in the present observations: after autolyzing for 6 hours, the kidneys of vitamin E-treated animals show distal tubules (plate 2, fig. 9, center and lower left) in a poorer state of preservation than the adjacent proximal tubules. The nuclei of the former have become fragmented, and the cytoplasm has become disorganized and separated from the basement membrane. On the contrary, and in contrast with this, the distal tubules of kidneys from vitamin E-deficient animals (fig. 10) are seemingly more resistant to autolysis than are the proximal tubules. After 6 hours the nuclei of the distal tubules are pyknotic but intact, and cellular structure is fairly well preserved; while in adjacent proximal tubules the cytoplasm is greatly disorganized and the nuclei are no longer stainable. Thus in the vitamin E-deficient animal the proximal tubules at 6 hours show more marked post-mortem changes than do the distal tubules, while in the vitamin E-treated animal the reverse is true.

During the 6-hour interval employed in these studies the structural differences in autolytic phenomena between vitamin E-deficient and vitamin E-treated animals were limited mainly to the convoluted portions of the proximal and distal tubules. The straight segments of the former as well as glomeruli and the medullary portions of the nephron remained essentially unaltered in appearance both in treated and in deficient animals.

Tissue non-protein nitrogen. Since protein degradation is a prominent feature of the autolytic process, the rise in tissue NPN during a 6-hour period of incubation at 37°C. was measured as an objective criterion of the amount of autolysis that had occurred. For this study kidneys were promptly removed, decapsulated and gently quartered with a razor. Hilar fat and connective tissue and the renal papilla were removed. The latter was eliminated because it undergoes no conspicuous structural changes during 6 hours; hence its disproportionate inclusion in various specimens might have intro-

duced corresponding variations when subsequent analytical results were calculated in terms of tissue weight.

For each animal specimens were taken from both kidneys to give a total weight of 300 to 400 mg. This combined specimen was then homogenized in a volume of 5% trichloracetic acid corresponding to 9 times the weight of the specimen (yielding an approximately 10% homogenate). A similar kidney sample was placed in a small sealed dish and incubated at 37°C. for 6 hours, then homogenized as above. After centrifugation the NPN content of duplicate 1 ml. aliquots of the supernatant solutions was determined by a semi-micro Kjeldahl method. The results were calculated as milligrams of NPN per 100 mg of fresh tissue, and for the initial specimens generally fell between 0.250 and 0.350. The difference between the initial value and that after 6 hours (Δ NPN) was interpreted as a measure of the amount of protein broken down by autolysis during this period.

The results of these analyses are shown graphically in figure 1, in which each point represents the data derived from a single animal. In the vitamin E-treated group it is apparent that the rate of autolysis remains essentially unchanged throughout the experimental period. Animals which had been on the diet for nearly two years, although not shown on the graph, had similarly low autolytic rates. The values obtained from 10 adult rats (fig. 1) fed a standard commercial ration were practically identical with those from the vitamin E-treated animals on the linseed fatty acid diet. In marked contrast to these two control groups, animals on the vitamin E-deficient regimen show a marked rise in renal autolytic rate. This rise begins rather abruptly after about 6 weeks on the diet, reaches a maximum between 12 and 15 weeks, and then remains distinctly elevated. No special significance is at present attributed to the exact shape of the curve which has been drawn; it was added mainly to call attention to the location of points representing the E-deficient animals and to emphasize the fact that they form a group distinctly different from the controls. The 5- or 6-week interval prior to onset of this renal alteration

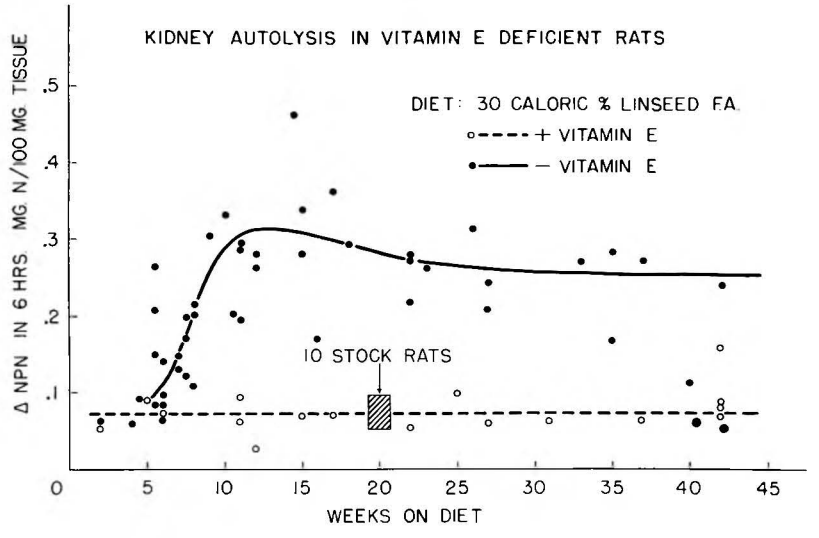

Fig. 1 Plot showing renal autolysis in terms of rise in tissue NPN during incubation for 6 hours at 37°C. Animals were started on the experimental diet when weaned at 21 to 28 days of age. Each point represents one animal. Vitamin E-treated animals received 25 mg of α-tocopheryl acetate twice weekly.

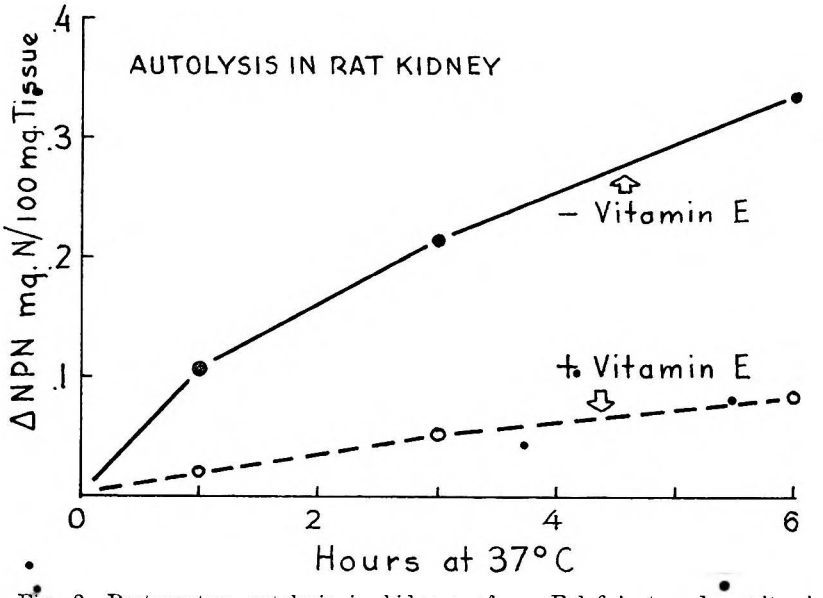

Fig. 2 Post-mortem autolysis in kidneys of an E-deficient and a vitamin E-treated rat. Animals had received a diet containing 20% by weight of cod liver oil for a period of 15 weeks after weaning.

is believed to represent the period during which the animal's stores of vitamin E are becoming depleted to a critical level. Determinations of renal tocopherol content currently in progress should help to clarify this matter.

A question arises as to whether the greater amount of NPN which appears during 6 hours in the vitamin E-deficient kidney is due to a more rapid rate of autolysis or to its earlier onset in these specimens. Figure 2 records the results obtained in serial analyses carried out on specimens allowed to autolyze for intervals up to 6 hours. It is apparent that autolysis begins promptly in the kidneys of both treated and deficient animals, but that it proceeds at a more rapid rate in the latter. The data illustrated were obtained from animals whose diet contained cod liver oil rather than linseed fatty acids, but the results are similar with either.

Comparison was also made of the autolysis occurring in distilled water homogenates of kidneys from vitamin E-deficient and vitamin E-treated animals (table 1). As might be anticipated, the amount of NPN liberated during incubation for 6 hours at 37°C. in homogenates of both groups is considerably greater than that liberated in the intact specimens. It is of interest, however, that in either type of preparation the rate of autolysis in the vitamin E-deficient group is distinctly greater than in the controls.

ADDITIONAL OBSERVATIONS

(a) General. Control animals receiving tocopheryl acetate do well on this regime and have remained healthy and vigorous up to the time of sacrifice at almost two years of age. The vitamin E-deficient animals generally do well for the first three or 4 weeks, although they seem to be more subject to respiratory infections. During the 5th and 6th weeks an increasing number of animals show a gradually spreading area of wetness in region surrounding the urethral orifice. The hair in this area becomes somewhat matted and discolored due in large part to the accumulation of solids from evaporating urine. The nature of this phenomenon has not been ascer-

tained, but it is presumed that either there is some change in the surface lubrication of the hair reducing its ability to shed water, or some change in the character of the urine which causes it to spread on the fur. The over-all adequacy of kidney function in vitamin E-deficient rats on this diet for 14 weeks or longer is indicated by the fact that their average blood urea concentration (46 mg %) is close to that for corresponding vitamin E-treated animals (44 mg %). Further detailed studies of renal function will be reported later.

TABLE 1

Autolysis in intact and homogenized rat kidney

ANIMAL NO.	DIET [1]	ΔNPN,[2] mgN/100 mg TISSUE	
		Intact	Homogenized [3]
5	Stock	0.045	0.441
6	Stock	0.070	0.335
373	+ E	0.058	0.377
374	+ E	0.037	0.358
Average		0.053	0.378
512	— E	0.214	0.594
514	— E	0.271	0.588
516	— E	0.222	0.587
519	— E	0.247	0.645
Average		0.239	0.604

[1] The vitamin E-treated and E-deficient animals received the synthetic diet containing 30 caloric % of linseed fatty acids. The + E animals had been on the diet for 59 weeks, and the — E for 34 weeks.

[2] Amount of NPN liberated during autolysis for 6 hours at 37°C.

[3] Twenty per cent homogenate in distilled water allowed to autolyze as above, then precipitated with an equal volume of 10% trichloracetic acid, and the supernatant used for NPN determination.

The ratio of kidney weight to body weight for the vitamin E-deficient rats was slightly increased over that for the control groups, although variations in body weight and total amount of adipose tissue complicate the interpretation of such a comparison.

(b) Depot fat and pigment. Depot fat in the vitamin E-deficient animals becomes markedly altered, as previously described by other investigators using diets similar to that in

the present study (Mason, Dam and Granados, '46; Granados, Mason and Dam, '47; Filer, Rumery and Mason, '46). During the 4th or 5th weeks numerous small pale yellow spots appear in the fat depots. These spots gradually become larger and more numerous until the entire mass assumes a mottled and increasingly darker yellow, opaque and firm character. With increasing age the fat darkens and eventually becomes a deep orange brown. These changes usually appear first in the fat associated with the uterus, ovaries and testis. Subsequently the dorsal retroperitoneal and perirenal fat and finally the subcutaneous, mesenteric and omental fat becomes involved. There may be considerable variation in this sequence. This altered fat is characterized by the presence of material with acid-fast staining properties (Mason et al., '46) and fat peroxides and pigments (Dam, '49). The altered fat evidently contains a considerable amount of material metabolically unavailable to the animal, since it persists in animals fasted almost to the point of death. Depot fat in the vitamin E-treated animals remains essentially normal in amount and appearance.

(c) Hemorrhagic kidneys. Between the 6th and the 10th or 12th weeks on the diet a variable number of the vitamin E-deficient animals have died suddenly with acute renal failure, hemorrhagic kidneys, hematuria and uremia (Emmel, '54). Blood urea may rise to terminal values of over 400 mg %. The number of animals in which this condition develops has varied considerably in different groups, being completely absent in many and occasionally having an incidence approaching 50% in others. Animals which survive beyond the 12th week generally continue in fairly good condition for periods up to 40 weeks or more. The kidneys of these surviving animals acquire a light tan or somewhat olive color, and the medulla usually shows a light outer zone. The papilla remains pale and the urine clear.

The kidneys of animals with acute renal failure appear dark purplish brown, are smooth, firm and somewhat swollen. The renal papilla generally is brownish, rather than its normal

white or light pink color. Microscopically there are glomerular hemorrhages with blood in the capsular spaces and tubular lumens, and occasionally small peritubular hemorrhages. Changes in the tubular epithelium may be minimal or absent unless the animal is in a comatose terminal state, at which time there is usually some intracellular disorganization and sloughing of cells of the proximal tubules.

The relationship of this form of renal disorder to the state of vitamin E-deficiency is not clear, although this condition has not been encountered in vitamin E-treated animals nor in animals on stock diets. The appearance of the kidneys in this condition is always quite uniform, and the changes are always bilateral. This, coupled with the fact that the incidence is higher among cage-mates and in some groups of animals than in others, suggests the possibility of an underlying infectious or toxic process. Some of these animals have shown evidence of pulmonary disease at autopsy, but its relationship to the onset of the renal disorder is not clear. Bacterial cultures of blood, lungs, kidneys and body cavities have failed to give a definite answer. However, the possibility remains that this hemorrhagic renal lesion is a result of sensitivity to bacterial toxin. This matter warrants further study under experimental conditions in which the incidence of respiratory infections can be kept at a minimum.[5] It is of interest that the period of highest incidence of this acute renal failure coincides with the time at which the rate of renal post-mortem autolysis is rising above normal and reaching its maximum value.

Observations on kidneys damaged by parenteral administration of uranium, chromium and mercury salts, and by sucrose, showed that these kidneys autolyze at the same rate as normal kidneys. This indicates that the increased rate of autolysis observed in the vitamin E-deficient kidneys is not due simply to kidney damage *per se*. Similarly, kidneys from animals with the hemorrhagic nephritis described above do

[5] The incidence of hemorrhagic kidneys has been essentially nil during the past 8 months since adequate temperature control has been established in the animal quarters.

not autolyze at a rate greater than would be anticipated on the basis of their period of vitamin E-deficiency.

DISCUSSION

The histological and chemical data presented demonstrate that the kidneys of rats raised on a diet deficient in vitamin E and rich in long-chain unsaturated fatty acids undergo post-mortem changes much more rapidly than do the kidneys of animals receiving the same diet supplemented with vitamin E. The inclusion of unsaturated fats in the diet seems to be necessary for the development of this renal abnormality. The effects of varying the dietary lipids will be reported later; but results thus far have indicated that cod liver oil is effective in producing the above abnormality, while a fat-free diet or one containing considerable amounts of saturated fats does not produce this type of abnormality in the kidney. Whether the differences between the effects of the saturated and unsaturated fats may be related to a greater need for vitamin E or its more rapid depletion in the presence of high dietary intake of the unsaturated fats cannot yet be stated, but it is hoped that renal tocopherol and lipid analyses currently in progress will contribute to an understanding of this question.

Abnormality of the kidney is completely prevented if vitamin E is administered throughout the experimental period. However, preliminary observations indicate that after this abnormality has become well established it is not readily reversible. Animals on the vitamin E-deficient regimen 15 weeks or longer and subsequently treated for periods up to 12 weeks with tocopheryl acetate (25 mg twice weekly) have shown autolytic rates comparable to those of untreated animals. Supplementary choline (3.5 gm/kg) and vitamin K (10 mg/kg) added to the diet did not alter the results in the vitamin E-deficient animals. Studies in progress clearly indicate that this renal abnormality, like certain other manifestations of vitamin E deficiency (Christensen, Dam and Gortner, '56), can be prevented by the addition of methylene blue to the linseed fatty

acid diet.[6] In animals maintained to adulthood on this regimen, withdrawal of the methylene blue is followed by development of the increased rate of renal autolysis and changes in the adipose tissue, uterus and testis characteristic of vitamin E deficiency. Observations on the prophylactic and therapeutic actions of tocopherol and other antioxidants in this connection will be reported elsewhere.

The nature of the underlying structural or metabolic alterations which lead to the increased rate of autolysis in the vitamin E-deficient kidney is not known. The seeming irreversibility of this abnormality suggests the possibility of some stable structural alteration or one involving components whose turnover rate in the kidney is very low. The fact that unsaturated fat in the diet appears necessary to produce this change would seem to direct attention to the lipid constituents of protoplasm and the role of vitamin E as an antioxidant. There is evidence in the literature (Jobling and Petersen, '14; Godlowski, '55, p. 29) that unsaturated fatty acids may be involved in the inhibition of proteolysis by forming a complex with proteolytic enzymes, and that the state of unsaturation of the fatty acids may here be of great importance. It is possible that some defect in an inhibitory mechanism of this sort, perhaps some change in mitochondrial phospholipids or lipoproteins, may occur in the vitamin E-deficient kidney. Certainly the depot fat becomes markedly altered in these animals, and it is possible that related changes may occur in the lipids incorporated into protoplasmic structure. Studies are in progress with these questions in mind.

The present observations are of interest in relation to the renal lesions reported by Martin and Moore ('36, '39). In rats kept for 10 months or longer on a vitamin E-deficient diet containing 20% by weight of lard, these investigators

[6] Data obtained since this manuscript was submitted indicate that, whereas changes in kidney and adipose tissue may be held in abeyance indefinitely by methylene blue, the onset of changes in the uterus and testis is merely delayed by some two to three months.

described (but did not illustrate) somewhat spotty but progressive tubular damage leading to sloughing of epithelium and eventually involving practically every nephron. In the present study no such condition was observed in promptly fixed kidneys from the vitamin E-deficient rats. It is possible that rapid post-mortem changes contributed to the findings of Martin and Moore, but this cannot be stated with certainty since their diet contained relatively little unsaturated fat in comparison with that used in the present study. However, it should be apparent that in evaluating histologic changes in the kidneys of vitamin E-deficient animals it is important that the organs be removed and fixed without delay.

Preliminary observations on organs other than the kidney have indicated that when examined by the same technique the testis, skeletal muscle and liver from vitamin E-deficient animals do not consistently show the increased autolytic rate found in the kidney. It is of interest that in the livers of rats receiving certain types of vitamin E-deficient diets (Schwarz, '51; Chernick, Rodnan and Schwarz, '54) there has been demonstrated a metabolic defect which leads to premature decline in oxygen uptake by liver slices. Although this liver defect is promptly relieved by administration of tocopherol, it may still have a basis in common with the renal abnormality herein described. Attention might also be called to the description of unusually rapid gross putrefactive changes which Blaxter et al. ('52) observed in calves moribund with a syndrome apparently related to inadequate dietary vitamin E. Irving and Budtz-Olsen ('55) have reported "kidney lesions" occurring as early as 30 days after placing rats on a vitamin E-deficient diet low in protein and containing 20% by weight of cod liver oil; however, these lesions have not been fully enough characterized to evaluate them in relation to the present findings.

SUMMARY AND CONCLUSIONS

1. Weanling rats were transferred to a vitamin E-deficient diet in which 30% of the caloric value was supplied as the free fatty acids obtained from linseed oil. Controls receiving

25 mg of α-tocopheryl acetate twice weekly showed no abnormalities during a period of nearly two years.

2. A newly recognized renal manifestation of vitamin E deficiency is described: in animals kept for 6 weeks or longer on the vitamin E-deficient regimen the rate of post-mortem autolysis in the kidney is markedly increased. This abnormality is most conspicuous in the proximal convoluted tubules and is characterized by more extensive histologic changes and more rapid rise in tissue NPN.

3. A relatively high intake of unsaturated fatty acids appears to be necessary for the development of this renal abnormality, since the latter was not found in animals whose diet contained lard in place of the linseed fatty acids.

4. The renal abnormality is completely prevented by the prophylactic administration of α-tocopheryl acetate; but when it has become established the abnormality is not readily reversible by tocopherol therapy.

5. These findings indicate an interrelationship between vitamin E and the role of unsaturated lipids in the structural and functional organization of the kidney.

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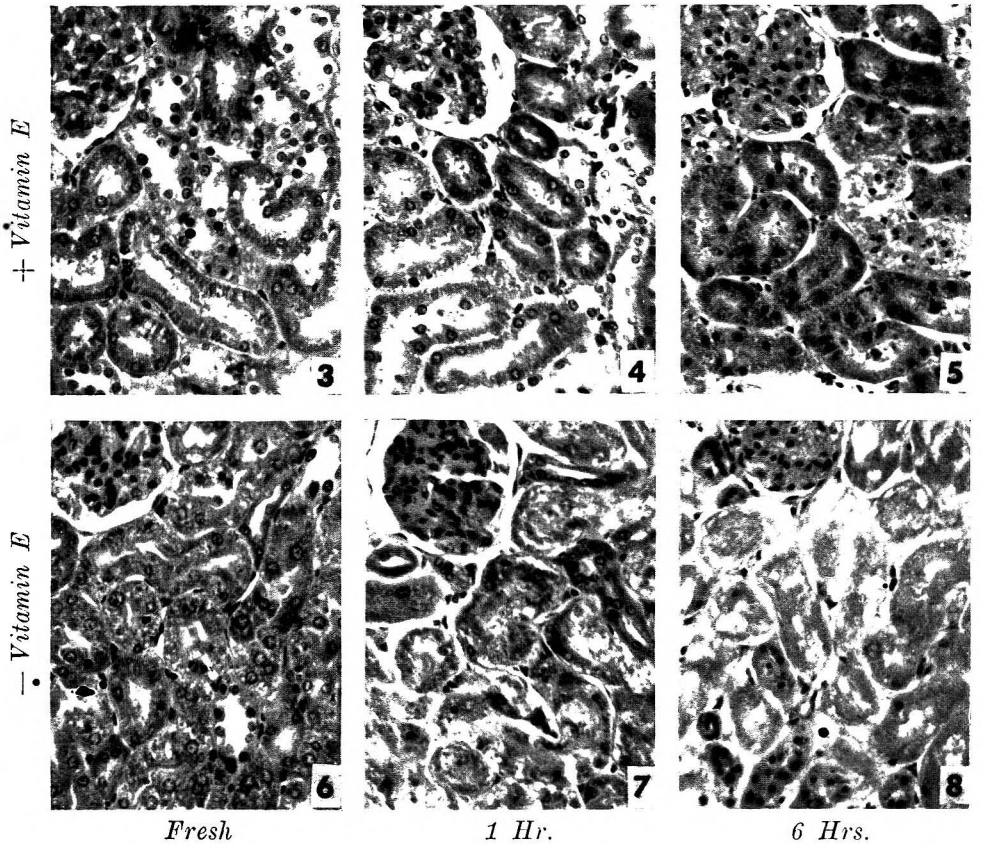

+ Vitamin E
− Vitamin E
3
4
5
6
7
8
Fresh
1 Hr.
6 Hrs.

