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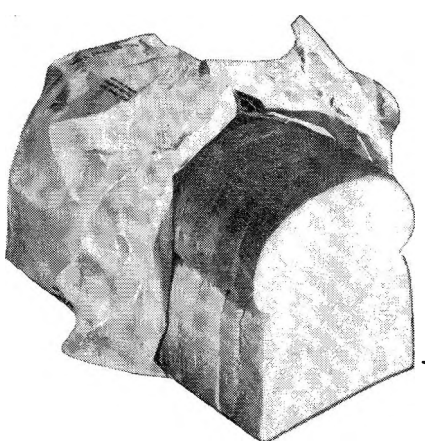

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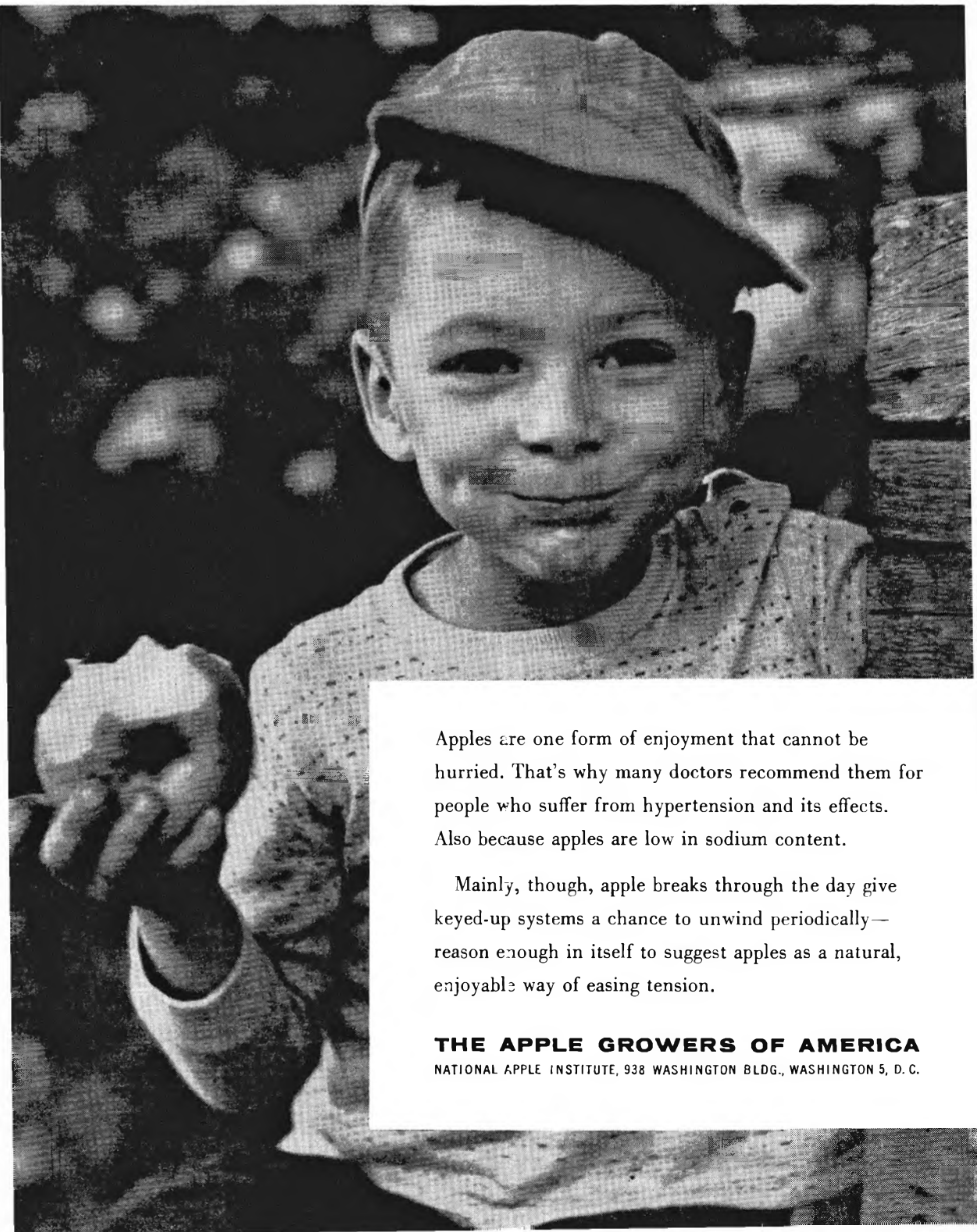

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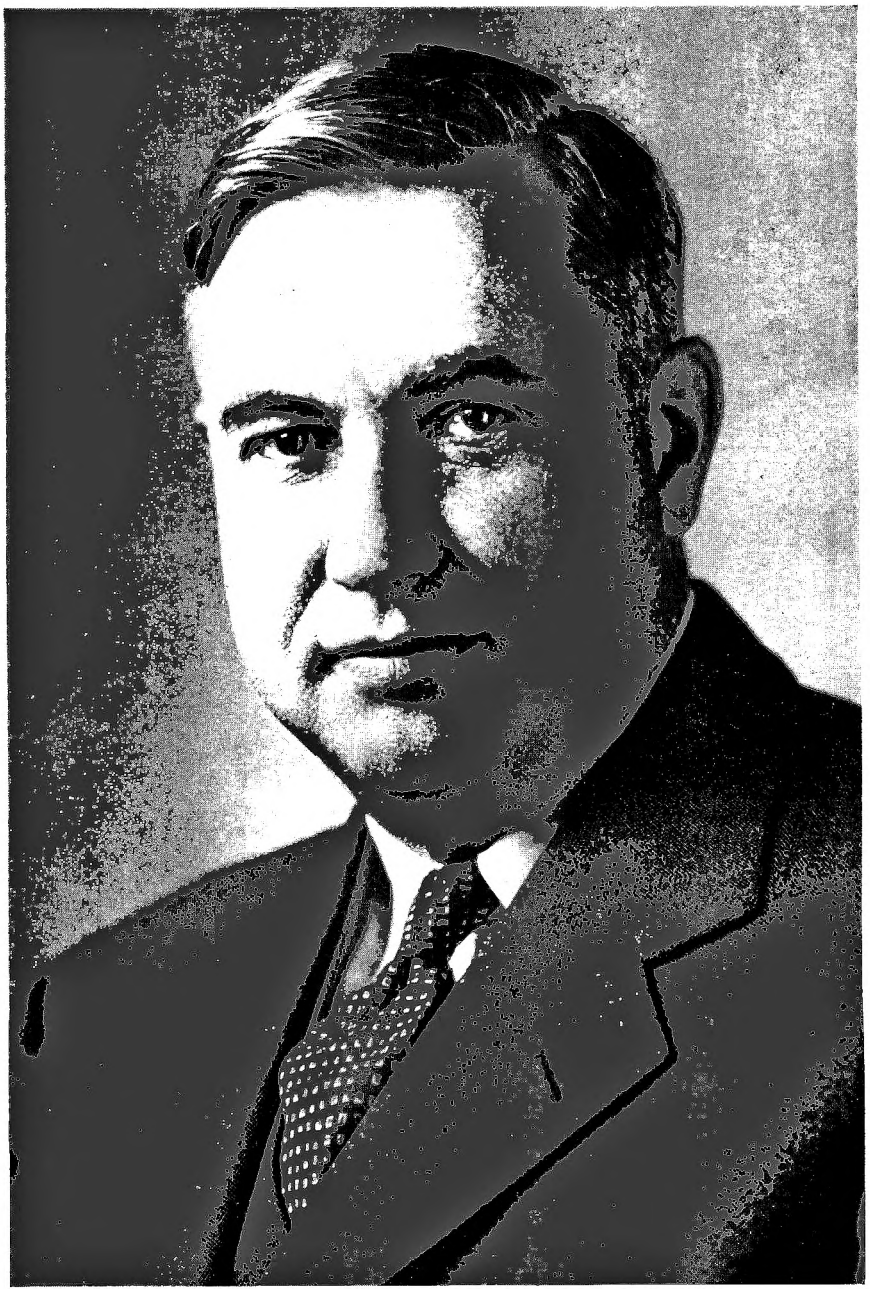

HARRY JAMES DEUEL, JR.

HARRY JAMES DEUEL, JR.

(October 15, 1897 — April 17, 1956)

In common with most of us, Harry James Deuel, Jr. frequently suffered from distortions of his surname. However, he generally regarded these occurrences with more amusement than annoyance, and according to his version of the event it was such an error which got him his first job. A secretarial error may then have been responsible for the beginning of a distinguished career in nutrition. In 1917, C. F. Langworthy, Chief of the Office of Home Economics of the Department of Agriculture, had an opening for a Junior Chemist. One of his associates, looking over the list of applicants, asked him how he would like to have a "Devil." The "Devil" was the son of Harry James and Myrtle Lillian Deuel, born in St. Paul, Minnesota, on October 15, 1897. Raised and educated in St. Paul, he had completed three years at Carleton College, where he had been a scholarship student.

There must certainly have been other factors involved in this appointment to the post of Junior Chemist, because his first scientific publication, in which he was the junior author with Dr. Langworthy, appeared the following year in the Proceedings of the National Academy of Sciences. This paper dealt, as did 11 others from this period, with problems of digestibility. Although these were largely joint publications with Drs. Langworthy and Arthur D. Holmes, Deuel was sole author of one. The capacity for effective and lucid exposition which characterizes his later writings is clearly evident at this time. It is also evident that these studies had a considerable effect in shaping his ultimate interests, since "The Digestibility of Certain Miscellaneous Vegetable Fats" and "The Digestibility of Some Hydrogenated Oils" were subjects to which he returned in 1946 and subsequent years.

During his employment in the Office of Home Economics, Harry Deuel found time to take the courses at George Washington University which permitted Carleton College to grant the A.B. degree in 1918. In 1920 he left the Department of Agriculture to undertake full time graduate work at Yale University. During three years at Yale, he worked with Oscar Baudisch, Arthur H. Smith, and George R. Cowgill on a variety of problems which resulted in several publications. The problem for his Ph.D. dissertation was carried out under the direction of Professor Lafayette B. Mendel. In the resulting paper in the Journal of Biological Chemistry on "The Metabolism of Some Pyrimidines," it was shown that thymine and uracil are in part metabolized to urea and that, although pyrimidines may be in part excreted without alteration when administered in large amount, they are not normally detectable as end-products of metabolism.

After receiving his Ph.D. in 1923, Dr. Deuel was appointed Instructor in the Department of Physiology of Cornell University Medical College where he continued until 1928, being promoted to Assistant Professor in 1927. He was, of course, one of the many who valued his association with Professor Graham Lusk, and was co-author of 7 papers in the series on "Animal Calorimetry" from Lusk's laboratory. In addition he always considered it a great privilege to have been able to read the proof and prepare the index for the 4th edition of Lusk's "Science of Nutrition."

Much of the work done at Cornell was in association with Dr. William H. Chambers, then a National Research Fellow in Medicine, and their studies of several aspects of metabolism in phlorhizin diabetes appear to represent the beginning of an interest in carbohydrate metabolism which continued through much of Dr. Deuel's scientific career.

It was also while at Cornell that Dr. Deuel married Grace Cutting, to whom he had become engaged during his last year at Yale. Miss Cutting had come from California to take an M.S. in Columbia's famed school of education, and through a school friend in California had gone to New Haven on a blind

date for the Harvard-Yale game. This resulted in a good many week-end trips to New York by Harry Deuel, which belied the story with which he used to like to embarass Mrs. Deuel — to the effect that she had proposed to him in the New York subway, on the pretext of asking directions, and when he was so nearly asleep that he didn't realize he was saying, "yes." Because of financial problems, Grace returned to California to teach for a year before they were married at the Cutting home in Riverside in 1924.

During the summer of 1925, Dr. Deuel was a special student at the Mayo Clinic, and served as a co-investigator and subject on a study of the nitrogen minimum which is a classic in this field. For this research, which was carried on with the collaboration of Irene Sandiford, Kathleen Sandiford, and Walter Boothby, Dr. Deuel subsisted on an essentially protein-free diet for 63 days. During most of the first 30 days the nitrogen intake was reduced to 0.26 and 0.24 gm per day, and a urinary nitrogen excretion of 2.10 gm per day was reached. The effects of thyroxine administration were then studied during two periods totalling 24 days, and it was demonstrated that the usual response in metabolic rate was produced, together with a rise in urinary nitrogen excretion. It could be concluded that thyroxine was still able to accelerate the breakdown of "deposit" protein in the severely depleted individual. After returning to a nitrogen intake of 7.12 gm per day for one week, Dr. Deuel resumed the restricted diet, with a nitrogen intake of about 0.7 gm per day for 9 days. During this period the urinary nitrogen excretion fell very rapidly and had reached the lowest value on record, 0.0241 gm per day per kilo, at the termination of the experiment. In discussing the results it was stated, "At least the nitrogen lost was not vital in the sense that it was an indispensable part of the living organism without which the organism as a whole could not function in an apparently normal manner, since the subject of this experiment readily carried on his daily laboratory work as well as many extraneous duties." And, at the end of the paper, "No noticeable physiologic disturbances

resulted from the prolonged ingestion of protein-free food other than the nausea occasioned toward the end of the period by the monotony of the food." I am sure that the others associated with this experiment would attest to the fact that these words were more a reflection of the characteristic enthusiasm with which Dr. Deuel undertook a project than of the response which would have been the result in most subjects.

By 1928 Dr. Deuel had established himself an an investigator and teacher and was appointed Professor of Physiology in the University of Maryland Medical School. After one year, however, he left to take the Professorship of Biochemistry in the School of Medicine of the University of Southern California, which was just being reopened. During the course of the next 20 years, he was to develop his department into one with a full time faculty of 6, an active graduate program, and a responsibility for biochemistry and nutrition in the College of Letters, Arts, and Sciences as well as the School of Medicine. Despite his many activities in research, and in various University functions in addition to teaching, Professor Deuel seemed always to be able to find time to get to know students well. For many years he and Mrs. Deuel regularly entertained the first year class in medicine in small groups. In part, he felt that the faculty had a responsibility to help make the students feel at home in the University and School of Medicine, but these social contacts also reflected a warm interest in people which characterized all of his associations.

During the first 8 or 9 years at the University of Southern California, Deuel and his associates concerned themselves primarily with aspects of fat and carbohydrate metabolism relating to ketosis; and with the investigation of sex differences in carbohydrate metabolism which were originally noted by Deuel and Gulick as an earlier and more severe fasting ketonuria in women than in men. In the studies on ketosis it was found that, although the rat is not normally subject to a starvation ketosis, a ketosis could be produced by the ingestion of sodium acetoacetate which appeared to be

equivalent to a ketosis of endogenous origin. This permitted a more extensive investigation of the ketolytic action of various carbohydrates than would have been possible with the dog. The important conclusion was reached that the formation of liver glycogen and ketolytic activity go hand in hand. With respect to the mechanism of ketone body formation, studies with various fatty acids demonstrated the formation of an approximately equal amount of ketone bodies from sodium butyrate and caproate as was obtained from sodium acetoacetate, while an equimolar amount of caprylate yielded about twice as much. These experiments of Butts, Cutler, Hallman, and Deuel, together with the *in vitro* studies of Jowett and Quastel on liver slices (also appearing in 1935) made it impossible any longer to consider the oxidation of fatty acids as adequately described by the original β-oxidation theory of Knoop. In other experiments it was shown that rats will develop a spontaneous fasting ketonuria if fatty livers have previously been induced by diet.

Although papers in the series on ketosis and on the sex differences in carbohydrate metabolism continued to appear in subsequent years, two new interests began to develop around 1938. These were closely related to an association with The Best Foods, Inc., which was established at this time and continued until Dr. Deuel's death. The representatives of Best Foods were dissatisfied with sources for bioassay of vitamin A on the west coast, which was required on margarine coming on to the market, and Dr. Deuel was interested in beginning work on vitamin A. Support by Best Foods made it possible for Dr. Deuel to develop and maintain a large rat colony for vitamin A biossay. As a result of this association, a broader program of research support developed,— particularly in the field of the nutritive value of various fats and fatty acids. For Professor Deuel, these studies represented a return to the field of interest of his first work in nutrition with Dr. Langworthy.

As early as 1939, Dr. Deuel had initiated a multi-generation experiment, patterned after those of Sherman and Campbell,

and using Sherman diet B in which butter fat had been replaced by a margarine fat. The results obtained with the first 10 generations were reported in 1945, and provided further confirmation of earlier studies which had shown margarine fat to be entirely satisfactory as a dietary fat. The earlier, shorter-term experiments had demonstrated an equal nutritive value for margarine and vegetable fats as for butter fat, and had originally represented one side of a "butter-margarine controversy" in which experiments at the University of Wisconsin had indicated a lower nutritive value for margarine fats than butter fat. This disagreement was eventually resolved by the finding of the Wisconsin group that the poorer response on margarine was only observed when the diet contained lactose as the sole or principal carbohydrate. Deuel's studies, demonstrating the high nutritive value of vegetable fats and their partial hydrogenation products, were not only an important factor in the eventual elimination of the discriminatory taxes then levied on margarine; but were also of very fundamental importance in proving that there are no peculiar requirements for saturated fatty acids (as had been suggested by some), and confirming the essential nutritive equivalence of the higher fatty acids,— except for the essential, unsaturated fatty acids.

Vitamin A studies included the demonstration that the intestine, rather than the liver, is the principal site of conversion of carotene to vitamin A in the rat. This conclusion was foreshadowed by the finding of little or no provitamin A activity of carotene when administered parenterally, published with E. L. Sexton as the senior author in 1946. In the following year, work carried out principally by F. H. Mattson for the Ph.D. degree was reported in which the intestinal conversion was quite clearly demonstrated. Although there were many other aspects of carotene and vitamin A metabolism which were investigated, perhaps the most significant contribution is a series of papers resulting from over 10 years of collaboration with L. Zechmeister and his associates. It was a fortunate circumstance which found the outstanding

authority on the chemistry of the carotenoids at the California Institute of Technology is Pasadena in close proximity to Deuel's unexcelled laboratory for the study of vitamin A bioassay in Los Angeles. The effectiveness of the relationship was further fortified by the fact that the Deuels lived in South Pasadena and Pasadena during much of this period, and by the close personal friendship which developed with Dr. Zechmeister. Eleven papers were published dealing specifically with stereochemical configuration and provitamin A activity, and others dealing with the effectiveness of conversion of various natural carotenoids to vitamin A.

It is impossible to do justice to the variety of other researches with which Deuel concerned himself,— such as the original demonstration that the cerebroside in the spleen in Gaucher's disease is not only abnormal in amount but also in the fact that it contains glucose rather than galactose. However, mention should be made of the interest which he developed in the question of optimum requirements for fat in the diet. It would be possible to infer that, except for the requirement for essential fatty acids and fat-soluble vitamins, fats are not necessary in the diet. A large number of experiments, carried out with Scheer, Greenberg, Alfin-Slater and others, showed that optimum performance with respect to severe exercise, growth, reproduction and lactation can not be obtained on low-fat diets. As a result of such studies, Professor Deuel developed a real concern that limitations of fat intake in an attempt to reduce the incidence or severity of atherosclerosis might have seriously deleterious effects in other directions.

Another significant contribution to applied nutrition is represented by Professor Deuel's approach to the problem of toxicity of food additives. He recognized, and emphasized very effectively, the fact that a knowledge of the metabolism of the material in question can greatly simplify the evaluation of possible toxicity, and place conclusions on a much sounder basis than would otherwise be possible. This approach was

employed effectively by him in studies relating to the use of citric acid derivatives and sorbic acid.

Much of the work which has been discussed was carried out after Professor Deuel had been appointed Dean of the Graduate School in 1949. At this time he had relinquished the chairmanship of the Department of Biochemistry and Nutrition, but of course retained his Professorship in the Department. He continued to devote a large part of his time to research and writing, and continued to supervise the research of graduate students. It was also during this period that he undertook the writing of "The Lipids." Volume I, on the chemistry of the lipids, was published in 1951. It had originally been intended that a second volume would cover biochemical aspects, but it was found that Volume II would be required just to complete the presentation of digestion, absorption, transport, and storage. This was published in 1955. While working on this volume, and on Volume III, dealing with the metabolism and nutritional importance of lipids, Dean Deuel began to suffer with a painful hip. The difficulty was originally associated with a fracture of the hip which he had suffered a few years earlier while ice skating, and with a calcification defect in the joint. The condition soon became so severe that walking became very painful and difficult, and for a considerable period even sleep became almost impossible. Despite this handicap, he refused to limit his activities, and his suffering certainly did not show in his relations with others or his optimistic enthusiasm for new problems. Late in 1954 it was found that he was the victim of an osteogenic sarcoma. Despite the realization that metastases to the lung were already present, Dean Deuel insisted on a hemipelvectomy in the hope that he would have sufficient pain-free time in which to complete Volume III of the Lipids and carry out plans to spend a year lecturing in England and on the continent. After an amazingly rapid recovery from the surgery, Dean Deuel was back at the University and at his book early in 1955. His attitude appeared to be that his problem had been solved, except for the loss of a leg, and I am sure that many of us

really believed that he was unaware of the probable prognosis at this time. Indeed, his principal concern seemed to be for the embarrassment which he sensed in others in adjusting to the fact that he had lost a leg.

He and Mrs. Deuel did go to England under the auspices of the Fulbright program in the summer of 1955, after attending the meetings in Europe. For most of the year he carried on a very active program of lecturing. His efforts to do justice to his commitments were rewarded by large and distinguished audiences, and the warmth of his reception was a great satisfaction to him. Although his health began to fail seriously during the winter, he was also able essentially to complete the writing of the last volume of The Lipids. It became necessary for him to return to this country in the spring, and to be hospitalized immediately on his return. He lived only a few weeks longer,— but long enough to know that he had been elected President of the American Institute of Nutrition, of which he had been a member since its origin. He died in Pasadena on April 17, a few hours after the announcement of his election at the Nutrition Dinner.

Among the many other societies of which he was a member were the American Society of Biological Chemists, the American Physiological Society, and the Harvey Society. He had been a member of the board of directors of Annual Reviews Inc. since 1946, and became President of the Board in 1953. He had been Chairman of the Oil Seeds and Peanut Committee of the Research Marketing Administration of the U.S. Department of Agriculture since 1949. Dean Deuel was the recipient of the Borden Award from the American Institute of Nutrition in 1949, the Monsanto Presentation Award of the Institute of Food Technologists in 1954, and an Honorary Award by the University of Brussels in 1955.

In an effort to perpetuate the memory of his contributions to the developement of biochemistry and nutrition at the University of Southern California and the memory of a distinguished scientific career, the Harry J. Deuel, Jr. Award has been established with funds contributed by friends and

former students. This will be awarded annually at Commencement to a Ph.D. candidate. In addition, the University has dedicated the laboratories in which he carried on his work after becoming Dean of the Graduate School as the Harry J. Deuel, Jr. Laboratories. Speaking at the dedication of these laboratories on May 15, 1957, Professor Wendell H. Griffith said, "This dedication to Harry James Deuel, Jr., Dean and Professor, is wholly reasonable and natural because this great University can have no other thought than righteous pride and appreciation of his loyal and earnest service to the institution to which he gave such eager devotion, to medical science whose mysteries and opportunities for good captured his imagination and impelled his energies and skills, and to his fellow men in whom he found never-ending pleasure and to whom he returned in abundance inspiration for their own endeavors."

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Los Angeles

DEPOSITION IN TISSUES AND FECAL EXCRETION OF *TRANS* FATTY ACIDS IN THE RAT [1,2]

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(Received for publication August 8, 1957)

INTRODUCTION

The unsaturated fatty acids in natural fats, with few exceptions, are of the *cis* configuration, while those in hydrogenated fats are to a considerable extent of the *trans* configuration (Hilditch and Vidyarthi, '29). It has been suggested (Sinclair, '56) that there may be a relationship between the consumption of hydrogenated fats and the increased incidence of atherosclerosis and that the unnatural *trans* fatty acids in hydrogenated fats may contribute to the formation of atheroma. On the other hand, it has been assumed that the metabolism of *trans* fatty acids proceeds normally (Barbour, '33).

Recently Allen et al. ('56) have shown that rats fed *trans* fatty acids for a two-week period are capable of metabolizing the *trans* fatty acids from margarine stock as well as those from synthetic triglycerides which had the *trans* double bond in either the Δ 8 or 9 position. However, this study merely showed that the rat is capable of metabolizing *trans* fatty acids. In the present study specific amounts of *trans* fatty acids were fed for a longer period of time and the amounts of *trans* fatty

[1] This study was supported by a grant-in-aid from the National Livestock and Meat Board.

[2] Portion of a thesis presented by P. V. Johnston as partial fulfillment of the requirements for the degree of Doctor of Philosophy in Food Technology.

acids deposited in the carcass measured in order to determine: (1) whether a relatively constant or a maximum level of *trans* fatty acids was deposited in the tissue, (2) if the *trans* fatty acids which were deposited in the carcass fat disappeared when *trans* fatty acids were removed from the diet.

EXPERIMENTAL

A basal diet which consisted of 64% glucose,[3] 31% casein and 5% Wesson ('32) salt mix was used in all the feeding experiments. A margarine stock which contained 40% of *trans* fatty acids provided the source of *trans,* and was added at the expense of the glucose. Two percent of soybean oil served as a source of essential fatty acids and the test fat was added at a 10% level. Four grams of a water-soluble vitamin mix were added to each kilogram of food. This mixture was composed of choline 93.5 mg, thiamine 1.24 mg, riboflavin 1.24 mg, pyridoxine 1.24 mg, calcium pantothenate 2.48 mg, and folic acid 0.30 mg per 100 mg. The fat-soluble vitamins were given by dropper once each week.[4]

One hundred and three weanling female rats were used; three were sacrificed and all but 10 of the remainder were divided into two major groups. The diet for group I contained 10% margarine stock; for group II, 5% margarine stock and 5% olive oil. Each major group was divided into 5 subgroups. The animals in subgroup A were fed the test diet for a period of one month, those in subgroup B for two months and those in subgroup C for three months (table 1). The animals in subgroups D and E were fed 10% margarine stock for one month and then transferred to a diet free of *trans* fatty acids. This diet contained 2% of soybean oil as the only fat source (table 2). The animals in subgroup D were sacrificed after being on the fat-free diet for one month and those in subgroup E after

[3] Cerelose.