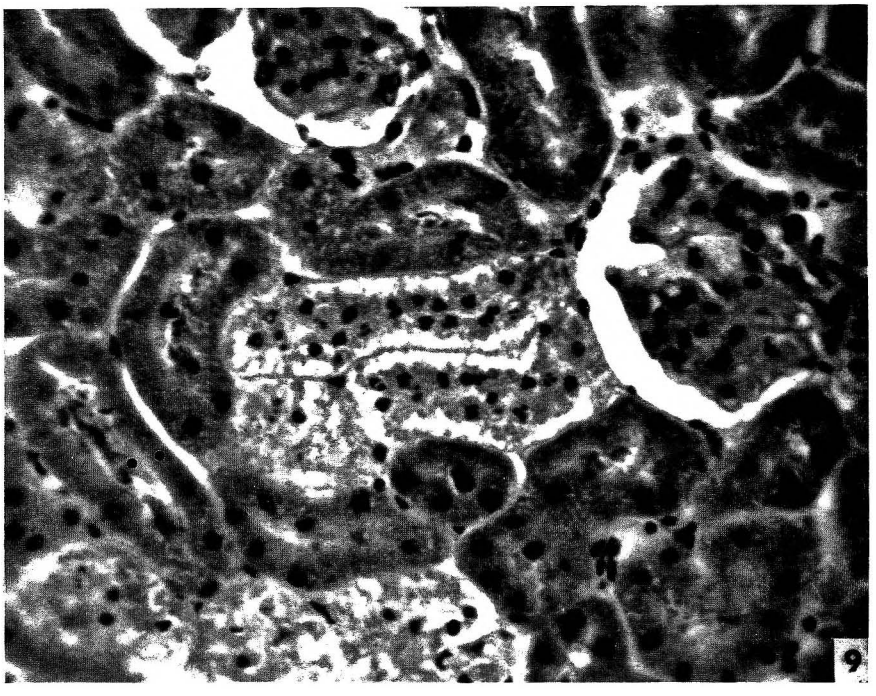
PLATE 2

EXPLANATION OF FIGURES

High power views from same kidney specimens as figures 5 and 8. Six-hour autolysis at 37°C. Bouin, H and E, 400 ×.

9 Vitamin E-treated animal. Distal tubules at center and lower left show nuclear fragmentation and more extensive cellular disorganization than do adjacent proximal tubules.

10 Vitamin E-deficient animal. Nuclei of distal tubules are pyknotic, but cellular structure is fairly well preserved. Nuclei of proximal tubules are no longer stainable, and cellular structure is extensively disrupted.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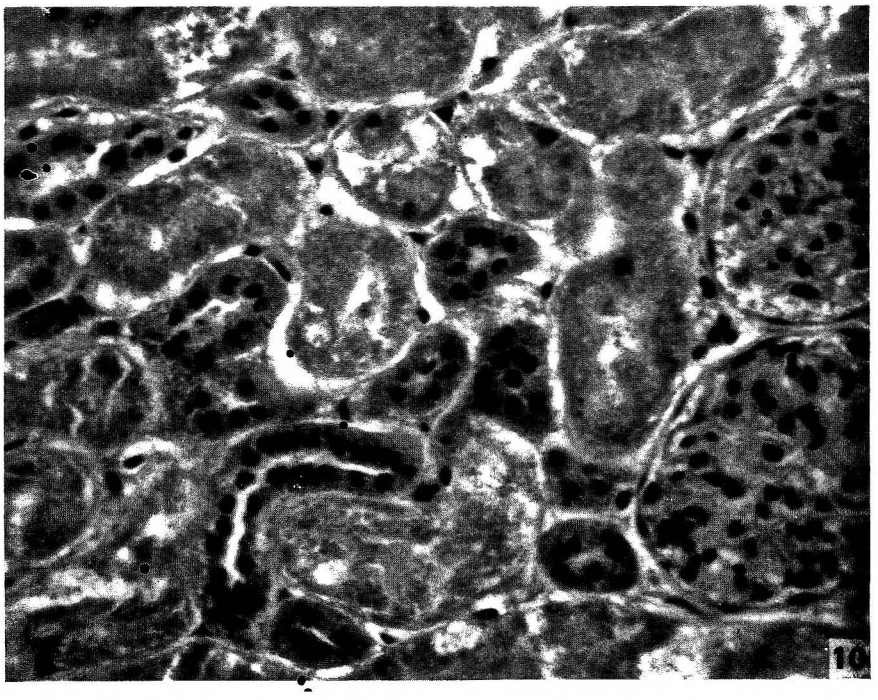
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APPARENT DIGESTIBLE ENERGY AND NITROGEN IN THE FOOD OF THE WEANLING RAT

INFLUENCE ON FOOD CONSUMPTION, NITROGEN RETENTION AND CARCASS COMPOSITION [1,2]

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The available energy content of a ration appears to exert a definite influence on food intake. Hill and Dansky ('50) reported that "the productive energy level of a ration was a major factor in controlling feed intake" of chicks, while Dansky and Hill ('51) noted that the chick has a "remarkable ability to compensate for reduced dietary energy level by increasing feed consumption." Sibbald et al. ('56) noted that the food intake of weanling rats was significantly influenced by the apparent digestible energy content of the ration and postulated that, within physiological limits, these animals eat to satisfy their energy requirements.

The influence of dietary protein level on feed consumption in chicks has been discussed in two recent reports. Hill and Dansky ('54) observed that feed intake was primarily controlled by the productive energy content of the ration while the "protein level had little or no effect on rate of feed consumption." Peterson et al. ('54) noted that the feed intake, within physiological limits, was governed by the energy

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needs, while "the protein intake limited the need for feed energy by limiting growth."

In several species the energy intake has been shown to influence nitrogen utilization. Working with rats, Swanson ('51) noted the protein-sparing action of energy. Calloway and Spector ('55) reported that the urinary nitrogen excretion of adult rats became progressively greater as the energy supply was reduced. Working with weanling rats, Meyer ('56) noted that increasing the indigestible portion of a food increased the total fecal nitrogen excretion though no change in endogenous urinary nitrogen excretion was observed. Sibbald et al. ('56) in an experiment in which two levels of digestible energy and one level of nitrogen were fed to weanling rats observed that 69% of the variation in nitrogen retention was associated with digestible energy consumption.

The ratio of protein to available energy has been shown to exert a considerable influence upon the fat deposition of animals. Hill and Dansky ('54) added oat hulls to a 20% protein basal diet for chickens and observed that as the level of indigestible material increased the carcass fat content decreased. Peterson et al. ('54) reported that low protein diets resulted in chickens yielding carcasses high in fat content. When increasing levels of celluflour were included in the feed the fat content of the carcasses decreased, but there was no significant change in the protein content.

The experiment reported herein was designed to study the following relationships in the weanling rat: (a) digestible energy and food consumption, (b) nitrogen intake and food consumption, (c) digestible energy and nitrogen retention and (d) the digestible energy:nitrogen ratio of the diet and carcass composition.

METHODS

Two male and two female rats were allotted to each of the 16 rations listed in table 1. In the formulation of these

rations 4 levels of a non-nutritive cellulose,[3] were combined with 4 levels of nitrogen. Additions of Alphacel or the nitrogen source or both were made at the expense of the sucrose portion of the ration. The choice of Alphacel as a diluent was based on an earlier experiment (Sibbald et al., '56) in which it was found that the addition of 10% of this material resulted in a proportional decrease in the overall digestibility of the ration.

The nitrogen source used in this experiment was identical with that used by Sibbald et al. ('56). As indicated in a footnote to table 1 the source consisted of a mixture of casein, lactalbumin, DL-methionine, L-histidine·HCl and DL-threonine calculated to supply the indispensable amino acid requirements of the rat in such a ratio that lysine was most limiting (Rose, '38; Block and Bolling, '51).

Following a 7-day ration acclimatization period the rats were placed in individual metabolism cages. The subsequent experimental period was limited to 7 days, for the rats were growing very rapidly and variations in weight, resulting from the feeding of rations differing in nutritive value, would have been magnified if a longer experimental period had been employed. Unpublished data[4] indicate that variations in weight have an important bearing upon energy intake.

Prior to use the metabolism cages were sprayed with a hot 2% boric acid solution. On termination of the experiment the cages were rinsed with distilled water, the washings being added to the urine which was collected in 50% sulphuric acid. The urine and washings from each cage were brought to 500 ml with distilled water prior to analysis. Feces were collected daily, dried in an air oven at 105° C. and then ground to a fine powder. Throughout the experiment the rats were fed ad libitum.

The rations and feces were analyzed for gross energy content using a Parr Oxygen Bomb Calorimeter while all

[3] Alphacel, a "non-nutritive cellulose" obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

[4] Bowland, J. P. ('56).

nitrogen determinations were by the method of Kjeldahl, using mercury as a catalyst. Individual analyses of urine and feces were made for each rat on experiment. Apparent digestible energy (hereinafter referred to as ADE) consumption was determined by subtracting total fecal energy from

TABLE 1

Rations [1] fed during acclimatization and metabolism periods

RATION	ALPHACEL [2]	NITROGEN [3] SOURCE "A"	SUCROSE	ANALYSIS	
				Gross energy	Gross nitrogen
	%	%	%	*Cal/100 gm*	*mg/100 gm*
1a	10	10.2	69.8	420	1424
1b	10	15.2	64.8	425	2031
1c	10	20.3	59.7	432	2664
1d	10	25.4	54.6	439	3312
2a	20	10.2	59.8	412	1363
2b	20	15.2	54.8	420	2122
2c	20	20.3	49.7	429	2716
2d	20	25.4	44.6	435	3400
3a	30	10.2	49.8	418	1490
3b	30	15.2	44.8	435	2092
3c	30	20.3	39.7	428	2811
3d	30	25.4	34.6	458	3428
4a	40	10.2	39.8	426	1418
4b	40	15.2	34.8	426	2072
4c	40	20.3	29.7	433	2686
4d	40	25.4	24.6	448	3349

[1] Each ration contained 5% Mazola oil, 4% salts (Jelinek et al., '52) and 1% vitamin mix (Sibbald et al., '56).

[2] Alphacel "non-nutritive cellulose." Nutritional Biochemicals Corporation, Cleveland, Ohio.

[3] Nitrogen source "A." Casein 58.7 gm (~ 8 gm N), lactalbumin 68.8 gm (~ 8 gm N), DL-methionine 0.5 gm, L-histidine·HCl 1.5 gm, DL-threonine 1.0 gm.

the gross energy intake; apparent digestible nitrogen (ADN) consumption was calculated in a similar manner. Nitrogen retention was determined by subtracting gross urinary nitrogen from the ADN consumption.

On termination of the experiment all rats were killed. The carcasses were cut into small pieces and dried in an air

oven at 105° C. for 4 hours. The semi-dry carcasses were then ground in a small Wiley mill to pass through a 20-mesh sieve, and returned to the oven for a further 4 hours. An earlier experiment (Sibbald, '55) indicated that for rats up to 150 gm in weight the above method of drying was very satisfactory, for heavier rats a longer period of drying was necessary.

The dry carcasses were analyzed individually for protein (N × 6.25) by the method of Kjeldahl and for fat by extracting in a Soxhlet apparatus for 20 hours with alcohol followed by 20 hours with ether (A.O.A.C., '55).

Since most of the variables studied were influenced by body weight it was necessary to remove any variation associated with this factor prior to determining the magnitude of the various relationships under consideration. This could have been accomplished by covariance but the simpler method of expressing the variables on a 100 gm body weight basis was used. The body weight referred to was the average of the initial and final body weights during the experimental period.

RESULTS

Food consumption. The principal mean values, relative to food consumption, obtained in the experiment are shown in table 2, while a summary of the correlations obtained during statistical treatment of the data is presented in table 3. The derivation of the r values listed followed the method outlined by Snedecor ('46).

Examination of the results indicated that as the level of Alphacel in the ration was increased (10, 20, 30 and 40%) the ADE content decreased and the food consumption per 100 gm body weight progressively increased. The increase was essentially proportional to the Alphacel level in the ration. Calculation of the ADE consumption per 100 gm body weight indicated that the increased food consumption compensated for the decreasing ADE content of the food. Thus the ADE consumption per 100 gm body weight was essentially the same at each Alphacel level. This indicates

TABLE 2

Mean values for data relating to the effect of apparent digestible energy and nitrogen on food consumption

RATION	NO. RATS	BODY WT. OF RATS	FOOD CONSUMPTION/100 GM BODY WT.	GROSS N CONSUMPTION/100 GM BODY WT.	APPARENT DIGESTIBLE				APPARENT DIGESTIBILITY OF FOOD
					Energy/100 gm food	Energy consumption/100 gm body wt.	Nitrogen/100 gm food	Nitrogen consumption/100 gm body wt.	
		gm	*gm*	*mg*	*Cal.*	*Cal.*	*mg*	*mg*	*%*
1a	4	76	84	1205	374	314	1299	1091	88
1b	4	83	84	1723	380	319	1888	1586	89
1c	4	87	85	2256	384	326	2483	2110	88
1d	4	85	88	2914	391	334	3056	2689	88
2a	4	79	100	1362	327	327	1192	1192	79
2b	4	86	93	1968	336	312	1911	1777	79
2c	4	96	98	2657	342	335	2400	2352	79
2d	4	87	92	3124	354	326	3146	2894	78
3a	4	81	106	1579	300	318	1286	1363	70
3b	4	86	110	2282	318	350	1864	2050	70
3c	4	82	104	2911	317	330	2542	2644	71
3d	4	85	105	3576	340	357	3066	3219	70
4a	4	78	120	1702	279	335	1189	1427	61
4b	4	79	116	2413	275	319	1777	2061	62
4c	4	77	124	3342	282	350	2259	2801	61
4d	4	83	112	3761	283	317	2950	3304	58

that on diets varying in ADE from 275 to 391 Cal. per 100 gm, weanling rats ate to satisfy their energy requirements.

The relationship between food consumption per 100 gm body weight and the ADE content of the ration is depicted graphically in figure 1. Each point represents the food consumption per 100 gm body weight of individual rats plotted

TABLE 3

Correlation coefficients relative to the influence of apparent digestible energy and nitrogen, and gross nitrogen on food consumption

		CORRELATION	D.F.	SIGNIFICANCE
				%
Gross correlations				
	r_{12}	0.858	62	1
	r_{12} (within replicates)	0.861	59	1
	r_{12} (within sex)	0.858	61	1
	r_{13}	0.119	62	..
	r_{14}	0.082	62	..
	r_{23}	0.247	62	..
	r_{24}	0.171	62	..
Partial correlations				
	$r_{12.3}$	0.861	61	1
	$r_{12.4}$	0.859	61	1
	$r_{13.2}$	0.088	61	..
	$r_{14.2}$	— 0.127	61	..
Multiple correlations				
	$R_{1.23}$	0.863	61	1
	$R_{1.24}$	0.860	61	1

1 is the food consumption, gm/100 gm body weight.
2 is the reciprocal of the apparent digestible energy, Cal./100 gm food.
3 is the reciprocal of the apparent digestible nitrogen, mg/100 gm food.
4 is the reciprocal of the gross nitrogen, mg/100 gm food.

against the reciprocal of the ADE per 100 gm of food. The reciprocal of the ADE content of the food was used in preference to the natural values because of the nature of the relationship involved. If a decrease in the ADE content of the ration resulted in a proportionate increase in the food consumption then the relationship would be:

$$Y = \frac{K}{Z} \qquad (1)$$

where, Y is the food consumption in grams per 100 gm body weight,

Z is the ADE content of the food (Cal. per 100 gm)

K is a constant

If $\frac{1}{Z} = X$, the theoretical equation (1) becomes:

$$Y = KX \qquad (2)$$

therefore the relationship of food consumption to the reciprocal of the ADE content of the ration was studied.

A straight line was plotted (fig. 1) by the method of least squares. The regression equation obtained was:

$$Y = -0.7 + 33188X \qquad (3)$$

$$\text{or } Y = a + bX \qquad (4)$$

where $a = -0.7 \pm 7.68$ gm

$b = 33188 \pm 2484$

and $s_{y.x}$ (the standard error of estimate for deviations from regression) = 7.28 gm

If a is equal to zero and b = K then the theoretical equation (2) is essentially the same as the derived regression equation (3). A "t" test indicated that a was not significantly different from zero, consequently equation (4) becomes:

$$Y = bX \qquad (5)$$

The best estimate of K (equation 2) may be obtained from:

$$K = \overline{yx} = 33064 \qquad (6)$$

where, $\overline{y}$ is the mean of Y

and $\overline{x}$ is the mean of X

A "t" test between b (33188, equation 4) and K (33064, equation 6) indicated no significant difference, thus the theoretical relationship shown in equation (2) is compatible with the regression equation obtained from the actual data (3).

A gross correlation (r_{12}) between food consumption per 100 gm body weight and the reciprocal of the ADE per 100 gm of food (table 3) yielded a highly significant r value of 0.858. Removing the influence of replication (r_{12} within replicates) and sex (r_{12} within sex) from the above correlation resulted in no change in the r values obtained (0.861 and

0.858) indicating that the gross correlation (r_{12}) was in no way forced by either replication or sex differences. It may therefore be seen that in this experiment approximately 74% of the variance in the food consumption of weanling rats was associated with the ADE content of the food. The original standard deviation in food consumption was 14.10 gm

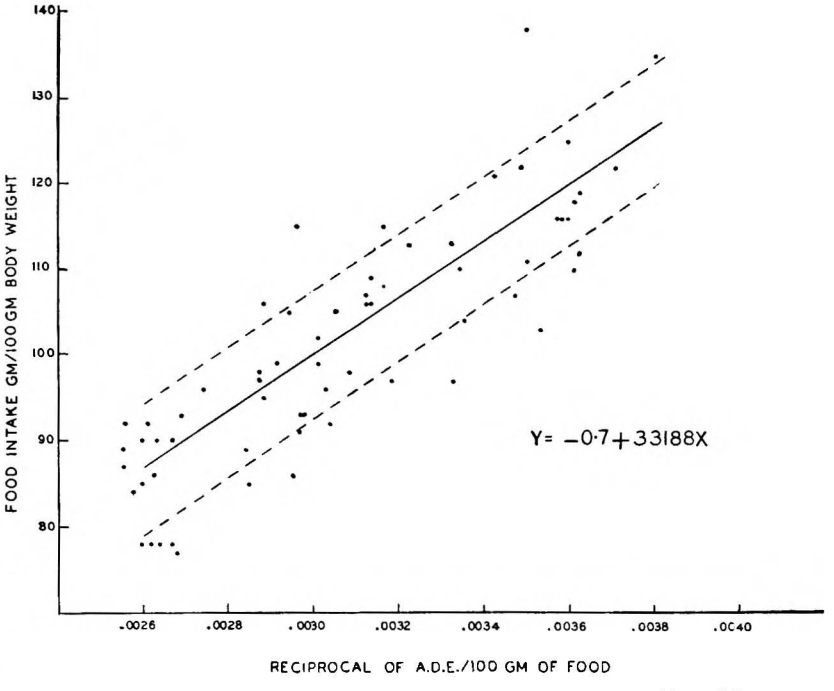

Fig. 1 Food intake versus the reciprocal of the apparent digestible energy content of the food. The broken lines represent the standard error of estimate for deviations from regression.

and the standard error of estimate for deviations from regression ($s_{y.x}$) 7.28 gm which indicates the variation remaining after removing that associated with the ADE content of the food.

The gross nitrogen (GN) content of the rations is listed in table 1, while the nitrogen consumption, both gross and digestible, per 100 gm body weight is recorded in table 2. These results indicate that there was no relationship between

GN or ADN consumption and food or ADE intake. Statistical support for these observations was obtained from a series of correlations. When the reciprocals of the ADN and GN content of the rations were correlated with food consumption non-significant r values of 0.119 and 0.082 were obtained. The partial correlations $r_{12.3}$ and $r_{12.4}$, in which the relationship between food consumption per 100 gm body weight and the ADE content of the rations was measured, the influence of the ADN and GN content of the foods being removed, were not different from the gross correlation r_{12} (0.861, 0.859 and 0.858 respectively). These findings, together with the partial correlations $r_{13.2}$ and $r_{14.2}$, measuring the relationships between food consumption and ADN and GN respectively when the influence of ADE was removed, which were of negligible size (0.088 and -0.127 respectively) further emphasize that neither the GN nor the ADN content of the rations exerted any effect upon food consumption.

Peterson et al. ('54) noted that the protein content of a ration exerted an influence upon the feed consumption of chickens through its ability to limit growth. In the present experiment the influence of weight changes was controlled by considering the average weight throughout the experiment. Therefore, a relationship such as that reported by Peterson et al. ('54) was not measured. It is suggested that in a short period in which change in weight, relative to total body weight, is small, the nitrogen level of a ration exerts no measurable influence on food consumption.

Nitrogen retention. The principal mean values, relative to nitrogen retention, obtained in this experiment are shown in table 4 A. The nitrogen consumption per 100 gm body weight increased as the ADE:GN ratio of the rations decreased, since the rats ate to satisfy their energy requirements. The percentage of GN apparently digested decreased ($P < 0.01$) as the level of Alphacel in the rations increased. This supports the work of Meyer ('56) who reported an increase in the metabolic fecal nitrogen of the rat when the

TABLE 4

Mean data relating to (A) the influence of apparent digestible energy on nitrogen retention and (B) carcass composition of experimental animals

RATION	A					B		
	NITROGEN CONSUMED /100 GM BODY WT.	NITROGEN DIGESTED	NITROGEN RETAINED	DIGESTIBLE NITROGEN RETAINED	DIGESTIBLE ENERGY/GM DIGESTIBLE NITROGEN IN FOOD	MOISTURE	FAT	PROTEIN
	mg	*%*	*%*	*%*	*Cal.*	*%*	*%*	*%*
1a	1205	91	80	87	288	69.6	11.4	15.5
1b	1723	92	73	78	201	70.7	10.2	16.0
1c	2256	93	60	64	155	70.4	10.6	16.6
1d	2914	92	47	51	128	69.6	11.9	16.4
Mean		92				70.1	11.0	16.1
2a	1362	87	76	87	274	69.7	11.5	15.4
2b	1968	90	67	74	176	70.4	9.5	16.1
2c	2657	88	58	66	143	70.9	10.5	16.8
2d	3124	92	47	51	112	70.7	9.8	16.5
Mean		89				70.4	10.3	16.2
3a	1579	86	76	88	233	71.7	8.4	15.6
3b	2282	89	59	66	170	71.5	8.4	16.3
3c	2911	90	53	58	125	71.4	8.6	16.2
3d	3576	89	45	50	111	71.9	8.7	16.3
Mean		88				71.6	8.5	16.1
4a	1702	84	68	81	235	71.7	8.9	15.4
4b	2413	86	56	65	155	71.3	9.0	16.2
4c	3342	84	44	52	126	70.8	10.1	15.8
4d	3761	88	41	46	96	72.1	8.6	16.9
Mean		86				71.7	9.2	16.1

cellulose content of the food was increased. Similar relationships for the pig have been reported by Crampton et al. ('55) and Lloyd and Crampton ('55).

The percentage of GN and ADN retained was not related to the nitrogen intake but was found to be largely influenced by the ADE content of the rations. It was anticipated that the relationship between the percentage ADN retained and the ADE per gram ADN in the food would be curvilinear as on high nitrogen rations some of the nitrogen would be used to supply energy resulting in a large urinary nitrogen excretion, while on low nitrogen diets the nitrogen intake would be limited and hence the endogenous nitrogen excretion would represent a larger percentage of the ADN as the intake of the latter decreased.

Subjecting the nitrogen retention data to statistical analysis (Snedecor, '46) yielded the following information. The association between the percentage ADN retained and the ADE per gram ADN in the food yielded a highly significant r value of 0.906 at 62 D.F. A test for curvilinearity of regression demonstrated a significantly ($P < 0.01$) improved relationship; a highly significant R value of 0.932 at 61 D.F. indicates an association of 87% between the variables. The quadratic regression equation for the curvilinear relationship is:

$$Y = -4.3 + 0.6768X - 0.001223X^2$$

where Y is the percentage ADN retained and X the ADE per gram ADN in the food. This equation yielded a maximum value for Y of 89.3% when X equalled 276.7 Cal. ADE per gram ADN. The original standard deviation in the per cent ADN data was 14.8% and the standard error of estimate for deviations from curvilinear regression ($s_{y.x}$) 5.36% which indicates the variation remaining after removing that associated with ADE:ADN ratio of the food.

Carcass composition. The mean values relating to the moisture, fat and protein content of the carcasses of the rats used in this experiment are reported in table 4 B. It is of interest to note that although the rats were fed the

experimental rations for a period of two weeks (one week acclimatization, one week experimental), during which time they increased their body weights by as much as 200%, there was very little variation in carcass composition between ration groups. A slight tendency for the moisture content of the carcasses to increase with the Alphacel content of the diets was noted, while the fat content tended to decrease. The protein content of the carcasses was very uniform between Alphacel levels although within Alphacel levels the low nitrogen rations tended to result in lower protein carcasses.

DISCUSSION

The results of this experiment have demonstrated that 74% of the variation in food consumption was associated with the ADE content of the ration. The data of table 2 indicate that in 7 days weanling rats eat approximately 330 Cal. ADE per 100 gm body weight. The statistical findings associated with the nitrogen retention data indicate that a level of 276.7 Cal. ADE per gram ADN in the food resulted in a maximum percentage of the ADN being retained. These results would suggest that in the formulation of rat rations the bulk should be so controlled that it is physiologically possible for the animals to consume 330 Cal. ADE per 100 gm body weight per week and that the ADE per gram ADN of the food should be in the region of 277 Cal. At energy levels lower than 277 Cal. the nitrogen fraction of the diets will be inefficiently utilized while at higher energy levels no better use of the nitrogen will be made and, as the food intake is largely controlled by the ADE content of the ration, the rate and efficiency of growth could be conceivably reduced due to sub-optimum nitrogen intake. At exceedingly high ADE levels relative to ADN, nitrogen starvation of the animals is a possibility.

Working with chicks Hill and Dansky ('54) and Peterson et al. ('54) have shown that the energy: protein ratio of the feed exerts a considerable influence upon fat deposition in

the carcass. As the ratio of energy to protein decreased a decrease in body fat was observed. Although the results of the present experiment demonstrated no major changes in carcass composition relative to changes in the ADE:ADN ratio of the food it is believed that such changes would have occurred had the animals been exposed to the experimental rations for a longer period.

SUMMARY

It has been demonstrated that on rations varying in apparent digestible energy content from 275 to 391 Cal. per 100 gm, weanling rats ate to satisfy their energy requirements. The nitrogen content of the ration appeared to exert a negligible influence upon food consumption. Mean values indicate that weanling rats will consume approximately 330 Cal. per 100 gm body weight per week.

Nitrogen retention was largely controlled by the ratio of apparent digestible energy per gram of apparent digestible nitrogen in the food. Statistical treatment indicated that a level of 277 Cal. apparent digestible energy per gram of apparent digestible nitrogen was optimal for maximum nitrogen utilization.

No differences in carcass composition resulted from maintaining weanling rats for a period of two weeks on the experimental diets. It was assumed that the short experimental period limited the manifestation of such differences.

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STUDIES ON GOITROGENIC AGENTS IN FOOD

I. GOITROGENIC ACTION OF GROUNDNUT

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The deficiency of iodine in the diet in relation to simple goiter is now well known. Also, the incidence of goiter in animals and in human subjects with normal dietary intake of iodine has been noticed often (Nutrition Rev., '50), thereby indicating the possibility of existence of factors other than iodine-lack in the etiology of simple goiter. This has led to an extensive search for goitrogenic agents in foods all over the world with many interesting results. The finding of Chesney, Clawson and Webster ('28), Webster and Chesney ('30) and Marine et al. ('30) that cabbage feeding produced thyroid hyperplasia in rabbits must be considered as the first milestone towards progress in this field. Subsequently a large number of foodstuffs like cauliflower, rape, mustard and cabbage seeds, turnip, rutabaga, Brussels sprouts, etc., were found to possess goitrogenic properties (Kennedy and Purves, '41; Greer et al., '48). Most of the workers have shown that among these various foodstuffs, those belonging to the Brassica family possessed the greatest goitrogenic effect. The isolation of 1,5-vinyl 2 thio-oxazolidone by Astwood et al. ('49a, b) from ground rutabaga was of considerable significance and gave a great impetus to the search for similar goitrogenic agents in foodstuffs more commonly used in various diets. The role of soybean as a goitrogenic agent even in the presence of large amounts of iodide in the diet was described by McCarrison ('33b) and later on by various other investigators (Sharpless, Pearsons and Prato, '39;

Wilgus, Gassner, Patton and Gustavson, '41; and Halverson, Zepplin and Hart, '49).

McCarrison ('33b) has incriminated groundnut (Arachis hypogaea) also as a goitrogenic agent even in the presence of large amounts of iodine in the diet, especially when the diet is poor in essential ingredients like vitamins. This is of special significance to India, where groundnut cake is one of the main nutrient materials fed to cows. Groundnut either raw or fried is also used in large amounts for human consumption in various parts of India. The original work of McCarrison (loc. cit.) on groundnut was not very comprehensive and it was therefore considered worthwhile to study this goitrogenic effect in greater detail.

EXPERIMENTAL

The effects of groundnut supplements on the growth of albino rats and also on the size, iodine content of thyroids, on the uptake of radioactive iodine (I^{131}) by the thyroid and on the urinary excretion of radioactive iodine were studied. Analyses of groundnut for the presence of well-known dietary goitrogenic agents like 1,5-vinyl, 2 thio-oxazolidone, cyanogenetic glycosides, calcium and *p*-aminobenzoic acid were also carried out.

The basal diet used in these experiments was similar to that of Halverson, Zepplin and Hart ('49), except that the calcium content was reduced by 75%, so that the goitrogenic effect of calcium would not be pronounced. The percentage composition of the basal diet was as follows: wheat gluten 30; ground yellow corn 68; and salt mixture 2. The percentage composition of the salt mixture prepared from reagent grade chemicals was: $Ca_3(PO_4)_2$, 24; NaCl, 45; KCl, 15; $MgSO_4 \cdot 7H_2O$, 10.0; $FeCl_2 \cdot 4H_2O$, 4.8; $MnSO_4 \cdot H_2O$, 0.8; $ZnSO_4 \cdot 7H_2O$, 0.2; and $CuSO_4 \cdot 5H_2O$, 0.2. The water-soluble vitamins were dry mixed with the wheat gluten before the diet was prepared. The vitamin levels added per kilogram of diet were as follows: 6.0 mg thiamine hydrochloride, 9.0 mg riboflavin, 9.0 mg pyridoxine hydrochloride, 60.0 mg cal-

cium pantothenate, 20.0 mg nicotinic acid, 300.0 mg *p*-amino-benzoic acid, 1.0 gm inositol, 2.0 gm choline chloride, 0.1 mg biotin and 0.3 mg folic acid. The fat-soluble vitamins were given as a commercial preparation,[1] to an ounce of which 100 mg of α-tocopherol and 10 mg of 2-methyl, 1,4-naphtha-quinone were added. The rats were given this vitamin solution by dropper once a week at the rate of 12 drops per rat per week. The average iodine content of the diet used (without the salts) was 280 µg per kilogram.

Experimental animals. For each set of experiments 28 albino rats with an average body weight of about 44 gm were divided into 7 groups of 4 each, two males and two females in each group. The animals were housed in individual cages and care was taken in keeping the cages clean, in order to eliminate the factor due to coprophagy in the production of goiter as stressed by McCarrison ('33a).

The diets of the different groups in each batch were as follows: group I, basal ration only; group II, basal ration 80 parts and groundnut cake 20 parts; group III, basal ration 80 parts and defatted groundnut cake 20 parts; group IV, basal ration 85 parts and defatted groundnut cake 15 parts; group V, basal ration plus 300 mg of pure groundnut protein per rat per day; group VI, basal ration plus a water extract of groundnut cake equivalent to 1.5 gm of groundnut cake per rat per day and group VII, basal ration 80 parts with groundnut cake 20 parts and an iodine supplement of 1 µg of iodine per rat per day as potassium iodide. All rats were fed ad libitum. Distilled water was used in the preparation of the foods and also for drinking purposes. The growth rate of the rats was followed by weighing the individual rats at weekly intervals. The food consumed was determined frequently and was found to be 10 to 12 gm per rat per day.

Preliminary experiments showed that a minimum of 7 weeks was necessary for the goitrogenic effect of groundnut to become marked. The animals were therefore kept on the

[1] Adexoline vitamin A and D concentrate, Glaxo.

experimental diets for 7 weeks after which they were put in metabolic cages and each given 100 microcuries of iodine^{131} in KI carrier intraperitoneally. The urine was collected for 24 hours at the end of which the animals were sacrificed under ether anaesthesia and the thyroids dissected out. The abdominal cavity was also opened and any urine in the bladder aspirated in a syringe and added to the urine collected earlier. In another set of similar experiments the thyroid glands of rats in each group were separately pooled and analysed for their iodine content.

Methods. The method of Karns ('32) as modified by Von Kolnitz and Remington ('33) was employed for the determination of iodine content of the rations excluding the salt mixture as recommended by Halverson et al. ('49). The procedure described by Taurog and Chaikoff ('46) was followed for the separation of different iodine-containing fractions from the thyroid gland and the colorimetric method of Walaszek-Piotrowski and Koch ('52) was adopted for the idoine determinations. The radioactivity in the different fractions namely, the thyroxine, diiodotyrosine and inorganic iodine fractions of the thyroid glands were determined according to the method of Perlman, Chaikoff and Morton ('41).

Tests for the presence of the following known dietary goitrogenic agents in groundnut cake were also carried out: for thio-oxazolidone by the method of Astwood et al. ('49a, b); for cyanogenetic glycosides by the method of Koenig ('20); for calcium by the A.O.A.C. procedure ('50) and for *p*-aminobenzoic acid by the method of Eckert ('43).

RESULTS AND DISCUSSION

The average body weights and thyroid weights of rats in different groups are given in table 1. The weights of thyroid glands of rats fed groundnut preparations were considerably increased. In the group fed groundnut protein, however, there was no appreciable increase in the thyroid weight. The thyroids of rats fed with a water extract of the groundnut

cake were markedly enlarged, thereby showing that the goitrogenic factor was water soluble.

The distribution of iodine among the three fractions of the thyroid gland hydrolysate namely, inorganic, thyroxine and diiodotyrosine fractions are given in table 2. The addition of groundnut cake either raw or defatted to the basal ration did not significantly alter the total iodine content of the gland; however, there was a difference between the inorganic

TABLE 1

Influence of different groundnut rations on body weight and thyroid weight of albino rats

GROUP [1]	AVERAGE INITIAL WEIGHT	AVERAGE FINAL WEIGHT	INCREASE IN WEIGHT	AVERAGE WEIGHT OF THYROID GLAND (FRESH)	RANGE OF THYROID WEIGHTS
	gm	*gm*	*gm*	*mg*	*mg*
I	45	91	46	9	8–10
II	44	97	53	19	16–20
III	44	108	63	24	19–28
IV	45	103	58	20	17–23
V	44	98	54	12	9–13
VI	45	95	50	20	19–22
VII	45	88	43	10	9–11

[1] Group I received the basal ration; group II, 80 parts basal ration and 20 parts groundnut cake; group III, 80 parts basal ration and 20 parts defatted groundnut cake; group IV, 85 parts basal ration and 15 parts defatted groundnut cake; group V, basal ration and 300 mg groundnut protein per rat per day; group VI, basal ration and water extract of groundnut cake equivalent to 1.5 gm of groundnut cake per rat per day and group VII, 80 parts basal ration and 20 parts defatted groundnut cake plus 1 μg of potassium iodide per rat per day.

and organic fractions, the former having a higher value. In the organic fraction the percentage distribution of iodine between diiodotyrosine and thyroxine fractions appears to be more or less constant. In the iodine-supplemented group the total iodine was considerably increased, the main increase being in the inorganic fraction. The groundnut protein feeding does not alter the iodine distribution between the fractions, while the water extract of the groundnut cake has the same effect as that of whole groundnut cake. The radioactivity

measurements in different fractions of the thyroid gland given in table 3, confirm the results obtained with iodine determinations mentioned above. The percentage radioactivity excreted in the urine in 24 hours (table 3) was found

TABLE 2

Iodine content of the thyroid glands in different groups of rats

GROUP [1]	IODINE CONTENT OF THYROID GLANDS AVERAGE OF EACH GROUP			PERCENTAGE OF INORGANIC IODINE	PERCENTAGE OF ORGANIC IODINE
	Total	Thyroxine iodine	Diiodotyrosine iodine		
	μg	μg	μg		
I	6.30	1.64	4.36	4.8	95.2
II	6.08	1.38	3.61	17.9	82.1
III	6.10	1.35	3.50	20.5	79.5
IV	6.00	1.36	3.58	18.2	81.8
V	6.20	1.61	4.37	5.4	94.6
VI	5.84	1.35	3.58	19.8	80.2
VII	10.80	2.68	6.95	10.8	89.2

[1] See footnote 1, table 1 for description of diets.

TABLE 3

Radioactivity distribution in the thyroid glands and in the urine in different groups of rats at the end of 24 hours

GROUP [1]	PERCENTAGE OF ADMINISTERED ACTIVITY RECOVERED IN DIFFERENT FRACTIONS OF THYROID			PERCENTAGE OF ADMINISTERED I^{131} EXCRETED IN THE URINE
	Inorganic I_2 fraction	Thyroxine fraction	Diiodotyrosine fraction	
I	0.30	3.90	10.4	48 ± 2
II	3.50	3.00	9.1	56 ± 2
III	3.30	2.90	8.6	60 ± 4
IV	2.22	2.80	9.15	58 ± 1
V	0.32	2.96	9.82	47 ± 3
VI	2.84	2.61	9.08	57 ± 2
VII	0.40	4.80	13.60	61 ± 3

[1] See footnote 1, table 1 for description of diets.

to be greater in groups II, III, IV, VI and VII, revealing thereby a greater amount of iodine excretion in these groups. Feeding of groundnut protein (group V) did not increase the excretion of iodine^{131}.

As mentioned earlier, an equal number of male and female rats were used in each of these groups. No significant dif-

ference was noticed in the iodine content, radioactive iodine concentrating power or in urinary excretion of I^{131}, between the two sexes in the respective groups. However the thyroid weights were greater (15 to 20%) in the females in each of the groups.

All these results show that groundnut in the proportions used has a goitrogenic effect on albino rats and the factor concerned appears to be more concentrated in defatted cakes and is water soluble. Groundnut cake analysed for various known dietary goitrogenic agents showed that it was free from thio-oxazolidones and cyanogenetic glycosides. *p*-Amino-benzoic acid was found only in traces and the calcium content varied from 0.067 to 0.074%. Hence this goitrogenic effect of groundnut cannot be associated with any of these substances.

The goitrogenic effect of groundnut was counteracted by supplements of small amounts of iodine, a finding which is contradictory to that of McCarrison ('33b), who observed that both groundnut and soybean are goitrogenic even in the presence of large amounts of iodine in the diet. However, Halverson et al. ('49) have been able to show that the goitrogenic effect of soybean is reversed by the addition of small amounts of iodine to the diet. It must, however, be remembered that the experimental diet used by McCarrison in 1933 was lacking in "essential ingredients" like vitamins and the amount of iodine in the diet was enormous. Iodine in large amounts is known to have an antithyroid effect (Merton et al., '44). Thus in McCarrison's work, these two factors — high inorganic iodine and vitamin deficiencies — appear to have operated in addition to the mild goitrogenic action of groundnut.

SUMMARY

1. The goitrogenic effect of groundnut was studied using albino rats. The goitrogenic factor appeared to be water soluble and could not be identified with any of the known dietary goitrogens.

2. This goitrogenic effect was inhibited by dietary supplements of small amounts of iodine as potassium iodide.

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STUDIES ON GOITROGENIC AGENTS IN FOOD

II. GOITROGENIC ACTION OF ARACHIDOSIDE

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In an earlier communication (Srinivasan et al., '57) it was shown that groundnut possesses a mild goitrogenic effect. Preliminary studies revealed that the peanut meal containing a higher amount of the red skin, as estimated by the method of Stansbury and Hoffpauir ('52) was able to bring about a greater enlargement of the thyroid gland in albino rats, when compared with rats fed diets containing depigmented peanut meal. It was therefore considered worth while to investigate the effect of the glycoside from the red skin of groundnut on the thyroid gland of albino rats.

EXPERIMENTAL

Arachidoside, the glycoside from groundnut *(Arachis hypogaea)* was prepared by the method of Tayeau and Masquelier ('47, '48) and was used for (a) the study of its influence on the thyroid glands and (b) the study of its metabolism and urinary excretion in albino rats.

Twelve albino rats, weighing on an average 100 gm, were caged separately and were divided into three groups of 4 each, each group containing two males and two females. The basal ration used was the same as the one used in part I of this series (Srinivasan et al., '57). Group I was fed the basal ration only, while group II was fed the basal ration plus 10 mg of arachidoside per rat per day and group III was fed the basal ration with 10 mg of arachidoside and 10 µg of iodine as potassium iodide per rat per day. The rats

were fed 15 gm of basal diet daily, the average consumption being 12 to 14 gm per rat per day. Distilled water was given ad libitum.

The experimental feeding was continued for a period of 7 weeks as in the previous study, individual weights being taken at the end of every week. At the end of the 7th week, the animals were sacrificed under ether anaesthesia, the thyroids dissected out quickly and weighed in a Roller-Smith torsion balance. The glands were then examined histologically.

For the study of the urinary metabolites excreted after arachidoside feeding, 9 albino rats of about 150 gm body weight were kept separately in metabolic cages. They were divided into three groups of three each with group I serving as the control, with 15 gm of basal ration only, while in group II the rats were fed 15 gm of basal ration plus 50 mg of arachidoside and in group III, the iodine supplement of 10 µg of iodine as potassium iodide was included along with the 15 gm of basal ration and 50 mg of arachidoside for each rat. The urine was collected for 24 hours and was analysed for the following: unchanged anthocyanin by butanol extraction of acidified urine; free phenols and total phenols by the procedure of Bray, Thorpe and White ('50); ethereal sulfate by the method of Treon and Crutchfield ('42); glucuronide by the method of Bray, Humphris, Thorpe, White and Wood ('52) and total iodine by the method of Perkin ('33). The iodine content of the phenolic fraction obtained by the ether extraction of the hydrolysed urine (as in the procedure for total phenols) was also determined to see whether iodinated phenolic compounds had been formed.

The influence of iodination and bromination on the glycoside was also studied as follows: 25 mg of the glycoside were dissolved in 25 ml of phosphate buffer (pH 5.8). Iodine in KI (calculated in the ratio of 6 atoms per mole of the glycoside) was dissolved in 25 ml of phosphate buffer (pH 5.8). Both solutions were equilibrated for one hour at 37°C., mixed and let stand for 24 hours at 37°C. The chocolate-brown

precipitate that settled was collected in a centrifuge tube, washed well with ether and dried. There was practically no free iodine left over at the end of this period (5 ml of the supernatant medium would react with only 0.225 ml of N/200 $Na_2S_2O_3$). The precipitate weighed 25 mg and it contained 19.88% iodine, by analysis.

Similarly when the glycoside was treated with bromine water, an orange yellow precipitate was obtained, which was found to be a bromo derivative.

TABLE 1

Influence of arachidoside feeding on the body weights and thyroid weights of albino rats

GROUP [1]	AVERAGE INITIAL WEIGHT	AVERAGE FINAL WEIGHT	INCREASE IN WEIGHT	AVERAGE WEIGHT OF THYROID GLAND (FRESH)	RANGE OF THYROID WEIGHTS
	gm	*gm*	*gm*	*mg*	*mg*
I	100	165	65	9	8–11
II	102	206	104	24	18–26
III	100	182	82	11	9–13

[1] Group I, basal ration: group II, basal ration and 10 mg of arachidoside per rat per day; group III, basal ration and 10 mg of arachidoside and 10 μg of potassium iodide per rat per day.

RESULTS AND DISCUSSION

From table 1, it will be seen that arachidoside apart from increasing the thyroid weight has a good growth-promoting effect. Addition of small amounts of iodide as potassium iodide to the arachidoside supplemented diet is found to maintain the thyroid weight within the normal range. Histological examination of the thyroid slices showed that arachidoside feeding produced a typical colloid picture. Examination of thyroid slices of rats fed the above diet plus a supplement of potassium iodide, however, showed a normal thyroid picture. The results of the study of the phenolic excretion are given in table 2 and these reveal that the glycoside is well metabolised in the body. A greater percentage of both free and conjugated phenols are excreted in the urine of animals

fed with this glycoside. A significant amount of iodine is present in the phenolic fraction of the urine excreted by rats fed arachidoside (table 3). If this presence of iodine in the phenolic fraction of the urine is indicative of a preferential iodination of the phenolic metabolites of the glycoside in the thyroid tissue leading to the goitrogenic action of the glycoside, it would then bring the latter into the group of "aromatic

TABLE 2

Amounts of different phenolic products excreted in the urine in 24 hours by albino rats fed 50 mg of arachidoside

GROUP [1]	UNCHANGED GLYCOSIDE	FREE PHENOL	TOTAL PHENOL	GLUCURONIDE	ETHEREAL SULPHATE
	mg	*mg*	*mg*	*mg*	*mg*
I	Nil	12	39	32	7
II	Nil	19	82	65	14
III	Nil	20	64	62	12

[1] See footnote 1, table 1 for description of diets.

TABLE 3

Iodine excreted in the urine in different groups in 24 hours

GROUP [1]	TOTAL IODINE	IODINE IN PHENOLIC FRACTION
	µg	*µg*
I	0.2	Nil
II	0.5	0.2
III	1.2	< 0.2

[1] See footnote 1, table 1 for description of diets.

thyroid inhibitors" of Fawcett and Kirkwood ('53). This view is further supported by the ease with which this glycoside may be halogenated *in vitro*.

SUMMARY

Arachidoside, the glycoside isolated from groundnut is found to be goitrogenic and as a result of the study of phenolic excretion in the urine, it is suggested that the effect is due to preferential iodination of the phenolic metabolites in the thyroid tissue.