[4] One drop per rat of the following vitamin mixture was administered once each week. Five grams vitamin A (200,000 U.S.P. units, courtesy of Distillation Products), 0.0054 gm vitmain D_2 and 2.535 gm vitmain E (mixed tocopherols) in 100 ml of olive oil.

TABLE 1

The percentage of trans *fatty acids in the carcass fat of rats fed dietary fat in the form of 10% margarine stock or 5% margarine stock and 5% olive oil for one to three months*

GROUPS	NO. OF RATS	TOTAL FAT	TRANS FATTY ACIDS	TRANS IN LIVER FAT [1]	DIET. TRANS FOUND IN LIVER [1]	MEAN BODY WT. GAIN AFTER		
						3 Weeks	7 Weeks	12 Weeks
		gm	*%*	*%*	*%*	*gm*	*gm*	*gm*
I. 10% Margarine stock Total content in:								
A. 1 month	5	16.5 ± 2.5 [2]	16.7 ± 1.4 [2]	18.0	0.57	29.6 ± 3.1 [2]	—	—
B. 2 months	10	29.2 ± 3.7	18.9 ± 1.3	17.8	0.18	26.9 ± 6.0	164.2 ± 15.5	—
C. 3 months	10	47.8 ± 5.6	18.0 ± 0.9	16.8	0.09	31.1 ± 4.3	167.2 ± 14.2	209.8 ± 14.9
II. 5% Margarine + 5% olive oil Total content in:								
A. 1 month	5	19.7 ± 1.9	10.5 ± 0.6	10.8	0.82	30.2 ± 3.5	—	—
B. 2 months	10	30.8 ± 3.9	11.3 ± 0.7	9.8	0.13	29.6 ± 3.5	164.0 ± 8.3	—
C. 3 months	10	44.3 ± 5.7	10.8 ± 0.9	12.5	0.12	27.6 ± 2.6	167.0 ± 10.4	194.4 ± 11.4
Control	10	40.7 ± 6.5	0	0	—	29.2 ± 2.3	172.6 ± 12.1	204.8 ± 14.3

[1] Data for the liver fat are calculated from the pooled samples for each group.

[2] Standard deviation of the mean.

two months. The remaining 10 rats were fed the basal diet plus 10% of olive oil and served as the *cis* or "natural" fat group.

All animals were individually housed, the food consumption was noted, feces samples collected, and the animals weighed at least once each week. The rats were fasted for 24 hours and anesthetized. The livers were removed immediately after death and weighed. All samples were stored at —20°C while awaiting analysis.

The carcass was transferred to a large beaker and digested with the aid of concentrated hydrochloric acid. The beaker and contents were heated in a steam cabinet at 60°C for 24 hours; the fat floated to the surface and was readily removed with petroleum ether (Skellysolve F). The remaining solution was extracted three times by shaking with Skellysolve F in a separatory funnel and the combined extracts washed free of acid with water, dried over anhydrous sodium sulfate and freed from solvent under vacuum. All extractions were carried out quantitatively.

The fat was extracted from the livers by grinding them in a mortar with anhydrous sodium sulfate and then extracting the mixture in a Soxhlet apparatus for 24 hours with acetone followed by Skellysolve F. The solvents were removed under vacuum after drying over anhydrous sodium sulfate.

The feces were treated with 30% hydrochloric acid before extraction in order to break down any soaps present, and the solution extracted with Skellysolve F. The combined extracts were dried over anhydrous sodium sulfate and freed from solvent under vacuum.

The analysis for *trans* double bonds was carried out with the aid of a Beckman IR2A infrared spectrophotometer. The Jackson and Callen ('51) baseline method was used for calculating percentage of *trans* double bonds in the fat. A 5% solution of the samples in carbon disulfide was used for the analyses, and all results were based on a trielaidin standard.

RESULTS

Trans fatty acids were only detected in the tissues of the animals which had received a diet containing *trans* fatty acid. No *trans* fatty acids were found in the tissues or feces of the three animals sacrificed at the beginning of the experiment. Only traces of absorption at 10.3 μ in the animals which had received 10% olive oil as a source of dietary fat were noted. These traces of *trans* fatty acid may have been due to the limitation of the method of analysis or may have been due to the transfer of *trans* fatty acids from the breeding colony [5] during the weanling period. At the end of the first month of feeding the animals contained between 15.5 and 18.8% of *trans* fatty acids in the carcass fat; at the end of the second and third month the *trans* fatty acid content of the carcass increased less than 2 and 1% respectively (table 1).

The total amount of carcass fat increased considerably during the three-month period. After one month the animals contained approximately 16 gm, after two months 29 gm, and after three months 48 gm, of total carcass fat respectively. However, despite the increase in total fat, the percentage of *trans* fatty acids in this fat remained virtually unaltered. During this period the animals had consumed 12.5 to 15 gm of diet per day or 0.5 to 0.6 gm of *trans* fatty acids per day.

In order to maintain the percentage of fat existing in the *trans* form at a constant level, the actual weight of *trans* fatty acids in the body naturally increased in proportion to the increase in total carcass fat. For example, a rat having 14 gm of total carcass fat which contained 18.8% of *trans,* had an actual weight of 2.6 gm of *trans* fatty acids in its carcass; whereas an older animal having 43.2 gm of total fat which contained 18.7% of *trans* had 8.0 gm of *trans* fatty acids in its carcass. Hence, the rat organism selected a specific amount of the dietary *trans* acids to deposit in the fat stores and thus maintain a constant concentration of these acids in its body fat. The

[5] Holtzman Rat Company, Madison, Wisconsin.

amount of dietary *trans* fatty acids found in the tissues seemed to depend on three major factors:

1. The amount of dietary *trans* fatty acids which were immediately metabolized.
2. The rate at which the already deposited *trans* fatty acids were mobilized from the fat stores and metabolized.
3. The amount of dietary *trans* fatty acids which were excreted in the feces.

Since the food consumption per rat was noted every day, a balance study could be made to determine the efficiency of metabolism of *trans* fatty acids by the rat. As the larger portion of the deposited *trans* fatty acids was found in the carcass body fat the individual figures for total body fat and percentage of *trans* fatty acids were determined and used in the calculations. The total fat content and percentage of *trans* fatty acids of both the feces and liver were based on pooled samples, since only small amounts of the ingested *trans* fatty acids were found in these samples. The analysis of the liver fat for each group is reported in tables 1 and 2. The calculations revealed that during the first, second and third months respectively the animals which received 10% of margarine stock deposited between 25.6 and 19.0, 24.5 and 14.2, and 22.0 and 15.4% of the dietary *trans* fatty acids in their carcass fat; 0.54, 0.18 and 0.09% in their liver fat, and throughout the entire feeding period excreted between 1 and 4.5% of the ingested *trans* fatty acids. The remainder of the *trans* fatty acids were apparently metabolized.

Trans fatty acids seemed to be metabolized in essentially the same manner as a labeled fatty acid (Lovern, '55). That is, a certain percentage of the labeled fat was selected to make up the total fat composition. The percentage of labeled fat was maintained at a constant level provided that the food intake remained unaltered. The remainder was metabolized and a small amount excreted.

The animals which had received 10% of dietary fat in the form of 5% margarine stock and 5% olive oil contained approximately 20 gm of carcass fat after one month, 31 gm after

TABLE 2

Decrease of trans *fatty acid in carcass fat of rats which had been transferred for one to two months to a diet free of* trans *fatty acids*

GROUPS	NO. OF RATS	TOTAL FAT	TRANS FATTY ACIDS	TRANS IN LIVER FAT [1]	DIET. TRANS FOUND IN LIVER [1]	MEAN BODY WT. GAIN AFTER 3 WEEKS
		gm	%	%	%	*gm*
I. From 10% margarine stock, Decrease to in:						
D. 1 month	10	27.4 ± 5.1 [2]	6.5 ± 0.68 [2]	9.1	0.17	30.3 ± 4.0 [2]
E. 2 months	10	30.3 ± 4.8	4.4 ± 0.87	8.3	0.11	28.1 ± 2.3
II. From 5% margarine + 5% olive oil, decrease to in:						
D. 1 month	10	28.3 ± 4.3	4.9 ± 0.57	10.3	0.19	27.2 ± 4.1
E. 2 months	10	32.5 ± 4.2	2.8 ± 0.98	6.4	0.22	26.0 ± 3.5
Control	10	40.7 ± 6.5	0	0	—	29.2 ± 2.3

[1] Data for the liver fat are calculated from the pooled samples for each group.
[2] Standard deviation of the mean.

two months, and 44 gm after three months (table 1). They utilized the ingested *trans* fatty acids in a similar manner to those fed 10% of margarine stock. That is, a constant level of *trans* fatty acids was maintained in the carcass fat (table 1). However, the percentage of *trans* fatty acids in the carcass fat was slightly higher than half of that found in the carcass fat of animals which had received 10% of margarine stock. This discrepancy can be explained by the fact that the inclusion of olive oil in the diet appeared to facilitate the absorption of the *trans* fatty acids. This observation was confirmed by the fact that the animals which had received olive oil and margarine stock excreted only small quantities of the dietary *trans* fatty acids. In no case did this amount exceed 1% and in many cases the amount of absorption at 10.3 μ was too small for the calculation of *trans* faty acids with any degree of accuracy. The higher absorption of *trans* fatty acids from the gastrointestinal tract evidently led to a higher percentage deposition of *trans* fatty acids in the depot fat.

The balance studies on the animals receiving 5% of olive oil and 5% of margarine stock showed that during the first month 39.6 to 29.7% of the dietary *trans* fatty acids were deposited in the carcass fat and 0.82 in the liver fat. These figures seem strikingly higher than those found for the animals receiving 10% of margarine stock. However, this is merely a reflection of the higher absorption by the animals of the *trans* fatty acids due to the inclusion of olive oil. During the second and third months when the concentration of *trans* fatty acids did not appreciably increase, 27.9 to 19.9 and 22.5 to 17.9% of the dietary *trans* fatty acids were deposited in the carcass fat and 0.12 to 0.22% in the liver fat. The amount of dietary *trans* fatty acids excreted by the animals in this group was extremely small and in most cases below 1%. The remainder of the dietary *trans* fatty acids were apparently metabolised.

The percentage of *trans* fatty acids in the carcass fat decreased when *trans* fatty acids were removed from the diet. They did not completely disappear from the tissues even at the end of two months on a diet free of *trans* fatty acid (table 2).

After one month on the diet free of *trans* fatty acid, the carcass fat of the rats which had received 10% of margarine stock (table 1) had decreased to 6.5% and after two months to 4.4% of *trans* fatty acids (table 2). The carcass fat of the animals which had received margarine stock and olive oil contained approximately 11% of *trans* fatty acids (table 1). After one month on a diet free of *trans* fatty acid, this had decreased to 4.9% and after two months to 2.8% of *trans* fatty acids (table 2).

The results are again in agreement with the behavior of labeled fat. That is, once the labeled fat is removed from the diet, it gradually disappears from the tissues. The depletion of *trans* fatty acids appeared to be somewhat slower than may be expected. However, the present study is in apparent agreement with the work of Kohl ('38) who found that over 30 days were required to deplete animals which had been fed elaidic acid for only three days. The latter study was made before the perfection of the infrared method of analysis for *trans* double bonds. As recent reports have pointed to the relative inaccuracy of the older technique (lead salt-alcohol method) for the determination of *trans* acids (Jackson and Callen, '51), a direct comparison between the study of Kohl and the present one cannot be made.

The depletion of *trans* fatty acids from the liver was slower than from the carcass. This slower rate of depletion was probably due to the fact that the *trans* fatty acids in the depot fat were mobilized and entered the metabolic pool. Hence, the amount of *trans* fatty acids in the liver fat remained higher than in the carcass while this mobilization was in progress.

Holman and Aaes-Jorgensen ('56) indicated that the isomers of *trans* fatty acids inhibit growth. However, in the present study, no evidence of such inhibition was observed. The mean body weight gains of each group after three weeks on the diets are reported in tables 1 and 2. After 7 weeks the animals in groups IB IC, IIB and IIC showed an average mean body weight gain of 166 gm while the animals in the control group which received olive oil as the fat source showed

an average gain of 172.6 gm. The animals in groups IC and IIC which received the diet for a further 5 weeks showed an additional average gain of 35 gm during this time while the animals in the control group gained another 32.2 gm (table 1). Application of the "t" test to the weight data indicated that the differences were not significant (Snedecor, '55). This conflict with the results of Holman and Aaes-Jorgensen may be explained by the fact that in the present study the rats received an adequate amount of essential fatty acids whereas those used by Holman et al. had been depleted of essential fatty acids.

It can be concluded from the present study that rats are capable of efficiently metabolizing *trans* fatty acids over a three-month feeding period. However, the hypothesis of Sinclair ('56) cannot be rejected solely on the basis of the efficiency of metabolism of these fatty acids by the rat. A recent report of Johnston et al. ('57) has established that *trans* fatty acids are present in human tissues. The *trans* fatty acids found in human tissue are no doubt of dietary origin. The results of the present study indicate that if *trans* fatty acids do contribute to the onset of any degenerative disease, this apparently does not occur due to an inability of the animal to metabolize *trans* fatty acids. The effect of the *trans* fatty acids on other metabolites remains unknown. Before Sinclair's hypothesis is considered completely unfounded, further investigations appear essential.

SUMMARY

Trans fatty acids in the form of hydrogenated margarine stock were fed to rats. *Trans* fatty acids were found to be deposited in the tissues only when they were present in the diet. The largest amounts of the deposited *trans* fatty acids were found in the carcass fat, smaller amounts in the liver, and only small quantities were excreted in the feces; the major portion of the ingested *trans* fatty acids were metabolized. When the *trans* fatty acids were removed from the diet

they gradually decreased in amount in the tissues. The presence of *trans* fatty acids in the diet did not appear to inhibit growth.

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THE EFFECT OF DIETARY ENERGY CONCENTRATION AND AGE ON THE LYSINE REQUIREMENT OF GROWING CHICKS [1]

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Determinations of the lysine requirement in the chick by various investigators have yielded inconsistent results. When expressed as per cent of the diet, values ranging from 0.9% (Almquist and Mecchi, '42) to 1.1% (Hill, '53) have been recommended. In an attempt to explain the variability of these results, Edwards et al. ('56) limited the growth of their birds by feeding diets containing graded levels of bulk and concluded that the lysine requirement was influenced by the rate of gain. Griminger ('55), who had selected birds according to their natural ability to grow rapidly and slowly, did not, however, find any difference in the lysine requirement.

An optimum ratio between dietary energy and the requirement for nitrogen has been demonstrated repeatedly (Combs, '56, Sibbald et al., '56). Baldini and Rosenberg ('55) found that the chick required more methionine in the diet when fat was included. This response was explained by the dominant influence dietary energy has on feed intake as was demonstrated by Hill and Dansky ('54). March and Biely ('56),

[1] Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers University, The State University of New Jersey, Departments of Agricultural Biochemistry and Poultry Husbandry, New Brunswick. The experimental data in this paper are taken from a thesis submitted by the senior author to the Graduate School of Rutgers University in partial fulfillment of the requirement of the Ph.D. degree in Agricultural Biochemistry.

however, reported that the addition of fat to poultry diets increases the requirement for both choline and methionine so that it is also conceivable that the participation of this amino acid in fat metabolism has contributed to these results. A study of the influence of dietary energy (fat) on the requirement for lysine should avoid this complication since Mitchell ('47) showed that lysine is primarily needed for rapid protein synthesis as found in the growing rat, while it is practically dispensable in the adult.

The effect of age on nutrient requirements has been investigated mostly with respect to protein. In the pig less protein is required as the animal grows older (Reber et al., '53, Mitchell et al., '36–'37). Since the protein requirement represents primarily the sum of requirements for the indispensable amino acids, the requirement for the latter should also decrease with progressing age. For the turkey poult Fisher et al. ('56) and Kratzer et al. ('56) independently showed that the concentration of protein and lysine in the diet could safely be reduced when the birds grow older. Direct evidence for a decreasing requirement for lysine during the growth period of the chick and direct evidence to establish the rate at which it decreases are lacking, although the results of Bird ('53) are suggestive that the chick at 8 weeks requires less lysine than during the first few weeks of life. The lysine requirement during various periods was, therefore, investigated.

EXPERIMENTAL

Groups of 10 Indian River males or New Hampshire ♂ × Columbian ♀ crossbreeds of both sexes received ad libitum diets primarily composed of corn, sesame meal, and various ratios of cellulose and fat (table 1). With the exception of experiment 3, all birds were allotted according to body weight and the 5- to 12-day weight gain on a commercial starter ration.[2] Dietary treatments were started at 12 days of age. In experiment 3 two groups, one of each sex, were used in each

[2] G.L.F.

treatment starting at one day of age. For experiments 4 and 5 the amino acid level was lowered stepwise at various rates during a two- to 11-week growth period as indicated in table 4. In each experiment one group serving as the positive control received 1.2% of lysine continuously. Where the lysine concentration was reduced more than once during the test period, such dietary changes were repeated only when the level fed previously maintained growth within 5% of that of the group fed 1.2% lysine. Individual body weights and group feed consumptions were recorded at intervals as shown in the tables.

TABLE 1

Composition of diets

INGREDIENTS	DIET B	DIET C	DIET D	DIET E	DIET F
	%	%	%	%	%
Corn meal	54.77	54.77	47.05	47.05	47.05
Sesame meal	32.00	32.00	32.00	32.00	32.00
Wheat gluten	—	—	6.00	6.00	6.00
Minerals	2.50 [1]	2.50 [1]	3.70 [2]	3.70 [2]	3.70 [2]
Vitamins	0.73 [3]	0.73 [3]	0.22 [4]	0.22 [4]	0.22 [4]
Cellulose [5]	10.00	—	10.00	5.00	—
Fat	—	10.00 [6]	—	5.00 [7]	10.00 [7]
Productive energy Cal./lb.	815	1115	815	965	1115
% Protein, calculated	19.4	19.4	23.1	23.1	23.1
analyzed (N × 6.25)	20.3	20.5	23.4	23.4	23.4
Energy: protein ratio	40	54	35	41	48
% Lysine, calculated	0.57	0.57	0.65	0.65	0.65

[1] Bone meal 1, limestone 1, iodized salt 0.5, manganese sulfate (70%) 0.01 gm per 100 gm diet.

[2] Dicalcium phosphate 2.2, Mico Concentrate (Limestone Prod. Corp., Newton, N. J.) 1.0, iodized salt 0.5 gm per 100 gm diet.

[3] Choline Cl (25% dry mix) 600, inositol 50, *p*-aminobenzoic acid 15, niacin 2, Ca-pantothenate 1.5, riboflavin 1.0, pyridoxine 0.5, thiamine 0.25, menadione 0.05, vitamin B_{12} 0.05, biotin 0.0125 mg and vitamin A 200, vitamin D 120 units per 100 gm diet.

[4] Choline Cl 150, ascorbic acid 25, niacin 15, inositol 10, thiamine 2.5, Ca-pantothenate 2, riboflavin 1.6, pyridoxine 0.6, menadione 0.5, folic acid 0.4, *p*-aminobenzoic acid 0.2, biotin 0.06, vitamin B_{12} 0.02 mg and vitamin A 1000, vitamin D 60 units per 100 gm diet.

[5] Solka Floc.

[6] Stabilized beef tallow.

[7] Corn oil.

All data are calculated on the basis of lysine itself although the monohydrochloride of the L-isomer [3] was used to raise the concentration of this amino acid in the basal diets to the treatment level. In experiment 3 all rations were made isonitrogenous by the addition of diammonium citrate. The term energy is used to indicate productive energy based on values reported by Fraps ('46) with the exception of fat for which a value of 2900 Cal/lb. was assumed as suggested by Hill et al. ('54).

RESULTS

Preliminary results of feeding graded levels of lysine in diets containing 815 and 1115 Cal. of productive energy per pound, each energy level being tested in a separate experiment, are summarized in table 2. Since the dietary treatment affected growth and feed utilization alike, the data were combined in the index of performance value (gain/feed × gain) as proposed by Baldini and Rosenberg ('55).

TABLE 2

Effect of energy level on the lysine requirement of the chick [1]

(Experiments 1 and 2)

DIET	LYSINE	WEIGHT GAIN		FEED UTILIZATION		INDEX OF PERFORMANCE	
		0–4 wk.[2]	4–9 wk.	0–4 wk.	4–9 wk.	0–4 wk.	4–9 wk.
	% of diet	*gm*		*gain/feed*		*gain × feed utilization*	
B	0.6	209 [3]	488 [4]	0.200	0.240	40	120
Experiment 1	0.8	522	1015	0.337	0.282	176	286
(815 Cal./lb.)	1.0	602	1027	0.368	0.277	221	285
	1.2	563	976	0.347	0.278	195	272
C	0.8	459	807	0.331	0.211	152	170
Experiment 2	1.0	674	1277	0.451	0.299	304	382
(1115 Cal./lb.)	1.2	729	1304	0.501	0.315	365	410
	1.4	718	1297	0.489	0.310	351	402

[1] Indian River males, 10 birds per group.

[2] The birds were 12 days old at the start of the experiment.

[3] Standard error of the mean: ± 38.

[4] Standard error of the mean: ± 19.

[3] The lysine was reported to contain 95% of L-lysine HCl and 5% of D-lysine HCl. This value was confirmed by microbiological assay and by optical rotation ($\alpha_D = 22.9°$ at 22°C in 6N HCl).

The requirement was calculated from the first derivative of the quadratic equation $\bar{Y} = a + bx + cx^2$ fitted to the index value for the entire test period. Thus it was found that 1.02% and 1.24% of lysine have to be fed in diets containing 815 and 1115 Cal./lb. respectively to promote optimum growth and feed utilization under the experimental conditions (20% protein, two to 11 weeks of age). A comparison of the performance indexes calculated separately for the first and second half of the experimental period (table 2) shows that birds after 6 weeks of age perform equally or better at 0.2% less lysine than is found to be optimum during early growth.

To define more precisely the lysine requirement on diets of various energy contents, 5 lysine levels were fed at 0.1% increments in experiment 3. Also the protein content of the basal diet was raised to 23% to insure adequate protein intake with the high energy diet. Table 3 gives the mean 25-day body weights, the total feed and lysine intake as well as feed, energy, and lysine utilization data. A statistical analysis indicated a highly significant response ($P < 0.01$) to either lysine or energy supplementation. From performance index values, mathematically treated as before, the lysine requirement was calculated to be 1.13, 1.24 and 1.33% of diets containing 815, 965 and 1115 Cal./lb., respectively. Thus the chick needs 0.07% more lysine with each 100 Cal. added to diets containing between 800 to 1100 Cal./lb., the relation being linear in this range. A comparison between requirement values calculated from results reported in table 2 and table 3 also indicated that birds fed 3% more protein require about 0.1% more lysine on each level of energy. The magnitude of this value is in excellent agreement with the observations of Grau ('48) and Grau and Kamei ('50).

Table 3 further shows that growth is improved considerably by adding energy to the diet if sufficient lysine is available. Simultaneously the corresponding total feed intake decreases slightly, thus leading to a more efficient utilization of the feed consumed, and the lysine included therein, with increasing energy concentration. In contrast, energy utilization remains

nearly constant, with possibly slightly less efficient utilization in the high-energy diet. Such results agree with those reported by Hill and Dansky ('54), who found that calorie-rich diets promote weight gains that are proportionally higher in energy (fat), consequently showing less efficient conversion of energy and better utilization of protein or its components. Within each energy level, the utilization of lysine gradually decreases as its concentration in the diet increases towards the requirement level. Thereafter the efficiency of conversion declines sharply since any excess lysine is not only wasted but also creates an imbalance that impairs growth. In addition, poor lysine efficiency was noted at very low levels in experiments

TABLE 3

Effect of energy level on the lysine requirement of the chick [1]

(Experiment 3)

DIET	LYSINE	25-DAY BODY WT.	FEED INTAKE	LYSINE INTAKE	FEED UTILIZATION	NUTRIENT UTILIZATION	
						Energy	Lysine
	% of diet	*gm*	*gm*	*gm*	*gain/feed* [2]	*gain/Cal.* [2]	*gain/lysine* [2]
	0.8	263 [3]	554	4.43	0.401	0.223	50.1
D	0.9	298	571	5.14	0.448	0.250	49.8
(815 Cal./lb.)	1.0	327	588	5.88	0.483	0.269	48.3
	1.1	336	596	6.56	0.494	0.275	44.8
	1.2	327	582	6.98	0.492	0.274	41.0
	0.9	300	542	4.87	0.476	0.224	52.9
E	1.0	341	573	5.73	0.522	0.246	52.2
(965 Cal./lb.)	1.1	363	574	6.31	0.559	0.263	50.8
	1.2	379	575	6.90	0.588	0.278	49.0
	1.3	367	566	7.35	0.578	0.272	44.4
	1.0	346	544	5.44	0.557	0.226	55.4
F	1.1	344	511	5.61	0.593	0.240	53.7
(1115 Cal./lb.)	1.2	384	555	6.66	0.616	0.251	51.4
	1.3	409	566	7.35	0.648	0.264	49.9
	1.4	384	553	7.75	0.619	0.252	44.3

[1] Averages of two groups of 10 New Hampshire × Columbian chicks (one group of each sex) during a 25-day experimental period.

[2] Total gain in grams/total feed, energy or lysine consumed in grams or Calories.

[3] Standard error of the mean: ± 15; least significant difference at $P < 0.05 = 29$ and at $P < 0.01 = 39$.

1 and 2, probably because the deficiency interfered with the economy of metabolism in general.

Performance indexes reported in table 2 for early and later growth had indicated that, in the older bird, good growth and feed efficiency could be obtained at a lower level of lysine than was required by the very young chick. Table 4 shows the results of two experiments designed to evaluate the lylsine requirement for successive growth periods from about two to

TABLE 4

Effect of age on the lysine requirement of the chick [1]

(Experiments 4 and 5)

GROUP	LYSINE	MEAN BODY WEIGHTS					RELATIVE BODY WT.[3]
		Initial [2]	2 wks.	4 wks.	6 wks.	9 wks.	
	% of diet	*gm*	*gm*	*gm*	*gm*	*gm*	*%*
1 (4)[4]	1.2	136	403	780	1110	1909 [5]	100
2 (5)	1.2	138	398	773	1241	1938	100
3 (4)	1.2	136	398	788	—	—	
	0.8	—	—	—	1124	1924	+ 1
4 (5)	1.2	138	390	—	—	—	
	0.8	—	—	713	1107	1868	— 4
5 (4)	0.8	136	357	677	1013	1814	— 5
6 (5)	1.2	138	394	—	—	—	
	1.0	—	—	792	—	—	
	0.8	—	—	—	1192	—	
	0.6	—	—	—	—	1720	— 11
7 (4)	1.2	136	389	755	—	—	
	0.6	—	—	—	1024	1514	— 21
8 (5)	1.0	138	392	—	—	—	
	0.8	—	—	732	—	—	
	0.6	—	—	—	994	1478	— 24

[1] 10 Indian River males per group.

[2] The birds were 12 days old at the start of the experiment.

[3] Figures are per cent deviations of the final body wt. from the values obtained in the respective lot fed 1.2% lysine throughout the test.

[4] Figures within parenthesis after the group number indicate the number of the experiment.

[5] Standard error of the mean: ± 36; least significant difference at $P < 0.05 = 108$ and at $P < 0.01 = 148$.

11 weeks of age on diets containing a medium level of energy (975 Cal./lb.). Inspection of the 9-week body weights shows that 1.2% lysine is not required throughout the entire growth period in order to reach optimum final weights. Reducing the lysine level to 0.8% at 4, two or zero weeks (at this point the birds are 12 days old and were reared on a commercial starter ration calculated to contain 1.1% lysine) leads to final weights that deviate only + 1, —4 and —5%, respectively, from the lots fed 1.2% lysine for the entire test period. Feeding 0.6% lysine, however, decreased the final weight by 21 or 24% when this low level was introduced at 4 weeks and by 11% when the change was made at 6 weeks. Any reduction of the amino acid level has noticeably less effect on the 9-week body weight the later the change is introduced, again indicating that the optimum level decreases with age.

The fact that the final weight on the 0.8% level (group 5, table 4) was not significantly different from that obtained on the 1.2% level should not be interpreted to mean that the requirement is actually lower than stated. Examination of the data for the intermediate periods of two and 4 weeks shows in each case a significant difference ($P < 0.01$) between the two levels, the difference becoming insignificant during the last three weeks of the experiment. From the practical standpoint, then, it might not be so vital to furnish the high level of lysine, but this belief does not invalidate the original conclusions.

To establish the lysine requirement during growth up to 11 weeks of age, mean daily weight gains for two- or three-week intervals in response to graded levels of lysine are summarized in table 5. This table also includes data from experiment 3 to cover the zero- to two-week growth period, after the values were corrected for the difference in protein level of the basal diet according to the results of Grau ('48) and the relative requirement values established for these rations previously. A direct comparison of data between experiments is considered justified since the experimental conditions were identical and the growth response to 1.2% lysine was practically the same in

each experiment. Although the differences used in establishing the requirements usually were not statistically significant when comparing individual groups, it is believed that the large number of tests adds considerable weight to the decisions. It is concluded, therefore, that the rapidly growing chick from day-old to 4 weeks of age requires about 1.1% lysine in diets containing 20% protein and 975 Cal./lb. Thereafter this level should be decreased at a rate of 0.05% per week until a level of 0.8% of lysine is reached at 8 weeks of age. Further reductions were found incompatible with optimum growth to 11 weeks and, in a single experiment, to 14 weeks of age. It

TABLE 5

Mean daily weight gains obtained in experiments 3, 4 and 5

AGE OF BIRDS	LYSINE CONTENT OF DIET, %						SUGGESTED REQUIREMENT
	0.6	0.8	0.9	1.0	1.1	1.2	
wks.	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*% of diet*
0– 2	—	6.7 (2) [1]	8.0 (2)	8.9 (2)	9.3 (2)	8.8 (2)	1.1
2– 4	—	15.2 (3)	17.1 (2)	18.0 (3)	19.0 (2)	18.5 (8)	1.1
4– 6	—	23.4 (3)	—	28.4 (1)	—	26.9 (4)	1.0
6– 8	19.0 (2)	26.2 (4)	—	—	—	28.5 (2)	0.9
8–11	23.8 (3)	37.5 (3)	—	—	—	35.6 (2)	0.8

Figures within parenthesis indicate the number of lots of 10 birds each used in the computation of the average value.

should be noted that when lysine is substituted in the crystalline form up to 50% of the requirement, the indicated reductions are mandatory for optimum growth, since diets containing an excess of 0.1 to 0.2% of lysine above the requirement consistently depressed growth up to 5%.

DISCUSSION

Basic elements for any synthesis are required in proportion to production rate which in turn, within limits of the system, depends on the ready supply of all needed components. During growth, therefore, the requirement for individual nutrients must correlate with the rate of gain that can be maintained with respect to a certain diet composition. The effici-

ency of a ration, however, is improved with any change of its composition that brings the concentration of all its components in closer proportion to the immediate need of the animal fed, thus, by eliminating waste, accommodating the highest concentration of each dietary requirement within a given amount of feed. Consequently for optimum production single nutrient requirements depend on total nutrient balance.

This concept is demonstrated by the data of table 3 where an increase in energy concentration shows no response in diets that are deficient in lysine while higher levels of lysine are well utilized only if more energy becomes available. These results agree with those of Sunde ('56) and Davidson ('56) who found that well-balanced supplementations of fat and protein consistently improved growth and feed conversion while no response or even retardation was observed when only one of the nutrients was increased. This interdependence between diet components, however, is found only when the nutrients under study approach balance. In the range of more severe deficiency Williams and Grau ('56) reported better growth on any lysine level with a reduction of dietary energy simply through stimulation of feed intake which relieves the deficiency but does not improve the disproportional ratio of lysine to other nutrients.

The relationship between dietary energy and optimum lysine concentration was found to be linear within the range tested, namely 0.07% lysine per 100 Cal. This observation is in contrast to the change of the lysine requirement in response to higher protein levels (Grau, '48). The difference probably is due to the fact that in the present studies energy was added in place of an inert diet component (cellulose), while Grau substituted sesame meal for glucose in order to raise the protein content. In doing so, he simultaneously reduced the energy concentration of his diets, thus having two effects on the lysine requirement opposing each other.

The rate of change with age in the requirement for lysine was pointed out in table 5. A comparison of the data with the requirement in the growing turkey (Kratzer et al., '56)

shows that the poult sharply reduces its initially high demand for lysine during the first 4 weeks of life while the chick indicates no reduction during this period. At 4 weeks the lysine requirement for both species is approximately the same and continues to decrease at a similar rate. The decrease in the lysine requirement with age is readily explained by a shift in the ratio of amino acid to energy expenditure. In the growing bird the rate of gain constantly decreases in proportion to body weight while at the same time the enlarging system continuously increases its need for energy. Since optimum growth is best produced by diets that contain the nutrients in closest proportions to the immediate need of the body, the necessity for reducing the lysine content of the feed with progressing age is obvious.

The demonstrated reciprocal dependence between optimum dietary lysine and the energy or protein level in the diet has shown the importance of other diet components on single nutrient requirements. The effect of age was also pointed out. Recommendations of a lysine requirement, therefore, must be stated on the basis of a specified dietary composition and age of the animal. The expression as a percentage of the diet is then perfectly adequate.

SUMMARY

With fast growing broiler chicks, the lysine requirement and its variability in nearly practical type corn-sesame rations was investigated. It was found:

1. Up to 4 weeks of age, the chick requires 1.1% lysine in diets containing 20% protein of good quality and 975 Cal. of productive energy per pound of feed. Thereafter the level of lysine should be reduced at a rate of about 0.05% per week to a minimum of 0.8% since excess dietary lysine was found to impair growth and feed efficiency. These tests were run until the birds were about 11 weeks of age.

2. Changes in dietary energy concentration alter the lysine requirement at a rate of 0.07% per 100 Cal. An increase of

3% in the protein of the diet (20 to 23%) raises the lysine requirement about 0.1%.

3. The relationships under (2) are indicated to be linear but only if the ratio of all other diet components remains constant. The effect of the total nutrient pattern on single nutrient requirements is discussed.

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SHORT-TERM RAT FEEDING STUDIES WITH GAMMA-IRRADIATED FOOD PRODUCTS

I. FROZEN STORED FOODS [1]

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Recently attention has been focused upon the utilization of ionizing radiation for the preservation of foodstuffs. It has been demonstrated that either accelerated electrons (Brasch and Huber, '47; Huber, '48) or gamma rays (Duffy, '53; Goldblith and Proctor, '54) may be satisfactorily used for this purpose on a laboratory scale. Since this process can, potentially, yield a product which may be stored for extended periods of time at ambient temperature, even though the product is essentially raw, it has considerable economic importance. Before irradiated foods can be used for human consumption, however, it is necessary to demonstrate the safety of radiation processed foods for human consumption. Lehman and coworkers (Lehman and Laug, '54; Lehman et al., '55) of the Food and Drug Administration have outlined procedures to be followed for doing this.

In evaluating the safety of irradiated foods, two primary factors must be considered and, insofar as possible, studied independently. These are nutritional adequacy and toxicity. An excellent review of the question of nutritional adequacy of

[1] This paper represents work done on a collaborative project between the University of Colorado and the U. S. Army Medical Nutrition Laboratory in cooperation with the Office of The Surgeon General under Contract no. DA-49-007-MD-549.

irradiated foods has been prepared by Johnson ('57). Toxicity encompasses a variety of factors such as carcinogenicity, the action of an antimetabolite, or the presence of acutely lethal or "poisonous" substances. For the purposes of this paper, toxicity is defined in the general sense of causing deleterious effects upon biological development, excluding the factor of nutritional adequacy. In this context, weight gain will serve as a fairly critical index of toxicity, provided the nutritional needs of the experimental animals have been satisfied.

The final determination of the suitability of irradiation preservation for food items must rest upon biological evaluation. Even though the irradiated products do not exhibit appreciable loss of nutritional values, low levels of radiation by-products which are foreign to animal metabolism may establish toxic conditions due to repeated ingestion of the treated food. Only a few studies have been reported concerning this problem. Poling et al. ('55) have found that irradiated (2×10^6 rep)[2] beef does not exhibit any toxic properties when fed to rats for 104 weeks. It was necessary, however, to supplement the diet with vitamin E, as was indicated by the earlier work of DaCosta and Levenson ('51). Similarly, irradiated organ meats (Bubl and Butts, '56) have been successfully fed to rats for extended periods of time. A more thorough review of the question of wholesomeness has been presented by Read and Kraybill ('57).

Because each food item, or closely related group of items, represents a different chemical system which may respond to irradiation in a unique way, it is necessary that animal feeding experiments with irradiated foods be extended to a larger group of items. The present investigation reports studies on 14 food items fed to rats for 8 to 12 weeks. The emphasis in these experiments has been placed on potential toxicity rather than nutritional adequacy. The separation of these factors has been achieved by the use of a diet containing sufficient amounts of a nutritionally adequate basal ration for normal growth in

[2] Rep: Roentgen equivalent physical is the amount of ionizing radiation which will result in the absorption in tissue of 93 ergs per gram.

the absence of toxic components in the irradiated food supplement. By employing a relatively high level of the irradiated food item in the diet it was believed that low levels of toxic compounds would be found by measuring weight gain over an 8- to 12-week period. The problem of palatability for the rat has been corrected for by incorporating caloric consumption in the statistical evaluation of the results. Two irradiation levels were studied, one in the range anticipated for use in commercial procedures, and a second twice as great to heighten the production of irradiation by-products. The foods studied were stored frozen following irradiation in order to investigate the products of the irradiation process itself rather than post-irradiation changes arising from storage at elevated temperatures. Other experiments using some of these products stored at 70°F will be reported in a subsequent paper. These investigations also served another purpose: to provide clearance, if possible, for further short-term human feeding studies at this laboratory.

METHODS

Fourteen foods were used in these studies: ground beef, fresh ham, haddock fillet, boned turkey (mixed light and dark meat), sliced bacon, whole kernel corn, leaf spinach, sliced peeled beets, green snap beans, sliced peaches, whole strawberries, canned bread, military sustenance cereal bar, and whole powdered milk. The foods were packed in no. 2 tin cans in Chicago and shipped frozen to Arco, Idaho, where they were gamma-irradiated in a "swimming-pool" type of reactor utilizing a mixed fission source (spent fuel rods) having an average energy output of 1 Mev. The temperature of the pool was 60°F during irradiation. The total dosages used were 3×10^6 rep and 6×10^6 rep which were administered at average dose rates of 2.8 to 6.8×10^6 rep per hour except for the following items: (a) green beans: 12.1 to 13.7×10^6 rep per hour; (b) bacon: 8.0 to 14.2×10^6 rep per hour; (c) haddock: 11.0 to 14.2×10^6 rep per hour; and (d) ground beef: 9.3 to 10.0×10^6 rep per hour. Following irradiation the treated foods were

shipped frozen to our laboratory where they were stored at 8°C until they were compounded into the diets. The control samples were treated in identically the same way as the irradiated samples except that they were not irradiated.

The experimental diet consisted of 65% of a nutritionally adequate basal ration including the water-soluble vitamins (see table 1) plus 35% (dry weight) of the food under investigation. Proximate analysis (moisture, protein, nitrogen, fat and bomb calories) of the completed diets are presented in table 1. The completed rations were prepared once each week, packaged in heat-sealed polyethylene bags, and stored at —8°C until immediately prior to use.. The fat-soluble vitamins were administered in the following solution:

A and D mixture (1 ml contains 60,000 U.S.P. units A plus 12,000 U.S.P. units D)	1 ml
α-tocopherol	70 mg

Each rat received orally one drop (0.05 ml) once a week.

Weanling male albino Holtzman or Sprague-Dawley rats, initially weighing 45 to 50 gm, were used in these studies. After receipt from the supplier, they were maintained on commercial laboratory pellets [3] for 4 days before initiating the experiment.

Each diet group consisted of 10 rats housed individually. The duration of the feeding study was from 8–12 weeks. Each animal was fed ad libitum with fresh diet offered daily and a record was kept of the daily food consumption. The weight gain was determined by weighing each animal twice a week.

Statistical analyses of the data were performed using multiple covariance analysis (Snedecor, '50) on the initial weights, caloric consumption and total weight gains. The inclusion of initial weight in these analyses did not appear to influence greatly the interpretation of the results so that this step could probably have been omitted. The inclusion of caloric consumption, which is partially dependent upon the palatability of the product for the rat, proved helpful in distinguishing between decreased weight gain due to decreased palatability and that

[3] Purina Laboratory Chow, Ralston Purina Company, St. Louis, Mo.

TABLE 1

Formulation and analyses of diets used

FORMULATION OF DIETS

Main mixture:	%
Vitamin-free casein	13.00
Cornstarch	34.07
Salt-mixture (Hawk-Oser)	2.61
Roughage: Cellu-B	0.97
Gum Acacia	0.97
Corn oil (Mazola)	13.00
Vitamin-methionine mixture (below)	0.28
Choline	0.10
Experimental food item (dry solids)	35.00
Vitamin-methionine mixture:	*gm*
Pyridoxine	1.0
Thiamine-HCl	1.0
Niacin	5.0
Calcium pantothenate	4.0
Riboflavin	1.0
Folic acid	0.2
Biotin	0.002
Vitamin B_{12} (0.1% in mannitol)	2.0
Inositol	10.0
p-Aminobenzoic acid	50.0
Menadione	3.3
DL-Methionine	195.0

PROXIMATE ANALYSIS OF COMPLETED DIETS

Diet	Moisture	Fat	Protein	Kilocalories per gram of wet diet
	%	%	%	
Bacon	14.3	28.5	15.9	5.02
Beans, snap	82.0	2.4	3.4	0.88
Beef	29.2	27.1	13.8	4.96
Beets	70.5	4.0	4.6	1.41
Bread	15.2	17.4	13.8	4.29
Cereal bar	3.0	20.8	15.8	4.97
Corn	51.0	7.9	8.5	2.36
Haddock	62.4	5.5	15.8	1.98
Ham, fresh	43.5	15.1	13.5	3.23
Milk, powdered	2.6	24.6	20.2	5.22
Peaches	64.1	6.0	4.1	1.71
Spinach	73.2	3.6	7.5	1.30
Strawberries	50.4	7.8	6.5	2.45
Turkey	46.1	12.5	19.6	2.77

Methods of analysis

Moisture: 8 hour vacuum at 60°C.

Fat: Mojonnier method, A.O.A.C.

Protein: Kjeldahl-macro digestion, semi-micro distillation. Factors: Milk, 6.38; others, 6.25.

Calories: Bomb calorimeter.

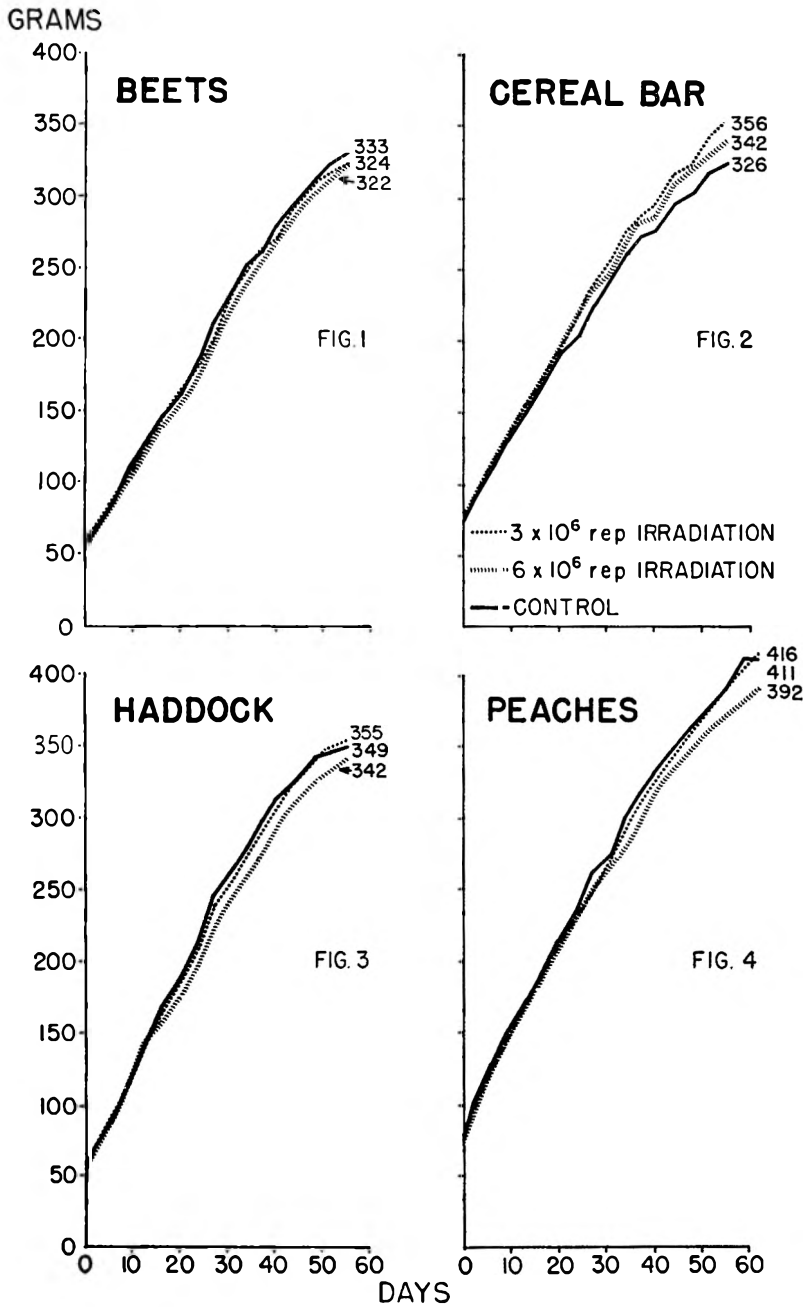
due to true toxicity. In the case of two foods the feeding experiments were repeated to clarify certain facets of the data. In these cases (peaches and turkey), an analysis of variance was performed on the combined 8-week feeding data for the two experiments. Before combining the data, tests for homogeneity in treatment response were employed. In both cases a non-significant treatment and test interaction showed that treatment response in the two experiments was essentially the same.

Gross examination was made of the internal organs of all the animals surviving the feeding period and appropriate histologic examinations were performed.

RESULTS AND DISCUSSION

Growth, food consumption and caloric efficiency data are presented in table 2. In all of the studies an average daily weight gain of 5 to 7 gm was the rule rather than the exception. Growth curves for 4 representative foods are presented in figures 1 through 4.

In all of the experiments the animals were large, healthy in appearance and lively, following the feeding period. No gross symptoms of toxicity of the irradiated foods were found in any of the animals. Statistical analysis of the data from 12 of the 14 foods further confirmed this interpretation. These foods are bacon, snap beans, beef, beets, bread, cereal bar, corn, haddock, fresh ham, powdered milk, spinach and strawberries. In the case of two of these foods significant increases in weight gain of rats raised on the irradiated diets were observed, namely 3×10^6 rep irradiated corn and 6×10^6 rep irradiated cereal bar (table 3). The growth curves for the cereal bar experiment are given in figure 2. Similar curves were obtained from the corn diets. These results might be attributed to a breakdown of the cellular walls by ionizing radiation, thus making the nutrients more available for utilization by the rat. This would be consonant with the findings of Lawton et al. ('51) and Seaman et al. ('52) that high-energy electrons can degrade various forms of cellulose. Certainly the corn, as well



Figs. 1–4 Growth of rats on diets containing irradiated foods.

TABLE 2

Average weight gains, caloric consumption and caloric efficiency of male albino rats raised on diets containing irradiated food items

(All weight gains are for an 8-week period unless otherwise noted)

FOOD	RADIATION DOSE	INITIAL WEIGHT	WT. GAIN	CALORIC CONSUMPTION	CALORIC EFFICIENCY [1]
	rep	*gm*	*gm*	*kilocalories*	
Bacon	0	55	255	3439	0.074
	3×10^6	55	268	3599	0.074
	6×10^6	54	263	3391	0.078
Beans	0	57	257	3600	0.071
	3×10^6	57	260	3461	0.075
	6×10^6	58	254	3751	0.068
Beef	0	59	280	4399	0.064
	3×10^6	62	307	4622	0.066
	6×10^6	61	269	4259	0.063
Beets	0	55	279	3860	0.072
	3×10^6	55	269	3838	0.070
	6×10^6	55	267	3851	0.069
Bread	0	68	296	4211	0.070
	3×10^6	68	298	4365	0.068
	6×10^6	67	297	4406	0.067
Cereal bar	0	76	250	3636	0.069
	3×10^6	77	278	3921	0.071
	6×10^6	77	265	3967	0.067
Corn	0	67	301	4437	0.068
	3×10^6	67	313	4831	0.065
	6×10^6	65	301	4606	0.065
Haddock	0	54	296	3423	0.086
	3×10^6	54	302	3649	0.083
	6×10^6	54	288	3457	0.083
Ham, fresh	0	77	294	4043	0.073
	3×10^6	78	299	4133	0.072
	6×10^6	77	306	4224	0.072
Milk, powdered	0	77	277	3578	0.077
	3×10^6	76	273	3621	0.075
	6×10^6	77	277	3929	0.071
Peaches	0	76	301	4532	0.066
(1st test)	3×10^6	76	281	4213	0.067
	6×10^6	78	270	4290	0.063
Peaches	0	76	315	3930	0.080
(2nd test	3×10^6	72	317	3810	0.083
9 weeks)	6×10^6	72	302	4157	0.073
Spinach	0	87	234	2926	0.080
	3×10^6	86	236	2641	0.089
	6×10^6	86	229	2926	0.078
Strawberries	0	69	294	4553	0.065
	3×10^6	67	309	4758	0.065
	6×10^6	70	302	4602	0.066
Turkey	0	66	307	4320	0.071
(1st test)	3×10^6	65	287	4240	0.068
	6×10^6	68	285	4254	0.067
Turkey	0	72	314	4795	0.065
(2nd test	3×10^6	72	340	4962	0.069
12 weeks)	6×10^6	71	316	4798	0.066

[1] Grams gained per kilocalorie consumed.

as the other vegetables studied, exhibited a softened texture as a result of irradiation. Kertesz et al. ('56) have reported an apparent relationship between the softening of several fruits and vegetables and irradiation-induced alterations in their constituent polysaccharides.

In preliminary spinach-feeding studies the high oxalate content of the spinach was inadvertently overlooked. Irradiation of the spinach did not prevent the onset of oxalate-poisoning

TABLE 3

Results of tests of significance applied to total weight gains of male albino rats raised on diets containing selected irradiated foods

FOOD	TEST PERIOD	COMPARISON	
		3×10^6 rep vs. control	6×10^6 rep vs. control
Cereal bar	8 weeks	n.s.	$P < 0.05$ [2]
Corn	8 weeks	$P < 0.05$ [1]	n.s.
Spinach	8 weeks	n.s.	n.s.
	12 weeks	n.s.	n.s.
Peaches	8 weeks, 1st test	n.s.	n.s.
	8 weeks, 2nd test	n.s.	n.s.
	8 weeks, combined tests	n.s.	$P < 0.05$ [2]
	9 weeks, 2nd test	n.s.	n.s.
Turkey	8 weeks, 1st test	n.s.	$P < 0.05$ [2]
	8 weeks, 2nd test	$P < 0.05$ [1]	n.s.
	8 weeks, combined tests	n.s.	n.s.
	12 weeks, 2nd test	$P < 0.05$ [1]	n.s.

[1] Growth of animals fed irradiated food significantly greater.
[2] Growth of animals fed irradiated food significantly less.

in the animals. Chemical analysis of the irradiated and non-irradiated spinach futher demonstrated that the irradiation did not decrease the oxalate content of the spinach. However, supplementation of the spinach diet with a quantity of calcium gluconate stoichiometrically equivalent to the oxalate content prevented the onset of oxalate poisoning. The results of this second spinach study clearly indicated that the irradiation of spinach did not produce any toxic compounds demonstrable by 12-week feeding tests with rats.