ACKNOWLEGDMENT

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AGE DIFFERENCES IN THE EFFECTS OF L-CYSTINE AND DL-METHIONINE ON LIVER CHOLESTEROL STORAGE IN THE RAT[1]

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A short-time feeding experiment with adolescent rats (Okey and Lyman, '54) gave some indication that the lipotropic effect of protein toward cholesterol might be due largely to the methionine furnished by the protein but that L-cystine feeding tended to facilitate liver cholesterol storage. In that study it was also noted that the rats fed an L-cystine supplement with a "low adequate" level of dietary protein (15%) tended to eat more and to gain more weight than did the DL-methionine-supplemented rats given the same basal diet.

Later, the data from a number of series of rats fed, from weaning, levels of protein varying from 15 to 30% were evaluated. These data showed that, in males at least, the lipotropic effect of protein above the level required for a "normal" rate of growth might vary markedly with the age and sex of the animal (Okey and Lyman, '56). The highest liver cholesterol values were observed at 7 to 8 weeks after weaning and in males fed the basal diet containing 15% protein. They were associated with a high food intake and a rapid growth rate. It seemed desirable, therefore, to try to find whether there was a relationship between the effects of methionine and cystine on appetite and adolescent growth, on the one hand, and on liver cholesterol retention on the other.

[1] Supported in part by research grants from the National Heart Institute, Public Health Service, United States Department of Health, Education and Welfare H-1013.

EXPERIMENTAL

The diets were those previously used (Okey and Lyman, '54), i.e.: B, the basal 15% casein-egg albumin diet with 1% cholesterol; HP (B with 15% extra egg albumin substituted for 15% sucrose); BM (B plus extra DL-methionine approximately equivalent to that furnished by 15% egg albumin (0.9%); BC (diet B plus cystine approximately equivalent to that furnished by 15% egg albumin (0.3%). The basal diet (B) furnished about 0.6% methionine and 0.3% cystine. Included in the 10-week series were rats fed diets I (B without cholesterol), II (HP without cholesterol), and BCM (B plus both cystine and methionine).

Rats were placed on diet at weaning and sacrificed at (a) the 7- to 8-week periods at which maximal differences had been observed between the animals fed 15% and those fed 30% protein and (b) the 10-week period at which differences had been markedly diminished (Okey and Lyman, '56).

The rats sacrificed at 7 to 8 weeks were from the same lot of litters. One series of 7-week animals of the B, HP, and BC groups were given, each day, only the mean amounts of food consumed by a BM group the previous day. Other B, HP, and BC groups of rats of like size were fed ad libitum, and careful food intake records were kept. A 5- to 7-day delay in autopsy time of the latter groups was necessary because of the limited facilities available for liver nitrogen determinations. The 10-week rats were from a different lot of litters and were all fed ad libitum.

Except for the animals fed the control diets without cholesterol, each age and diet group consisted of 10 males and 11 or 12 females. Control groups were half those sizes.

The care of animals at autopsy and the preparation and analysis of tissues have been previously described (Okey and Lyman, '54). Liver nitrogen was determined by a semimicro Kjeldahl procedure using about 50 to 75-mg samples. Food cups were removed at 10:00 P.M. preceding the day of autopsy.

RESULTS AND DISCUSSION

Food intake and growth. Weanling rats, in contrast to the half-grown animals studied previously, were found to eat the methionine-supplemented diet readily. The attempted "pair-feeding" to the BM group actually resulted in series of BC, B, and HP groups of rats with slightly lower total food intakes than those of the BM groups (table 1).

TABLE 1

Mean total weight gains and food intakes

DIET [1]	MALES		FEMALES	
	Total gain	Total intake	Total gain	Total intake
	gm	*gm*	*gm*	*gm*
7 weeks [2]				
B "pair-fed" [3]	166 ± 10.6 [4]	512 ± 14	121 ± 5.4	443 ± 5
ad lib.	177 ± 9.5	507 ± 21	131 ± 3.0	447 ± 10
HP "pair-fed" [3]	173 ± 13.1	512 ± 12	118 ± 4.8	433 ± 10
ad lib.	166 ± 13.4	480 ± 12	118 ± 8.8	428 ± 22
BM ad lib.	197 ± 7.3	551 ± 23	130 ± 7.0	467 ± 18
BC "pair-fed" [3]	174 ± 8.3	520 ± 11	120 ± 6.2	448 ± 8
ad lib.	206 ± 9.5	564 ± 10	127 ± 9.0	445 ± 18
10 weeks				
I	235 ± 13.4	836 ± 55	158 ± 5.5	726 ± 38
II	212 ± 7.2	778 ± 35	136 ± 7.2	643 ± 25
B	185 ± 9.6	702 ± 36	133 ± 7.8	668 ± 34
HP	200 ± 11.1	700 ± 20	129 ± 11.3	627 ± 11
BM	200 ± 8.0	731 ± 31	129 ± 5.0	650 ± 23
BC	248 ± 9.8	843 ± 34	147 ± 6.3	691 ± 24
BCM	200 ± 3.2	705 ± 26	147 ± 6.2	689 ± 21

[1] Diet B = basal 15% casein-egg albumin diet with 1% cholesterol; HP = B with 15% extra egg albumin in place of 15% sucrose; BM = B + extra DL-methionine approximately equivalent to that furnished by 15% egg albumin (0.9%); BC = B + cystine approximately equivalent to that in 15% egg albumin (0.3%); I = B without cholesterol; II = HP without cholesterol; BCM = B + both cystine and methionine.

[2] The ad libitum data were for the first 7 weeks of the study only, and are therefore strictly comparable with the data for the "pair-fed" groups.

[3] The term "pair-fed" is used for the rats offered daily the mean amounts of food consumed by the BM group of the same sex on the previous day.

[4] Standard error.

Week to week analyses of the comparative food intakes and weight gains of the groups of male rats showed that only the ad libitum group fed the BC diet ate more than the BM males consistently and throughout the entire experimental period. Mean food intakes of all the other groups were, however, as high or higher than those of the BM rats for the first three to 5 weeks and then tended to drop below them. For the ad libitum rats these drops were usually smaller than for the rats which had been offered only the amount of food eaten by the BM rats, and therefore had had lower food intakes during the early weeks of the experiment. The resulting differences in growth rates of the ad libitum and "pair-fed" groups at the time of autopsy were considered sufficient to justify separation of the two series in evaluation of the data. Females showed the same trends as males, but the actual differences between groups were so small as to be insignificant.

The ad libitum-fed rats constituting the 10-week groups were, on the average, 3 to 5 gm smaller at weaning than were the 7- to 8-week series. They gained weight somewhat more slowly for two to three weeks thereafter. Consequently, they usually reached a maximum rate of gain per week during the 7th rather than the 6th week after weaning. Growth rates had, however, dropped sharply by the 9th week. At the time of sacrifice most of the *10-week males* were gaining at about 10 gm per week as contrasted to more than 15 gm for the 7- to 8-week males. The exceptions were the cystine-supplemented males which were still gaining more than 15 gm per week. *Females* showed small differences in food intake and weight gains, but the trends of variation with diet were in the direction observed in males.

For the 10-week period, males fed the cystine-supplemented diet had significantly higher total weight gains and food intakes than did males fed the basal diet (B), the high protein diet (HP), or the methionine and the methionine-cystine supplemented diets (BM) and (BCM) ($p < 0.01$). Cholesterol-fed females given extra cystine (BC and BCM) usually gained

more weight than did those fed the high protein diet (HP) or the methionine diet (BM) but not significantly more than those fed the basal diet with cholesterol (B). For some reason, the controls without cholesterol (diet 1) gained somewhat more, and the rats fed 15% protein with cholesterol (B) gained a little less during the 10-week study than did comparable rats in other, similar studies. Taking this into consideration, however, it seems fair to conclude that:

1. Methionine added to a 15% diet did not inhibit the food intake or growth when fed to weanlings, for periods up to 10 weeks.
2. Extra cystine tended to increase food intake and weight gain — effects which became more noticeable after the 8th week.

Cystine-supplemented rats, especially females, appeared at autopsy to have a greater proportion of fat than did those on the high protein or methionine-supplemented diets. A small number of nitrogen determinations and Soxhlet extractions of decapitated and eviscerated carcasses also gave some indication of an increase in body fat. Standardization of technique and more samples would be necessary to make the data conclusive.

Liver lipid percentages, at 7 weeks, were about the same in the "pair-fed" males given the basal diet (B) only, and in those given cystine (BC) (table 2). Percentages in both groups were much higher than those in the groups given extra protein (HP) and methionine (BM). The ad libitum males given diet B had higher liver lipid percentages than the BC group, but the latter had slightly larger livers. (It will be remembered that all ad libitum rats were a few days older than the "pair-fed" groups at autopsy.) At 10 weeks, however, the liver lipids in the B group were much lower than in the BC group. At that time there was comparatively little difference in lipid values for the B, BM, and BCM groups, but values for the HP group were significantly lower than any others except the controls on cholesterol-free diets.

TABLE 2

Liver lipids, cholesterol and nitrogen

(Means with standard errors)

DIET AND GROUP [1]	LIVER WT.	TOTAL LIPIDS	TOTAL CHOLESTEROL	NITROGEN	LIPID N RATIO [2]
	gm	*% moist wt.*	*% moist wt.*	*% moist wt.*	
7–8 wks. Males					
B, "pair-fed" [3]	11.4	16.5 ± 2.2 [4]	3.62 ± 0.38	2.74	6.24
ad lib.	12.9	22.9 ± 2.8	3.68 ± 0.34	2.75	8.78
HP, "pair-fed" [3]	9.1	6.5 ± 0.5	1.11 ± 0.17	3.07	1.93
ad lib.	8.4	6.6 ± 0.4	1.16 ± 0.16	3.56	1.87
BM	10.4	8.7 ± 0.9	1.72 ± 0.29	3.13	2.74
BC, "pair-fed" [3]	10.6	17.3 ± 2.5	2.71 ± 0.08	2.98	6.52
ad lib.	13.4	16.3 ± 2.1	2.69 ± 0.28	3.20	5.40
10 wks. Males					
B	9.5	10.3 ± 0.5	2.08 ± 0.20	3.14	3.18
HP	9.1	5.9 ± 0.3	0.90 ± 0.13	3.38	1.78
BM	9.1	9.1 ± 0.7	1.69 ± 0.23	3.34	2.75
BC	12.1	15.6 ± 0.8	3.00 ± 0.40	2.91	5.52
BCM	9.4	9.3 ± 0.3	1.81 ± 0.15	3.09	3.09
7–8 wks. Females					
B, "pair-fed" [3]	6.5	8.6 ± 1.8	1.59 ± 0.24	3.37	2.57
ad lib.	6.5	9.8 ± 1.0	1.63 ± 0.26	3.51	2.87
HP, "pair-fed" [3]	6.1	6.9 ± 0.7	1.54 ± 0.33	3.55	1.94
ad lib.	5.6	5.4 ± 0.4	0.80 ± 0.15	3.79	1.44
BM	6.4	6.7 ± 0.5	1.53 ± 0.28	3.65	1.84
BC, "pair-fed" [3]	5.9	9.9 ± 1.0	1.73 ± 0.23	3.51	2.84
ad lib.	6.5	8.7 ± 1.3	1.40 ± 0.29	3.60	2.49
10 wks. Females					
B	5.8	7.6 ± 0.9	1.26 ± 0.19	3.48	2.23
HP	5.7	5.6 ± 0.5	1.93 ± 0.20	4.18	1.56
BM	5.7	6.9 ± 0.8	1.37 ± 0.21	3.39	2.01
BC	6.5	8.0 ± 0.7	1.65 ± 0.26	3.43	2.36
BCM	6.1	6.4 ± 0.5	1.05 ± 0.14	3.72	1.78

[1] Diet B — basal 15% casein-egg albumin diet with 1% cholesterol; HP = B with 15% extra egg albumin in place of 15% sucrose; BM = B + extra DL-methionine approximately equivalent to that furnished by 15% egg albumin (0.9%); BC = B + cystine approximately equivalent to that in 15% egg albumin (0.3%); BCM = B + both cystine and methionine.

[2] Means of ratios for individual animals.

[3] The term "pair-fed" is used for the rats offered daily the mean amounts of food consumed by the BM group of the same sex on the previous day.

[4] Standard error.

In females, mean liver lipid variations with age and diet followed the same pattern as in males but were not sufficiently great to be of statistical significance with the numbers of animals used (10 per group).

Liver cholesterol percentages varied with age and diet in much the same fashion as did total lipids (table 2). Highest liver cholesterol values were observed at 7- to 8-weeks in both the "pair-fed" and the ad libitum-fed males given basal diet B. But at 10 weeks, the values for the BC group had been maintained and even increased, and those for the basal diet had fallen. The originally lower percentages for the HP groups had also fallen and values for the BCM group were as low as for the rats given methionine only, — facts which made the high values for the BC males seem the more remarkable.

Nitrogens were determined and lipid/N and cholesterol/N ratios were computed (fig. 1 and table 2), partly to test the extent to which variations in liver glycogen were influencing percentages of liver lipid and cholesterol in rats sacrificed by the technique used, and partly to test whether the cystine and methionine supplements had any specific effect on liver protein.

Variations in nitrogen were found to be comparatively small, but since they were in the opposite direction from the variations in total lipids, the variations in lipid/N and in cholesterol/N ratios were considerable. Ratios of cholesterol to nitrogen showed patterns of variation with diet which were essentially similar to, but less exaggerated than, those obtained when cholesterol was computed to "% fat free dry weight," (ratio of cholesterol to non-fat solids). This might indicate that variability in liver glycogen was not very great in rats killed, as these were, 12 to 15 hours after removal of the food cups from the cages.

As a whole, the data from this study give further evidence that, even when a methionine-supplemented diet is eaten in equivalent amounts, it is somewhat less effective as a lipotropic agent for cholesterol than is the equivalent amount

of protein. This is in agreement with previous findings in work with adolescent rats, and with the conclusion of Lucas and Ridout ('55) that protein may furnish lipotropic factors other than methionine.

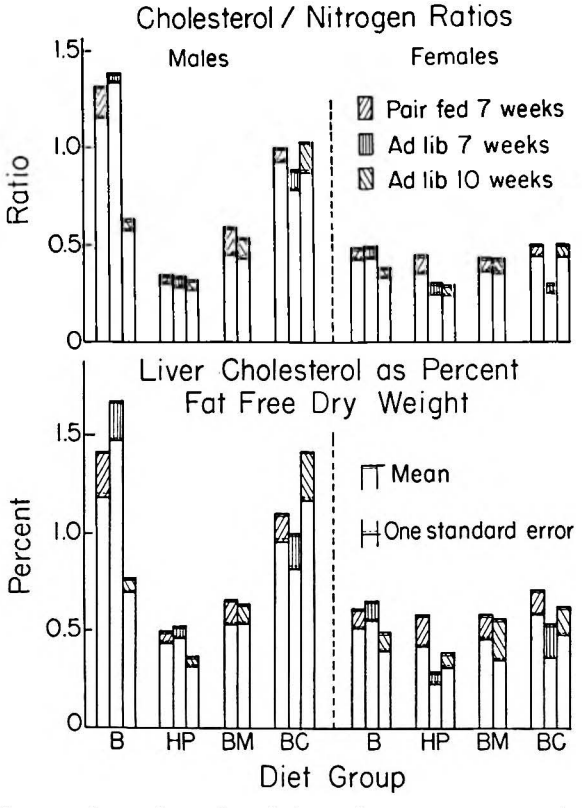

Fig. 1 Comparative values for cholesterol as percentage of fat-free dry weight and cholesterol/nitrogen ratios in rat livers.

The finding that cystine exerts some lipotropic effect toward cholesterol during the period of most rapid growth in male rats, but that it seems almost to augment liver lipid and cholesterol storage at a later period, is harder to explain. The data suggest that cystine may in some way promote accumulation of fat and that, possibly, the accumulation of

cholesterol is a corollary associated with its solubility in fat or with its promotion of synthesis of fatty acids required for esterification. The comparatively high gains of the older cystine-fed animals cannot be overlooked. However, the fact that, at the 7- to 8-week interval, the BC animals with restricted food intake had higher liver cholesterol percentages than did the ad libitum rats of the cystine-supplemented groups would indicate that the accumulation of liver fat and cholesterol was more likely to be due to a change in the path of intermediary metabolism than solely to quantity of food ingested.

SUMMARY

Rats were fed, from weaning: (1) an adequate, cholesterol-rich, synthetic diet containing 15% protein (2) that diet supplemented with 15% extra albumin; (3) with equivalent methionine; (4) with equivalent cystine; (5) with both cystine and methionine. The methionine diet was eaten so readily by weanlings that an attempt at "pair-feeding" to this group was only partially successful.

Methionine supplementation, in rats sacrificed both at 7 and at 10 weeks, resulted in a somewhat smaller lipotropic effect toward both fat and cholesterol than did the equivalent amount of intact protein. Cystine had evidently lowered liver cholesterol in the rats killed at 7 to 8 weeks, but at 10 weeks the cystine-supplemented males without methionine had higher liver fat and cholesterol than did those fed the unsupplemented, low protein diet. Liver nitrogen data are reported together with cholesterol/N ratios.

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THE METABOLIC FATE OF SULFUR^{35} IN SHEEP [1,2]

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The results of several radioisotope studies which have been reported in recent years indicate that labeled organic sulfur is found in proteins formed after feeding labeled inorganic sulfur to ruminants. Block and Stekol ('50) reported that milk proteins contained appreciable amounts of labeled cystine and methionine after feeding labeled sodium sulfate to a cow. In a subsequent study Block, Stekol and Loosli ('51) demonstrated the synthesis of cystine and methionine in milk proteins and serum albumins of a goat after feeding labeled sulfate. The specific activity of sulfur in the cystine and methionine of these proteins was equal. This was also found to be the case for the rumen proteins of an ewe which had been fed labeled sulfate. Hale and Garrigus ('53) reported that labeled cystine appeared in wool proteins following the feeding of labeled elemental sulfur and labeled sodium sulfate. Keener, Teeri, Harrington and Baldwin ('53) reported that sulfur^{35} from sulfur dioxide used as a silage preservative was detected in milk and blood proteins after feeding the silage to cows. The purpose of the studies which are to be reported here was to obtain information relative to the extent that inorganic sulfur is utilized by sheep.

[1] This project was supported in part by the U. S. Atomic Energy Commission.

[2] Partial reports on this work have been presented at the Federation of American Societies for Experimental Biology Meetings, 1953, and the American Society of Animal Production meeting, 1954.

EXPERIMENTAL

Two 9-month-old Hampshire wethers and an 11-month-old Southdown-Hampshire crossbred ewe were used in experiment I. The animals were kept in metabolism stalls and urine and feces collected quantitatively. The ration, which was pelleted, consisted of 75% ground alfalfa and 25% barley, and contained 0.043% methionine sulfur, 0.032% cystine sulfur, and 0.132% sulfate sulfur. Each animal was given approximately 5 millicuries of sulfur^{35} as sodium sulfate with 13 mg of carrier sulfate by gelatin capsule. Blood samples were obtained at various times during the 4-day collection period and the animals were sacrificed 96 hours after dosing. Radioactivity was determined in duplicate samples of selected tissues, gastrointestinal tract contents, feces and urine, using procedures which have previously been described (Kulwich, Pearson and Lankenau, '54).

Tissues samples of liver, spleen and skin were hydrolyzed in sealed tubes with 6 *N* HCl at 110°C. The liver and spleen samples were hydrolyzed for 7 hours, and skin for 16 hours. The hydrolysates were evaporated to dryness in vacuo below 40°C., and the residue was taken up in distilled water. The hydrolysates were fractionated by means of ion exchange chromatography.[3] The fractions from the ion exchange column were evaporated to dryness in vacuo below 50°C. and then taken up in distilled water; 1.0 ml aliquots, together with one drop of 0.2% benzalkonium chloride, were evaporated in stainless steel planchets for determining the radioactivity in a windowless flow counter. Corrections were made for decay and self absorption when necessary. In order to determine the total amount of cystine and methionine in these fractions, as well as in the feed used, microbiological assays were carried out (Horn and Blum, '56; Horn, Jones and Blum, '50).

In experiment II repeated doses of labeled sulfate were administered to lambs over an extended period of time. The two crossbred lambs used in this study were three months old

[3] Using 0.9 × 100 cm columns of Dowex 50 resin in the hydrogen form, and eluting with hydrochloric acid of varying normality (Stein and Moore, '51).

at the start of the experimental period. Prior to and during the experimental period lamb A, a wether, received a pelleted ration (ration A) in which soybean protein was the chief source of protein while lamb B, an ewe, received a pelleted ration (ration B) in which 1.2% urea replaced part of the soybean protein. The percentage composition of ration A was: modified cornstarch, 7.5; glucose, 3.5; bentonite,[4] 1.0; ground yellow corn, 12.1; ground orchard grass hay, 7.0; ground oat straw, 33.5; soybean protein, 8.0; ground barley, 25.0; sodium chloride, 1.0; dicalcium phosphate, 1.0; vitamin A and D oil, 0.4. Ration B was similar in percentage composition except that it contained urea, 1.2; corn oil, 2.0; soybean protein, 4.5; and ground oat straw, 33.8. Ration A contained 13.0% protein equivalent, 0.24% total sulfur, 0.045% cystine sulfur and 0.041% methionine sulfur, while the corresponding values for ration B were 13.3, 0.19, 0.037 and 0.032%, respectively.

A total of 11 doses of sulfur^{35} in the form of sodium sulfate were administered in gelatin capsules to each lamb on successive Mondays, Wednesdays and Fridays. Each dose contained 0.7 mg of carrier sulfate and about 0.6 millicuries of radiosulfur. The animals were kept in metabolism cages during this period and were sacrificed 7 days after receiving the last dose.

RESULTS AND DISCUSSION

The data for urinary and fecal excretion of sulfur^{35} by the lambs of experiment I are presented in table 1. It is evident from these data that much of the radiosulfur was rapidly absorbed from the alimentary tract and then quickly excreted. Urinary sulfur^{35} excretion for the first day averaged about 35%, and the total 4-day urinary excretion was about 49%. A rapid fecal excretion of radiosulfur was also observed, with an average of about 17% of the dose recovered in the first day's feces and a total 4-day fecal excretion of about 31% of the dose. This rapid appearance of radiosulfur in the feces

[4] Colloidal clay, used as a binding agent.

is far in advance of when it would have been excreted if it had passed through the gastrointestinal tract at a rate similar to food residues. It is probable that most of the rapidly excreted radiosulfur found in the feces was rapidly absorbed from the rumen or other portions of the upper alimentary tract and then secreted in the bile and also perhaps directly into the

TABLE 1

Excretion of sulfur^{35} after oral administration of labeled sodium sulfate to sheep

(Expressed as percentage of dose)

	EXPERIMENT I				EXPERIMENT II [1]	
	Wether 9	Wether 45	Ewe 890	Average	Wether A	Ewe B
Urine:						
Day 1	49.3	26.1	30.4	35.2	45.2	41.5
Day 2	9.6	8.0	9.2	8.9	7.9	5.4
Day 3	3.2	4.1	3.4	3.6	6.3	1.5
Day 4	1.2	1.4	1.9	1.5	3.1	1.8
Day 5					3.1	1.7
Day 6					3.1	1.9
Day 7					3.4	1.4
Total	63.3	39.6	44.9	49.2	72.1	55.2
Feces:						
Day 1	15.7	20.6	13.4	16.6	23.4	34.9
Day 2	9.5	9.2	7.2	8.6	7.5	9.3
Day 3	3.1	5.5	2.6	3.7	3.0	5.2
Day 4	1.4	2.9	2.2	2.2	1.2	2.8
Day 5					1.1	2.1
Day 6					1.3	2.0
Day 7					1.3	2.2
Total	29.7	38.2	25.4	31.1	38.8	58.5

[1] Values for experiment II are for sulfur^{35} excretion following the final dose and are expressed as percentage of a single dose.

intestines. Determination of the sulfur^{35} content of the blood gave further evidence of the rapidity with which the ingested radiosulfur was absorbed. The peak concentration of radiosulfur in the blood occurred within 6 hours of the time of dosing. In the case of ewe 890, blood samples were obtained at more frequent intervals and it was established that the peak sulfur^{35} concentration occurred between one and three hours

after dosing (fig. 1). At two hours after dosing 6.99% of the dose was in the blood, after which the concentration diminished so that at 96 hours after dosing 1.86% was in the blood.