Peaches were a food in which a retest was indicated and performed. The statistical analysis of the data obtained from the first 8-week feeding study yielded results of borderline significance with the 6×10^6 rep irradiated product. Simple analysis of variance of the weight gains alone showed a significant ($P < 0.05$) decrease in growth on this diet. Repetition of this experiment did not yield a significant ($P < 0.05$) decrease in weight gain of rats raised on the 6×10^6 rep peach diet. Analysis of the combined data for the two 8-week tests gave a significant ($P < 0.05$) difference between the control and 6×10^6 rep irradiated peach diet. It would appear, then, that 6×10^6 irradiation of peaches may cause the production of some compound(s) which exert a mildly deleterious effect upon the rat when ingested for 8 or more weeks. No difference between the control and 3×10^6 rep irradiated peaches was found. The observed differences between the non-irradiated and the 6×10^6 rep irradiated peaches might be explained by the results obtained by Teply and Kline ('56) with irradiated dessert powder. They reported that the rat growth depressant effect of irradiated dessert powder was due solely to the sucrose component. Very high levels of irradiation have also been shown to alter carbohydrates, including sucrose and glucose, in aqueous solution (Wolfrom et al., '56). Inasmuch as the peaches used in our studies were commercially prepared in a sugar syrup, the latter component might be the source of the indicated low-level toxicity. It is interesting that in successive-generation studies at this laboratory employing a composite diet of 9 irradiated foods treated with 6×10^6 rep, no adverse effects of irradiation sterilization on growth have been found even though the diet contains 20% of peach solids. Further studies of 6×10^6 rep irradiated peaches are planned.

Turkey was another product on which a retest was performed. In the first 8-week study a significant depression in weight gain resulted from feeding the 6×10^6 rep product. In order to determine more accurately whether the decreased

growth was due to decreased food consumption or to the presence of some other growth depressant, Carpenter's ('53) graphical "appetite quotient" method was applied. The results of this test also suggested the presence of a growth depressant in the more highly irradiated turkey. Repetition of the experiment did not confirm these findings. Analysis of the data from the combined tests also did not verify the presence of an irradiation-induced toxic by-product in 6×10^6 rep irradiated turkey.

No deleterious effects from 3×10^6 rep irradiated turkey were encountered at any time. It is therefore believed that this food item may be considered satisfactory from the toxicological viewpoint.

From these studies, 13 food items would appear to be non-toxic following irradiation with either 3×10^6 rep or 6×10^6 rep. Peaches, the 14th item, may have some low-level toxicity following 6×10^6 rep irradiation, but are non-toxic following irradiation with only 3×10^6 rep.

SUMMARY

Fourteen foods irradiated with three or 6 million rep and subsequently stored in the frozen state have been fed ad libitum to rats for 8 to 12 weeks as 35% of the dry weight of the diet. The remaining 65 % of the diet was a non-irradiated nutritionally adequate semi-synthetic ration. Comparable non-irradiated foods were used as controls. Covariance analysis of the weight gains, caloric consumption, and initial weights was performed to determine low-levels of toxic products in the irradiated foods.

Thirteen foods (bacon, snap beans, beef, beets, bread, cereal bar, corn, haddock, fresh ham, powdered milk, spinach, strawberries and turkey) have been found to be non-toxic by this technique. A suggestion of low level toxicity of 6×10^6 rep irradiated peaches has been obtained; 3×10^6 rep irradiated peaches have been shown to be non-toxic.

The interpretation of these results has been discussed.

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THE INFLUENCE OF DIET UPON TISSUE CONCENTRATION OF VITAMIN B_6 [1]

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A change in the tissue concentration of a vitamin may provide information concerning need for the vitamin, possible body stores and functions which the vitamin performs in metabolism.

Certain variations in the diet have been shown to affect the content of vitamin B_6 in liver and blood. In rats and monkeys, tissue levels of that vitamin reflected vitamin B_6 intake (Greenberg and Rhinehart, '49; Sheppard and McHenry, '46). When the protein in the ration of mice was increased, the need for vitamin B_6 was greater (Miller and Baumann, '45). The tissue concentration of vitamin B_6 in rats decreased when protein was increased in the ration (Tulpule and Williams, '55). After feeding various rations ad libitum and in restricted amounts to rats, Tulpule and Williams ('55) ascertained that caloric intake may also influence the concentration of vitamin B_6 in the liver.

The observations reported herein provide information concerning the influence of variations in diet upon the concentration of vitamin B_6 in the liver and blood of rats.

EXPERIMENTAL

Three experiments, which are referred to as A, B, and C, were conducted. In all three, albino weanling rats of the

[1] This study was part of a Northeast Regional Project (NE 37, Relationships between protein and other selected nutrients and their metabolism and utilization); a cooperative study involving agricultural experiment stations in the Northeast Region and supported in part by regional funds.

Wistar strain were randomly divided into groups consisting of three males and three females and housed in individual cages. The rations for all animals contained Jones-Foster salt mixture 4% [2]; sugar, vitamin-free casein [2] and vegetable oil in various amounts; and a mixture providing the following amounts of vitamins per gram of ration: 20 units of vitamin A, 2.2 units of vitamin D, 0.11 mg α-tocopherol, 1 mg of vitamin C, 1.65 mg of choline chloride, 0.05 mg of menadione, 0.11 mg of p-aminobenzoic acid, 0.1 mg of niacin, 0.022 mg of riboflavin, 0.022 mg of thiamine hydrochloride, 0.066 mg of calcium pantothenate, 0.44 µg of biotin, 2.0 µg of folic acid and 0.03 µg of vitamin B_{12}. When given, vitamin B_6 was fed separately as a 3-ml solution of pyridoxine hydrochloride.[3]

Vitamin B_6 determinations were conducted on freshly drawn blood at one- or two-week intervals. Blood was obtained during the experiment by snipping the tail of the animal and at the termination of the experiment by heart puncture. Blood filtrates were prepared for analysis according to the method of Marquez ('55). Analysis for vitamin B_6 was performed by the microbiological method of Parrish et al. ('56), with certain modifications. The modifications consisted of decreasing the volume in the assay tubes to 4 ml, shaking 17 hours without a glass bead and adding tryptophan to the basal medium at a level of 0.015%. In earlier experiments, 100% recoveries of pyridoxine hydrochloride standards added to blood filtrates were obtained when tryptophan was added to the basal medium.[4]

Vitamin B_6 in the liver was determined at the end of each experiment. Liver filtrates were prepared according to the procedure of Tulpule and Williams ('55) and assayed as the blood filtrates. Weight and food consumption records were kept for all animals.

In experiment A, each group of animals was fed a different amount of vitamin B_6. Animals weighing approximately 75 gm

[2] Purchased from Nutritional Biochemicals Corporation, Cleveland.

[3] In the text of this paper, pyridoxine hydrochloride will be referred to as pyridoxine.

[4] Unpublished data.

were divided into 5 groups and maintained for 4 weeks on a ration containing 18% casein and 10% corn oil. Each group of animals received 0, 2.5, 5.0, 7.5, or 15 μg of pyridoxine per rat per day. During the 4 weeks of experiment A, the blood concentration of vitamin B_6 had not reached a constant value for a given pyridoxine intake, so a second experiment, experiment B, was carried out repeating various levels of pyridoxine intake for longer periods of time.

For experiment B, the effects of different intake levels of both vitamin B_6 and protein were studied. Five groups of animals received an 18% casein ration and a supplement containing 0, 2.5, 5.0, 10 or 20 μg of pyridoxine per rat per day. A 6th group of animals received a 9% casein ration and 10 μg of pyridoxine per rat per day. All the rations in this experiment contained coconut oil at a 4% level.[5] The animals were maintained on these rations for 7 weeks. In experiments A and B, restricted feeding was employed so that all animals received amounts of food that represented the average food consumption of those receiving no pyridoxine on the previous day of the experiment.

For experiment C, three series of weanling animals (consisting of 18 each) were fed ad libitum a 9, 18 or 36% casein ration containing 4% of coconut oil. Each series was further divided into three groups, so that, for each level of protein intake, 6 animals received 0, 5 or 10 μg of pyridoxine per animal per day for a period of 9 weeks. Because no vitamin B_6 could be detected in the blood of any of these animals on the 47th day of the experiment, the highest level of vitamin supplement was increased to 20 μg per day from the 49th day to the termination of the experiment. Two additional groups of animals (groups 10 and 11) were given 10 μg of pyridoxine and fed the same amount of ration that was consumed by the group receiving the 18% casein pyridoxine-free ration. Group 10 received a 4% coconut oil ration and group 11, a 10% corn oil ration. These two groups were included so that

[5] The oil was changed in order to collaborate with studies being conducted at the New Jersey Agricultural Experiment Station.

any effect of food restriction or change of oil in the ration could be noted.

RESULTS AND DISCUSSION

The mean concentration of vitamin B_6 in the blood and liver for each group of animals at various intervals of time is presented in table 1. In experiments B and C, the vitamin B_6 intake and the concentration of vitamin B_6 in the liver per milligram of liver nitrogen are given as there was a significant positive correlation between the two.

As mentioned above, restricted feeding was employed in experiments A and B. Food consumption did not vary from animal to animal within an experiment, except for the group receiving no pyridoxine, and the variation within this group was small.

In order to determine if a statistical difference existed among values obtained for various groups of animals, variance analysis (Snedecor, '45) and multiple range tests (Duncan, '55) were applied to the observations of vitamin B_6 content of the blood for experiments A and B. A comparison of various groups of animals in experiments A and B demonstrated that in most cases a significant difference in blood concentration of vitamin B_6 existed with increasing pyridoxine intake. Only in a few cases were there no differences, namely, between the groups which received 0 and 2.5 μg of pyridoxine in experiments A and B, and those which received 5.0 and 7.5 μg of pyridoxine in experiment A.

The concentration of vitamin B_6 in the liver exhibited little variation with different vitamin B_6 intakes during experiment A. Both Tulpule and Williams ('55) and Beaton and McHenry ('53) demonstrated that changes in vitamin B_6 content of the liver of rats due to dietary treatment were evident in two weeks. Perhaps 4 weeks of restricted feeding in experiment A was not sufficiently long to manifest such a change. In experiment B, there was a significant positive correlation, $r = 0.896$, between vitamin B_6 intake and vitamin B_6 content of

TABLE 1

Average concentration of vitamin B_6 in blood and liver

Experiment A

WEEKS ON EXP.	FOOD CONSUMPTION	PYRIDOXINE HYDROCHLORIDE INTAKE ($\mu g/rat/day$)				
		0	2.5	5.0	7.5	15
	gm	*Vitamin B_6 in blood, $\mu g/100$ ml*				
1	7.0	0.75	1.38	1.16	0.93	1.90
2	7.0	0.50	1.38	2.80	2.67	2.84
3	8.5	0.43	0.47	1.77	2.20	2.88
4	10.0	0.75	0.80	1.33	1.57	2.22
Vitamin B_6 in liver (μg/mg liver nitrogen)		0.094	0.148	0.149	0.133	0.129

L.S.D. $_{(t = 0.05)}$ = 0.351 for vitamin B_6 in blood.

Experiment B

WEEKS ON EXP.	FOOD CONSUMPTION	PYRIDOXINE HYDROCHLORIDE INTAKE ($\mu g/rat/day$)					
		0	2.5	5.0	10.0	20	10 [1]
	gm	*Vitamin B_6 in blood, $\mu g/100$ ml*					
1	6.0	0.27	0.25	1.83	3.91	4.07	4.72
3	7.0	0.75	1.13	1.68	2.72	3.03	3.21
5	6.5	0.08	0.05	1.22	3.71	5.28	4.15
7	6.0	1.39	2.09	2.23	2.81	3.99	4.27
Vitamin B_6 in liver (μg/mg liver nitrogen)		0.118	0.110	0.199	0.317	0.277	0.330

L.S.D. $_{(t = 0.05)}$ = 0.358 for vitamin B_6 in blood.

Experiment C

GROUP	CASEIN IN RATION	PYRIDOXINE HYDROCHLORIDE INTAKE	DAYS OF EXPERIMENT				VITAMIN B_6 IN LIVER
			19	33	47	61	
	%	$\mu g/rat/day$	*Vitamin B_6 in blood, $\mu g/100$ ml*				*μg/mg liver nitrogen*
1	9	0	0.25	0.17	0	0	0.084
2	9	5	1.56	0.52	0	0	0.090
3	9	10 [2]	3.85	0.97	0	1.20	0.125
4	18	0	1.18	0.42	0	0	0.070
5	18	5	2.07	0.58	0	0	0.090
6	18	10 [2]	1.88	0	0	0	0.128
7	36	0	0.90	0	0	0	0.089
8	36	5	1.61	0	0	0	0.118
9	36	10 [2]	1.86	0	0	0	0.137
10	18	10 [2]	0.85	0.86	1.16	0.98	0.170
11	18	10 [2]	0.63	0.98	1.60	0.90	0.148

[1] This group of animals received a 9% casein ration. All other groups received an 18% casein ration.

[2] The pyridoxine hydrochloride intake was increased to 20 μg/rat/day on the 49th day of experiment and continued at this level to the end of the experiment.

the liver. The animals ate less and possibly stored more vitamin B_6 and had more circulating in the blood.

Table 1 also contains results from experiment C in which both vitamin B_6 and protein intake were varied. These animals were fed ad libitum, except groups 10 and 11 which were fed the average amount of food that the animals in group 4 consumed. As the food consumption of the animals which were fed ad libitum increased, the vitamin B_6 in the blood decreased and by the 47th day none could be detected.

The concentration of vitamin B_6 in the blood of the animals in groups 10 and 11 was less than that of animals which consumed similar diets in experiments A and B. The animals in group 10 received as much pyridoxine as the group which received 10 μg per rat per day in experiment B, but group 10 had a lower concentration of vitamin B_6 in the blood. The same comparison can be made for group 11 and the group that received 7.5 μg of pyridoxine in experiment A. The animals in groups 10 and 11 ate 12 gm of food per day at the beginning of the experiment and this was gradually reduced to 9 gm as the intake of group 4 decreased. The question arises as to whether it is food restriction, or decrease in protein intake because of food restriction which results in the detection of vitamin B_6 in the blood. A comparison of the group in experiment B which received 5 μg of pyridoxine per day and the 18% casein ration with group 2 of experiment C provides data for this purpose. The animals in these two groups were consuming nearly the same amount of nitrogen, 143 and 165 mg per day, at the termination of the experiments. The animals in the first group ate nearly 6 gm of food a day, while the animals in the latter group ate approximately 13 gm of food per day. The latter group of animals had lower blood and liver concentrations of vitamin B_6. Food consumption appeared to increase the need for vitamin B_6 in this experiment.

It is interesting that in the animals of group 3 of experiment C, which received the low-protein ration, no vitamin B_6 was detectable in the blood on the 47th day, although some was found on the 61st day. This might be due to the fact that the

pyridoxine supplement was increased to 20 μg on the 49th day and continued at this level to the end of the experiment. It became of interest to determine if animals, which were fed ad libitum a ration containing 18% casein and were given more pyridoxine, would have any vitamin B_6 in the blood. A group of animals maintained for 4 weeks on such a ration and given 50 μg of pyridoxine hydrochloride per rat per day had an average of 16.9 μg% of vitamin B_6 in their blood. Litter mates maintained for 4 weeks on the same diet, but given only 6 gm of food per day and 10 μg of pyridoxine per rat per day, had 4.0 μg% of vitamin B_6 in the blood. Both vitamin B_6 intake and food consumption influenced blood level of vitamin B_6.

The concentration of vitamin B_6 in the liver for experiment C varied significantly ($r = 0.871$) with vitamin B_6 intake. The animals that received more protein in their rations did not have higher amounts of vitamin B_6 in the liver. This was not the case in experiment B, where animals that received more protein and the same amount of vitamin B_6 had more vitamin B_6 in the liver. A true comparison cannot be made, however, because in one experiment restricted feeding was employed, and in the other, ad libitum feeding.

A note concerning the appearance of deficiency symptoms should be included. In general, dermal symptoms were more severe when vitamin B_6 was decreased and protein increased in the ration. The rations were designed to develop a vitamin B_6 deficiency, permitting little of the sparing effect of fat. Similarities have been observed in the syndromes which accompany rations deficient in vitamin B_6 and the essential fatty acids (Deuel, '54). Symptoms appearing with one may be alleviated by the other, and male animals are known to require more of the essential fatty acids than females. Most of the animals in experiments B and C received rations which contained 4% of coconut oil. Coconut oil contains about 2.5% of linoleic acid (Feiser and Feiser, '44). In experiment C, hematuria was observed in all animals that received no pyridoxine and appeared sooner in male animals and those receiving rations more generous in protein. Hematuria is a symptom

which accompanies fat deficiency (Deuel, '54). Because of the interdependence between vitamin B_6 and the essential fatty acids, a vitamin B_6 deficiency could hasten the development of symptoms of an essential fatty acid deficiency when these fatty acids are low in the ration. Likewise, a higher quantity of protein in the ration may aggravate a vitamin B_6 deficiency and this in turn may cause hematuria to appear sooner.

SUMMARY

Albino rats maintained on various intakes of pyridoxine hydrochloride and fed restricted amounts of food had concentrations of vitamin B_6 in the blood which varied according to intake.

When fed ad libitum and given 0, 5 or 10 μg of pyridoxine hydrochloride per rat per day, the animals had no detectable vitamin B_6 in the blood. Increasing the percentage of casein in the ration and holding the vitamin B_6 intake constant for rats receiving 0, 5 or 10 μg of pyridoxine hydrochloride per day resulted in a more rapid decline in blood content of vitamin B_6.

As food intake increased, the vitamin B_6 content of liver decreased when vitamin B_6 intake was held constant. The concentration of vitamin B_6 per milligram of liver nitrogen could be correlated with vitamin B_6 intake in two experiments which were longer than 4 weeks.

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APPLICATION OF THE PROTEIN DEPLETION-REPLETION TECHNIQUE IN BABY PIG FEEDING EXPERIMENTS

III. COMPARISON OF THE DEPLETION-REPLETION TECHNIQUE WITH CONVENTIONAL GROWTH FEEDING TRIALS [1]

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In previous papers in this series, Peo et al. ('57) have made use of the protein depletion-repletion technique in studying the protein requirement of the baby pig. However, they point out that in order to evaluate the technique direct comparisons with other acceptable methods are needed. Benditt et al. ('48) made an indirect comparison of the rat-repletion method and growth trial in measuring protein utilization. They made use of growth data obtained by Barnes and co-workers ('46) and compared the results to their data obtained with the rat-repletion method. The results obtained by the two groups using different methods were remarkably similar; however one would question the validity of such a comparison on the basis of the many complicating factors such as differences in environment, time, genetic makeup of the animals, etc., which would undoubtedly affect the final results. They also question the applicability of their results to other species, specifically human beings. Thus the three experiments reported herein were conducted to compare the depletion-repletion and conventional growth feeding trials as methods for evaluating the optimum level of protein for baby pigs. As pointed out in

[1] Journal paper no. J-3239 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa. Project no. 959.

earlier papers in this series, the primary purpose for testing the repletion technique was to find a method that would be less variable than standard ad libitum feeding trials, and still give valid results.

EXPERIMENTAL

Animals

All pigs used in these experiments were crossbred animals. They were taken from the sows at 7 to 14 days of age and fed for one week a common pre-starter ration containing 40% dried skim milk (Speer et al., '54). Feed and water were provided ad libitum during the preliminary and experimental periods.

Experiment 768. Twelve litters of six pigs each averaging 8.9 days of age and 6.9 lbs. body weight were used to evaluate three levels of protein (15, 20 and 25%) by the two different methods (conventional growth and depletion-repletion). A mixture of equal parts solvent-processed soybean oil meal (50% protein) and low heat spray-dried skim milk (34% protein) was used as the major source of protein in the test rations (table 1). The animals were confined to individual metabolism cages; the screen flooring was removed so that the pigs were on concrete floors with wood shavings for bedding. Six replications of the 6 treatments were conducted at each of two times due to the limitation of only 36 cages. The average initial ages and weights were maintained as similar as possible between the two time intervals. The animals were randomly allotted by weight within litters to the 6 treatments making use of a randomized block design in which each block was made up of a single litter of 6 pigs.

The pigs were housed in a controlled temperature, well ventilated baby pig nursery equipped with germicidal lamps around the room, at the entrance and at the air inlet. The floor was radiant heated by thermostatically controlled circulating water and the room temperature controlled by a forced-air heating system. Initially the room temperature was maintained at 80°F. and the floor temperature at 84°F. These

temperatures were reduced 4° each week to 68° and 72° respectively and were maintained for the remainder of the experiment.

At the end of the one-week preliminary period half of the pigs were fed a protein-free ration (Peo et al., '57) for a one-week depletion period and then fed the test rations for a two-week repletion period. The other half received the test rations for the three-week period. Individual weight changes and feed consumption data were recorded weekly. The total gain per pig during the repletion period or the total gain per pig during the three-week conventional-growth period was used

TABLE 1

Composition of basal rations

INGREDIENT	PROTEIN	EXP. 768	EXP. 754 AND 772
	%	%	%
Corn (ground yellow)	9.4	59.19	35.35
Sucrose		10.00	15.00
Whey (70% lactose)	13.0	2.50	15.00
Dried skim milk	34.0	9.06	—
Solvent extracted soybean oil meal	49.9	9.06	25.00
Lard (stabilized)		2.50	2.50
Dicalcium phosphate		2.32	2.00
Calcium carbonate		0.27	0.25
Salt (iodized)		0.50	0.50
Trace mineral premix [1]		0.10	0.15
Vitamin-antibiotic premix [2]		2.00	2.00
Beet pulp	8.0	2.00	2.00
Brewers' yeast	45.0	0.50	—
Pepsin (1: 3000)		—	0.25
Total		100.00	100.00

[1] Calcium Carbonate Co., Quincy, Illinois; 0.10% contributed the following in milligrams/pound of ration: Fe, 32.0; Cu, 2.2; Co, 0.8; Zn, 37.1; Mn, 25.8; K, 3.4.

[2] Soybean oil meal carrier; contributed the following per pound of ration: experiment 768: vit. A, 3000 I.U.; vit. D_2, 1000 I.U.; riboflavin, 3.5 mg; Ca pantothenate, 5.8 mg; niacin, 22 mg; choline Cl, 113 mg; vit. B_{12}, 20 μg; folic acid, 0.5 mg; thiamine·HCl, 5.0 mg; pyridoxine, 1.5 mg; para-amino benzoic acid, 8.0 mg; biotin, 20 μg; inositol, 250 mg; alpha-tocopherol acetate, 10 mg; menadione, 10 mg; ascorbic acid, 300 mg; chlortetracycline, 50 mg; experiments 754 and 772; riboflavin, 3.1 mg; Ca pantothenate, 3.0 mg; niacin, 22.7 mg; choline Cl, 0.0 mg; others same as in exp. 768.

as the observation in the statistical analysis of the gain data. Likewise the pounds of feed required per pound of gain for the repletion period or for the three-week conventional growth period was used in the analysis of the feed data.

Experiments 754 and 772. Similar procedures were used in allotting the pigs in these experiments as in the previous one, except that the animals were maintained in pens of 4 pigs each. The pigs were housed in a unit equipped with a forced-air heating system, but the floors were not heated. The room temperature was maintained at 72°F. and heat lamps were provided to insure adequate restricted space heat for the first two weeks. Wood shavings were provided as bedding. Also, in these experiments, the depletion-repletion portion (exp. 754) and the conventional growth test (exp. 772) were not conducted simultaneously, thus the data cannot be combined for statistical comparisons of the two methods. In each experiment 72 pigs were randomly allotted by weight within litters to three replications of 6 ration treatments. The test rations were the same for the two experiments, consisting of three levels of protein (19, 22 or 25%) with or without 0.05% supplemental DL-methionine. Solvent-processed soybean oil meal served as the major source of protein in these experimental rations (table 1). All pigs were fed the same preliminary rations as that used in experiment 768.

Initially the pigs in experiment 754 averaged 6.9 lbs. body weight and 8.9 days of age. They were carried through a one-week preliminary period, a one-week depletion period, a one-week repletion period, then the depletion-repletion cycle was repeated. The weight gains and feed consumption for the repletion periods were pooled for statistical analysis. The average pig repletion gain per pen was used as the observation and the pen was considered the experimental unit. The feed data were recorded and analyzed as the pounds of feed required to produce a pound of repletion gain.

The pigs in experiment 772 were carried through a similar one-week preliminary period and then on the test rations for a 4-week experimental period. Thus the time required to com-

plete the two experiments was the same (5 weeks). The average total 4-week gain was used at the observation and here again the pen of 4 pigs was considered the experimental unit. Initially the pigs in this experiment averaged 7.0 lbs. body weight and 10.6 days of age.

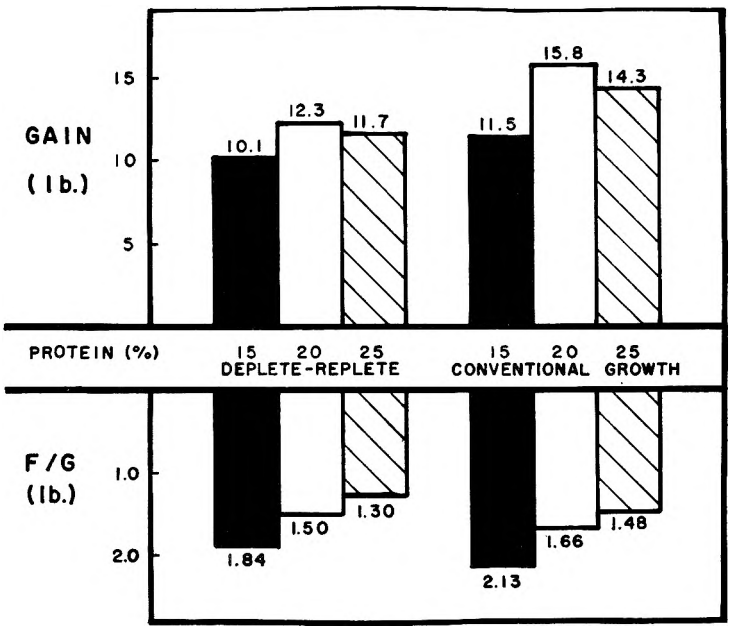

Fig. 1 Average gain and feed required per pound of gain in two-week repletion period and three-week conventional growth period (experiment 768).

RESULTS AND DISCUSSION

Experiment 768. A summary of gains and feed required per pound of gain is presented in figure 1. The response was similar in each method, that is, the 20% protein level produced the greatest gains with the higher level (25%) giving intermediate results and the low level (15%) being inadequate in either method. On the other hand, the feed required per pound of gain was least for the higher protein level and greatest for the low level of protein. Analysis of variance (table 2) showed the gain response to be a significant quadratic regression and the feed response to be a significant linear regression.

TABLE 2

Analysis of variance plan and observed mean squares for experiment 768

SOURCE OF VARIATION	COMBINED ANALYSIS			SEPARATE ANALYSIS				
					Conventional growth		Repletion	
	d.f.	Gain	Feed/gain	d.f.	Gain	Feed/gain	Gain	Feed/gain
		Mean squares			*Mean squares*		*Mean squares*	
Litters	11	19.9064	0.0720	11	16.0836	0.0790	7.4595	0.0302
Method	1	111.7513	0.7771					
Protein levels	2	66.0117	2.2532	2	57.6150	1.3723	15.2658	0.9118
Linear regression	1	56.3333	4.3200	1	46.2038	2.5676	14.5704	1.7876
Quadratic regression	1	75.6900	0.1863	1	69.0312	0.1770	15.9612	0.0360
Method × protein	2	6.8716	0.0310					
Error	55	4.8971	0.0365	22	6.3900	0.0490	4.0346	0.0236

The data were treated statistically in two ways. The combined analysis of variance (pooled depletion-repletion and conventional growth data) was computed to test for interactions between method of measuring and protein levels fed. This gives a measure of the validity of the results. Also the data from each method were analyzed individually to estimate the relative sensitivity of the two methods.

As can be seen in the combined analysis of variance plan (table 2) and by inspecting the data there appears to be no interaction of protein levels and method of measuring in this experiment. The mean square for interaction as measured by weight gains is only 1.4 times the mean square for error; likewise the data for feed/gain give a ratio of interaction mean square/error mean square of less than one. Obviously neither of these is statistically significant. This can be readily seen from the data and individual analyses of variance, as each method gave a quadratic regression for gains with the maximum gains observed on the 20% protein level, also each method resulted in a linear regression for feed/gain with the maximum feed required per unit of gain being observed at the low (15%) protein level.

The sensitivity of the two methods did not differ greatly in respect to rate of gain, the observed coefficients of variation being 18.6 and 17.7% for the conventional growth and depletion-repletion methods respectively. However, in the measure of feed required per pound of gain the conventional growth method resulted in a coefficient of variation of 12.6% as compared to 9.9% for the depletion-repletion method. This represented a 23% reduction of the coefficient of variation by using the depletion-repletion technique.

It should be pointed out that difficulties arise in making and interpreting direct comparisons of the two methods. The repletion data were taken over a two-week period, whereas the conventional growth data were collected over a three-week period; however if one attempts to keep the length of growth periods equal it is difficult to avoid an age difference because

of the necessary depletion period. Since on an ad libitum feeding program there is less labor involved in the conventional growth test, unless the depletion-repletion technique proves to be more sensitive or requires less time or material, there would be no advantage to using it. Thus it was decided to use the same total number of days for each method, namely, 28 days, including the 7-day preliminary feeding period.

TABLE 3

Repletion gains and feed required per pound of repletion gain

Experiment 754

LEVEL OF PROTEIN	19		22		25	
METHIONINE ADDED	–	+	–	+	–	+
TREATMENT NUMBER	1	2	3	4	5	6
			Repletion gains (lbs.)			
Rep. I	8.25 [1]	7.00	9.44	7.58	8.38	9.88
Rep. II	8.38	7.94	12.00	11.00	10.38	12.69
Rep. III	7.44	8.25	10.31	9.08	9.44	10.25
Average	8.02	7.73	10.58	9.22	9.40	10.94
			Feed/gain (lbs.)			
Rep. I	1.63	1.66	1.22	1.88	1.39	1.22
Rep. II	1.57	1.56	1.25	1.54	1.35	1.12
Rep. III	1.91	1.33	1.24	1.49	1.14	1.09
Average	1.70	1.52	1.24	1.64	1.29	1.14

[1] Each observation represents an average for a pen of 4 pigs.

Experiments 754 and 772. Summaries of gains and feed required per pound of gain are presented in tables 3 and 4. Also the analysis of variance plan and observed mean squares are presented in table 5.

In the conventional growth experiment the gains were greater on 19% protein than on 22 or 25% with the response being a significant linear regression of gain on protein levels. Gains on the methionine-supplemented groups were slightly down for the two lower levels of protein and up 1.53 lbs. per pig for the higher (25%) protein levels. This could be expected since increasing the protein level in the ration by

substituting soybean oil meal for corn increases the methionine deficiency relative to the other amino acids. However this apparent interaction of methionine × protein level was not significant.

TABLE 4

Gains and feed required per pound of gain

Experiment 772

LEVEL OF PROTEIN	19		22		25	
METHIONINE ADDED	−	+	−	+	−	+
TREATMENT NUMBER	1	2	3	4	5	6
			Gains (lbs.)			
Rep. I	20.00 [1]	16.12	17.50	14.06	15.75	20.25
Rep. II	17.25	19.33	16.31	16.94	12.81	12.44
Rep. III	20.38	20.25	21.06	20.94	16.75	17.19
Average	19.21	18.57	18.29	17.31	15.10	16.63
			Feed/gain (lbs.)			
Rep. I	1.82	1.84	1.79	1.70	1.82	1.67
Rep. II	1.99	1.76	1.82	1.80	1.70	1.79
Rep. III	1.93	1.92	1.76	1.89	1.92	1.75
Average	1.91	1.84	1.79	1.80	1.81	1.74

[1] Each observation represents an average for a pen of 4 pigs.

TABLE 5

Analysis of variance plan and observed mean squares for experiments 754 and 772

SOURCE OF VARIATION	d.f.	EXPERIMENT 754		EXPERIMENT 772	
		Gain	Feed/gain	Gain	Feed/gain
		Mean squares		*Mean squares*	
Replication	2	6.0109	0.0583	19.4982	0.0118
Protein levels	2	9.4320	0.2312	14.0721	0.0176
Linear regression	1	15.7781	0.4002	27.4216	0.0310
Quadratic regression	1	3.0859	0.0020	0.7225	0.0042
Methionine supplementation	1	0.0068	0.0020	0.0047	0.0103
Protein × methionine	2	3.2338	0.1620	2.7662	0.0030
Error	10	0.5276	0.0201	5.1698	0.0056

The depletion-repletion experiment gave a different picture for gains. The gain per pig increased as the level of protein in the ration increased. The increase in gain was greater for the protein interval 19 to 22% protein than for the 22 to 25% interval, resulting in a significant quadratic response. Here again the methionine supplementation appeared detrimental on the two lower levels of protein, but beneficial to the higher level of protein. In this experiment the apparent interaction was consistent enough through the replications to result in a significant methionine × protein interaction. This is probably largely due to the increase in soya protein relative to the increase in protein from other sources rather than due to the increase in protein *per se*. In the 19% protein rations, 71% of the protein is from soybean oil meal whereas in the 25% protein rations 84% of the protein is soya protein.

The apparent difference in level of protein required for gains as compared to repletion gains could prove a major criticism of the repletion method in measuring protein adequacy for growing animals. This difference was not apparent in the previous experiment in which both methods were conducted simultaneously. Thus the difference here may be a result of factors other than method of measuring.

The trends in response for the two methods were similar with the highest level of proteins resulting in the most efficient feed utilization in each experiment. The linear regression component was significant in each case. In experiment 754 (repletion data) the protein × methionine interaction was significant, however the pattern of response was erratic with an apparent response on the low and high levels of protein but not on the intermediate level. The trends were the same for experiment 772, with a saving of 0.07 lbs. of feed per pound of gain in the supplemented groups of high and low protein. On the other hand, the supplemented and unsupplemented groups of the 22% protein level required equal amounts of feed to produce a pound of gain. This erratic response to methionine supplementation cannot be explained on the basis of increasing levels of soya protein in the diet.

Statistically, one cannot compare these two experiments directly. However it should be pointed out that the reduction in variation by using depletion or repletion was not as evident in these experiments as in experiment 768. The coefficents of variation for gain data being 13.0 and 7.8% respectively for the conventional growth and depletion-repletion, and likewise for feed/gain the observed values were 4.1 and 10.0%. The variation of gains appeared to be decreased but the variation of feed required to produce a pound of gain appeared to be increased by depletion-repletion.

The results of these experiments indicate that the depletion-repletion technique may exaggerate the protein requirement, which could be expected as the depleted animal may be in a condition to utilize more protein than the animal of similar age in a repleted state. If this point is considered, the technique should be applicable to young growing animals equally as well as for adult animals. Cannon ('54) points out that the utilization rate for a particular essential amino acid in protein repletion of an adult rat is two to 5 times that for maintenance alone. Under the conditions of the experiments reported herein the protein or amino acid requirement should be magnified considerably more in order to meet the requirement for maintenance, repletion of amino acid pools and rapid growth. This may explain partially the differences in results obtained in experiment 768 as compared to 754 and 772. In experiment 768 a combination of milk and soya protein provided a diet quite well balanced in the essential amino acids and therefore the animals in the depleted state met their requirements by a more efficient utilization of the protein present in the diet, resulting in little or no difference in the optimum level of protein as meausred by the two methods. In the other experiments, soya protein alone was not as efficient in repleting baby pigs, thus an increased level of protein proved beneficial. If this is the case one should expect a more dramatic response to methionine which appears to be the most limiting amino acid in soya protein for growth of young pigs.

However, under these conditions, other amino acids may become limiting.

The observed response to methionine supplementation was more consistent for the depletion-repletion method, resulting in a statistically significant interaction of protein level × methionine supplementation. Although the trends were similar for the conventional growth method the response varied to such an extent that it was not statistically different.

SUMMARY

A direct comparison of protein depletion-repletion and conventional growth as methods of measuring the level of protein required for baby pigs gave comparable results when a combination of solvent extracted soybean oil meal and dried skim milk provided the major source of protein. The depletion-repletion method appeared to be the more sensitive technique as measured by the relative within treatment variation. Protein levels of 15, 20 and 25% resulted in a quadratic response as measured by gain, with maximum rate of gain being observed on the 20% protein level. When measured by feed required per pound of gain, each method resulted in a linear response with the 25% protein ration being the most efficient.

In an indirect comparison where the two methods were used to evaluate levels of 19, 22 and 25% protein, soya protein being the major source, the observed gain data differed between the two methods. In the conventional growth experiment 19% protein gave maximum gains, whereas in the depletion-repletion experiment 25% protein resulted in maximum gains. The response as measured by feed required per pound of gain was similar for the two methods with the higher protein level being the most efficient.

A consistent response to the addition of 0.05% methionine was observed on the 25% protein level but not on the other levels. This protein level × methionine supplementation interaction was statistically significant for the gain and feed data as measured by the depletion-repletion method, but not as measured by the conventional growth method.

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METABOLISM OF PTEROYLGLUTAMIC ACID AND LIVER NUCLEIC ACID LEVELS IN CERTAIN VITAMIN DEFICIENCIES

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In previous publications (Guggenheim et al., '56; Guggenheim and Halevy, '57) protein-deficient rats were shown to be less able to convert pteroylglutamic acid (PGA)[1] to citrovorum factor (CF) than well-fed controls. In the present study these observations have been extended to include the effects of certain vitamin deficiencies on the formation of CF in the liver, which is the main site of the conversion of PGA to CF in the body (Nichol, '53).

CF, after reaction with adenosine triphosphate (ATP), is capable of donating its formyl group for purine synthesis (Greenberg, '54), and is, therefore, important for the biosynthesis of nucleic acids. The effects of various vitamin deficiencies on hepatic levels of ribonucleic (RNA) and deoxyribonucleic acids (DNA) were, therefore, studied. Deficiency of vitamin B_{12}, another vitamin which plays an important role in the formation of nucleic acids (Arnstein, '55), has already been shown to decrease the concentration of RNA and DNA in the liver (Rose and Schweigert, '52; Schweigert, Scheid and Downing, '54; Wong and Schweigert, '56).

[1] As used in previous publications (Guggenheim et al., '56; Guggenheim and Halevy, '57), the term "pteroylglutamic acid" has been restricted to the synthetic compound and "citrovorum factor" to the substance (s) promoting growth of *Pediococcus cerevisiae* 8081 (*Leuconostoc citrovorum*); the term "folic acid" is a generic term applicable to substances which stimulate the growth of *Streptococcus faecalis R.*

METHODS

Young male rats were used throughout.

The normal diet has been described previously (Guggenheim and Halevy, '57). For the production of thiamine, pyridoxine, riboflavin and pantothenic acid deficiencies the respective vitamins were omitted from the control diet. In order to hasten the appearance of pyridoxine, riboflavin and pantothenic acid deficiencies, suitable metabolic antagonists, i.e.: 20 mg of desoxypyridoxine or 50 mg of galactoflavin were added to each kilogram of the respective vitamin-free diets. Rats on the pantothenic acid-deficient diet were given daily subcutaneous injections of 5 mg of pantoylaminoethanethiol after 5 weeks on the deficient diet. Folic acid deficiency was produced by adding 25 gm of succinylsulfathiazol (SST) per kilogram of control diet. For induction of vitamin A deficiency the supplement of vitamin A was omitted.

The animals were used for experiment only after they developed the typical signs of the intended nutritional deficiency (table 1). Rats on the folic acid deficiency-producing diet were examined after three weeks. Control animals received the full diet either ad libitum or restricted to the quantity of food consumed by the experimental animals.

The animals were killed by decapitation and their livers examined for folic acid (FA), CF, RNA and DNA as previously described (Guggenheim and Halevy, '57).

RESULTS

Vitamin deficiencies and metabolism of PGA

Several groups of young rats were kept on the respective vitamin-deficient diets. After the times indicated (table 1) about one-half of each group received an intraperitoneal injection of 1 mg of PGA per 100 gm body weight. On the following day, all animals were sacrificed and their livers examined for FA and CF. Since rats designated for experiments on thiamine and pyridoxine deficiencies had a similar initial weight, and since they happened to eat similar amounts of

TABLE 1

Effect of vitamin deficiencies on the folic acid (FA) and citrovorum factor (CF) content of the liver

(The figures indicate means and standard errors)

GROUP	NO. OF RATS	DIETARY TREATMENT	TIME ON DIET	DAILY FOOD INTAKE	WEIGHT			PGA [1]	LIVER				CF AS % OF FA
					Initial	Final	Change		Folic acid		Citrovorum factor		
									Per 100 gm body weight	Increase	Per 100 gm body weight	Increase	
			Weeks	*gm*	*gm*	*gm*	*gm*		*μg*	*μg*	*μg*	*μg*	
1	38	Normal controls	3–5	10.8	60	138	+ 78	−	7.2 ± 0.60		5.6 ± 0.43		
								+	15.8 ± 0.95	8.6 ± 1.12	13.2 ± 1.33	7.6 ± 1.40	79 ± 3.9
2	16	Vitamin A deficient	4–5	5.6	35	74	+ 39	−	8.0 ± 0.51		6.0 ± 1.24		
								+	11.3 ± 2.16	3.3 ± 2.22	8.8 ± 1.84	2.8 ± 2.22	76 ± 6.6
3	14	Pair-fed with 2	4–5	5.6	35	83	+ 48	−	8.4 ± 0.74		6.8 ± 1.20		
								+	13.8 ± 1.91	5.4 ± 2.04	11.6 ± 2.30	4.8 ± 2.59	80 ± 6.5
4	23	Thiamine deficient	4–5	6.9	95	101	+ 6	−	5.9 ± 0.43		3.3 ± 0.60		
								+	13.2 ± 1.67	7.3 ± 1.73	10.0 ± 1.55	6.7 ± 1.66	62 ± 6.0
5	20	Pyridoxine deficient	4–5	6.5	98	85	− 13	−	6.5 ± 1.34		5.1 ± 1.25		
								+	9.0 ± 0.93	2.5 ± 1.63	6.7 ± 0.68	1.6 ± 1.42	67 ± 4.5
6	22	Pair-fed with 4 and 5	4–5	6.7	93	110	+ 17	−	5.3 ± 0.75		4.0 ± 0.45		
								+	12.5 ± 1.03	7.2 ± 1.28	9.9 ± 1.12	5.9 ± 1.21	77 ± 5.2
7	22	Riboflavin deficient	6–10	7.7	61	90	+ 29	−	3.5 ± 0.56		1.9 ± 0.45		
								+	6.1 ± 1.33	2.6 ± 1.44	4.5 ± 0.99	2.6 ± 1.09	62 ± 6.1
8	11	Pair-fed with 7	6–10	7.7	65	143	+ 78	−	8.3 ± 1.30		5.0 ± 1.22		
								+	15.8 ± 1.68	7.5 ± 2.12	12.1 ± 1.72	7.1 ± 2.11	70 ± 5.5
9	18	Pantothenic acid deficient	6–9	8.4	42	88	+ 46	−	8.8 ± 0.95		4.5 ± 0.50		
								+	15.2 ± 1.96	6.7 ± 2.18	13.5 ± 2.04	9.0 ± 2.10	78 ± 5.8
10	12	Pair-fed with 9	6–9	8.4	42	154	+ 112	−	6.3 ± 2.21		5.8 ± 3.14		
								+	15.3 ± 3.31	9.0 ± 3.91	11.5 ± 3.23	5.7 ± 4.51	83 ± 5.8
11	22	Folic acid deficient	3	6.4	61	129	+ 68	−	2.4 ± 0.28		1.7 ± 0.27		
								+	8.8 ± 1.70	6.4 ± 1.71	7.5 ± 1.53	5.8 ± 1.55	78 ± 7.1

[1] Plus indicates one intraperitoneal injection of 1 mg of pteroylglutamic acid (PGA) per 100 gm body weight 24 hrs. before examination of liver. Minus indicates no injection.

food during the experimental period of 4 to 5 weeks, the results obtained with the pair-fed control rats of these two groups were treated as one group and are represented as group 6. Figures for normal rats fed the full diet ad libitum for three to 5 weeks (group 1) are presented for comparison.

Vitamin A deficiency. After injection of PGA smaller amounts of both FA and CF were found in the livers of deficient animals than in those of ad libitum-fed controls. The difference for FA only proved to be statistically significant. Thus, the livers of the deficient rats appeared to be less able to retain FA than the livers of normal animals.

Thiamine deficiency. Liver levels of FA and CF in both PGA-treated and untreated as well as CF in treated animals were slightly diminished. The percentage of FA converted to CF seems to be smaller than in normal rats. Assuming an equally impaired capacity of deficient rats to retain both FA and CF in their livers, this would indicate that less PGA is metabolized to CF than in normal rats. In comparison with pair-fed rats, however, this difference proved to be on the borderline of significance only.

Pyridoxine deficiency. PGA-treated rats suffering from this nutritional deficiency showed significantly lower liver levels of both FA and CF. Furthermore, conversion of PGA into CF was slightly diminished.

Riboflavin deficiency. Rats kept on a riboflavin-deficient diet exhibited significantly lower figures for both FA and CF contents in their livers. The increase following administration of PGA was smaller than normal. Conversion of PGA to CF was likewise depressed when compared with normal rats, but not in comparison with pair-fed controls.

Pantothenic acid deficiency. No marked deviation from normal values was noted.

Folic acid deficiency. As was to be expected, lower figures for both FA and CF were found in livers of FA-deficient rats. However, treatment with PGA induced a normal increase of both FA and CF. The capacity of the liver to convert PGA into CF does not seem to be affected in FA deficiency.

The livers of rats pair-fed with the deficient animals (groups 3, 6, 8, 10) did not show any significant impairment of either storage capacity of FA or CF or of the ability to convert PGA to CF. The reduced capacity for FA and CF retention of livers of pyridoxine- and riboflavin-deficient rats is therefore the result of the vitamin deficiency *per se* and not of the accompanying reduction of food intake. The impaired ability of livers of vitamin A-deficient rats to retain FA and that of thiamine-, pyridoxine- and riboflavin-deficient rats to convert PGA to CF, however, seems to be the combined effect of lack of dietary vitamins as well as of diminished food consumption.

Effect of biotin on metabolism of FA

Recently it has been reported that biotin is required for the biosynthesis of FA (Luckey et al., '55). Experiments were therefore performed in order to study the possible effect of biotin on liver levels of FA and CF. Forty-eight rats weighing about 40 gm were divided into 4 groups, each comprising 12 animals. One group received the control diet; the diets of groups 2, 3 and 4 were supplemented as follows: group 2 with 1 mg of biotin; group 3 with 25 gm of SST, and group 4 with 1 mg of biotin plus 25 gm of SST per kilogram of ration. After 4 weeks all rats were killed and their livers examined. As can be seen from table 2, SST significantly depressed both weight increase and liver concentrations of FA and CF. Biotin, however, had no marked effect on weight increase or liver levels of FA and CF, neither in rats maintained on the full diet nor in those supplemented with SST. Moreover, no effect of biotin on the conversion of PGA into CF could be observed.

Vitamin deficiencies and hepatic nucleic acids

Data on liver levels of RNA and DNA in deficiencies of vitamin A, thiamine, pyridoxine, riboflavin and pantothenic acid are presented in table 3. Since the nucleic acid content of liver varies with animal weight and energy and protein

TABLE 2

Effect of biotin on liver levels of folic acid (FA) and citrovorum factor (CF)
(The figures indicate means and standard errors)

SUPPLEMENT	WEIGHT		Weight	LIVER				CF AS % OF FA
	Initial	Increase in 4 weeks		Folic acid		Citrovorum factor		
				Per gm liver	Per 100 gm body wt.	Per gm liver	Per 100 gm body wt.	
	gm	*gm*	*gm*	*μg*	*μg*	*μg*	*μg*	
—	39 ± 0.65	109 ± 4.7	6.7 ± 0.35	2.8 ± 0.36	18.5 ± 2.3	2.2 ± 0.42	14.7 ± 3.2	80 ± 4.7
Biotin	38 ± 0.64	105 ± 4.3	6.3 ± 0.32	3.6 ± 0.28	22.7 ± 2.1	2.9 ± 0.18	18.6 ± 2.0	81 ± 5.8
SST [1]	41 ± 0.80	83 ± 4.9	6.0 ± 0.31	0.44 ± 0.07	2.69 ± 0.40	0.40 ± 0.09	2.48 ± 0.52	88 ± 7.1
Biotin + SST	39 ± 0.75	90 ± 4.0	5.9 ± 0.37	0.54 ± 0.13	3.09 ± 0.66	0.47 ± 0.14	2.65 ± 0.70	87 ± 4.7

[1] Succinylsulfathiazole.

TABLE 3

Effect of vitamin deficiencies on liver level of ribonucleic and desoxyribonucleic acids (RNA and DNA)
(The figures indicate means and standard errors)

GROUP	NO. OF RATS	DIETARY CONDITIONS	WEIGHT OF RATS	Weight	LIVER				RNA / DNA
					RNA		DNA		
					Per gm liver	Per 100 gm body wt.	Per gm liver	Per 100 gm body wt.	
			gm	*gm*	*mg*	*mg*	*mg*	*mg*	
1	20	Normal controls	135	5.6 ± 0.22	7.91 ± 0.16	32.8 ± 0.67	3.43 ± 0.10	14.2 ± 0.42	2.31 ± 0.053
2	12	Vitamin A deficient	74	2.9 ± 0.19	7.25 ± 0.10	28.8 ± 0.94	4.75 ± 0.12	18.6 ± 0.70	1.54 ± 0.068
3	10	Pair-fed with 2	83	3.4 ± 0.20	7.65 ± 0.20	31.9 ± 1.10	4.50 ± 0.19	18.3 ± 0.53	1.72 ± 0.098
4	12	Thiamine deficient	105	3.9 ± 0.24	6.28 ± 0.12	23.8 ± 0.87	4.81 ± 0.20	17.9 ± 0.57	1.42 ± 0.049
5	12	Pyridoxine deficient	96	4.2 ± 0.18	7.78 ± 0.10	34.0 ± 1.23	4.46 ± 0.09	19.5 ± 0.61	1.74 ± 0.050
6	9	Pair-fed with 4 and 5	112	3.8 ± 0.20	6.98 ± 0.06	23.5 ± 0.45	5.46 ± 0.15	18.5 ± 0.52	1.28 ± 0.037
7	10	Riboflavin deficient	66	3.3 ± 0.27	7.34 ± 0.14	36.7 ± 1.30	3.81 ± 0.13	19.1 ± 0.73	1.92 ± 0.072
8	10	Pair-fed with 7	140	4.6 ± 0.28	7.52 ± 0.15	24.7 ± 1.26	4.60 ± 0.12	15.1 ± 0.80	1.60 ± 0.061
9	10	Pantothenic acid deficiency	90	3.5 ± 0.25	8.37 ± 0.17	32.2 ± 1.59	4.64 ± 0.19	18.0 ± 0.62	1.79 ± 0.088
10	9	Pair-fed with 9	158	5.0 ± 0.29	8.51 ± 0.15	26.9 ± 1.27	4.81 ± 0.18	15.2 ± 0.34	1.78 ± 0.120

content of the diet (Campbell and Kosterlitz, '50; Leslie, '55), we decided to compare our results to the nucleic acid levels obtained in livers of normal rats of equal weight, fed the control diet ad libitum. Liver RNA and DNA were therefore determined in 36 normal rats weighing from 60 to 200 gm. The relationship between body weight and liver nucleic acids is presented in figure 1. A marked tendency for both RNA

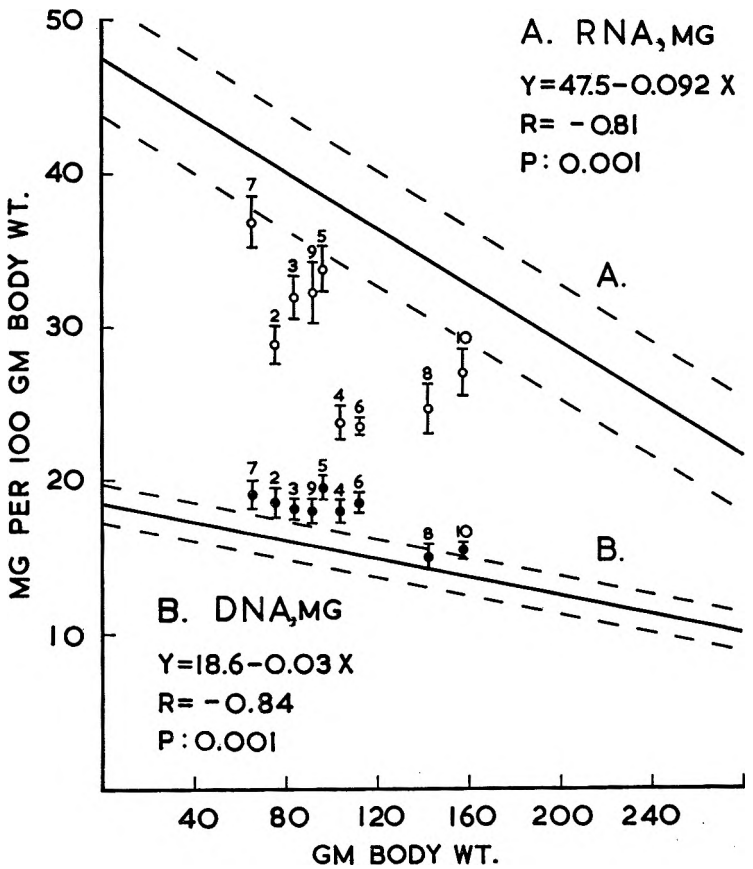

Fig. 1 The relation of body weight to liver levels of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). The straight lines indicate mean levels of RNA (a) and DNA (b) in normal rats, and the broken lines, their standard errors. Each point represents the mean value of RNA or DNA level in the deficient rats together with its standard error. The numbers refer to the groups of deficient rats as represented in tables 2 and 4.

and DNA to decrease with increasing body weight could be demonstrated. The slopes of the regression lines indicate that an average increase of 10 gm in body weight resulted in a decrease of 0.92 mg of RNA and 0.30 mg of DNA per 100 gm of body weight. The correlation coefficients between these variables are — 0.81 for RNA and — 0.84 for DNA, and the probabilities that these correlations are due to chance alone are 0.001. Figure 1 further shows that the RNA values of all groups of vitamin-deficient rats studied as well as those of their pair-fed controls were considerably lower than those of normal rats of equal weight, whereas figures for DNA were found to be higher. Consequently, the RNA : DNA ratio in all groups of deficient rats was lower: 1.28 to 1.92 as compared to 2.31 in well-fed rats weighing 135 gm. Hence, no correlation seems to exist between the disturbances in PGA metabolism caused by vitamin deficiencies and the hepatic concentration of nucleic acids.