Lofgreen, Weir and Wilson ('53) reported that rumen contents obtained from sheep from three to 5 hours after feeding a ration containing 0.15% total sulfur plus 0.2% sodium sulfate contained only a trace of inorganic sulfur. In the present

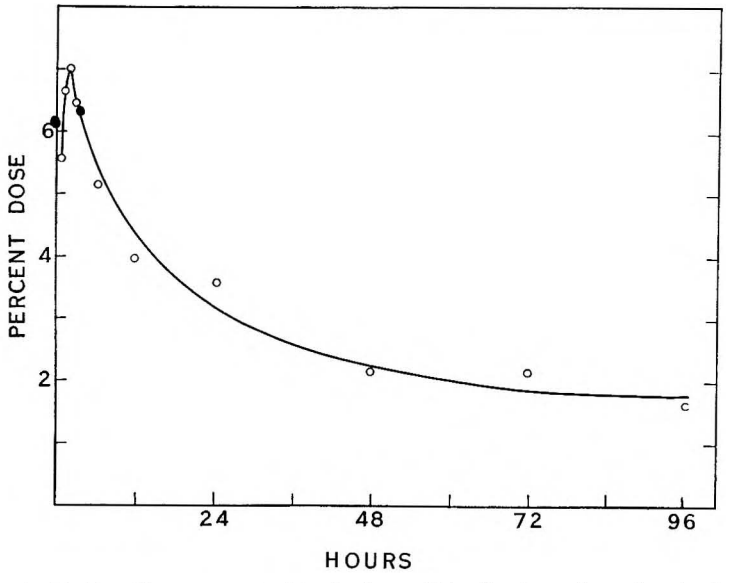

Fig. 1 Radiosulfur content in blood of ewe 890 after ingestion of a single dose of labeled sulfate. Blood volume was estimated as 6.3 ml per 100 gm body weight (Hansard, Butler, Comar and Hobbs, '53).

experiment the rapid uptake of radiosulfur by the blood, which reached a peak within 6 hours after oral administration of labeled sulfate, indicates that a considerable portion of the dose has been absorbed rapidly from the rumen or other portions of the upper alimentary tract. However, while part of the labeled sulfur was being absorbed into the blood, the microorganisms of the rumen were actively incorporating sulfur^{35} into cyst(e)ine and methionine. The rate of incorporation of sulfate sulfur by rumen microorganisms is probably

quite high. Emery, Smith and Huffman ('56) reported that, in an *in vitro* study, after three hours of incubation, the rumen microbiota from bovines incorporated from 10 to 40% of the sulfur from the labeled sulfate of the substrate. Labeled methionine and cyst(e)ine were isolated from a hydrolysate of the rumen microbiota. Anderson ('56) carried out in vivo and in vitro experiments which indicated that dietary sulfate is reduced to sulfide, part of which is utilized by rumen microorganisms while the remainder is absorbed into the blood from the rumen and then rapidly removed from the blood by the liver. The chemical form of blood radiosulfur was not

TABLE 2

Distribution of sulfur^{35} *in gastrointestinal tract contents 4 days after oral administration of labeled sodium sulfate to sheep*

(Expressed as percentage of dose)

	WETHER 9	WETHER 45	EWE 890	AVERAGE
Rumen contents	0.77	3.24	1.42	1.81
Reticulum contents	0.02	0.14	0.13	0.09
Omasum contents	0.02	0.13	0.02	0.06
Abomasum contents	0.09	0.07	0.11	0.09
Small intestine contents	0.11	1.11	0.44	0.55
Large intestine contents	0.36	0.37	0.32	0.35
Total	1.37	5.06	2.44	2.95

determined in the present experiment. However, in view of the fact that under normal feeding conditions Anderson ('56) could not detect sulfide in the blood, and our finding that a large portion of the dose was rapidly excreted by the urinary pathway, it is probable that at the time of peak radiosulfur uptake in the blood, most of the radiosulfur was in the form of sulfate. This labeled sulfur may have been absorbed into the blood from the rumen or other portions of the upper alimentary tract in the sulfide form and then have been oxidized to the sulfate form.

The data on distribution of radiosulfur in the gastrointestinal tract contents 4 days after ingestion of labeled sulfate are presented in table 2. Since microbial synthesis of cystine

and methionine occurs in the rumen, it would be expected that greater tissue uptake of labeled organic sulfur compounds would be associated with higher sulfur^{35} incorporation into the rumen contents. If the rate at which rumen contents moved down the alimentary tract were similar for the lambs of experiment I, then the amount of sulfur^{35} in the rumen contents of these animals might be expected to provide an indication of the tissue uptake of radiosulfur (table 3) in these animals, provided there were no great differences in efficiency of utilization of the labeled compounds. The amounts of sulfur^{35} found in the rumen contents at sacrifice of the first, second and third lambs (wether 9, wether 45, and ewe 890) in this experiment were 0.77, 3.24 and 1.42% of the dose, respectively. The corresponding values for percentage of dose in the liver at sacrifice were 0.94, 3.46, and 1.16. Blood sulfur^{35} concentrations from 48 to 96 hours after dosing also showed a similar trend. The blood sulfur^{35} values at sacrifice for lambs 45 and 890 were 3.19 and 1.98% of dose, respectively, and the corresponding values at 72 hours after dosing were 3.2 and 2.1% of the dose. The percentage of dose recovered in the kidneys of lambs 9, 45 and 890 was 0.13, 0.47 and 0.16%, respectively. A similar pattern, wherein sulfur^{35} uptake was lowest for number 9, highest for number 45, and intermediate for number 890 also appeared upon comparison of radiosulfur uptake in adrenals, thyroid, spleen, intestinal tissue, pancreas, lungs and heart. It was interesting to find that this pattern of sulfur^{35} uptake was reversed in the case of ear cartilage, so that the first lamb had the highest uptake and the second lamb the lowest uptake. The sulfur^{35} found in the cartilage probably was incorporated chiefly as ester sulfate in mucopolysaccharides. Boström and Åqvist ('52), in a study in which labeled sulfate was injected, reported that the chief tissue uptake in rats was in chondroitin sulfuric acid which was isolated from cartilage. In experiments carried out with rabbits (Kulwich, Pearson and Lankenau, '54), cartilage sulfur^{35} uptake was found to be far greater than uptake in soft tissues. For example, 4 days after oral administration of labeled

sulfate to rabbits, the sulfur^{35} concentration of ear cartilage was about 8 times as high as the uptake in liver. In the lambs the concentration of sulfur^{35} in cartilage was only about one-half the concentration found in liver, with the total sulfur^{35} uptake in the liver averaging about 1.85% of the dose while the sulfur^{35} uptake in rabbit liver averaged 0.46% of the dose.

TABLE 3

Distribution of sulfur^{35} in sheep 4 days after oral administration of a single dose of labeled sodium sulfate

(Per cent dose per gram wet tissue × 10^4 standardized to a body weight of 30.0 kg)

TISSUE	WETHER 9	WETHER 45	EWE 890	AVERAGE
Liver	1.57 (0.94)[1]	45.6 (3.46)	22.6 (1.16)	28.0 (1.85)
Kidney	16.7 (0.13)	41.5 (0.47)	16.4 (0.16)	24.9 (0.25)
Adrenals	17.7	18.2	27.8	21.2
Thyroid	15.7	23.4	..	19.6
Trachea	12.1	26.9	17.6	18.9
Spleen	12.4 (0.05)	26.7 (0.13)	14.7 (0.07)	17.9 (0.08)
Rumen	8.4 (0.38)	24.1 (1.35)	10.6 (0.53)	14.4 (0.75)
Reticulum	10.1 (0.13)	19.7 (0.21)	13.0 (0.15)	14.2 (0.16)
Omasum	10.9 (0.11)	29.4 (0.35)	10.7 (0.08)	17.0 (0.18)
Abomasum	10.0 (0.18)	23.0 (0.45)	9.1 (0.24)	14.0 (0.29)
Small intestine	15.1 (0.74)	28.2 (2.78)	20.3 (1.11)	21.2 (1.54)
Large intestine	13.5 (0.87)	19.0 (0.77)	13.5 (0.79)	15.3 (0.81)
Red bone marrow (femur)	15.9	30.7		17.5
Skin	14.8	23.0	12.7	16.9
Pancreas	10.2	24.0	15.4	16.5
Lung	12.3 (0.35)	15.8 (0.67)	18.9 (0.57)	15.7 (0.53)
Ovaries			15.3	
Intestinal lymph nodes	11.5	18.2	15.9	15.2
Blood[2]	..	16.9 (8.19)	10.5 (1.98)	13.7 (2.59)
Ear cartilage	18.1	9.2	12.4	13.2
Aorta	12.9	10.8	13.9	12.5
Sternum	18.6	13.3	4.6	12.2
Heart	7.0 (0.07)	16.3 (0.25)	12.3 (0.14)	11.8 (0.15)
Femur epiphysis (distal)	9.8	11.6	13.0	11.5
Incisors	6.3	4.3	4.1	4.9
Gastrocnemius muscle	3.3	4.7	4.6	4.2
White bone marrow (femur)		4.7	3.8	4.2
Brain	2.0 (0.01)	4.5 (0.03)	3.3 (0.03)	3.3 (0.02)
Femur shaft	5.4	3.0	3.0	3.2

[1] Values in parentheses represent per cent dose in entire tissue.

[2] Blood volume estimated according to data of Hansard et al. ('53).

The major portion of the radiosulfur in the livers was present as labeled cystine and methionine. By means of a chromatographic fractionation of hydrolysates of fresh liver it was found that about 84% of the total radiosulfur recovered from lamb liver hydrolysate was present as cystine and methionine. About 71% of the total radiosulfur recovered from the liver of an orally dosed rabbit sacrificed 4 days after dosing was present as cystine and methionine (Kulwich, Struglia, Jackson and Pearson, '54). Thus, if we consider liver sulfur^{35} as an index of the extent to which sulfate sulfur was incorporated into cystine and methionine of body proteins, we would conclude that radiosulfur from a tracer dose of labeled sulfate was incorporated into body proteins of sheep about 4 times as actively as it was in rabbits. The chromatographic fractionations of hydrolysates of sheep tissues that were carried out revealed that most of the activity emerged in the cystine and methionine peaks, and a smaller fraction of the sulfur^{35} emerged in a peak which was eluted early in the run. Sulfate is known to emerge within the volume of effluent included in this early peak, but on the basis of this fractionation the identity of this peak was not definitely established. Forty-six percent of the labeled sulfur in the liver was in the methionine and 38% in the cystine. The corresponding values for spleen were 45 and 28%, and for skin 12 and 66%. Of the total labeled sulfur in these tissues, about 80% was present as cystine and methionine. The activity in the cartilage of these sheep was only about 25% of the level we observed for cartilage in rabbits (Kulwich, Pearson and Lankenau, '54). The differences observed in the fate of labeled sulfate in the two species may be accounted for, at least in part, by the fact that in the sheep the dose went directly to the site of microbial activity while in the rabbit considerable absorption may have occurred before sulfur^{35} reached the cecum.

During the 30-day feeding period of experiment II lambs A and B gained an average of 0.46 and 0.63 lb. per day. The average daily feed consumption was 2.0 and 3.0 lb. per day and pounds of feed required for pound of gain 4.3 and 4.8, respec-

tively. During the 7-day collection period following the final dose the total urinary sulfur^{35} excretion of lamb A and lamb B was equivalent to 72 and 55% of a single dose, respectively (table 1). The corresponding values for total sulfur^{35} in the feces of lambs A and B were 39 and 59%, respectively. During this 7-day period lamb B consumed about 30% more feed than lamb A and throughout the entire experimental period lamb B consumed 50% more feed. Perhaps the presence of more feed in the gastrointestinal tract was a factor which tended to lower the amount of radiosulfur which was absorbed into the blood and then excreted in the urine as sulfate, and tended to increase the amount of radiosulfur incorporated by rumen microorganisms. Another factor which would tend to increase the incorporation of labeled sulfate by the microorganisms, and therefore in turn, the uptake of labeled cystine and methionine in the tissues of the sheep, was the fact that lamb B received 1.2% urea in the ration. This animal also gained more weight than did lamb A. Any urea nitrogen that was utilized for bacterial protein synthesis would be accompanied by incorporation of sulfur since it is also a component of these proteins. The tissue sulfur^{35} uptake was calculated in terms of percentage of the total of the 11 radiosulfur doses administered (table 4). The sulfur^{35} uptake of these tissues was generally lower than in those obtained 4 days after single dose of labeled sulfate in experiment I. It will be noted that wool samples showed the greatest uptake of sulfur^{35}. The wool was obtained from an area which had been sheared at the start of the 30-day experimental period, and therefore contained a high percentage of recently synthesized fiber. By the use of the radioautographic technique it was demonstrated that radiosulfur was distributed throughout a half inch length of wool fibers which were formed during the 30-day experimental period. Individual fibers were pressed against no-screen X-ray film for 17 days and upon development the radioactive zone of the fiber showed up very distinctly. When some of this wool was hydrolyzed and the labeled cystine was isolated by means of chromatography, it was found that the

specific activity of the cystine radiosulfur was about 0.03% dose per millimole of sulfur.

Tarver and Morse ('48) reported that during a 14-day period following oral administration of labeled methionine to rats the active tissues lost most of their radioactivity and and tissue sulfur^{35} concentration became rather stabilized at a

TABLE 4

Tissue sulfur^{35} content of sheep 7 days after final oral doses of labeled sodium sulfate

SAMPLE	WETHER A		EWE B	
	Per cent [1] dose per gram × 10^4	Per cent dose in entire tissue	Per cent dose per gram × 10^4	Per cent dose in entire tissue
Liver	11.9	0.59	15.7	0.81
Kidneys	5.99	0.05	7.26	0.08
Spleen	4.01	0.02	8.41	0.04
Lungs	8.03	0.20	6.46	0.22
Pancreas	2.50		6.54	
Adrenal	6.43		8.32	
Gastrocnemius muscle	2.40		4.97	
Heart	7.66		7.27	
Intestinal lymph nodes	5.12		5.20	
Ear cartilage	8.73		13.3	
Aorta	3.01		6.78	
Rumen tissue	4.71		5.33	
Brain	2.62		4.14	
Kidney fat	0.68		1.60	
Wool	65.2		66.7	
Skin	7.98		9.50	
Blood [2]	4.49	0.85	9.14	1.73

[1] Values are calculated as per cent of the total activity of the 11 doses, and are standardized to a body weight of 30.0 kg.

[2] Blood volume estimated at 6.3 ml per 100 gm body weight (Hansard et al., '53).

lower level. That there is a similar tendency in sheep is apparent when we compare the activity of various tissues from animals given a single dose with those given multiple doses of sulfur^{35}. This tendency for a lowered sulfur^{35} uptake was more pronounced in the more active tissues such as blood, kidney, liver and spleen than in muscle. The muscle radiosulfur content for lambs A and B was 2.40×10^{-4} and

4.17×10^{-4}% dose per gram, respectively, compared to an average value of 4.2×10^{-4} for a single dose (experiment I). The sulfur^{35} content in the livers of lambs A and B constituted 0.59 and 0.81% of the total dose, compared to an average value in the single dose study of 1.85%; kidney values were 0.05 and 0.08, compared to an average of 0.25% for a single dose; and spleen values were 0.02 and 0.04, compared to 0.08% for a single dose.

Fractionation studies were carried out with plasma samples obtained after the 9th dose was administered. It was found that sulfur^{35} concentration in the 80% ethanol precipitate accounted for over 98% of the plasma radiosulfur three hours after the 9th dose, and that during the following three days the radiosulfur concentration in the 80% ethanol insoluble fraction decreased 39% for lamb A and 21% for lamb B. The sulfur^{35} in the filtrate represented 1.5% of the total activity for lamb A and 1.2% for lamb B and dropped rapidly so that after 72 hours the filtrate activity was only 0.34% of the total for lamb A and 0.16% of the total for lamb B. Cystine isolated from a hydrolysate of the 80% ethanol precipitate of plasma obtained from lamb A 24 hours after the 9th dose had a specific activity of 0.022% dose per millimole of sulfur and methionine had a specific activity of 0.027% dose per millimole sulfur. The corresponding values for lamb B were 0.038 and 0.041% dose per millimole of sulfur in cystine and methionine, respectively. About 20% of the radiosulfur recovered from the hydrolysate of the serum precipitate from lamb A and about 7% of that recovered in the corresponding sample from lamb B were in the sulfate-containing fraction.

SUMMARY

Data on the tissue uptake and excretion of radiosulfur by sheep after oral administration of single and multiple doses of labeled sulfate are presented. Absorption of labeled sulfur into the blood was rapid, with the peak occurring within 6 hours. Excretion of the major part of the dose was fairly rapid, with an average of 49% excreted in the urine and 31%

in the feces during the 4-day collection period. Appreciable amounts of labeled sulfur were found in all tissues analyzed. Liver, kidney, adrenals, thyroid, spleen, skin, intestinal tissues and cartilage showed greater uptake than muscle tissue. Fractionation studies revealed that most of the labeled sulfur in liver, spleen and skin was in the form of cystine and methionine.

When labeled sulfate was given three times weekly over a period of 24 days the excretion pattern of the individual doses appeared to be similar to that observed for a single dose. Wool fibers contained radiosulfur in the half inch of new growth.

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VITAMIN B_6 DEFICIENCY IN RABBITS [1]

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The rabbit has been thought to be independent of dietary sources of B vitamins, since it consistently consumes a special portion of its excreta, rich in vitamins and proteins, termed "soft" or "night" feces. The adequacy of this source of B vitamins for the total needs of the animal is questionable. Indeed, a niacin requirement was shown by Wooley ('47), and a critical need for dietary choline was demonstrated by Hove, Copeland and Salmon ('54). From the consideration of cecectomized rabbits, Herndon and Hove ('55) suggested that the high nutritive value of "soft" feces resulted, in part, from a concentration of the residual dietary nutrients into this portion at the expense of the remaining fecal material rather than a production of such nutrients, *de novo,* in the cecum.

It is the purpose of this paper to report studies on the nutrition of vitamin B_6 in the rabbit, and on the interrelation between vitamin B_6 and vitamin E.

EXPERIMENTAL

Weanling 4-week old rabbits of the California-White strain were placed upon a purified diet made up as follows: Meth-

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anol-extracted casein, 25%; mineral mixture (Salmon, '47), 5%; nonnutritive fiber[2] (cellulose), 10%; sucrose, 43%; vitamin pre-mix, 5%; lard, 9%; cod liver oil, 1%; KHCO3, 1.2%; and $MgSO_4$[3] 0.8%. The vitamin pre-mix contained dry, ground, pure vitamins added to sucrose to give the following levels as micrograms per gram of diet: thiamine, 5; riboflavin, 5; calcium pantothenate, 30; i-inositol, 200; niacin, 40; 2-methyl-1, 1,4-naphthoquinone, 2 vitamin B_{12}, 0.05; folacin, 2; biotin, 0.1; and choline, 2000. α-Tocopheryl acetate was added to this diet at 0.01%. In the experiment where fat was left out of the diet, synthetic vitamin A and vitamin D_2 were added to the vitamin pre-mix at levels to give 5 μg and 2.2 I.U/gm diet, respectively.

Vitamin B_6 in liver and in feces was assayed by the method of Atkin et al. as reported by Snell in Gyorgy ('50), using *Saccharomyces carlsbergensis*. Soft and hard feces were collected from individually stanchioned animals. The feces were separated, dried at room temperature, and ground to pass a 24-mesh screen. Whole blood clotting time was determined in tube and in drop; prothrombin time was estimated by adding a lung extract as a source of thromboplastin. Urinary xanthurenic acid was determined by the method of Rosen et al. ('51); because of a lack of a standard, the values are reported on an arbitrary scale of 0 to 4. Urinary creatine and creatinine were determined as previously indicated (Hove and Herndon, '55). The vitamin E requirement of the rabbits was determined by the cure-of-creatinuria method as given by Hove and Harris ('47).

RESULTS

After 35 days on the depletion diet, the rabbits (two for each level) showed a graded growth response to increasing levels of pyridoxine administered once a day by pipette (fig. 1). The daily requirement for optimum growth on this

[2] General Biochemicals, Inc., Chagrin Falls, Ohio.

[3] Magnesium sulfate dried powder (*ca* three water of hydration), 0.8%.

diet was 39 μg/day, or, since the animals ate about 40 gm of food daily, the requirement may be said to be about 1 μg/gm food.

Other growth data are shown in table 1. The antimetabolite desoxypyridoxine did not interfere with growth when ample pyridoxine was in the diet; nor did penicillin (0.01%) improve growth. On the simple vitamin B_6-deficient diet, rabbits grew less than 5 gm/day and more than half of them died

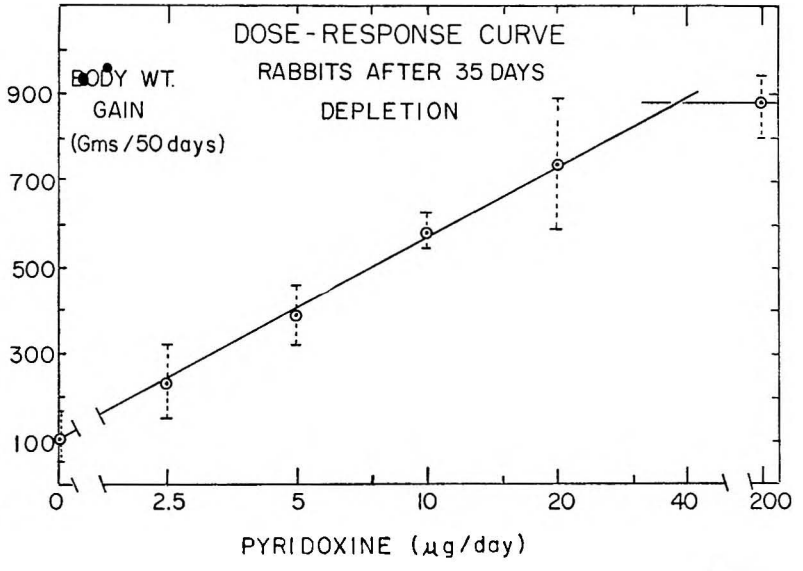

Fig. 1 Response of rabbits to graded oral daily doses of pyridoxine. Young weanling animals depleted 35 days on basal diet prior to test.

at an average time of about 100 days. The addition of desoxypyridoxine at 10 μg/gm of diet greatly aggravated the deficiency, the growth rate was even less and all animals died at an average time of 76 days. The addition of penicillin to the vitamin B_6-deficient diet had no effect on growth. Four rabbits were fed a fat-free pyridoxine-deficient diet; their growth was slightly lower and all rabbits died at an average time of 99 days.