DISCUSSION

Various forms of vitamin deficiencies appear to affect the metabolism of PGA. Rats deficient in vitamin A, pyridoxine and riboflavin retain less FA and CF, respectively, in their livers than normal rats. Furthermore, conversion of PGA to CF appears to be impaired in deficiencies of thiamine, pyridoxine and riboflavin. Folic and pantothenic acid deficiencies, on the other hand, are apparently without any influence on PGA metabolism. Rats receiving a qualitatively full diet, however, but quantitatively restricted to the food intake of the deficient animals, did not show any impairment of either storage capacity of FA or CF, or the ability to convert PGA to CF.

The disturbances of PGA metabolism are not accompanied by corresponding changes in nucleic acid concentrations of the liver. Moreover, in both vitamin-deficient and pair-fed rats liver RNA was found to have decreased and liver DNA to have increased, irrespective of whether the nutritional conditions affect PGA metabolism or not. The common cause of this

uniform response in both deficient and control rats is probably the diminished consumption of food and consequently of protein (table 1). Deficiency in calories or in protein involves a decrease in liver cell volume and a loss of cytoplasm but little or no change in cell number or DNA content of the nuclei (Campbell and Kosterlitz, '52; Thomson et al., '53). The result is a relative reduction of hepatic RNA and an increase of DNA (Mandel, Jacob and Mandel, '50; Thomson et al., '53; Guggenheim and Halevy, '57). The levels of liver nucleic acids therefore seem to be rather independent of vitamin deficiencies in spite of the disturbances in PGA metabolism which they may produce. A similar conclusion was reached previously (Guggenheim and Halevy, '57). Various procedures affecting PGA metabolism, such as protein deficiency, or incorporation of either SST or 4-aminopterin into the diet, did not result in a depression of hepatic levels of RNA or DNA.

A certain independence of liver nucleic acids of PGA metabolism has recently been reported by Dinning, Cosgrove and Day ('57). Ascorbic acid deficiency was found to depress the utilization of formate in the synthesis of serine and methionine by guinea pig liver homogenates, probably resulting from an impairment of the conversion of PGA to CF (Nichol and Welch, '50; May, Nelson, Lowe and Salmon, '50). The deficiency did not affect, however, either the liver levels of DNA and RNA, or the incorporation of formate into purines. It may therefore be assumed that the biosynthesis of nucleic acids in the liver is more resistant to disturbances in PGA metabolism than other PGA-dependent reactions.

SUMMARY

1. Young rats were subjected to diets producing deficiencies of vitamin A, thiamine, pyridoxine, riboflavin, pantothenic and folic acids. Rats deficient in vitamin A, pyridoxine and riboflavin were less able to retain folic acid (FA) and citrovorum factor (CF), respectively, in their livers than normal rats. Conversion of pteroylglutamic acid (PGA) to CF seems to be

impaired in deficiencies of thiamine, pyridoxine and riboflavin. Folic and pantothenic acid deficiencies had no influence on PGA metabolism. Pair-fed control rats did not exhibit any impairment of PGA metabolism.

2. Biotin had no effect on hepatic levels of FA and CF nor on the conversion of PGA into CF.

3. Liver ribonucleic acid (RNA) per unit body weight was uniformly decreased and liver deoxyribonucleic acid (DNA) increased in both vitamin deficient and pair-fed animals. This effect is attributed to the diminished consumption of calories and protein under these conditions.

4. It is concluded that hepatic concentration of nucleic acids is rather independent of disturbances in PGA metabolism resulting from vitamin deficiencies.

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THE EFFECT OF HIGH VERSUS LOW PROTEIN EQUICALORIC DIETS ON THE HEAT PRODUCTION OF HUMAN SUBJECTS [1,2]

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The concept that protein is the dominant factor responsible for the dynamic effect of diets dates back to the time of Rubner ('02). His conclusions and those of his immediate successors were based on results obtained during short-time observations with dogs or humans when a food component (protein, fat or carbohydrate) was fed to an experimental subject maintained in a basal or fasting condition just prior to the feeding test. Protein produced a greater dynamic effect than did an equicaloric amount of carbohydrate or fat. In determining the dynamic effect of protein in this manner, the amounts of the three nutrients oxidized in the two periods thus compared were quite dissimilar. A serious error of interpretation was the assumption that the dynamic effect of a single nutrient so determined was characteristic of that nutrient when it formed a part of a complete diet. This assumption has since been shown to be untenable (Forbes, '33; Mitchell, '34; Maynard, '37; Forbes et al., '38a).

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[2] This study was part of a Northeast Regional Project (NE-37, Relationships between protein and other selected nutrients and their metabolism and utilization); a cooperative study involving agricultural experiment stations in the Northeastern Region and supported in part by regional funds. Department of Animal Nutrition Publication no. 238. College of Home Economics Research Publication 154.

Some of the reasons why dynamic effects should be determined by difference between results obtained from established states of nutrition rather than from single feedings of test nutrients have been set forth by Forbes et al. ('46). In an extensive series of experiments with albino rats during 70-day feeding periods, Forbes and associates ('35, '38b, '39, '40, '44) and Black, Maddy and Swift ('50) showed conclusively that there was a decrease in heat production as the protein content of equicaloric diets increased from 6 to 45%. This was found to be true with mature rats as well as with young rapidly growing animals. As the percentage of protein in the diet increased from 6 to 25%, the successive lesser heat productions were accompanied by increasing gains of body tissues. At levels of dietary protein from 25 to 45%, however, it was found that although heat production continued to decrease, the body gain also decreased. This decrease in body gain was greater than the decrease in heat production, so the utilization of the energy of the diets that contained more than 25% protein became poorer. Since approximately 78% of the gross energy of protein is metabolizable, the diets high in protein furnished less metabolizable energy. However, it is to be emphasized that the utilization of the metabolizable energy decreased consistently with the increase of dietary protein above 25%. This general observation may be considered as an example of the statement of optimum utilization as set forth by Forbes ('33) and by Mitchell ('34).

The consistent decrease in heat production with increased protein intake on equicaloric diets is opposed to the inference drawn from the short-time experiments previously mentioned in which a single food component was fed. From a practical standpoint, diets and not single nutrients are the units consumed and oxidized, a fact which has not been fully recognized in the field of human nutrition. In view of the findings with small animals, it seemed important to undertake corresponding work with human subjects.

Two experiments which were designed to study this phenomenon in humans have been reported recently by Swift et al.

('57). The first involved measuring the resting heat production of 18 college men at intervals throughout the day and the second consisted of measuring the entire daily metabolism of three pairs of subjects during a 48-hour period in the respiration calorimeter. The results in both studies indicated a slightly higher heat production from the high-protein diet. The authors felt however that adaptation might have been an unknown factor and that if the subjects had been maintained on a constant diet throughout a period of several weeks, results would have more accurately represented the energy utilization of the diets. It was planned therefore in the present investigation to measure the metabolism very soon after the subjects first received the experimental diets and also for several weeks thereafter.

It was found in sampling the items used in the diets, especially those cooked in deep fat, that the sample taken for analysis might be somewhat different (especially in energy content) from the one consumed. In the work here reported, special care was taken in sampling such items.

EXPERIMENTAL

Four male college students 21 years of age were selected as subjects for a study of the dynamic effects of two diets which were of equal energy content but were widely different in protein content. The subjects were selected on the basis of normal basal metabolism, uniform weight (approximately 150 lbs.), acceptance of the diets and interest in the study. They were housed and fed at a cottage used exclusively for the purpose.

The purchase and storage of foods for the diets were consistent throughout the experiment. Sufficient quantities of ground beef and case lots of canned fruits and vegetables were purchased before the experiment began. The ground beef was analyzed for nitrogen, and the individual servings were then weighed, wrapped in aluminum foil and stored in a freezer. All dairy products were purchased from the University creamery, and other foods were obtained at a local grocery.

The basic foods of the low- and high-protein diets were the same. The low-protein diet was compounded to include the commonly used foods which were low in protein and easy to sample. The addition of ground meat, eggs, milk and ice cream to the low-protein diet provided the basis for the high-protein diet. In order to avoid a wide difference between the fat contents of the two diets, less butter was used in the high-protein diet. Furthermore, mashed potatoes were used in the place of French-fried potatoes. Some variation in the diets was provided by the alternate use of two different fruits and two different vegetables at the noon meal, and two different vegetables at the evening meal. Except for the alternate use of tomato juice and green beans and the omission of pineapple at the noon meal, the individual foods eaten by the subjects were the same as in the study recently reported (Swift et al., '57). Candy (fudge), sugar and one carbonated beverage each day were the high carbohydrate, non-protein foods used to increase the caloric value of either the low- or the high-protein diet to the desired level of intake. During the low-protein periods, supplementary diacalcium phosphate, vitamins A and D and B complex vitamins were given daily to each subject.

One of each of the meals to be used in the experiment was prepared and analyzed for nitrogen and energy before the experiment began. This was necessary to assure that the two diets were equicaloric and of definite protein content. Each diet furnished 3111 Cal. daily. The protein supplied by the low-protein diet was 38.0 gm per day, and the high-protein diet furnished 128.6 gm. The daily calculated intakes of fat and carbohydrate on the low-protein diet were 151.2 gm and 469.5 gm, respectively (calculated from U. S. D. A. Handbook No. 8, Watt and Merrill, '50). For the high-protein diet these values were 174.8 gm and 286.4 gm for the fat and carbohydrate, respectively. The intake of all nutrients, as calculated, exceeded the National Research Council Allowances except for the daily intake of protein on the low protein diet (65 vs. 38 gm).

The meals were served each day at 7:15 A.M., 12:15 P.M. and 5:30 P.M. The cooked foods were prepared by standardized procedures and all foods were weighed to the nearest gram on a Toledo scale. Each subject was required to eat all the food served him, and bread was used to wipe the last traces from the plate. To minimize any possible undesirable psychological responses, the subjects on the low- and high-protein diets were served in separate dining rooms.

In an extension of the work recently reported (Swift et al. '57), the respiration calorimeter (Braman, '33) was used to measure the 24-hour metabolism. Minor modifications in the interior of the chamber were made to adapt the apparatus for use with humans. The usual preliminary tests were made for tightness of the chamber and ventilation lines and for quantitative recovery of known amounts of CO_2. Prior to the actual test the subjects entered the chamber several times and remained for a few hours to become accustomed to the routine. The subjects were paired, (R. B. and G. K.; F. K. and G. S.), each pair serving as a unit in the calorimeter. The use of two subjects as a unit provided an appropriate amount of CO_2 and undobutedly helped to prevent apprehension on the part of the subject. One of the objectives was to determine whether evidence would be found of an adaptation to a given protein intake as concerned the energy metabolism. The first calorimetric measurement was therefore made soon after the subjects were placed on their respective diets.

The first meal of the low-protein diet preceded the 24-hour metabolism measurement in the respiration calorimeter by 54 hours. Following the first calorimeter period, while the subjects continued to receive exactly the same diet for a total of 63 days, the 24-hour heat productions were measured at the end of two, 5 and 7 weeks, respectively. Exactly one week after the beginning of the experiment on the low-protein diet, the feeding of the high-protein diet to the second pair of subjects was initiated in which exactly the same sequence was followed. The schedule of experimentation is indicated in table 1.

After the subjects R. B. and G. K. had completed the last calorimeter test on the low-protein diet (period 7), they continued to receive this same diet for several days while the other pair of subjects, F. K. and G. S., underwent their final test on the high-protein diet (period 8). This schedule was designed to provide uniform time intervals of 54 hours between the time of the first meal on a given diet and the beginning of the first calorimeter test on that diet. The assignment of the diets was reversed and three more calorimeter periods took place during the ensuing 38 days. A total of 14 calorimeter periods was scheduled of which 13 took place as planned. It was necessary to omit period 6 because of a death in the family of one of the subjects. The dietary intake, however, was maintained constant during this period even though a calorimeter test could not be made.

After a preliminary period of 6 hours in the respiration chamber, total metabolism was measured continuously for a period of 24 hours. During this time the subjects were free to read or study, and three times a day each was required to take 5 minutes of exercise in the form of knee-bends, push-ups, etc. They obtained about 7 hours of sleep, as it was mandatory that they retire at a given hour at which time all lights were turned off. They were required to arise at a definite hour when the lights were turned on. The heat generated by the lights was measured as a part of the "blank run" and was subtracted from the direct heat measurement of each experimental period. Two operators were on duty at all times, one of whom was always visible to the subjects. This fact plus the ability to communicate with the operator at any time by means of an intercommunication system prevented any feeling of claustrophobia on the part of the subjects. While in the respiration calorimeter the subjects continued to receive three meals per day in accord with the dietary regime under study. Meals were prepared in the calorimeter room, and served immediately at the same hours each day.

The heat of radiation and conduction was measured by the rise in temperature of the quantity of water which flowed

through the calorimeter absorbers at a constant rate. Temperatures of the water as it entered and left the chamber were recorded at 4-minute intervals throughout a 24-hour period.

The heat represented by evaporation of water by the subjects was measured as a part of the ventilation. The water and carbon dioxide were determined by continuous aliquoting of ingoing and outcoming air throughout the entire test period.

In addition to the direct calorimetric measurement, the daily energy output was also measured by indirect calorimetry using the nitrogen-carbon-energy balance method (Swift and French, '54). This required a 5-day collection period (feces and urine) just prior to each calorimeter period. The first calorimetric measurement began 54 hours after the first meal of a given diet was fed. It was therefore not possible to employ the indirect heat measurement during the first periods in which a given diet was fed. Aliquots of the daily urine excretion were composited for anlysis. A small amount of a mixture of sodium fluoride and copper sulfate served as a preservative for the urine samples. Total daily fecal excretion was collected in polyethylene buckets, composited, and stored in a freezer at —30°F. Analyses were made of the composites after thawing and thoroughly mixing the material in a large commercial type Waring blendor. Nitrogen was determined on food and excreta by the Kjeldahl method, and energy was determined by the use of a bomb calorimeter.[3] The wet combustion method (Furman, '39), with minor modification, was employed for the determination of carbon. Respiratory carbon dioxide, the carbon of which was used in the computation of the heat production by this indirect method, was determined during the direct calorimetric test period.

DISCUSSION OF RESULTS

The essential data and sequence of the periods of metabolism measurements are shown in table 1. The details of computation of the daily heat production by the nitrogen-carbon-

[3] Emerson Apparatus Company.

energy balance method are given by Swift and French ('54). This procedure, which involves a measure of the protein and fat gained or lost by the subject, revealed in the periods of low protein intake that the subjects were in slight negative nitrogen balance, ranging from 2.9 to 7.8 gm of protein per subject per day. In previous work (Swift et al., '57) involving three periods of 10 days duration on low-protein intake (40.6 gm daily), the protein balance ranged from —4.4 to +2.2 gm per day. It was anticipated in the present experiment that nitrogen equilibrium would be attained at the end of 52 days or earlier. It appears that a longer period would be necessary. The subjects were in positive nitrogen balance during the periods of high protein intake, the average daily protein storage being 10.6 gm per individual.

The subjects were in positive energy balance in all 9 periods in which indirect calorimetry was employed and presumably in the other 4 periods as well. The daily energy balance for these calorimeter days ranged from 383 to 686 Cal. per subject. It is assumed that the heat production of the subjects when not in the calorimeter was greater, due to the added activity of attending classes and other incidental body exercise. The positive daily energy balances were, for the entire period, undoubtedly less than was found for the day spent in the calorimeter.

The agreement between the results of direct and indirect calorimetry with subjects F. K. and G. S. (table 1) was very satisfactory, the average difference between the two methods being only 1.1%, with a maximum of 2.0%. With subjects R. B. and G. K. the average difference was 4.8%, with a maximum of 6.2%. The authors have no explanation for this apparent difference in degrees of concordancy between direct and indirect calorimetry as found with the two pairs of subjects. All subjects were unquestionably more active during the days spent outside the calorimeter than on the day of the actual heat measurement. This fact, however, does not bear directly on the point, because the production of CO_2 and heat were measured throughout the same 24-hour interval. It is hardly

reasonable to suggest that moderate activity could affect digestibility and fecal output and therefore the indirectly measured heat production. The average difference in daily heat productions as measured by direct and indirect calorimetry for all 9 periods was 3.2%. Considering the possibility of the accumulation of experimental errors of both methods, the direct heat measurement is the more accurate.

A major point of interest in this work concerns the difference between the total 24-hour heat productions resulting from ingestion of diets of equal energy content but differing markedly in protein. With subjects R. B. and G. K. it is clear that no difference was found. Using the values as determined by direct calorimetry in periods 1 and 10 and the average of the direct and indirect in the other periods for these subjects, we find that the average daily heat productions were 4746 and 4764 Cal. for the low- and high-protein diets respectively. If we use only the directly determined heat productions, the average values are 4849 and 4830 Cal. for the low- and high-protein diets, respectively.

With subjects F. K. and G. S. the average heat production obtained from the low-protein diet was about 5% less than that from the diet high in protein. Using values as determined by direct calorimetry in periods 2 and 11 and the average of the direct and indirect in the other periods for these subjects, we find that the average daily heat productions were 4660 and 4925 Cal. for the low- and high-protein diets, respectively. Using the directly determined values only, these values are 4680 and 4938 respectively. The average of all 12 values (table 1) representing the heat production of the low-protein diet for all subjects as measured by direct and indirect calorimetry is 4699 Cal. The average of the 10 corresponding values for the high-protein diet is 4824 Cal., an increase of 2.7%. If the values for direct calorimetry only are used, the low-protein diet is represented by a heat production of 4777 Cal. and the high-protein diet by 4884 Cal., an increase of 2.2%.

Under the conditions of the experiment, it appears that a given diet is as correctly represented by the heat production

TABLE 1

Daily heat production of pairs of subjects on equicaloric diets

	SUBJECTS R. B. AND G. K.							SUBJECTS F. K. AND G. S.					
	Days on diet	Period no.	Heat production		Indirect as % of direct	Heat as water vapor		Days on diet	Period no.	Heat production		Indirect as % of direct	Heat as water vapor
			Indirect	Direct						Indirect	Direct		
			Cal.	*Cal.*	%	%				*Cal.*	*Cal.*	%	%
Low-protein diet	3	1		4857		31.7	High-protein diet	3	2		4983		32.0
	17	3	4663	4902	95.1	30.8		17	4	4955	4982	99.5	30.3
	38	5	4508	4796	94.0	29.5		38	6				
	52	7	4542	4842	93.8	29.6		52	8	4795	4850	98.9	31.8
	63							63					
High-protein diet	3	10		4907		32.0	Low-protein diet	3	11		4681		29.3
	17	12	4591	4744	96.8	29.8		17	13	4700	4730	99.4	31.9
	38	14	4591	4840	95.6	34.6		38	15	4538	4630	98.0	30.0

measured at the end of a few days as when measured after several weeks. No evidence was found of any trend involving adaptation of the metabolism to the continued constant dietary intake. These results with humans do not coincide with the finding with small animals referred to earlier in which a statistically significant but small decrease in heat production occurred with increasing dietary protein. However, the finding that no difference in total heat productions was found with one pair of subjects and slightly more than 5% with the other pair seems to permit the conclusion that if there exists any difference in the overall energy utilization of equicaloric diets of widely different protein contents with humans, such difference must be very small and should serve to bring into proper perspective the distortion that has resulted from interpretation of very short periods of metabolism measurements.

There is frequently a tendency to overemphasize the importance of the dynamic effect of diets in setting up caloric requirements. In referring to the misunderstanding and perplexity of many students and teachers regarding specific dynamic effect of food, Hawkins ('52) points out that the subject is often presented in such a way that one might ask, "Why do we not waste away because we eat?" He also calls attention to the fact that, "Practically all our knowledge of specific dynamic action has been derived from studies of animals kept warm in the fasting state, but otherwise under basal conditions. Practically nothing is known about it in human beings under ordinary conditions of activity and everyday living, and under normal food consumption at the usual intervals."

Using 6 human subjects, Glickman and co-workers ('48) made a careful study of the total dynamic effect of two diets, containing 7 and 37% of protein calories, respectively. In conclusion they state, "Comparing these rates with even the lightest types of muscular activity of men, it is evident that the S. D. A. of food is an inconsiderable item in the energy metabolism of active men, and in particular that high-protein meals as compared with low-protein meals exert inappreciable effect in enhancing the thermal load of men working in a hot

environment, or in protecting men against a cold environment.''

It is of some interest to note the percentage of the total heat which was eliminated by the evaporation of water (table 1). According to Rubner ('10), the manner of disposal of the increase in heat due to mechanical work is determined by the composition of the diet. He reported that in the case of pure carbohydrate, 62% of the increased heat was eliminated by the increased evaporation of water and 38% by an increase in radiation and conduction from the skin. Following the ingestion of meat, the entire extra heat production due to work was thrown upon the activity of the sweat glands. Lusk ('28) concluded from this work that, ''a high protein dietary is therefore contraindicated in athletic contests, especially when the weather is hot and humid.'' The work reported here did not involve any appreciable mechanical work but it is apparent (table 1) that the protein content of the diet had no definite effect on the method of heat disposal. The average percentage of the total heat eliminated by the evaporation of water in the case of the low-protein diet was 30.4%; the corresponding value for the high-protein diet was 31.8%.

SUMMARY

Using the respiration calorimeter, the 24-hour metabolism of two pairs of male college students was measured at intervals during a period of 63 days throughout which time the daily intake of energy and protein remained constant. The two diets were equicaloric but differed markedly in protein. At the end of 63 days the assignment of the diets to the two pairs of subjects was reversed and the experiment continued for 38 days with further measurements of the 24-hour metabolism by direct and by indirect calorimetry.

With one pair of subjects, the average daily heat production from the diet high in protein (128.6 gm per subject per day) was was about 5.0% greater than that obtained from the equicaloric diet of low protein content (38.0 gm per subject per day). With the second pair of subjects, no difference in

daily heat productions was found. This finding emphasizes the insignificant dynamic effects of high protein diets in contrast to that of meals composed entirely of protein.

Under the conditions of the experiment, it appears that a given diet is as correctly represented by the heat production measured at the end of a few days as when measured after several weeks. The percentage of the total heat which was eliminated by the evaporation of water was approximately 31% and was inappreciably affected by the composition of the diets.

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EFFECTS OF THE PREVENTION OF COPROPHAGY IN THE RAT

II. VITAMIN B_{12} REQUIREMENT [1]

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The laboratory rat is undoubtedly the most extensively used experimental animal in nutrition research today. In spite of the vast store of information regarding the specific nutrient requirements of this species that has accumulated over the years, the contributions of intestinal synthesis of vitamins and the role of coprophagy in making these products available to the animal remain poorly understood as pointed out in the exceptionally well presented review of this subject by Mickelson ('56). Most animals undoubtedly practice coprophagy to some degree. Estimates that have been made with the rabbit (Eden, '40) and the rat (Barnes et al., '57) indicate that approximately one-half of the excreted feces are eaten by these two species, even though they are maintained on raised wire screens. With very minor exceptions man does not practice coprophagy and in this respect, products of intestinal synthesis may not be available to him as they are to other non-ruminant species.

Information concerning the importance of coprophagy in making available to the rat products of intestinal synthesis is of fundamental interest and in so far as such information may be transferred to another species, is of practical significance in understanding the nutrient requirements of man.

[1] This research has been supported in part by a grant from the National Science Foundation.

Vitamin B_{12} is known to be synthesized in the intestine of the rat (Johansson et al., '53) as well as in other animals (Kulwich et al., '53). However, this vitamin has a highly specialized absorption mechanism and there is good reason to postulate that it might not be absorbed from that region of the intestine in which it is synthesized. Because of these special characteristics, Vitamin B_{12} was chosen for study in the rat in which coprophagy was prevented.

EXPERIMENTAL

Weanling rats [2] were used in all studies. Coprophagy prevention was accomplished by the use of the feces collector cup that has been described previously (Barnes et al., '57). The rats were maintained in individual cages with raised wire screen bottoms and were given food and water ad libitum. Diets that were used in these studies are described in table 1. The main deviations from a purified diet that has been found in this laboratory to give a very rapid growth rate, were the removal of vitamin B_{12} [3] and the change in the fat content with compensatory changes in carbohydrate. Body weight measurements were made at weekly intervals. At the termination of a given group, the rats were anesthetized with ether and blood was drawn by heart puncture. Serum cholesterol was determined by the Sperry and Webb ('50) modification of the Schoenheimer-Sperry method.

RESULTS

Rate of growth. Three separate experiments in which weanling, male rats have been fed diets with and without vitamin B_{12} provide information on growth rate. In experiment 1 (fig. 1) 5 groups of 10 rats each were fed either the control diet or the same diet minus vitamin B_{12}. Two groups (one with and one without vitamin B_{12}) were handled in the conventional manner while two similar groups were prevented from eating their feces by use of the plastic feces collectors.

[2] Holtzman, Madison, Wisconsin.

[3] Crystalline cobalamine, Merck.

A 5th group received the vitamin B_{12}-deficient diet and was maintained with a feces collection cup, but the fecal output for each rat was placed in a separate feeder jar and returned to that rat's cage. Thus feces were available for consumption even though the feces collection cups were maintained on the rats throughout the experiment. The growth curves for 14 weeks that are given in figure 1 show (1) that dietary vitamin B_{12} was not required for optimal growth in those rats with access to their feces; (2) the presence of the fecal collection

TABLE 1

Diet composition

MAJOR COMPONENTS			
INGREDIENT	DIET DESCRIPTION		
	Control	Low fat	High fat
	%	%	%
Casein [1]	25.0	25.0	25.0
Cerelose	53.0	68.0	38.0
Hydrogenated fat [2]	15.0	0.0	30.0
Salts [3]	4.0	4.0	4.0
Choline dihydrogen citrate	0.3	0.3	0.3
B vitamins (in sucrose) [4]	2.0	2.0	2.0
Fat soluble vitamins (in corn oil) [5]	1.0	1.0	1.0

VITAMIN COMPONENTS			
B vitamins in 2.0 gm sucrose		Fat soluble vitamins in 1.0 gm corn oil	
	mg		*mg*
Thiamine·HCl	0.40	Vitamin A acetate	0.31
Riboflavin	0.80	Vitamin D (calciferol)	0.0045
Pyridoxine·HCl	0.40	alpha Tocopherol	5.00
Ca Pantothenate	4.00		
Niacin	4.00		
Inositol	20.00		
Biotin	0.02		
Folic acid	0.20		
Vitamin B_{12}	1.00		

[1] Vitamin Test, General Biochemicals, Inc., Chagrin Falls, Ohio.

[2] Primex.

[3] Hubbell, R. B., L. B. Mendel and A. J. Wakeman, J. Nutrition, *14:* 273, 1937.

[4] In the vitamin B_{12}-deficient diet this vitamin was excluded. In the S.S.T. diets 2.0 gm of succinylsulfathiazole plus 3.0 gm cellu-flour was added to each 100 gm diet.

[5] Mazola.

cup inhibited growth in the typical manner as described in detail by Barnes and Fiala ('58); (3) the absence of vitamin B_{12} from the diet of the rats in which coprophagy was prevented resulted in a growth depression and (4) feeding feces to the vitamin B_{12}-deficient rats with fecal collection cups

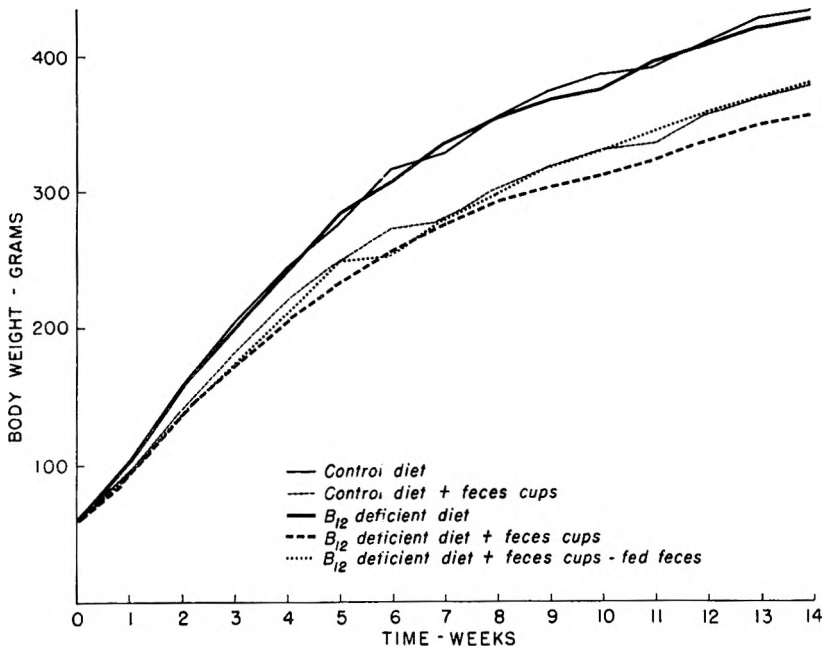

Fig. 1 The effect of a vitamin B_{12}-deficient diet upon the growth of rats that either had access to their feces or were prevented from practicing coprophagy.

returned their growth rate to the control level, but there was a delay of about 4 weeks before this effect became evident.

Since some depression of growth rate appeared quite early in the vitamin B_{12}-deficient rats in which coprophagy was prevented it was surprising that a more serious picture of deficiency did not appear as time went on. The minimal growth depression may have been due to partial access to feces or to a small absorption of newly synthesized vitamin B_{12} from the intestine.

A second experiment was set up in an attempt to produce a more serious deficiency. Bosshardt et al. ('50) and McCollum and Chow ('50) had shown that the requirement for vitamin B_{12} was apparently increased when a low-fat diet was fed. Therefore, for comparative purposes both a low-fat and a high-fat diet were used.

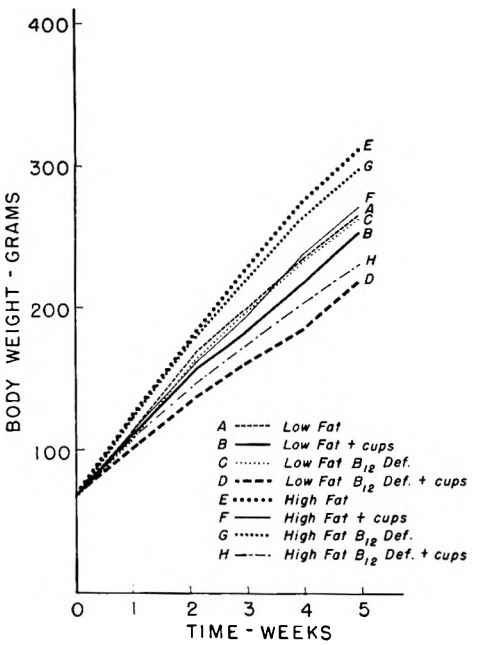

Fig. 2 The effect of vitamin B_{12}-deficient diets either high or low in fat content upon the growth of rats and the influence of coprophagy.

Growth curves of all groups with necessary controls are shown in figure 2. Again, it is seen that lack of vitamin B_{12} in the diet of the conventionally treated rats had no appreciable effect upon growth rate. As would be expected, the high-fat groups grew much more rapidly than the low-fat groups. The important control groups are F and B; the rats with feces collectors receiving the diet high or low in fat respectively. Comparing these two growth curves with H and D respectively (the vitamin B_{12}-deficient groups) an ap-

preciable growth depression is seen. This is evidence that the rat obtains enough vitamin B_{12} from his feces to prevent decreased growth even though none of the vitamin is in the diet. The curves do not show a difference in vitamin B_{12} requirements in the high-fat compared to the low-fat groups. However the rats receiving the low-fat diets developed a behavior pattern that suggests a more marked deficiency of vitamin B_{12} than is apparent from the growth curves. These rats continuously gnawed at their feces collection cups. They would bite through the rubber sleeve and get at the exposed edge of the plastic cup. The net result was that each night most of these rats had succeeded in dislodging their cups and, of course, for a short period of time had access to feces. Close inspection of the curves in figure 2 shows a slight upward inflection in the growth curve for the vitamin B_{12}-deficient, low-fat, coprophagy-prevented rats between the 4th and 5th weeks. This is suggestive that in this group, at least, the extent of vitamin B_{12} deficiency may have been limited by the degree to which coprophagy had been prevented.

A third experiment was then set up in an attempt to provide more careful attention to the construction and attachment of the feces collectors in the hope that better control of coprophagy prevention could be achieved. In addition, succinylsulfathiazole was included in two groups to assess again the influence of this intestinal sulfonamide on the development of vitamin B_{12} deficiency. Since interest had narrowed down to rats in which coprophagy was prevented no normal control groups were included. This proved to be a mistake for the growth curves shown in figure 3 are lower than has been observed previously with comparable diet groups. It would have helped in understanding this general depression of growth rates if normal control groups without feces collector cups had been included. The two groups receiving succinylsulfathiazole developed a diarrhea that lasted for about 10 days. This has been observed before and has been described in a previous communication (Barnes and Fiala, '58). The group receiving vitamin B_{12} slowly caught up with its counter-

part group that had succinylsulfathiazole in the diet, so that growth rates for these two can be considered equal. Marked lowering of growth rate was observed in the two groups not receiving vitamin B_{12} in the diet. Succinylsulfathiazole apparently enhanced the vitamin B_{12} deficiency under these conditions. At the end of 5 weeks 6 of the 10 rats in each group were bled for serum cholesterol determination and the remaining 4 were given 0.1 mg of crystalline vitamin B_{12} by

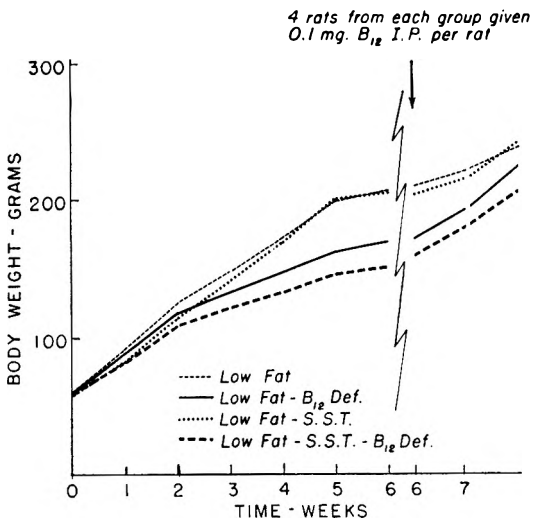

Fig. 3 The effect of vitamin B_{12}-deficient diets in combination with succinylsulfathiazole upon the growth of rats and the influence of coprophagy.

intraperitoneal injection. As was expected, the two vitamin B_{12}-deficient groups responded immediately by a sharp increase in growth rate while the two groups that had been receiving vitamin B_{12} in the diet were not affected.

Hematologic response. Red cell and hemoglobin were measured only in experiment 1 at the termination of the study. Since a mild deficiency of vitamin B_{12} had been imposed for 14 weeks, chronic effects upon the blood picture should have become evident. As seen in table 2 no obvious deviations from the controls were observed.

Blood cholesterol changes due to vitamin B_{12} deficiency were not outstanding (table 3). In experiment 2, two instances of lower cholesterol values and in experiment 3 one instance of decreased serum cholesterol were noted. In common with

TABLE 2

Hemoglobin and erythrocyte values at the termination of experiment 1

EXPERIMENTAL GROUP	HEMOGLOBIN	RED CELL COUNT
	gm per 100 ml	*Millions/mm* ^{3}
Control	15.3	9.7
Control plus feces cups	13.2	7.7
Vitamin B_{12}-deficient	14.9	9.0
Vitamin B_{12}-deficient plus feces cups	13.9	8.6
Vitamin B_{12}-deficient plus feces cups (fed feces)	14.0	8.3

TABLE 3

Blood serum cholesterol (total)

TREATMENT	SERUM CHOLESTEROL
	mg/100 ml
Experiment 1	
Control	69 ± 4.13 [1]
Vitamin B_{12}-deficient	68 ± 3.64
Control plus feces cups	60 ± 1.85
Vitamin B_{12}-deficient plus feces cups	62 ± 3.17
Vitamin B_{12}-deficient plus feces cups (fed feces)	72 ± 3.28
Experiment 2	
Low-fat control	88 ± 3.14
Low-fat vitamin B_{12}-deficient	94 ± 2.76
Low-fat control plus feces cups	91 ± 3.17
Low-fat vitamin B_{12}-deficient plus feces cups	81 ± 4.55
High-fat control	93 ± 1.98
High-fat vitamin B_{12}-deficient	83 ± 1.89
High-fat control plus feces cups	87 ± 3.07
High-fat vitamin B_{12}-deficient plus feces cups	80 ± 2.83
Experiment 3	
Low-fat control plus feces cups	62 ± 2.21
Low-fat vitamin B_{12}-deficient plus feces cups	67 ± 2.13
Low-fat, SST [2] plus feces cups	74 ± 1.24
Low-fat, SST, vitamin B_{12}-deficient plus feces cup	67 ± 1.97

[1] Standard error of the mean.

[2] Succinylsulfathiazole.

many other reports in the literature there were large variations as reflected by the magnitude of the standard errors. However, it seems clear that a rise in blood cholesterol was not obtained as has been reported by Hsu and Chow ('57).

DISCUSSION

Although it has been well established that a variety of animal species require vitamin B_{12}, mammalian experimental animals such as the rat and mouse must be subjected to rather rigorous procedures in order to demonstrate a need for the vitamin. For example, in order to demonstrate a requirement for vitamin B_{12} (animal protein factor) in the mouse, it was necessary to feed a vitamin B_{12}-free diet to pregnant females and then measure the growth rate of the young. More striking deficiency of the vitamin in the young mice was observed if they represented the third or 4th litter from the females that had been fed continuously on a vitamin B_{12}-deficient diet (Bosshardt et al., '49). Prior depletion of adult female rats has been found necessary in order to demonstrate a reduced growth rate in the offspring on a vitamin B_{12}-deficient diet (Sherman et al., '55). In order to avoid the rigorous and time consuming depletion of females during gestation and lactation, the development of thyrotoxicosis by feeding desiccated thyroid was found to aid in the production of a vitamin B_{12} deficiency in animals taken from stock colonies as demonstrated in mice by Bosshardt et al. ('49) (animal protein factor) and in the rat by several groups (Ershoff, '47; Register et al., '49; Frost et al., '49).

The necessity of employing such extreme procedures in order to demonstrate a vitamin B_{12} deficiency in the mouse and rat has led to the general concept that mammals including the mouse, rat, and probably man, have the capacity to store vitamin B_{12} in quantities large enough to prevent the development of deficiency symptoms for long periods of time even though exposed to vitamin B_{12}-deficient regimens. The present studies tend to refute this belief inasmuch as the weanling rat, taken directly from a stock colony without previous attempts

at depletion, showed a depression in growth rate indicative of a vitamin B_{12} deficiency that was evident within one or two weeks after starting on the experimental regimen. This certainly suggests that the amount of vitamin B_{12} that is stored in the weanling rat that is taken from a normal stock colony is not sufficient to last for any length of time when no vitamin B_{12} is available from exogenous sources. The important exogenous source that normally provides enough vitamin B_{12} to prevent a deficiency even though the diet is devoid of the vitamin is freshly excreted feces. If these deductions are correct one must find an answer to the question of why the weanling rat that is born of a mother that has been given a vitamin B_{12}-deficient diet during gestation and lactation shows immediate growth depression that is due to the lack of vitamin B_{12}. This rat has access to its feces and therefore, one would expect coprophagy to provide sufficient vitamin B_{12} to prevent deficiency symptoms. One explanation that appears to fit all of the facts is that the prior depletion of the mother deprives the young of nutrients that are essential for the intestinal synthesis of vitamin B_{12}. If this is true, moderate stores of the vitamin in the young animal are not sufficient to take care of tissue needs during rapid growth as long as coprophagy is prevented. Such a deduction must be subjected to experimental verification, but this consideration fits well the various reports in the literature that indicate a beneficial effect of vitamin B_{12} in the growing child who for various reasons is suspected of receiving an inadequate diet (Wetzel et al., '52; Crump and Tully, '55; Jolliffe, '55). There is evidence that in man the vitamin B_{12} that is produced by intestinal synthesis may not be available to the tissues (Hausmann et al., '53). The present observations suggest that this probably is the case in the rat in which coprophagy is prevented. However, since the addition of succinylsulfathiazole to the diet of such rats had an additive effect in enhancing depression of growth there is a possibility that some vitamin B_{12} arising in the large intestine is absorbed directly. Further work will be necessary to check this point. On the other hand

the major deduction to be made from these studies is that normal body stores of vitamin B_{12} may be rapidly depleted during active growth.

The serum cholesterol values that have been obtained in this study refute the claim of Hsu and Chow ('57) that hypercholesterolemia develops in vitamin B_{12} deficiency. If any change was obtained in the present investigation, a lowering of cholesterol was associated with vitamin B_{12} deficiency. This is more in line with the reports of Muller ('30) and of Williams et al. ('37) that hypocholesterolemia is found in human pernicious anemia patients in relapse.

SUMMARY

Stock rats of weanling age, without prior depletion, when placed on a highly purified diet that is deficient in vitamin B_{12} do not show any evidence of deficiency during the rapid growth period. On the other hand rats treated in the same manner, but prevented from eating their feces show a growth depression due to the lack of vitamin B_{12}. This growth depression is evident within the first week or two after being transferred to the deficient diet. The interpretation of this observation in terms of mammalian requirements for vitamin B_{12} during the period of rapid growth has been discussed.

The reported hypercholesterolemia in the vitamin B_{12}-deficient rats has not been confirmed under the conditions of deficiency induction that have been utilized in the present study.

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CAROTENE BALANCES ON BOYS IN RUANDA WHERE VITAMIN A DEFICIENCY IS PREVALENT

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In the course of a survey of avitaminosis A undertaken in Ruanda-Urundi (Roels, Debeir and Trout, '58), a high incidence of this deficiency was found among the population and the serum carotene levels of the Africans in the area were found to be very low.

As it is known that the inhabitants of this region eat very little food of animal origin, they must find their dietary source of vitamin A in the vegetable carotenoids they consume. For this reason, we thought it advisable to undertake carotene balance studies on a group of boys from the area, some of whom presented physical signs of vitamin A deficiency. Boys were chosen because the highest incidence of avitaminosis A was found in this group of the population.

It was found that the addition of a small quantity of fat to the diet greatly improves the absorption of vegetable carotenoids.

GENERAL PROCEDURE

Subjects and diets. Twenty-two boys, between 9 and 16 years of age, chosen from three schools in the region of Astrida (Ruanda) were housed in a ward of the hospital at Astrida for 41 days. Seventeen of them presented clear physi-

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cal signs of vitamin A deficiency (Bitôt spots) and 5 of them were chosen because they appeared normal from that point of view. Their activities were kept as near as possible to those in their normal life: a schoolroom was equipped at the hospital where their usual classes were given, games and walks were organized, etc.

The boys were kept under constant supervision day and night to avoid extraneous additions to their diet. In the mornings, they ate two slices of white bread with tea. At noon and at 6:00 P.M., they ate ad libitum from two of the following dishes: boiled potatoes, beans, white fleshed plantains, cassava flour, rice and sweet potatoes (carotene-free variety), and raw bananas. The only seasoning used was salt. They drank water. Once a week, they received, in addition, boiled lean beef and boiled peas. Apart from the bread, tea, rice, beef and peas which were "extras," these dishes make up their usual diet. Occasionally at home boiled greens and home-brewed beer would have been included in the diet. They certainly would not have had the regular meal times in their families nor the quantities of food they managed to consume in the hospital.

The boys were weighed and measured at the beginning, middle and end of the experiment, but there were no unusual changes. Stool specimens were examined for intestinal parasites with the following results: nos. 11 and 12, negative; no. 18, *Taenia;* nos. 2, 5, 7, 10, 17, 21, 22, *Trichocephales,* and nos. 1, 2, 3, 4, 6, 8, 9, 10, 13, 14, 15, 16, 19, 20 and 22, *Ascaris.* Treatment for these parasites was not given until after the end of the balance periods.

All stools were collected in individual pans for each boy. After an initial period of 8 days, during which the boys became accustomed to their environment and the techniques of the balance, the experiment proper was started. For this the boys were divided in 5 groups: two of 5 and three of 4 boys each. In each group, one "normal" boy was included.

The boys in group A received a supplement of 100 gm raw grated carrots with the midday meal and the same supplement

with the evening meal; those in group B, 100 gm of grated carrots and 9 gm (10 ml) of olive oil twice a day with the meals; in group C, 14 mg of carotene in 9 gm (10 ml) of olive oil with the meals; those in group D received twice a day 9 gm (10 ml) of olive oil with the meals and those in group E received twice a day a placebo, consisting of some cassava flour in a gelatin capsule.

The carrots contained on an average 9.4 mg of carotene per 100 gm. Serum carotene and vitamin A levels were determined twice before the boys were started on the diet, and afterwards on the 5th, 12th, 19th, 26th and 31st days of the diet. All of the deficient boys were given a prolonged course of vitamin A therapy after the end of the balance studies.

Determination of vitamin A and carotene (a) In serum. Three milliliters of serum were used for the determinations, which were done very soon after the blood was taken and the serum was separated. The utmost care was taken in avoiding light and oxidation during the short period the serum samples were kept. The usual procedure of protein precipitation with ethanol, saponification and petroleum ether extraction was followed. The total carotene was calculated from a transmission reading at 452 mμ in petroleum ether (40 to 60°C). Vitamin A was determined according to the Carr-Price method, reading transmission at 620 mμ and correcting for carotene interference. All solvents used were specially purified in our laboratory to give a transmission reading of 100.0 at the required wave lengths. For calibrating purposes, we used the standard vitamin A solution.[2] For carotene standard, we used all-trans beta-carotene.[3] This product, after drying in a high vacuum pistol, gave an extinction value (1 gram/litre) of 251.8 at 451 mμ in *n*-hexane. It had absorption maxima at 451 and 479 mμ and an absorption minimum at 468 mμ in the same solvent.

[2] Prepared for the first International Collaborative Experiment, organized in 1955 by the Vitamin Commission of the Food Division of the Applied Chemistry section of the International Union of Pure and Applied Chemistry. We are very grateful to this organization for donating this standard.

[3] We are grateful to Hoffman-LaRoche (Switzerland) for this material.

(b) In carrots. A small sample approximately equal to the amount of grated carrots fed to the boys was taken each day. The samples were mixed and stored in the dark in nitrogen at —35°C. When the balances were finished, the carotene content of the carrots was determined in the following manner: about 5 gm of the grated carrots were ground in a mortar with 0.1 gm of $MgCO_3$ and successive small amounts of a mixture of hexane and acetone (60/40 V/V) until all color had left the carrots. The combined extracts were washed 5 times with twice-distilled water and dried overnight in the presence of anhydrous sodium sulphate. The solution was then transferred to a 200 ml volumetric flask and brought up to the mark with hexane (A.O.A.C., '55). Ten-milliliter aliquots of this solution were used for chromatography. For this, the method of Thompson, Ganguly and Kon ('49) was used. The activated alumina was suspended in a solution of 8% (V/V) ethanol in *n*-hexane. A chromatographic column was formed by pouring this suspension into the chromatographic tube (about 1.5 cm diameter) to form a cylinder of about 4 to 5 cm high. The column was rinsed with a small amount of *n*-hexane and the 10-ml aliquot of the carotene solution was pipetted into it. The carotene was adsorbed near the top of the column and eluted with a solution of 2% (V/V) acetone in *n*-hexane. Xanthophylls were retained on the column. The eluate was evaporated on a water bath (not above 60°C), the last few milliliters by blowing nitrogen at room temperature. The residue was taken up in 10 ml of petroleum ether (40–60°C) and the transmission was determined on the Coleman 14 Universal Spectrophotometer at 452 mμ. The solvents and standards used were the same as those described in the method for serum.

(c) In stools. The stools for 24-hour periods were weighed, mixed and a sample equal to 10% of the total was taken and kept in the dark in a well-stoppered glass jar at —35°C. The samples of three or 4 days together were mixed with an equal amount of distilled water (W/V) for 5 minutes in a high-speed blendor. About 5 gm of this mixture was accu-

rately weighed in a glass-stoppered saponification flask. Five milliliters of aqueous 60% KOH (160 gm KOH + 106 gm H_2O) and 10 ml 95% ethanol were added and the mixture was heated for 30 minutes at 60°C in a water bath, under reflux. Fifty milliliters of twice-distilled water were added and the solution was transferred quantitatively to a separating funnel where it was extracted repeatedly with ethyl ether until the extract remained colourless. The combined ether extracts were washed with water until neutral and dried over 5 gm of anhydrous sodium sulphate for 45 minutes. The ether extract was decanted and the ether was evaporated in a water bath at 60°C, the last few milliliters being removed with a stream of nitrogen at room temperature. The residue was taken up in 10 ml of *n*-hexane. One milliliter of this solution was used for chromatography and spectrophotometry as described for the carrots.

The calibration curve for determining the carotene content was established under exactly the same conditions as those for the stools: increasing quantities of beta-carotene standard were added to several amounts of a mixed sample of stools (a) from the boys in group E, and the extraction, chromatography and spectrophotometry procedures described above were applied. Allowance was made for the carotene content of (a) itself by first establishing a graph giving the density of (a) as a function of its weight and correcting the densities of the calibration curve for this basic carotene content. The curve best fitting the experimental points was calculated by the method of least squares, and the expression thus obtained was used for determining the carotene content of the stools. The solvents and standards used were the same as those described in the method for serum.

RESULTS

A. Serum carotene and vitamin A levels. The serum carotene and vitamin A levels are given in table 1. It should be noted that the diet of boy no. 4 in group B was discontinued after 19 days because he developed diarrhoea. There is a

great irregularity in the serum carotene levels of boy no. 13 in group A. His serum carotene levels showed a sharp rise on the 5th and 12th days of the diet. For his meals, he sat next to boys of group B, who were receiving a supplement of carrots + oil. It is possible that he may have shared some of the supplement of his neighbors, despite the strict supervision.

B. Carotene in the stools. The amounts of carotene excreted in the stools are listed in table 2. It was impossible for us to do determinations on the stools of all the boys during the first 10 days of the experiment; the analyses were done on only three boys of each group during that period. Starting with the 11th day, the carotene excreted in the stools was determined for all the boys. The determinations for boy no. 4 were discontinued after the 19th day because of his attack of diarrhoea and one sample of boy no. 9 was lost.