The gross pathology of the simple vitamin B_6 deficiency in rabbits may be classified, first, as skin disorders, and second,

as neurological abnormalities. The skin disorders occurred in all deficient animals to some degree. This symptom appeared first as a marked scale formation with eventual thickening of the skin of the ears. Next an inflammation about the eyes and nose appeared. In a few cases, the full acrodynia occurred

TABLE 1

Growth and pathology of rabbits fed the vitamin B_6-deficient diet

EXPERIMENT NO.	TREATMENT [1]	NO. RABBITS	WEIGHT AT START	AV. GAIN PER DAY (50 DAYS)	DEATHS		SYMPTOMS		
					No.	Days	Skin lesion	Convulsions	Paralysis
			gm	*gm/day*					
I	$-B_6$	6	480 (330–660)	3.6 (1.70– 5.1)	2	70	5	1	3
	$-B_6$ + desoxy B_6	4	558 (310–630)	1.3 (0.0 – 2.4)	4	76	4	0	2
	$+B_6$	5	388 (300–610)	16.1 (12.0 –18.6)	0	0	0	0	0
	$+B_6$ + desoxy B_6	3	555 (310–780)	14.6 (10.8 –21.0)	0	0	0	0	0
II	$-B_6$	4	450 (360–530)	7.5 (2.6 –13.8)	3	115	4	2	2
	$-B_6$, no fat	4	390 (340–500)	5.0 (2.2 – 8.6)	4	99	3	2	1
	$+B_6$	4	470 (440–490)	17.5 (14.6 –20.8)	0	0	0	0	0
III	$-B_6$	2	625	4.5	1	145	2	0	1
	$-B_6$ + penicillin	2	610	5.0	0	0	2	0	0
	$+B_6$	2	485	15.0	0	0	0	0	0
	$+B_6$ + penicillin	2	450	17.3	0	0	0	0	0

[1] + B_6 indicates 17μg pyridoxine/gm diet; + desoxy B_6 indicates 10 μg of desoxypyridoxine/gm of diet; penicillin added at 0.01% level in diet, where indicated. Figures in parenthesis are ranges.

with marked incrustations and inflammations of the nose, eyes and paws. Loss of hair along the fore-legs with inflammation and desquamation was evident.

The neurological symptoms occurred as characteristic convulsions, and as paralysis with terminal contractural deformities. The incidence of this pathology is outlined in table 1. The convulsions in the vitamin B_6 deficiency appeared to

be triggered by shrill noise. There was a wild racing about the cage for 30 seconds, followed by collapse with marked thrashing of the limbs and twitching of the face muscles. The animals then relapsed into a coma, but with eyes open and the eyeballs pulled down. Recovery occurred slowly and was complete in about 10 minutes.

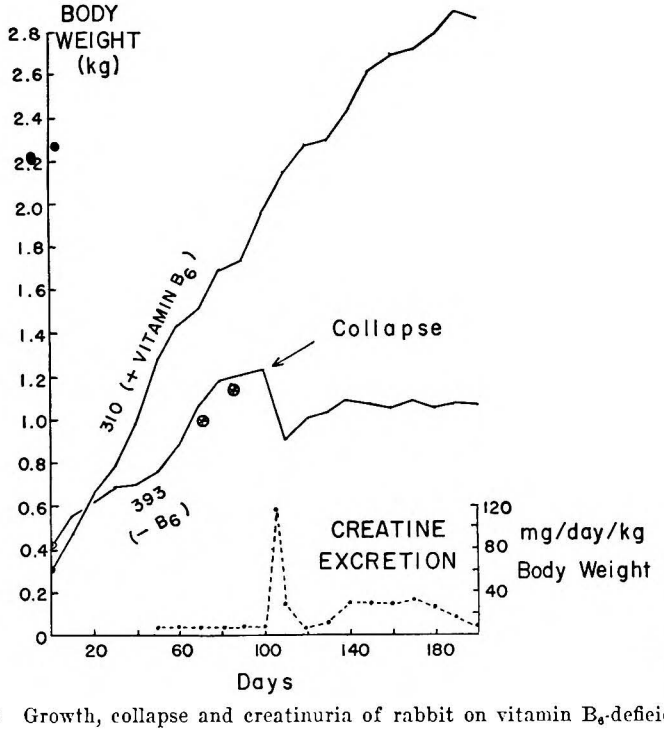

Fig. 2 Growth, collapse and creatinuria of rabbit on vitamin B_6-deficient diet. Convulsions indicated by circled star.

When paralysis occurred, it began as a sudden collapse. The excretion of creatine, which previously had been normal, rose rapidly to more than 100 mg/day within two to three days. Many animals died at this stage. The survivors stabilized in body weight and stopped excreting creatine. The paralysis, which had been evident after the first day as "ankle-drop" and loss of all fine movements of the limbs, became gradually worse over several months until death. Complete

loss of the use of the hind limbs occurred. The history of one of the three rabbits that survived the initial collapse is shown in figure 2 in comparison with a control animal. At death, the muscles of the hind legs were paper-thin as a result of the extreme atrophy. The spinal cord showed no gross lesions, but certain demyelinated areas appeared under histological examination, similar to those described by Follis ('48).

TABLE 2

Vitamin B_6 content of liver and feces of vitamin B_6-deficient rabbits

(Average of two animals per group after 100 days on diets)

PYRIDOXINE FED DAILY	AV. BODY WEIGHT	DRY FECES (24 HR.)		VITAMIN B_6 CONCENTRATION			FECAL EXCRETION OF VITAMIN B_6	
		Total wt.	Soft	Liver (wet basis)	Hard feces (dry basis)	Soft feces (dry basis)	Total	Soft feces
µg	*kg*	*gm*	*% of total*	*µg/gm*	*µg/gm*	*µg/gm*	*µg/day*	*% of total*
0	0.86	16.6	27.5	1.41	0.34	1.06	8.91	54.1
20	1.92	37.6	24.0	3.00	0.21	0.78	13.05	54.0
200	2.38	27.1	32.0	6.84	1.10	4.47	59.1	65.6

TABLE 3

Some characteristics of the vitamin B_6 deficiency in rabbits

CATEGORY OF INTEREST	NUMBER OF DEFICIENT RABBITS	DEFICIENT VALUES	CONTROLS VALUES
Liver fat, % on dry basis	8	9.71	10.83
Hemoglobin, gm/100 ml blood	14	8.84	12.91
Clotting time of whole blood, seconds	8	140	24
Prothrombin time of blood, seconds	8	12	10
Urinary xanthurenic acid, 0 to 4 rating	16	3	0
Vitamin E requirement, 20 mg tocopheryl acetate/kg body weight divided by days cured of creatinuria	4	0.27	0.29

The vitamin B_6 content of the liver (table 2), as determined on two rabbits from each group killed after 100 days on experiment, varied from 1.41 μg/gm in the deficient animals to 6.84 μg/gm in the normal controls. Analysis of the soft and hard feces from the rabbits (table 2) revealed that the concentration of vitamin B_6 in the soft feces was three to 4 times greater than in hard feces, whether the animal was deficient or not. The total vitamin B_6 excreted in the feces ranged from 8.9 μg daily by the deficient rabbits to 59.1 μg daily by the normal controls. However, the fraction of the total contributed by the soft feces ranged from 54% to 66%.

Some incidental data on the rabbits with the vitamin B_6 deficiency are reported in table 3. The liver fat was normal. A mild anemia was evident. The clotting time of whole blood was prolonged to 140 seconds in the deficient animals, as compared with 24 seconds for the normal controls. This difference was completely eliminated when a source of thromboplastin (10% water extract of normal lung) was added. Urinary creatine and creatinine were usually normal, with the exceptions noted previously. Xanthurenic acid was abundantly excreted. The vitamin E requirement of rabbits deficient in vitamin B_6 was not changed from the normal value of about 0.25 mg/day/kg body weight of the tocopheryl acetate. The data in table 3 indicate that development and cure of the vitamin E-deficiency muscular dystrophy was not influenced significantly by a simultaneous vitamin B_6 deficiency.

DISCUSSION

The data indicate that severe vitamin B_6 deficiency can be produced in rabbits by omission of this factor from the diet. The dermal and neurological pathology was essentially the same as observed in rats and in pigs (Follis, '48). The requirement of rabbits for pyridoxine was calculated to be 39 μg daily under the conditions used. This did not include the vitamin B_6 that may have been consumed *via* the soft feces. Under the conditions used, little synthesis of vitamin

B_6 appeared to have occurred in the cecum or gastrointestinal tract of the rabbits. The soft feces (normally consumed) from the rabbits on the deficient diet contained about 4 μg daily; this was about half of the total daily fecal excretion of vitamin B_6. As dietary pyridoxine increased, the excretion of vitamin B_6 increased, and the proportion in the soft feces remained close to 50%. This would indicate that the vitamin B_6 in the feces was principally of dietary or metabolic source.

No relation between vitamin B_6 and vitamin E was noted in the rabbits, either as measured by the vitamin E requirements or by the development of muscular dystrophy. Severe atrophy and some degeneration of muscles did occur in the paralyzed vitamin B_6-deficient animals, but this was an event secondary to the spinal cord lesions and the resultant paralysis. In this respect, the metabolism of vitamin B_6 apparently is different in the rabbit and the rat, since several reports have indicated an interrelation between these two vitamins in the rat. A sparing effect of vitamin E on low levels of pyridoxine for rat growth was reported by Harris, Hove, Mellott and Hickman ('47). It was noted that a statistically significant growth improvement occurred when α-tocopherol was fed along with either two or 4 μg of pyridoxine daily to depleted rats. However, at 8 μg of pyridoxine, no benefit due to tocopherol was evident. It had been reported by Sure and Ford ('42) that vitamin B_6-deficient rats excreted larger amounts of creatine than normal. This has been confirmed and extended by Dinning ('55) and Young, Dinning and Day ('55). These workers noted high levels of creatine excretion in rats deficient in both vitamins E and B_6. Either vitamin, when given alone, markedly lowered the creatinuria. Earlier, Mackenzie ('49) had announced that he had greatly intensified muscle lesions in rats subjected to a dual deficiency of vitamin B_6 and vitamin E, and that this damage frequently approached in extent that seen in rabbits deficient only in vitamin E. In rabbits, as handled under conditions of this laboratory, nothing similar to this has been noted. Routine

creatine and creatinine determinations on the rabbits deficient in vitamin B_6 revealed no creatinuria, even though a severe deficiency existed as shown by repressed growth, skin lesions and convulsions. Even when vitamin E was omitted from the vitamin B_6-deficient diet, the creatine excretion did not rise above the normal value of 10 mg/day/kg until about 60 days, after which muscular dystrophy set in rapidly. When 20 mg of *dl*, α-tocopheryl acetate was given to these doubly deficient rabbits, the creatine excretion returned to normal and remained normal for 65 to 75 days. However, the severe and sudden creatinuria that occurred in rabbits at the time they underwent the paralytic collapse after long periods of vitamin B_6 deficiency may be related to the phenomenon noted in rats.

SUMMARY

A vitamin B_6 deficiency has been produced in rabbits fed a diet devoid of this factor. The symptoms were lowered growth rate; death in about 100 days; scaly skin of the ears; some acrodynia of the nose, eyes and forepaws; convulsion similar to those described for other species; mild anemia; prolonged blood clotting time; and a sudden paralytic collapse accompanied by very marked creatinuria; creatine excretion increased from normal values of about 10 mg/day/kg of body weight to over 120 mg in less than three days. Death usually occurred during this collapse. In the few animals that survived, the paralysis of the hind quarters increased to complete immobility and contractural deformities and creatine excretion returned to normal.

The vitamin B_6 requirement of rabbits, under these conditions, was found to be 39 µg daily, or about 1 µg/gm of diet, as measured by growth. Soft feces contained three to 4 times as much vitamin B_6 as hard feces, and accounted for 54% of the total fecal vitamin B_6. These ratios were approximately the same in deficient as well as normal rabbits, although the total excretion increased from 8 µg/day/kg up to 59 µg/day/kg.

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IMPROVING THE NUTRITIVE VALUE OF FLOUR

VII. SUPPLEMENTING THE PROTEIN IN FLOUR WITH AMINO ACIDS [1]

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An adequate food supply is one of the paramount problems facing the steadily increasing world population. Of all the food components, protein is the most difficult to produce. The less expensive sources of protein, including cereals, are poor in nutritional quality. Animal proteins containing the necessary levels of the essential amino acids are more expensive because of the increased cost of production, and therefore are not available in adequate quantities for much of the world's population.

Cereals are also notably low in B vitamins and minerals, but in the United States the addition of B vitamins and iron to wheat flour has partially met this lack. In countries where incomes are high, with plenty of meat and milk available, the diet usually is low in cereal content and the amino acid as well as the B vitamin needs are met easily. However in countries where meat and milk are not readily available or among people in our own country on low incomes, particularly elderly people living alone, bread makes up a large share of the diet. For such groups bread should be as nutritious as possible not only in B vitamins and iron but in amino acid content as well. Since cereals make up the largest part of the diets for undernourished people over the world, it seems worthwhile to improve the quality of cereal proteins.

[1] Contribution No. 196 Department of Home Economics, Kansas Agricultural Experiment Station, Manhattan.

The classic experiments of Osborne and Mendel ('14, '19) showed that wheat protein, and particularly flour protein, is deficient in lysine. The amino acid content of bread may be increased by the addition of non-fat milk solids or by amino acid supplementation, or by a combination of both these methods. The use of non-fat milk solids presents at least two problems. First, there are not enough milk solids available to supplement all the bread produced, and second, only about 3% of milk solids may be added in the preparation of bread and still have an acceptable product.

Several investigators have studied the effect of adding different amino acids to wheat flour or of adding amino acids with protein supplements. Cremer, Lang, Hubbe and Kulik ('51) reported improvement in the biological value of wheat protein by the addition of lysine. Experiments by Rosenberg and Rohdenburg ('51) showed that considerable loss of lysine, from 9.5 to 23.8%, takes place during the baking process. Toasting reduces the lysine content 5 to 10% and a similar loss occurred when the bread became stale and dry. Further studies by the same authors ('52) showed that increasing the amounts of lysine in bread brought about significant growth responses in weanling rats and doubled the value of bread protein. In other studies Rosenberg, Rohdenburg and Baldine ('54) used lysine, methionine, valine and threonine to supplement a diet of bread, fat, salts and vitamins. Their results indicated lysine was the only amino acid that stimulated rat growth. Jahnke and Schuck ('56) used non-fat milk solids at 3, 6, and 12% in bread. The rats fed bread with 12% milk solids made gains in between the gains made by those fed bread with 3% and 6% milk solids plus 0.25% lysine. The protein efficiency was greater for the two latter diets.

The purpose of the experiments reported here was to determine the nutritional effect of supplementing flour with certain amino acids and to study the interrelationships of such supplementation with the B vitamins and the deposition of fat in the liver. As previous investigators have used chiefly

a diet of bread supplemented with fat, minerals and vitamins, it was decided to use diets of natural food patterned after those used by human beings.

PROCEDURE

Albino rats three weeks of age and weighing between 40 and 55 gm were divided into groups of 12 with equal distribution as to sex and litter mates. The percentage composition of the diets is shown in table 1. The diets were high in cereal content and rather low in meat, milk and eggs. They were based on diets similar to those consumed by people with low incomes. Adelson and Blake ('50) studied the food habits of low income groups in Georgia and Ohio and Moser ('45) studied those in South Carolina. These data were used as a basis for the diets. The method of preparation has been described previously by Westerman, Linn, Templeton and Wells ('49). The animals were weighed weekly and food consumption was measured. Growth studies were carried over a period of 10 or 12 weeks. Then the animals were sacrificed and the livers taken for vitamin analyses. Fat content of the livers also was determined, as an imbalance of amino acids is likely to be manifest by the deposition of excess fat in the liver.

RESULTS AND DISCUSSION

First experiment: Comparison of meat and added lysine. In experiment 1 the chief sources of protein in diets A and B were enriched flour, meat, milk and eggs (table 1). Diet B also had 3.2 mg/gm of lysine added to the flour. This quantity was chosen as Block and Bolling ('44) recommended the addition of 3 gm of lysine per 100 gm of protein in bread to compensate for the deficiency of lysine when the consumption of meat and milk was lowered. The meat content of the diets was rather low, therefore in diet B, lysine was also added to determine whether additional lysine above that present in the meat, milk and eggs was needed. The protein foods were also excellent sources of the B vitamins.

To replace the meat, which was omitted in diets C and D, the cereal content was increased to 43.8% (table 1). Diet C had 3.2 mg/gm of lysine added to the flour but diet D had neither meat nor added lysine. Analysis of the diets for total protein showed diet A with 15.25%, diet B, 15.13%, diet C, 12.25% and diet D, 12.06%. Control animals were fed a stock ration of laboratory chow.[2]

TABLE 1

Composition of the diets

INGREDIENT	EXPERIMENT 1		EXPERIMENT 2		
	Diets A and B [1]	Diets C [1] and D	Diet I	Diet II [2]	Diets III,[3] IV,[4] V,[5] and VI [6]
	%	%	%	%	%
Flour	37.0	43.8	37.5	44.1	48.9
Sugar	3.8	3.8	3.8	3.8	3.8
Fat	4.6	4.6	4.6	4.6	4.6
Meat	6.8	..	6.9	..	..
Dried milk	3.9	3.9	3.9	3.9	..
Eggs	1.4	1.4	..	..	..
Potatoes	17.5	17.5	17.8	17.8	17.8
Carrots	5.5	5.5	5.5	5.5	5.5
Green vegetables	8.6	8.6	8.9	8.9	8.9
Apples	10.9	10.9	11.1	11.2	11.1

[1] Diets B and C had 3.2 mg/gm of lysine added to the flour.

[2] Diet II had 3.2 mg/gm of lysine added to the flour.

[3] Diet III had 3.2 mg/gm of lysine, 0.4 mg/gm of tryptophan, 1.9 mg/gm of valine added to the flour.

[4] Diet IV had 3.2 mg/gm of lysine added to the flour.

[5] Diet V was identical with diet III and had also 5.2 μg/gm of thiamine, 3.3 μg/gm of riboflavin, 44.0 μg/gm of niacin, 34.0 μg/gm of iron added to the flour.

[6] Diet VI had 3.2 mg/gm of lysine and the vitamins as in diet V added to the flour.

The growth test was carried over a 12-week period and the results are shown in figure 1. The animals on diet A, containing meat, made an average gain of 207 gm which was more than that made by any of the other groups except those on the stock ration. However, those on diet B, with meat and added lysine, gained approximately the same, 201 gm. Apparently lysine was not the limiting factor but the rats on

[2] Purina.

these two diets did not make as good gains as those on the stock ration, namely 266 gm. Those on diet C with lysine added but with no meat in the diet, gained on the average 181 gm or 26 gm less than those on diet A with meat. This

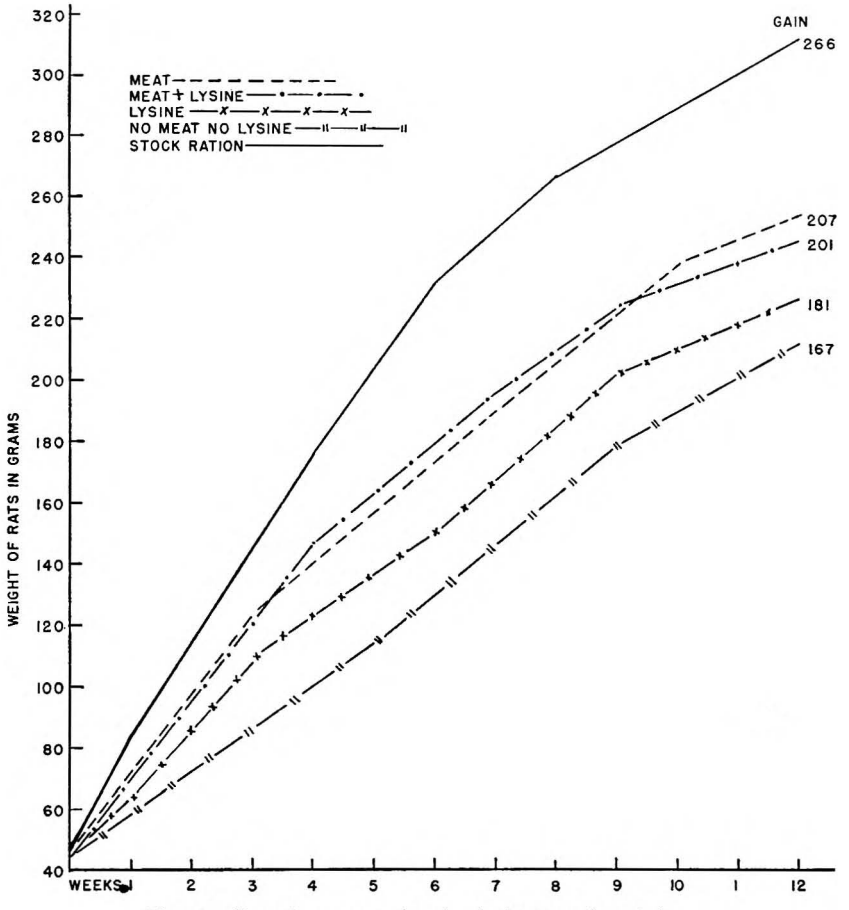

Fig. 1 Growth curves of animals in experiment 1.

was a significant difference ($P < 0.05$). Animals on diet D, without meat or added lysine, made an average gain of 167 gm, which was 40 gm less than those on diet A. This difference was significant at the 5% level as was also the difference of 34 gm between those on diet B, with meat and lysine, and diet D,

without meat and added lysine. A difference of 14 gm between those on diets C and D was non-significant. Evidently the quantity of lysine added in diet C, 0.14%, aided in increasing the growth rate somewhat but not enough to be significantly greater than that on diet D with the same quantity of milk and eggs. The addition of meat in diets A and B increased the total protein content, providing lysine and other factors that influenced the growth rate significantly.

The mean food efficiency, i.e., the number of grams gained per gram of food consumed, was calculated. There were no significant differences in food efficiency between diets A and B. However the food efficiencies of 0.241, 0.218 and 0.207 for diets A, B and C, respectively, were significantly greater ($P < 0.05$) than 0.156 for diet D. Diets A and B had a greater food efficiency ($P < 0.05$) than diet C. The meat in the diet probably increased the food efficiency but the addition of lysine to the diet containing meat brought about no improvement. Apparently the addition of lysine to the diet without meat improved the food efficiency above that with no supplement.

The livers of the rats were analyzed for thiamine, riboflavin, and fat content with the results shown in table 2. The mean thiamine content varied from 23 μg/gm for diet B to 15 μg/gm for diet C and the differences were non-significant. The riboflavin content, 49 μg/gm, was least in the livers of animals on diet D, without meat or lysine. Those on diets A and C with 77 μg/gm and 76 μg/gm had significantly higher amounts than those on diet D ($P < 0.05$). This might indicate that lysine has a role in riboflavin deposition.

The quantity of fat in the livers of the rats on diets A, B, and C averaged 2.9 while in those on diet D the average was 3.5%. The differences were statistically significant ($P < 0.05$). However, such small differences in fat deposition would indicate no imbalance in amino acid metabolism.

Second experiment: Addition of lysine, tryptophan, valine and B vitamins. Six diets were used in the second experiment and their composition is shown in table 1. Eggs were absent

from all the diets and only diet I had both meat and milk, diet II had milk and 3.2 mg/gm of lysine added to the flour. The percentage of flour was increased to 44.1% to make up for the lack of meat. The other diets were devoid of meat and milk and the quantity of flour was increased to 48.9%. In diet III the flour was supplemented with 3.2 mg/gm of lysine, 0.4 mg/gm of tryptophan and 1.9 mg/gm of valine. The quantities of tryptophan and valine were calculated to be the same as those occurring in the meat in diet I. Diet IV was the same as III except lysine only was added. Diet V was also similar to III, but in addition the flour was supplemented

TABLE 2

Effect of dietary supplements on the thiamine, riboflavin and fat content of liver

DIET	THIAMINE [1,2]	RIBOFLAVIN	FAT
	μg/gm	μg/gm	%
A	18	77	2.7
B	23	53	2.7
C	15	76	2.9
D	19	49	3.5

[1] Vitamin data reported on a dry fat-free basis.
Analysis of variance: no significant differences for thiamine.
Riboflavin: A and C > D at 5% level.
Fat: A, B and C < D at 5% level.
[2] All values are means.

with 5.2 μg/gm of thiamine, 3.3 μg/gm of riboflavin, 44.0 μg/gm of niacin and 34 μg/gm of iron. These amounts practically doubled the quantity of vitamins in the flour and alleviated the B vitamin and iron deficiencies in the diet without meat and milk. Diet VI contained no meat or milk, but the flour was supplemented with 3.2 mg/gm of lysine, and the same quantities of vitamins and iron as in diet III. The total protein contents were as follows: diet I, 13.13%; diet II, 11.44%; diet III, 10.88%; diet IV, 10.63%; diet V, 10.93%; and diet VI, 10.19%.

It was necessary to terminate the growth studies at 10 weeks as the animals on diets III through VI lost weight and some

died. The results of the growth test are shown in figure 2. The animals on diets I and II had a much healthier appearance and gained much more weight than those on the other diets. Their average total gains were 193 and 173 gm, respectively, which was not as much as the 229 gm gain made by those on

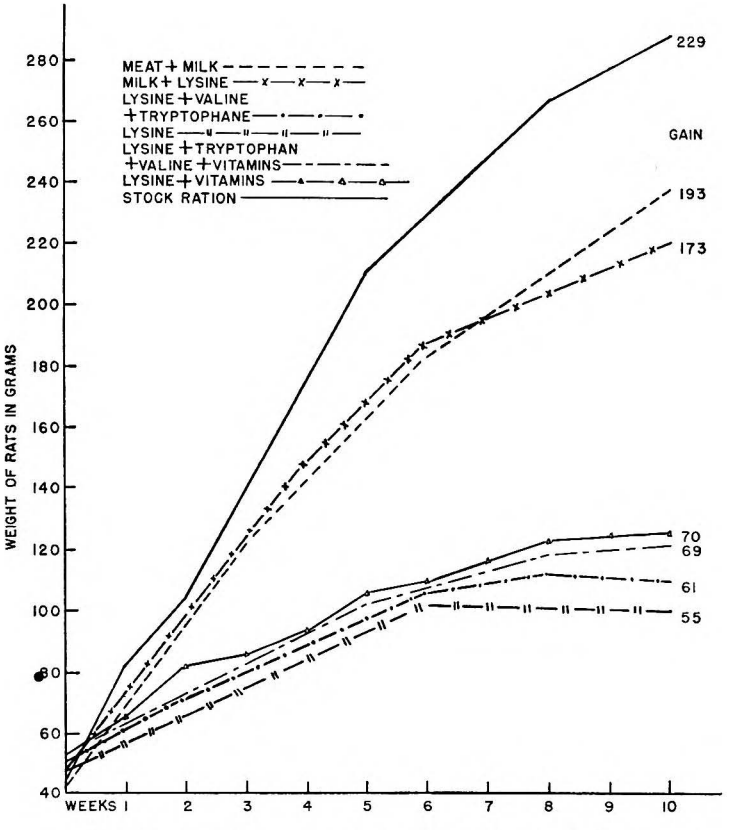

Fig. 2 Growth curves of animals in experiment 2.

the stock ration. The differences in weight gain in animals receiving diets I and II were non-significant statistically. However the differences between those on the stock ration and the two test diets were significant at the 5% level. The lysine added to the flour in the diet without meat seemed to compensate for the meat. When both meat and milk were

removed from the diets the rate of growth was quite slow even when lysine, tryptophan, and valine as well as B vitamins and iron were added. This is shown by the average gain of 69 and 70 gm respectively for the animals on diets V and VI. Without the addition of the vitamins and iron the gains were less, 61 and 55 gm for those on diets III and IV, respectively. The differences in weight gains between animals on diets III, IV, V and VI were non-significant, but when these were

TABLE 3

Effect of dietary supplements on B vitamins and fat in the livers of rats

DIET	THIAMINE [1,2]	RIBOFLAVIN	PANTOTHENIC ACID	NICOTINIC ACID	FAT
	μg/gm	*μg/gm*	*μg/gm*	*μg/gm*	%
I	21	59	55	428	2.5
II	29	54	63	536	2.3
III	32	46	87	571	2.2
IV	42	91	142	595	2.8
V	35	76	178	524	2.6
VI	34	68	200	466	2.2
Stock	29	75	51	468	2.6

[1] Vitamin data on a dry fat-free basis.

Analysis of variance: thiamine: I < all others, significant at 5% level; stock < IV and V at 5% level; III, V and VI no significant differences; V < IV at 5% level.

Riboflavin: I, II and III < stock at 5% level; all diets < IV at 5% level.

Nicotinic acid: I, VI and stock < II, III, IV and V, significant at 5% level; V < IV at 5% level; non-significant difference between I, VI and stock.

Pantothenic acid: I, II, III and stock < IV, V and VI, differences significant at 5% level; I, II, III and stock differences non-significant.

[2] All values are means.

compared with the weight gains made by those on diets I and II the differences were significant ($P < 0.05$).

The same differences were shown in food efficiency. Food efficiency values of 0.160 and 0.166 for animals on diets I and II were significantly higher ($P < 0.05$) than those of 0.113, 0.094, 0.107, and 0.096 for the animals on diets III, IV, V and VI. No significant differences between those on diets I and II or between those on diets III, IV, V and VI were found.

The vitamin and fat content of the livers are shown in table 3. The animals on diet IV had the highest quantities of

thiamine, riboflavin, and nicotinic acid in their livers. As these animals made the least gains, it may be that the vitamins were not utilized in normal growth processes, and were stored in the livers. The animals fed diets V and VI also stored fairly large quantities of thiamine and riboflavin. This might have been expected as these vitamins had been added to the food. Evidently there was a protein deficiency in these diets even though lysine, tryptophan and valine, or lysine alone had been added. This may have produced a condition whereby the vitamins were not utilized in normal metabolism.

Animals on diet I, with meat and milk, had on the average, less thiamine, 21 μg/gm, in the livers than those on diet II, without meat but with lysine added, or the stock diet, both of which had 29 μg/gm. This difference was significant ($P < 0.05$). The animals on diets IV and V had significantly more, 42 μg/gm and 34 μg/gm, respectively than did the stock rats ($P < 0.05$). Differences between those on diets III, V and VI were non-significant.

The quantities of riboflavin in the livers, 59 μg/gm, 54 μg/gm and 46 μg/gm, respectively, of animals on diets I, II and III were significantly less ($P < 0.05$) than 75 μg/gm for those on the stock diet. Those on diet IV, with 91 μg/gm, had a significantly greater quantity than those on the other diets ($P < 0.05$). Nicotinic acid content of the livers from those on diets I, VI and stock ration, 428 μg/gm, 466 μg/gm and 468 μg/gm, respectively, were significantly lower ($P < 0.05$) than those on diets II, III, IV and V, with 536 μg/gm, 571 μg/gm, 595 μg/gm and 524 μg/gm respectively. Non-significant differences were found between diets I, VI and the stock ration.

As no pantothenic acid was added, its only source was in the natural food materials. Diets I, II, III and the stock ration produced no significant differences in the amounts of this vitamin in the livers. However, animals on those diets stored significantly less quantities ($P < 0.05$) of this vitamin than those on diets IV, V and VI.

The fat content in the livers of the rats varied little for any of the diets, table 3. Evidently the balance of amino acids was sufficient to maintain normal fat deposition.

Under the conditions of these experiments the diets should contain either meat or milk or both, in order to promote fairly normal growth and food efficiency. The proper quantities of amino acids and B vitamins to be added to diets without meat and milk, but high in cereal content have not been found under the conditions of these tests.

SUMMARY

Experiments were conducted using natural foods to determine the effect of the addition of lysine, tryptophan, valine and B vitamins to diets high in cereal content and low in meat, milk and eggs on the growth rate, food efficiency, and deposition of B vitamins and fat in the livers of albino rats.

Animals on diets containing 12% meat, milk and eggs with 37% flour had a much better growth rate, food efficiency and healthier appearance than those on diets with 44% flour and no meat. The addition of lysine did not improve the diet containing meat, milk and eggs but when meat was omitted, the addition of lysine was beneficial.

When the animal protein was removed from the diets, even though lysine, tryptophan, valine, additional B vitamins and iron were incorporated into the flour, which then made up 49% of the diets, the growth rate and food efficiency were much below those with animal protein in the diet. The animals on the poorer diets deposited larger quantities of B vitamins in the livers, probably because they were not used in the growth processes. Deposition of fat was normal.

In diets of natural foods with a high cereal content evidently it is necessary to supply at least small quantities of animal protein along with additional amino acids in order to have healthy animals growing at a normal rate. The results indicate that the quantities of amino acids added to the flour were insufficient to meet the needs of the animals.

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NUTRITIONAL STUDIES ON RATS ON DIETS CONTAINING HIGH LEVELS OF PARTIAL ESTER EMULSIFIERS[1]

III. CLINICAL AND METABOLIC OBSERVATIONS

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During the chronic rat feeding studies of the partial fatty acid esters (Atlas emulsifiers) described in the initial report of this series (Oser and Oser, '56a) numerous observations of a biochemical and metabolic nature were made including examinations of the blood and excreta, digestibility studies, and short-term feeding tests of the polyol fraction of certain of these esters. The observations may be divided into three categories (a) physical appearance and behavior; (b) clinical tests; (c) metabolic studies, although in presenting and discussing the findings some overlapping will be inevitable.

PHYSICAL APPEARANCE AND BEHAVIOR

The laxative response. The only deviation from normal appearance or behavior in any of the rats was a tendency in some of the emulsifier groups, especially at the higher dosage levels, toward diarrhea accompanied by irritation of the anal region in the more severe cases. In respect to activity, condition of the fur, color and vascularity of the eyes, pinkness of the ears and toes, the rats in all groups appeared normal.

[1] This investigation was supported by a grant from the Atlas Powder Company, Wilmington, Delaware.

TABLE 1

Status of F_0 generation rats at 36th week in respect to laxative response [1]

FAT OR EMULSIFIER	LEVEL IN DIET							
	0		5%		10%		20%	
	D	A	D	A	D	A	D	A
None	—	—	—	—	—	—	—	—
Myrj 45	—	—	—	—	—	—	—	—
Myrj 52	—	—	—	—	most M = 1 [2] few M = 2 few F = 1	few M = 1–2	most M = 1–3	most M = 1–3
Span 60	—	—	—	—	—	—	—	—
Tween 60	—	—	—	—	most M = 1 few F = 1	few M = 1	most M = 1–2	most M = 1–2
Tween 65	—	—	1 M = 1	—	few M = 1	—	most M = 1 few M = 2	few M = 1
Tween 80	—	—	—	—	few F = 1	—	most M = 1–2	few M = 1 most M = 2–3
Mixture	—	—	—	—	—	—	most M = 1 few F = 1	few M = 1
Primex	—	—	—	—	—	—	—	—

[1] D = diarrhea; A = perianal irritation; M = males; F = females.

[2] Degrees of severity: 1 = trace or slight; 2 = mild or moderate; 3 = definite or marked; 4 = severe.

Only in the animals with pronounced diarrhea was there an unthrifty appearance due to the moist stools. The associated perianal alopecia and erythema extended well beyond the anal region and in the most severe cases the area appeared raw and serous, though never infected. The cages of these rats were changed two or three times a week.

The distribution of the laxative response among the various emulsifier and control groups is shown in table 1. Similar observations were recorded regularly but are summarized in this table only for the F_0 generation rats after 36 weeks on the test diets. This response was typical not only for this generation but for succeeding generations as well.

It will be seen that no laxative effect was noted in the basal control group, at the 5% level in any of the emulsifier groups, at the 10% level in the groups receiving Myrj 45, Span 60 or the mixture of emulsifiers, or at the 20% level in the groups receiving Myrj 45 or Span 60. In the case of the Myrj 52 and Tween groups the incidence and severity of the laxative reaction was greater at 20% than at 10, and appeared to affect males somewhat more than females. The condition of the stools of these rats varied from soft but well-formed to semi-fluid; only rarely were they frankly fluid. When the condition was of moderate or greater severity it was commonly associated with irritation and hypersensitivity around the anus sufficient to warrant considering lack of interest in copulation as a possible explanation for the reduced fertility frequently observed (Oser and Oser, '56b) in the 20% Myrj 52 and Tween groups.

At this point it is pertinent to cite a concurrent experiment in which the polyol moieties [2] of the entire series of emulsifiers were fed to groups consisting of three male and three female rats each, at levels equivalent stoichiometrically to 5, 10, and 20% of the intact esters.

The 12-week growth responses of these groups corresponded closely to those of the emulsifier groups. Of particular interest

[2] The polyol fractions were prepared by acid hydrolysis in the laboratories of the Atlas Powder Company.

was the similarity of the effects of the polyols to those of the emulsifiers on laxation, no laxation being observed in any of the Myrj 45 or Span 60-polyol groups, or in the Myrj 52- or any of the Tween-polyol groups at the 5 and 10% (equivalent) of Myrj 52-, Tween 60-, Tween 65-, and Tween 80-polyols.

It would thus appear that the intestinal reaction to these 4 emulsifiers at high feeding levels must be attributed not to the surface activity of the intact molecule, but to the molecular weight and concentration of the polyol moiety released upon hydrolysis. (Data on the structure of these compounds are published by the Atlas Powder Company, '48.) The laxative fatty acid emulsifiers were the esters of long-chain higher molecular weight polyoxyethylene groups (viz. 40 ethylene oxide moieties in the case of Myrj 52, and 20 in the case of the Tweens) whereas those causing no laxative response were the esters of either sorbitan (Span 60) or of polyoxyethylene alcohol with only 8 ethylene oxide groups in the chain (Myrj 45). Furthermore, in the case of the Tweens where the molecular weight of the polyol fraction was uniform, its stoichiometric concentration was less for Tween 65 (56.72%) than for Tween 60 or Tween 80 (77.23 and 76.44%, respectively) (table 6) and the degree of laxative effect induced by Tween 65 was likewise somewhat lower.

It was of interest however to determine whether there was any correlation between the degree of laxation, the moisture content of the stools and the voluntary water intake. Measurements were made in a series of rats on the 20% emulsifier diets and in control animals for a two-week period. Diarrhea occurred as usual in all groups except the basals, and the Myrj 45 and Span 60 groups. The fecal moisture content ranged from 64.7 to 81.0% in ascending order as follows: Basals, Span 60, Tween 65, Myrj 45, Tween 60, Tween 80, and Myrj 52. The water intake of these groups ranged from 7.6 to 13.5 ml per 100 gm body weight per day; except for reversal of the basals and the Span 60 group, the order of ranking was the same.

CLINICAL

Blood examinations. At three stages of the chronic feeding study, namely 12, 52, and 78 weeks, a sampling of rats from each group was selected at random for chemical and cytological blood tests, whereas at the end of the two-year period all survivors were so examined. Determinations were made of hemoglobin, sugar, and non-protein nitrogen levels, and of the erythrocyte, leukocyte and differential leukocyte counts. The results are presented (table 2) only for the 104-week period inasmuch as the data were consistently negative and too voluminous to justify the space for additional repetitive tables. Chronic effects would be most likely to be reflected in the terminal series of tests conducted on the 296 surviving rats, which were fairly evenly distributed among the test groups.

Since the number of animals subjected to blood examination varied with periods and among test groups, the data could not be subjected to statistical study by analysis of variance. However by taking a series of composite averages of data for each item determined, according to the factors which might affect variance, such as sex, age, generation, emulsifier, and test level, any trends or effects due to these factors should be disclosed. The data shown in table 3 were computed in this manner and reveal the following:

The blood hemoglobin levels were within the normal range throughout the entire test and for all groups, the mean values being slightly higher at 12 weeks than at later periods (as were also the erythrocyte counts) and for the initial generation than for succeeding generations. The composite mean hemoglobin values for the control and emulsifier groups (including all dosage levels) varied from 13.3 to 14.4% whereas the corresponding red cell counts varied from 8.08 to 8.50 $\times$ 10^6 per cubic millimeter.

Leukocyte counts and especially differential counts are subject to wider variation which was manifest in these observations. However the mean values for total counts fell

TABLE 2

Summary of hematological observations, including blood sugar and non-protein nitrogen levels, on rats in feeding experiments for 104 weeks

(Data shown are averages of values observed)

FAT OR EMULSIFIER	NO. AND SEX	HEMOGLOBIN	RED BLOOD COUNT	WHITE BLOOD COUNT	DIFFERENTIAL COUNT				CHEM	
					Polys [1]	Lymphs [2]	EOS [3]	BAS [4]	Sugar	NPN [5]
		gm/100 ml	*× 10³ per mm³*	*× 10³ per mm³*	*%*	*%*	*%*	*%*	*mg/100 ml*	
None	7 M	13.3	8.73	12.7	43.7	52.3	2.4	0	114.3	41.0
	13 F	12.6	6.78	11.5	40.0	54.0	1.5	0.3	115.8	44.3
5% Level										
Primex	5 M	14.2	8.31	23.4	29.6	60.2	2.4	1.5	107.8	34.8
	10 F	13.2	6.91	11.9	30.8	64.1	1.3	0.8	117.5	40.5
Myrj 45	4 M	11.9	7.36	16.1	44.8	50.3	1.8	0.3	119.2	38.8
	5 F	13.8	8.04	10.3	19.6	74.0	1.4	0	109.0	37.0
Myrj 52	7 M	12.7	7.19	17.2	41.0	53.7	1.5	0	107.9	33.7
	9 F	13.1	7.52	10.8	29.7	65.0	1.7	0	104.4	33.9
Span 60	5 M	13.1	7.87	14.9	33.2	60.6	1.4	0	121.2	32.6
	12 F	13.6	7.75	12.3	31.0	61.8	2.8	0	112.8	33.7
Tween 60	3 M	14.1	8.72	13.8	28.7	68.3	2.7	0	124.6	34.7
	9 F	13.2	8.34	12.5	35.0	55.6	4.9	0.2	96.9	40.7
Tween 65	5 M	13.1	8.04	15.0	43.2	51.0	0.8	0	112.4	30.2
	9 F	13.9	7.69	9.6	34.7	57.2	1.6	1.8	128.6	23.8
Tween 80	5 M	13.2	8.38	13.1	28.4	66.4	2.2	0	127.6	31.6
	7 F	13.7	7.98	11.7	32.6	62.1	1.0	0	96.2	32.5
Mixture	12 M	14.1	8.58	10.5	39.9	54.2	1.9	0.8	96.6	35.3
	8 F	13.6	7.37	11.1	39.9	50.3	3.6	1.5	111.9	43.9
10% Level										
Primex	4 M	11.9	8.09	16.8	46.3	49.0	0.5	0	121.8	42.8
	10 F	12.5	7.94	12.3	48.6	47.7	0.8	0	111.9	32.2
Myrj 45	6 M	14.1	7.99	19.3	46.5	50.3	1.0	0	109.0	33.5
	8 F	13.8	7.48	11.6	36.4	59.1	1.9	0	113.9	36.9
Myrj 52	5 M	12.9	8.22	11.4	45.2	48.8	1.8	0.2	82.2	27.3
	9 F	12.5	6.92	10.8	42.1	53.9	0.8	0.2	100.8	37.0
Span 60	5 M	12.3	7.86	8.8	49.6	45.6	1.4	0	102.4	35.8
	7 F	12.2	6.94	8.4	45.6	47.1	1.6	0.1	103.9	32.9
Tween 60	7 M	14.4	8.44	14.2	30.7	64.7	1.4	0.1	115.9	34.3
	6 F	13.8	7.03	15.5	20.2	76.5	0.2	0.5	120.2	37.8
Tween 65	4 M	12.6	7.85	15.5	39.5	56.5	0.8	0	121.0	34.5
	6 F	13.5	7.43	7.8	34.0	63.8	0	0	111.7	32.2
Tween 80	3 M	13.0	7.96	16.7	41.7	55.3	0.7	0	124.3	40.3
	6 F	13.6	7.34	13.1	27.5	69.8	0.3	0	104.8	38.2
Mixture	5 M	13.2	7.87	16.1	41.6	51.4	0.8	0	116.0	36.6
	7 F	13.9	8.31	10.9	41.1	54.3	0.1	0	112.3	43.0
20% Level										
Myrj 45	6 M	14.0	8.93	16.2	32.7	59.7	1.7	0.2	92.8	37.2
	11 F	13.7	7.71	11.4	29.2	63.9	1.1	1.3	104.0	37.8
Myrj 52	6 M	14.2	8.59	13.8	44.3	49.7	0.8	0.2	110.3	47.8
	6 F	15.2	8.37	10.1	43.7	53.0	0.5	0	98.3	40.0
Span 60	6 M	12.1	7.94	12.1	43.3	53.2	0.3	0.2	111.2	37.8
	7 F	12.5	7.44	9.4	33.9	63.0	0.9	0	109.4	35.1
Tween 60	6 M	14.3	8.47	10.9	38.8	56.5	0.5	0	105.0	40.5
	6 F	13.9	7.98	9.4	45.8	50.3	0.5	0.3	108.5	45.2
Tween 65	4 M	14.3	8.44	13.9	27.3	83.8	3.8	0	110.3	37.5
	6 F	12.3	8.02	15.3	35.7	60.2	1.5	0	102.0	40.4
Tween 80	6 M	14.7	8.40	12.9	34.3	61.7	0.7	0	120.3	44.2
	6 F	14.3	8.30	9.4	33.3	61.3	0.3	0	111.4	34.8
Mixture	5 M	13.8	7.61	13.3	28.2	63.2	2.2	0.4	122.0	35.0
	6 F	13.7	7.19	15.0	33.5	62.2	1.7	0	103.5	45.3

[1] Polys = Polymorphonuclear leukocytes.
[2] Lymphs = Lymphocytes.
[3] EOS = Eosinophiles.
[4] BAS = Basophiles.
[5] NPN = Non-protein nitrogen.

TABLE 3

Composite averages of blood cytological and chemical determinations

AVERAGES		NUMBER OF TESTS	HEMOGLOBIN	RBC [1]	WBC [2]	PMN [3]	EOS [4]	SUGAR	NPN [5]	PLASMA CHOLESTEROL [6]
			%	$\times 10^6$ per mm^3	$\times 10^3$ per mm^3	%	%	mg/100 ml	mg/100 ml	mg/100 ml
Overall		1044	14.3	8.39	13.1	26.1	1.4	102	40.7	90.8
By sexes	Males	496	14.3	8.60	14.3	27.1	1.3	103	40.2	88.1
	Females	548	14.0	8.18	12.1	25.2	1.4	101	41.2	92.9
By duration of test period	12 wk.	484	15.0	8.79	15.7	18.4	1.1	89	41.3	87.3
	52 wk.	132	13.9	8.55	13.0	26.7	2.2	95	47.3	(94.9)
	78 wk.	132	13.8	7.71	11.2	30.3	1.3	114	36.6	
	104 wk.	296	13.5	7.90	12.7	36.9	1.5	110	36.7	91.0
By generation	F_0	648	15.0	8.79	15.7	30.7	0.8	89	41.3	91.0
	F_1	132	14.4	8.77	15.5	17.2	1.0	112	45.7	
	F_2	132	14.1	8.28	11.3	19.0	1.4	93	38.1	94.9
	F_3	132	14.6	8.73	12.8	19.8	0.9	103	38.7	87.3
By test level	0%	54	13.7	8.08	13.2	29.8	1.9	105	43.6	91.0
	5%	338	14.0	8.26	12.9	26.5	1.6	103	39.3	91.6
	10%	322	14.0	8.25	12.2	25.6	1.3	102	38.7	94.2
	20%	330	14.2	8.40	13.3	25.6	1.3	100	42.0	86.3
By emulsifier or fat	None	54	13.7	8.08	13.2	29.8	1.9	105	43.6	91.0
	Myrj 45	142	14.1	8.26	13.3	25.5	1.4	104	40.5	84.4
	Myrj 52	149	14.2	8.25	12.8	29.1	1.4	99	41.3	92.8
	Span 60	144	13.3	8.08	13.4	25.5	1.5	103	39.4	90.7
	Tween 60	139	14.3	8.37	11.9	25.9	1.4	102	40.2	95.1
	Tween 65	136	14.0	8.32	13.0	25.6	1.2	102	38.3	88.2
	Tween 80	135	14.4	8.50	12.8	23.6	1.1	103	39.8	90.6
	Mixture	145	14.2	8.36	13.4	25.7	1.8	101	40.7	94.3

[1] RBC = Erythrocytes.
[2] WBC = Leukocytes.
[3] PMN = Polymorphonuclear neutrophiles.
[4] Eos = Eosinophiles.
[5] NPN = Non-protein nitrogen.
[6] Based on 123 rats at 12 weeks in F_3 generation; 88 rats at 36 weeks in F_2 generation (shown in parentheses) and 286 rats at 104 weeks in F_0 generation (cf. table 4).

within normal limits (Gardner, '47) regardless of emulsifier, dosage, age, or generation. It was interesting to note however, that the proportion of polymorphonuclear neutrophiles increased with the age of the rats, irrespective of the diet fed. Hence the apparently higher percentage of polymorphonuclears for the first generation rats as compared with their progeny is due to the fact that in the latter, blood counts were made only at the 12-week period. No evidence of eosinophilia was observed although the counts varied within rather wide limits, as is usually found in the rat.

No deviations from the ranges of normal blood sugar values were found in any of the composite means. Somewhat higher values were observed toward the latter part of the test period but these were nevertheless within normal limits, and, in any event, not related to the particular diets fed. The nonprotein nitrogen levels likewise were quite normal. These blood chemical findings suggest no evidence of impairment of carbohydrate metabolism or of renal function.

Plasma cholesterol values were obtained at several stages during the course of the study, but since many of the determinations were made in F_2 and F_3 generation rats they are summarized separately (table 4). No differences were observed between the sexes within groups in respect to plasma cholesterol levels. Hence the values shown in table 3 represent weighted averages for combined sexes. Analyses of these data by composite means according to the variable factors as described above show that average cholesterol levels were normal regardless of the duration of the feeding period or the diet fed and that no trend either upward or downward was observed from one generation to another.

Urine examinations. At half-yearly periods throughout this two-year feeding study, specimens of urine were collected from three male and three female rats in each group and examined with particular reference to the presence of albumin and reducing sugars and the microscopic appearance of the sediment. Similar observations were made on all surviving rats at or about the 104th week. Positive tests for albumin

were obtained sporadically at 26 weeks, the incidence bearing no relation to particular supplements or test levels. At 52 weeks the positive tests were more frequent and in some cases more intense than at any other period whereas at two years they diminished almost to the vanishing point, except in the 20% Myrj 52 group where the finding of albumin was more consistent. Microscopic examination revealed the presence of occasional pus cells but no more consistently in albumin-positive than in albumin-negative specimens. Casts

TABLE 4

Plasma cholesterol values of rats at various periods during chronic feeding study

DIET	12 WEEKS [1]				36 WEEKS [2]				104 WEEKS [3]			
	Level, per cent				Level, per cent				Level, per cent			
	0	5	10	20	0	5	10	20	0	5	10	20
	milligrams cholesterol per 100 ml plasma											
Basal	88	..	..	..	133	..	..	..	80	..	..	..
Myrj 45	..	87	72	78	..	87	77	..	..	92	85	87
Myrj 52	..	81	114	89	..	112	74	..	..	90	96	89
Span 60	..	90	114	76	..	79	92	..	..	97	97	86
Tween 60	..	73	135	97	..	69	106	..	..	91	96	94
Tween 65	..	71	84	76	..	99	110	..	..	103	94	73
Tween 80	..	70	88	95	..	96	111	..	..	94	85	83
Mixture	..	73	85	94	..	109	99	..	..	97	91	92
Primex	..	77	108	..	..	92	77	..	..	84	88	..

[1] F_3 generation, 123 rats tested.
[2] F_4 generation, 88 rats tested.
[3] F_6 generation, 286 rats tested.

were noted occasionally but not in significant numbers nor in particular groups. It is probable that some of the albumin reactions may have been due to contamination of the specimens with diet or debris. Positive tests for reducing sugars were obtained more frequently at two years than at any earlier period. Estimations of the incidence of the albumin and sugar reactions (which were generally of mild to moderate intensity) and of the occurrence of occasional oxalate crystals, are depicted in table 5. In the interest of conserving space the findings are shown only for the one- and two-year periods.

The plus signs do not imply that all specimens within a group were positive but indicate the frequency as explained in the footnote to this table. Thus albuminuria which was more marked at 52 weeks than at earlier or later periods, was also more common at higher than at lower dosage levels. However exceptions were noted (cf. the Mixture diets) and even the Primex groups and the basal controls showed positive reactions. No consistent pattern was observed with respect to glucosuria which, as previously noted, was more often seen in the aged rats and appeared in the control as well as the test groups.

In view of the chemical derivation of the polyoxyethylene glycol esters, interest has been directed toward the possibility of oxaluria and urinary calculi resulting from chronic ingestion of the emulsifiers. In the next paper in this series dealing with pathological findings the incidence of renal and bladder calculi will be discussed. However, the findings with respect to the presence of oxalate crystals in the urinary sediment are shown in table 5. The groups in which they were found included the basal controls. Only in a few instances were more than occasional crystals seen and the groups involved were not consistently associated with particular test substances, levels or periods.

It is of interest to note that the pH of the two-year urine specimens varied within the range 6 to 8 and was not significantly different in the rats fed high emulsifier or fat levels from that in rats fed lower levels of supplement or none at all. The contrasting behavior of rabbits reported by Eagle ('52) was confirmed in concurrent experiments designed to explain the clarification of the normally turbid urine of this species after feeding single massive doses of Myrj 45. Rabbits were given single intragastric doses of 20 ml of Myrj 45, Myverol 18-00, cottonseed oil, or lard. Urine was collected before and during the 24-hour post-dosage period. Prior to dosage the urine was very cloudy and the pH was usually 8 or higher; the neutral fats of Myverol 18-00 caused disappearance of the turbidity with concomitant reduction in

pH to 6.5 or less. The results with Myrj 45 were variable but when the urine was clear the pH was acid and when it was cloudy the pH was usually above 8.

Similar experiments in which 20 ml doses of the polyol moiety of Myrj 45 were administered failed to produce this effect. It would thus appear that the rabbit metabolizes the

TABLE 5

Principal urinary findings[1] *at one and two years*

EMULSIFIER OR FAT	LEVEL	ALBUMIN		SUGAR		OXALATE CRYSTALS	
		1 yr.	2 yr.	1 yr.	2 yr.	1 yr.	2 yr.
	%						
None	0	+	—	—	++	++	-
Myrj 45	5	—	—	—	—	—	—
	10	—	—	—	—	—	—
	20	—	—	+	—	+	—
Myrj 52	5	—	—	—	+—	—	—
	10	—[2]	+[2]	—	+	—	—
	20	+	++[2]	—	—	—	—
Span 60	5	+	—	—	+	—	—
	10	—	—	—	+—	—	—
	20	+[2]	—	—	—	—	+
Tween 60	5	-	-	—	—	—	—
	10	+	—	—	—	—	—
	20	++	—	—	—	—	
Tween 65	5	—	—[2]	—	+	—	—
	10	—	—	—	—	—	—
	20	++	—[2]	—	—	—	—
Tween 80	5	—	—	—		—	—
	10	+	—	—	—	—	- -
	20	++	—[2]	-	- -	—	—
Mixture	5	—	—	—	+	—	—
	10	—	—	—	—	—	—
	20	—	—	—	—	—	+
Primex	5	+	+[2]	—	+	—	—
	10	+	—	—	—	—	-

[1] — Indicates 0 to 2, + indicates 3 or 4, ++ indicates 5 or more mild to moderate reactions for albumin or sugar or instances of oxalate crystals.

[2] Accompanied by significant increase in pus cells.

fatty acid moieties of neutral fat, as well as of the partial esters, to yield acid metabolites whereas this phenomenon is not manifested by the rat.

METABOLIC STUDIES

Digestibility of fatty acid moieties. The emulsifiers under investigation were expected to function as sources of energy to an extent determined by their degree of gastrointestinal hydrolysis and absorption. Obviously it would be presumptuous to assume the partial ester emulsifiers to have caloric values, gram for gram, equal to that conventionally ascribed to fat. Since the true caloric value of each emulsifier was needed as a basis for estimating the efficiency of caloric utilization of the test diets (see the first report in this series, Oser and Oser, '56a) these values were computed for each emulsifier from the heat of combustion and coefficient of digestibility of its fatty acid moiety. It was assumed that the polyol fractions, whether or not absorbed and excreted, are not catabolized to yield energy. The heat of combustion of stearic and oleic acids was considered to be 9.4 Cal. per gram.

Fatty acid balance studies were carried out on F_1 generation rats and subsequently on F_3 generation rats, both having been on the experimental diets for 8 weeks at the time the tests were begun. The first series included not only all the emulsifiers, but also Primex (hydrogenated vegetable fat) and Myverol 18-00.[3]

Because of the softness of the stools of certain groups, the emulsifier groups used in the balance experiments were those receiving the highest feeding levels compatible with accurate collection of feces. Hence Myrj 45, Span 60, Myverol 18-00, and Primex were tested at the 20% level whereas the others were tested at the 10% level; additional groups received 5 and 10% of Myrj 45 and Primex.

Groups of three male and three female rats were picked at random from the large groups on the chronic experiment and

[3] "Distilled monoglycerides made from hydrogenated lard," purchased from Distillation Products Industries, Rochester, New York.

housed individually in metabolism cages. Diets were fed ad libitum but the intake was recorded each day. The daily output of feces from each rat in the first series of tests was collected in tared, screw-capped jars and refrigerated. At the end of the 7-day collection period the feces of the three rats of the same sex in each group were pooled, air-dried, and ground to provide composite samples. Fatty acid determinations were conducted on the feces (and also on representative samples of the emulsifiers and diets) according to the following procedure:

Five- to 10-gram aliquots of the sample were weighed into 250 ml round-bottom flasks and refluxed for two hours with 25 ml of 0.5 N alcoholic potassium hydroxide. After cooling, the mixture was acidified (congo red paper) with 1:1 hydrochloric acid, transferred to 250 ml separatory funnels and extracted 4 times with 50 ml portions of ethyl ether. The combined ether extract was washed to neutrality, transferred to clean beakers and the solvent evaporated on a steam bath. The residue in the beakers was extracted repeatedly (with warming) with small portions of redistilled petroleum ether which were decanted and combined and then filtered through dry paper into clean, tared beakers. After evaporation of most of the solvent on the steam bath, the beakers were dried to constant weight in vacuo at 105°C.

The procedure followed in the second series of tests (conducted on the F_3 generation rats) differed from the foregoing only in that each individual rat's output of feces was collected in 95% ethanol in order to avoid oxidation during air-drying; then homogenized in a small blendor. The resultant slurry was made to a definite volume with alcohol to provide aliquots for analysis.

The coefficients of digestibility of the fatty acid moieties were calculated as the percentage ratio of unexcreted fatty acid to the dietary intake. Since these experiments were designed to measure the digestibility of the emulsifier acids, *per se*, the intake figures were corrected for the amounts of fatty acids contributed by the basal diet alone. For the same

reason, excretion figures for rats in the experimental groups were corrected for the proportional amounts of fatty acid excreted by animals of the same sex receiving the basal diet.

The values found by this method for the fatty acid content of the emulsifiers and of the test diets are shown in table 6. Considering the number of different batches of emulsifier diet

TABLE 6

Fatty acid content of diets

EMULSIFIER OR FAT	FATTY ACID CONTENT [1]	LEVEL IN DIET	FATTY ACID CONTENT OF DIETS		
			Total found	Attributable to fat or emulsifier	
				Found [2]	Calculated [3]
	%	%	%	%	%
None		0	4.18		
Myrj 45	43.88	5	6.38	2.41	2.19
		10	8.33	4.57	4.39
		20	12.43	9.09	8.78
Myrj 52	14.00	10	5.28	1.52	1.40
Span 60	69.62	20	17.45	14.11	13.92
Tween 60	22.77	10	6.20	2.44	2.28
Tween 65	43.28	10	8.59	4.83	4.33
Tween 80	23.56	10	6.40	2.64	2.36
Myverol 18–00	77.20	20	20.60	17.26	15.44
Primex	90.00	5	8.64	4.67	4.50
		10	13.65	9.89	9.00
		20	23.72	20.38	18.00

[1] Determined by direct saponification. The remainder is considered to be the polyol moiety.

[2] Equal to total found minus respective corrections for fatty acids derived from basal diet: $0.95 \times 4.18 = 3.971$; $0.90 \times 4.18 = 3.762$; $0.80 \times 4.18 = 3.44$.

[3] Five, 10, or 20% of the values determined by analysis of emulsifiers as shown in column 2.

represented in this table and the limit of precision in mixing diets, the agreement between the calculated and observed fatty acid values is reasonably good.

In table 7 are summarized the data from which the coefficients of digestibility were computed. Except for a low value found in the second Myrj 45 test at the 20% level, the agreement among the coefficients of the different levels of either

Myrj 45 or Primex indicates that there was no relation between the dietary levels and efficiency of absorption.

Figure 1 illustrates the relationship between the coefficients of digestibility and the melting points of the various

TABLE 7

Coefficients of digestibility of fatty acid moieties

EMULSIFIER OR FAT	LEVEL IN DIET	TEST	FATTY ACID		COEFFICIENT OF DIGESTIBILITY	
			Intake [1]	Output [2]	Individual test	Mean
	%		gm	gm	%	%
None	0	(a)	27.97	4.45	84.1	83.75
		(b)	34.69	5.76	83.4	
Myrj 45	5	(a)	16.24	2.71	83.3	
		(b)	12.82	2.66	79.3	
	10	(a)	32.45	6.08	81.3	80.35
		(b)	27.03	4.56	83.1	
	20	(a)	71.72	11.53	83.9	
		(b)	64.62	18.59	71.2	
Myrj 52	10		10.56	0.42	96.0	
Span 60	20		98.20	45.68	53.5	
Tween 60	10		16.03	0.37	97.7	
Tween 65	10		30.05	4.77	84.1	
Tween 80	10		18.75	0.0	100.0	
Myverol 18-00	20		122.55	69.77	43.1	
Primex	5	(a)	30.96	1.91	93.8	
		(b)	25.97	1.81	92.9	
	10	(a)	64.09	3.44	94.6	93.46
		(b)	61.34	4.45	92.7	
	20	(a)	121.88	8.14	93.3	

[1] Corrected for contribution of basal diet.

[2] Fecal output corrected for basal excretion.

emulsifiers, all of which, it may be noted, are stearates except Tween 80 which is an oleic acid ester melting below 20° C. Similar correlation between the digestibility of fats and their melting points has been noted by Crockett and Deuel ('47) and by others. Myrj 52 was the only exception to the rule, the observed digestibility coefficient being high in relation

to its melting point. This may have been due to the fact that Myrj 52, which contains only 14% of stearic acid, accounted for only a small proportion of the total fatty acid of the diet. It is interesting to note, however, that the fatty

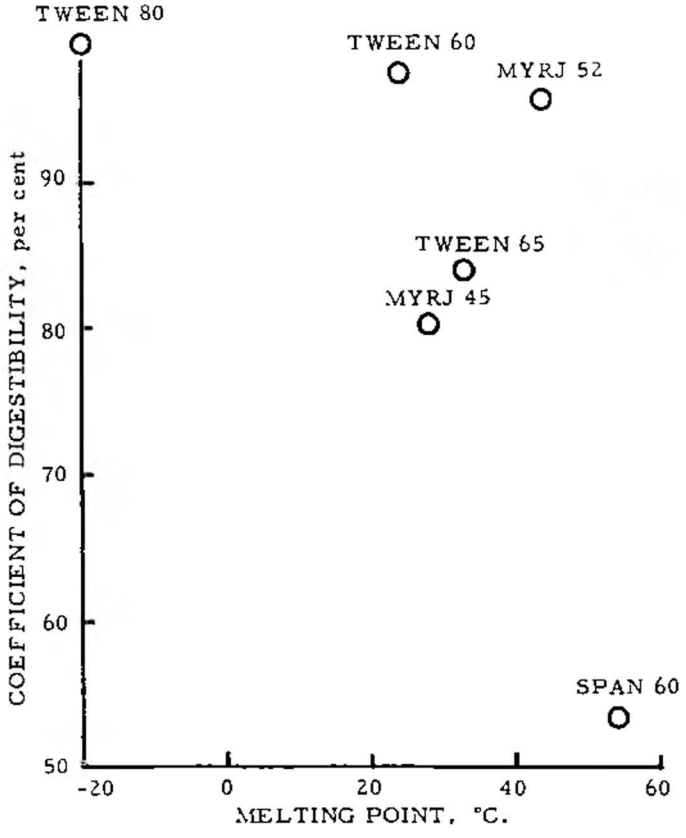

Fig. 1 Relation between coefficients of digestibility of fatty acids of emulsifiers and their titers.

acid moieties were most completely absorbed in those emulsifiers (Myrj 52 and the Tweens) which showed the greatest tendency to promote laxation. These particular emulsifiers are also characterized as the most hydrophilic of the series and, as shown above, are the ones whose polyol fractions proved most laxative.

SUMMARY AND CONCLUSIONS

The external appearance and behavior of the rats at all levels of partial ester emulsifier were quite normal throughout the two-year feeding test, except for those whose diarrheal condition resulted in inflammation and unthrifty appearance around the anal region. The animals thus affected were in the 20% Myrj 52 and Tween groups. No evidence of anemia, cachexia, or of abnormal neurological behavior was seen in any of the animals, particularly nothing to indicate the existence of any of the recognized nutritional deficiencies. Attention was directed to symptoms of thiamine shortage in view of the report that Myrj 45 at a 2% level caused destruction of dietary thiamine (National Research Council, '53); no such evidence was found in any of the emulsifier diets even at the 20% feeding level. Six-week storage tests at room temperature with diets containing up to 20% of Myrj 45 showed thiamine retention of 90% or better.

The laxative action was seen at the 20% level of Myrj 52 and the Tweens, and to a lesser extent at the 10% level, but not at all in the Myrj 45 or Span 60 groups. The emulsifiers responsible for this effect were those with long-chain polyoxyethylene groups (20 or 40 ethylene oxide moieties) whereas those which did not induce this response were the sorbitan stearate Span 60 and the polyoxyethylene (8) stearate Myrj 45. Laxation could be induced equally well by the longer chain polyols themselves.

Hematological tests at 12 weeks, one, one-and-a-half, and two years in all F_0 groups and at 12 weeks in 3 succeeding generations yielded consistently negative results. Blood chemical tests performed on representative rats at these periods included determinations of blood sugar and non-protein nitrogen and (in F_2 and F_3 generation rats) plasma cholesterol. A tendency toward an increase in blood sugar level was noted as the rats reached old age but the values were nevertheless within normal limits as, indeed, were all other blood chemical values. A transient albuminuria was seen in all

groups (including the controls) at one year and sporadic positive tests for reducing substances were obtained especially at two years, but the distribution of these findings among the groups, and the normal blood sugar and non-protein nitrogen values, suggest that they had no significance attributable to the emulsifiers. Microscopic examination of urine sediment at one year revealed the presence of occasional oxalate crystals in some of the emulsifier groups but in general fewer were found at the 20% levels than at 10%. Except possibly for the Span 60 group, the presence of oxalates in the two-year urine specimens was not significantly increased. These findings will be discussed in a subsequent report in connection with renal and bladder pathology.

Coefficients of digestibility of the fatty acid moieties of the partial ester emulsifiers were determined and used in computing the caloric value of the supplemented diets described in the first paper of this series (Oser and Oser, '56). The mean values found for Myrj 45, Myrj 52, Span 60, Tween 60, Tween 65, Tween 80, and Primex were 80.4, 96.0, 53.5, 97.7, 84.1, 100.0, and 93.5, respectively. These coefficients appeared to be correlated with the melting points of the emulsifiers except in the case of Myrj 52 which contains only 14% of fatty acid.

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