DISCUSSION

It is obvious, both from the amounts of carotene excreted in the stools and from the serum carotene levels, that carotene is much more easily absorbed from the vegetables when some fat is added to a diet ordinarily very low indeed in fat content. The boys in group A, who received a supplement of 18.8 mg of carotene per day in 200 gm of carrots, excreted on an average 18.8 mg of carotene per day in their stools. When we compare this with the 0.9 mg of carotene excreted by the boys in group E, who received the same diet except for the carrots, it is evident that the net absorption of carotene from the carrots by the boys of group A may have been on an average around 0.9 mg per day which is less than 5% of the carotene content of the supplement. This seems to be reflected in a slow rise in the serum carotene level of the boys in group A and a slight rise in their serum vitamin A content when compared to the serum levels of the boys in group E. The serum carotene level (and perhaps the serum vitamin A) on the 5th and 12th days for boy no. 13 in group A presents an anomaly for which we have no explanation. The serum carotene level of the boys in group B, who received a supplement of 200 gm

of carrots and 18 gm of olive oil per day, showed a very rapid increase, reaching, after 31 days of the diet, levels 5, 6, or even 7 times higher than their initial level. They excreted, on an average, 15.1 mg of carotene per day in their stools, while receiving the basic diet plus 18.8 mg of carotene a day. This should be compared with the average of 1.0 mg of carotene excreted per day by the boys in group D who received the basic diet with a supplement of 18 gm of olive oil. It appears that the net absorption of the carotene from the carrots by the boys of group B may have been around 4.7 mg per day (18.8 mg — 15.1 + 1.0 = 4.7 mg), or 25% of the supplement. This is a remarkable increase indeed over the less than 5% absorption of carotene from carrots when no oil was added. The serum vitamin A level of the boys in this group also increased.

The boys in group C who received a daily supplement of 28 mg of carotene in 18 gm of oil showed a still higher increase in serum carotene levels: after 31 days on the diet, they reached levels of up to 10 times their serum carotene content before they began to receive the supplement. They also showed an increase of their serum vitamin A level. The amount of carotene excreted in their stools was, on an average, 16.5 mg per day. Comparing this with the carotene excreted by the boys of group D (supplemented with oil alone), we estimate that the amount of carotene absorbed from the supplement per day was around 12.5 mg (28.0 mg — 16.5 + 1.0 = 12.5 mg), or about 45% of the supplement of carotene given daily.

The control groups D and E, receiving respectively a supplement of 18 gm of olive oil or a placebo, showed little variation in the levels of serum carotene and vitamin A. There was perhaps a little tendency towards a lowering of the carotene level. The carotene excretion per day was about 1.0 mg for both groups. This carotene must have come from the potatoes, bananas, sweet potatoes and peas in the diet. All those foodstuffs contain small amounts of carotene. Van Zeben ('45) reported that the absorption of carotene from raw grated carrots was about 30%, but his subjects received diets quite rich

TABLE 1

Carotene balance studies on boys in Ruanda

(Serum carotene and vitamin A levels)

TIME OF DETERMINATION	GROUP A			GROUP B			GROUP C			GROUP D			GROUP E		
	200 gm carrots			200 gm carrots + 20 ml olive oil			28 mg carotene + 20 ml olive oil			20 ml olive oil			Placebo		
	Boy no.	Carotene µg/100 ml serum	Vitamin A µg/100 ml serum	Boy no.	Carotene µg/100 ml serum	Vitamin A µg/100 ml serum	Boy no.	Carotene µg/100 ml serum	Vitamin A µg/100 ml serum	Boy no.	Carotene µg/100 ml serum	Vitamin A µg/100 ml serum	Boy no.	Carotene µg/100 ml serum	Vitamin A µg/100 ml serum
2nd day after arrival	19 [1]	53.8	52.1	4 [1]	59.3	28.4	2 [1]	82.8	44.6	6 [1]	66.0	55.3	20 [1]	87.7	38.0
Day before diet started		49.3	37.8		45.8	25.3		72.8	38.0		59.3	31.3		73.2	52.1
5th day of diet		53.8	39.6		98.7	28.9		240.5	47.4		54.8	30.3		68.5	47.9
12th day of diet		84.7	40.9		207.3	31.5		401.6	38.0		51.1	28.0		66.6	54.5
19th day of diet		102.7	61.4		278.8	29.1		529.5	50.8		56.6	35.4		71.2	57.8
26th day of diet		118.4	62.8		187.8 [2]	31.8 [2]		577.6	51.0		37.9	38.5		53.8	56.0
31st day of diet		103.7	51.4		150.4 [2]	39.5 [2]		584.6	74.0		55.7	25.3		62.9	58.4
2nd day after arrival	1	68.5	36.8	9	54.8	16.2	11	63.9	27.2	3	37.9	17.2	7	39.6	35.9
Day before diet started		54.8	35.2		53.8	28.0		55.7	34.9		32.8	24.4		34.4	32.3
5th day of diet		54.8	31.5		174.4	39.5		177.8	43.9		24.3	32.0		37.1	36.1
12th day of diet		73.2	37.0		305.2	30.3		305.2	40.9		26.7	27.0		36.2	26.7
19th day of diet		88.7	40.0		390.8	41.6		401.6	35.2		33.6	31.5		60.2	40.3
26th day of diet		69.4	41.4		354.2	38.3		494.8	49.8		23.4	32.0		41.4	44.4
31st day of diet		88.7	43.4		354.2	37.0		508.8	43.6		20.2	32.2		50.2	45.4

2nd day after arrival	21	46.6	25.3	18	72.2	24.4	12	69.0	22.1	15	92.8	41.0	17	48.4	30.5
Day before diet started		29.3	20.0		55.7	25.3		87.7	38.0		78.0	50.5		42.2	35.8
5th day of diet		54.8	42.0		139.1	62.3		189.1	32.0		68.5	48.5		42.2	37.3
12th day of diet		78.8	41.9		227.9	56.3		329.9	29.5		59.3	41.4		41.4	28.6
19th day of diet		92.7	41.6		322.7	82.6		462.9	45.9		68.5	47.5		28.5	27.0
26th day of diet		91.7	49.3		364.1	62.5		557.8	61.9		54.8	38.0		24.3	32.0
31st day of diet		87.7	41.4		336.4	70.8		560.8	52.0		60.2	50.0		23.4	37.0
2nd day after arrival	10	35.3	46.9	8	41.4	33.5	14	28.5	13.1	5	37.1	28.9	22	37.1	19.4
Day before diet started		37.1	40.6		37.1	35.6		37.9	27.5		37.9	41.2		46.6	10.1
5th day of diet		37.1	41.6		92.7	45.6		133.5	45.9		40.5	48.8		42.2	15.0
12th day of diet		45.8	45.6		162.2	33.5		246.5	42.8		49.3	38.0		45.8	21.2
19th day of diet		51.1	53.6		237.7	47.5		319.1	47.4		42.2	48.9		54.8	23.0
26th day of diet		54.8	61.4		272.4	34.9		362.1	51.6		45.8	29.8		45.8	16.7
31st day of diet		64.8	63.6		288.5	53.2		350.4	52.4		51.1	48.0		35.3	10.6
2nd day after arrival	13	58.4	37.4	16	63.9	39.5									
Day before diet started		45.8	46.6		46.6	40.6									
5th day of diet		189.1	32.3		121.5	36.3									
12th day of diet		331.7	26.0		227.9	34.9									
19th day of diet		67.5	51.4		263.0	43.6									
26th day of diet		64.8	57.0		306.9	40.9									
31st day of diet		77.0	57.5		360.1	49.0									

[1] Top section = normal boys (numbers 19, 4, 2, 6 and 20). The others presented physical signs of vitamin A deficiency.

[2] Diet discontinued because of diarrhoea.

TABLE 2

Carotene balance studies on boys in Ruanda
(Carotene excreted in stools)

GROUP	BOY NO.	3, 4, 5TH DAY OF DIET	7, 8, 9, 10TH DAY OF DIET	11, 12, 14, 15TH DAY OF DIET	16, 17, 18, 19TH DAY OF DIET	22, 23, 24, 25TH DAY OF DIET	26, 27, 29, 30TH DAY OF DIET	TOTAL CAROTENE EXCRETED	NO. OF DAYS	AVERAGE EXCRETED PER DAY	AVERAGE EXCRETED PER GROUP PER DAY	CAROTENE CONTENT OF SUPPLEMENT PER DAY
		mg	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*		*mg*	*mg*	*mg*
A	19 [1]	48.7	88.2	50.4	55.0	66.4	88.9	397.6	23	17.3	18.8	18.8
	1	66.5	102.3	60.8	62.8	84.1	76.0	452.4	23	19.7		
	21	69.9	63.1	69.0	85.0	97.2	62.2	446.3	23	19.4		
	10	—	—	68.4	68.1	77.4	53.5	267.4	16	16.7		
	13	—	—	88.9	68.4	92.7	88.4	338.4	16	21.1		
B	4	4.2	61.4	59.8	69.8	— [2]	— [2]	195.1	15	13.0	15.1	18.8 (+ oil)
	9	—	—	62.8	76.3	—	83.5	222.6	12	18.5		
	18	40.4	55.4	52.0	56.0	75.6	82.6	362.0	23	15.7		
	8	44.7	49.4	37.0	59.5	70.8	70.2	331.6	23	14.4		
	16	—	—	48.7	53.5	64.5	54.2	220.9	16	13.8		
C	2	4.4	56.8	94.5	88.8	60.1	85.9	390.5	23	17.0	16.5	28.0 (+ oil)
	11	—	—	7.9	68.5	109.3	96.9	282.6	16	17.7		
	12	8.6	66.3	51.9	49.3	66.3	73.2	315.6	23	13.7		
	14	5.1	71.0	74.6	91.0	62.7	99.4	403.8	23	17.5		
D	6	0.4	2.8	12.7	4.6	1.9	3.7	26.0	23	1.1	1.0	none (+ oil)
	3	1.8	2.7	4.4	2.1	2.6	4.8	18.4	23	0.8		
	15	0.2	1.8	3.9	4.4	2.8	3.0	16.1	23	0.7		
	5	—	—	12.3	4.0	3.4	6.7	26.4	16	1.6		
E	20	0.5	2.1	2.3	3.8	2.4	4.7	15.8	23	0.7	0.9	none
	7	0.7	1.4	14.3	4.4	2.5	6.6	29.9	23	1.3		
	17	—	—	2.0	4.4	2.4	3.4	12.2	16	0.8		
	22	0.2	2.2	3.7	4.1	1.6	3.5	15.3	23	0.7		

[1] The first line in each group gives data for a control boy.

[2] Diet discontinued because of diarrhoea.

in fats (e.g., 600 ml milk + 20 gm butter per day for a subject weighing 7.4 kilos).

Kreula ('50) found that three adult subjects on a low-fat diet excreted about 90% of the carotene content of finely grated carrots, and that the same amount was excreted when 60 gm of carotene-free margarine was added to the diet. It should be noted, however, that for the fat-enriched diet, he worked on two subjects and only on the stools of the third and 4th days after the beginning of the diet. Hume and Krebs ('49) found fairly good carotene absorption from canned carrots, but reported considerable individual variations. Their subjects received a diet low in carotene and vitamin A but otherwise well balanced. James and Hollinger ('54) report the utilization of about 50% of the carotene from sweet potatoes by three adult women and 5 adult men. These subjects also received a diet low in carotene and vitamin A but complete in every other way, including fatty foods such as bacon, ham, pork, cold meats, pork sausage and margarine.

We believe that the high incidence of vitamin A deficiency in Ruanda-Urundi is primarily linked with the very low intake of foods of animal origin in that region (meat is eaten, on an average, about three times a year and very few other products of animal origin are eaten) and secondarily with the very low fat content of the diet in this area. It is known that very little fat as such is consumed by the Africans in this country, and that the fat content of their staple foods (sweet potatoes, cassava, beans) is low. It was found in the present work that the addition of massive doses of a carotene-rich vegetable to the basic diet over a long period did not greatly increase serum carotene and vitamin A levels and that most of the carotene was excreted in the stools. The addition of a small amount of oil to the carrot supplement improved the absorption of carotene from the carrots markedly, and caused an important and rapid elevation of the serum carotene level and a noticeable increase of the serum

vitamin A. Dissolving pure carotene in oil improved still further the absorption of carotene and resulted in greater increases of the serum carotene level and in a rise of the serum vitamin A level. This was already clearly shown in our survey of vitamin A deficiency in this region (Roels, Debeir and Trout, '58); every individual who had eaten palm oil on the day before his blood was sampled, showed an extraordinarily high serum carotene level. Unfortunately, the oil palm can be grown in only a very small part of the territory. It is thought that the introduction of an oil-producing crop may contribute to the relief of vitamin A deficiency in Ruanda-Urundi. Further work on these lines is in progress.

SUMMARY

1. Avitaminosis A is prevalent in Ruanda-Urundi, particularly among the boys. The serum carotene levels of the inhabitants are very low. Therefore, carotene balances were done for 31 days on 22 boys, 17 of whom presented physical signs of vitamin A deficiency. The basic menu they were given was very similar to their ordinary diet.

2. The boys who received a supplement of 200 gm of carrots a day absorbed very little (less than 5%) of the carotene content and showed only a slight increase in serum carotene and vitamin A levels.

3. Those receiving a supplement of 200 gm of carrots and 18 gm of olive oil per day, absorbed 25% of the added carotene and showed high and rapid increases in their serum carotene and a noticeable increase in their blood vitamin A.

4. When carotene in oil was given, 45% of the carotene was absorbed and a spectacular rise of the serum carotene level occurred.

5. A control group received the basic diet with a placebo and another control group was given a supplement of 18 gm of olive oil daily.

6. It is suggested that an addition of fats to the diet may contribute to the relief of vitamin A deficiency in this region.

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METABOLIC EFFECTS OF MOLYBDENUM TOXICITY IN THE RAT [1]

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Following the findings of Dick ('56) that the effects of molybdenum in inducing a conditioned copper deficiency in the ruminant are dependent upon the intake of sulfate, much interest has been aroused in the mechanisms underlying possible metabolic interrelationships of molybdenum and sulfur. The results of Gray and Daniel ('54) indicated that methionine supplements exhibit a protective effect against molybdenum toxicosis in the rat. The later suggestion of Van Reen and Williams ('56) that methionine exerts such an effect after oxidation to inorganic sulfate appears to be substantiated by subsequent experiments. Orally administered sulfate, thiosulfate, cystine or methionine all had a similar protective effect against dietary molybdenum levels as high as 1200 p.p.m. These compounds prevented the growth depression as well as elevation of liver alkaline phosphatase activity associated with molybdenum toxicity in the rat (Van Reen, '54).

It appears possible that sulfate and molybdenum may be related at two different metabolic stages—either during

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absorption and transport through biological membranes, as suggested by Dick ('56), or by the interference of molybdenum with the normal metabolic transformations of sulfur compounds in the body. The studies reported in this paper were designed to investigate the levels of activity of a number of enzyme systems concerned with the metabolism of sulfur-containing compounds during molybdenum toxicosis in the rat. A number of other enzyme systems whose activity *in vitro* or *in vivo* has been found to be related to molybdenum or to copper were also examined.

EXPERIMENTAL

Male albino rats of the Wistar strain were purchased from a commerical source [4] at 5 weeks of age. The basal diet used had the following percentage composition: vitamin-free casein, 20; glucose, 70; salt mixture, 6; corn oil, 4. The following vitamin supplement was included in the diet (quantities in milligrams per kilogram of mixed diet) thiamine, 5; riboflavin, 8; niacin, 40; pyridoxin, 5; Ca pantothenate, 45; biotin, 0.4; vitamin B_{12}, 0.03; folic acid, 2; menadione, 5; inositol, 100; *p*-amino-benzoic acid, 100; α-tocopherol, 1500; vitamin A alcohol, 8; vitamin D concentrate, 750 I.C.U. The salt mixture had the following percentage composition: $CaCO_3$, 27; K_2HPO_4, 17; Na_2HPO_4, 12.4; $Ca_3(PO_4)_2$, 23.2; NaCl, 14.6; $MgCo_3$, 4.35; ferric citrate, 0.65; $MnCl_2$, 0.62; KI, 0.069; $ZnCO_3$, 0.033; cupric acetate, 0.075. This diet was supplemented when required with sodium molybdate and sodium sulfate to provide the desired levels of molybdenum and sulfate. Diet and distilled water were offered ad libitum.

In the first experiment the influence of molybdenum and inorganic sulfate on certain rat liver and kidney enzymes was investigated. Molybdenum was fed at 800 p.p.m. and sulfate at 0.29% of the diet. Experimental treatments were continued for a 5-week period. The second experiment was designed to supplement findings from the first trial regarding the effects

[4] Albino Farms, Red Bank, New Jersey.

of high-molybdenum diets. Molybdenum was fed at 1200 p.p.m. for one week and subsequently at 800 p.p.m. for three weeks. Animals were stunned by a blow on the head, decapitated and exsanguinated. Tissues were removed rapidly and homogenized in ice-cold water in an all-glass homogenizer.

Cytochrome oxidase activity of 1: 10 liver homogenates was determined by the method of Smith ('54) and isocitric dehydrogenase by the method of Hogeboom and Schneider ('50). Liver alkaline phosphatase was determined by the method of Bessey, Lowry and Brock ('46) using disodium *p*-nitrophenyl phosphate as substrate in 0.05 M glycine/NaOH buffer at pH 9.45. In later experiments to study phosphatase activity over a range of pH values, glucose 6-phosphate was used as substrate in 0.1 M cacodylate buffer; liberated inorganic phosphate being determined by the Fiske and Subbarow ('25) method. Cysteine desulfhydrase activity was assayed by anaerobic incubation of liver homogenates in 0.4 M phosphate buffer (pH 7.4) with 20 μM of cysteine hydrochloride in Warburg vessels. After incubation for 120 min., 0.2 ml of approximately 2 N H_2SO_4 was tipped from a side arm and liberated sulfide absorbed in saturated $ZnCl_2$ solution contained in a polyethylene cup inserted in the center well. Sulfide was determined by the methylene blue method of Fogo and Popowsky ('49). Uricase activity was estimated by the spectrophotometric method of Schneider and Hogeboom ('52) and the hypoxanthine content of liver homogenates by the similar method of Plesner and Kalckar ('56) using a commercial preparation of xanthine oxidase. Sulfide oxidase activity was assayed after perfusion of rat livers with approximately 500 ml of 0.2 M sucrose. Liver homogenates were incubated with 10 μM of Na_2S in 0.1 M phosphate buffer for 1 hr. at 37°C, thiosulfate formation being measured iodimetrically by the method of Kurtenacker and Wollack ('27).

The standard medium used for respirometry experiments was as follows: 0.5 M KCl, 0.4 ml; 0.1 M $MgCl_2$, 0.1 ml; 4×10^{-4} M cytochrome c, 0.1 ml; 0.01 M adenosine triphosphate (ATP), 0.3 ml; 0.1 M diphosphopyridine nucleotide (DPN),

0.1 ml; 0.1 M triphosphopyridine nucleotide (TPN), 0.1 ml; 0.1 M phosphate buffer (pH 7.4), 0.3 ml; water to 3 ml. Where appropriate, 0.01 M malate (0.3 ml) was added to the system as "primer." Otherwise all substrates were tipped from Warburg vessel side arms as 0.2 ml of 0.15 M solution.

The aryl sulfatase activity of 1:3 kidney homogenates in water was assayed by the technique described by Dodgson and Spencer ('57) using *p*-nitrophenyl sulfate as substrate in 0.5 M acetate buffer (pH 6.2). Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) were determined by the method

TABLE 1

The effect of dietary molybdenum and sulfate level on rat growth during a 5-week experimental period

DIETARY TREATMENT	FINAL WEIGHT
	gm
Unsupplemented basal diet	264 ± 8.93 (15) [1]
Basal + 800 p.p.m. Mo	187 ± 8.69 (15)
Basal + 800 p.p.m. Mo + 0.29% $SO_4^=$	249 ± 9.25 (14)
Basal + 0.29% $SO_4^=$	274 ± 13.72 (14)

[1] Number of observations indicated within parentheses.

of Schneider ('45). DNA was determined with the diphenylamine reagent and RNA with the orcinol reagent by correcting for the DNA present.

The protein content of all tissue homogenates was determined using the method of Lowry et al. ('51).

RESULTS

As is indicated in table 1 the feeding of 800 p.p.m. of molybdenum in experiment 1 resulted in a 36% depression of growth during a 5-week period. The inclusion of 0.29% of sulfate in the molybdenum-supplemented diet largely prevented this effect on growth.

No differences were noted in the activities of liver isocitric dehydrogenase, cysteine desulfhydrase or cytochrome oxidase on comparing rats receiving normal and high-molybdenum diets (table 2). Liver uricase activity appeared to be slightly

TABLE 2

The effect of dietary supplements of molybdenum and sulfate on rat liver and kidney enzymes and on liver hypoxanthine level (experiment 1)

	DIETARY SUPPLEMENT			
	Nil	800 p.p.m. Mo	800 p.p.m. Mo + 0.29 % $SO_4^=$	+ 0.29 % $SO_4^=$
Liver				
Alkaline phosphatase (μM nitrophenol liberated/mg protein/30 min.)	0.0311 (2) [1]	0.1081 (2)	0.0380 (2)	0.0429 (2)
Sulfide oxidase (μM sulfide oxidized/mg protein/60 min.)	0.059 ± 0.006 [2] (4)	0.026 ± 0.006 [3] (4)	—	—
Hypoxanthine (μg hypoxanthine/mg liver protein)	1.82 ± 0.19 (4)	2.14 ± 0.13 (4)	2.78 ± 0.21 (3)	3.30 ± 0.20 (3)
Cysteine desulfhydrase (μM sulfide liberated/mg protein/120 min.)	0.0436 ± 0.0106 (4)	0.0486 ± 0.0107 (4)	0.0515 (2)	0.0505 (2)
Cytochrome oxidase (Δ OD_{550}/mg protein/min.)	1.78 ± 0.21 (4)	1.71 ± 0.18 (4)	—	—
Isocitric dehydrogenase (Δ OD_{340}/mg protein/min.)	0.160 ± 0.019 (4)	0.159 ± 0.010 (4)	0.168 (2)	0.184 (2)
Kidney				
Aryl sulfatase (μM nitrophenol liberated/mg protein/60 min.)	0.0381 ± 0.0058 (5)	0.0336 ± 0.0071 (4)	0.0294 (2)	0.0341 (2)

[1] Number of observations indicated within parentheses.

[2] Standard error of mean.

[3] Treatment difference significant with $P < 0.01$.

depressed during molybdenum toxicity (exp. 2, table 4) but owing to variability within groups this difference was not statistically significant. There were also no differences in kidney aryl sulfatase activities of rats receiving normal and high-molybdenum diets nor were there differences in the rates of coupled oxidation of succinate and L-cysteine sulfinate, or of oxidation of succinate, malate or β-hydroxybutyrate by liver homogenates (table 3). In the 4 animals examined a consistently lower endogenous Q_{02} was noted in liver homogenates prepared from rats receiving 800 p.p.m. molybdenum than in liver homogenates from unsupplemented control animals.

In experiment 1 a marked depression of sulfide oxidase activity was noted in homogenates of livers from rats suffering from molybdenum toxicosis (table 2). Recent work by Ichihara [5] has indicated that the sulfide oxidase system of rat liver has cofactor requirements for hypoxanthine and for copper. In view of the role of molybdenum in xanthine oxidase, it was considered possible that the feeding of excessive quantities of molybdenum might lead to an increased liver xanthine oxidase activity with a resulting decrease in liver hypoxanthine. No important difference in the level of this purine attributable to molybdenum supplementation was found (table 2), although the results suggest that the liver hypoxanthine level may be increased by prolonged feeding of sulfate supplements. In the second experiment a larger number of liver homogenates was assayed for sulfide oxidase activity (table 4). Previous results were confirmed, with the sulfide oxidase levels of livers from "high molybdenum" animals being depressed to about 56% of normal values (treatment difference statistically significant with $0.001 < P < 0.01$).

The elevation of liver alkaline phosphatase and depression of kidney alkaline phosphatase activity during molybdenum toxicity has already been noted by Van Reen ('54) and Van

[5] Unpublished data.

TABLE 3

The effect of dietary supplements of molybdenum on the oxidation of substrates by rat liver homogenates (experiment 1) [1]

DIETARY SUPPLEMENT	SUBSTRATE				
	Nil endogenous Q_{O_2}	Malate Q_{O_2}	Malate + β-hydroxybutyrate Q_{O_2}	Succinate Q_{O_2}	Succinate + L-cysteine sulfinate Q_{O_2}
Nil	2.485 ± 0.317 [2] (4) [3]	6.837 (2)	9.317 (2)	12.660 (3)	16.632 (2)
800 p.p.m. Mo	1.756 ± 0.232 (4)	7.418 (2)	9.624 (2)	13.683 (3)	16.485 (2)

[1] System as described in text.
[2] Standard error of mean.
[3] Number of observations.

Reen and Williams ('56). Results obtained in this present investigation (tables 2 and 4) add further confirmation to these observations and to the previously described effects of sulfate in preventing changes in alkaline phosphatase activity when this ion is administered simultaneously with molybdate.

In determining alkaline phosphatase/DNA and alkaline phosphatase/RNA ratios of normal and "high molybdenum" rat liver and kidney, it was found that marked differences existed in these ratios between the two dietary treatments (table

TABLE 4

The influence of molybdenum toxicity on liver sulfide oxidase and uricase activity and upon alkaline phosphatase/nucleic acid ratios in liver and kidney (experiment 2)

	DIETARY SUPPLEMENT	
	Nil	800 p.p.m. Mo
Liver		
Sulfide oxidase (μM sulfide oxidized/mg protein/60 min.)	0.059 ± 0.007 [1] (13) [2]	0.033 ± 0.005 [3] (17)
Uricase (μg uric acid oxidized/mg protein/10 min.)	1.72 ± 0.33 (5)	1.37 ± 0.04 (6)
Alkaline phosphatase (μM nitrophenol liberated/mg protein/30 min.)	0.0462 ± 0.0049 (8)	0.1051 ± 0.0130 [4] (8)
Alkaline phosphatase/DNA ratio	1.44 ± 0.17 (5)	3.14 ± 0.42 [3] (5)
Alkaline phosphatase/RNA ratio	0.76 ± 0.12 (5)	1.98 ± 0.14 [4] (5)
Protein/DNA ratio	38.40 ± 6.10 (5)	26.94 ± 1.49 (5)
Kidney		
Alkaline phosphatase (μM nitrophenol liberated/mg protein/30 min.)	0.0485 ± 0.0068 (8)	0.0220 ± 0.0033 [3] (8)
Alkaline phosphatase/DNA ratio	1.11 ± 0.20 (8)	0.40 ± 0.06 (8)
Alkaline phosphatase/RNA ratio	1.49 ± 0.20 (8)	0.59 ± 0.12 [4] (8)
Protein/DNA ratio	22.8 ± 1.4 (8)	18.1 ± 0.8 [5] (8)

[1] Standard error of mean.
[2] Number of observations.
[3] Treatment difference significant with $P < 0.01$.
[4] Treatment difference significant with $P < 0.001$.
[5] Treatment difference significant with $P < 0.02$.

4). The lack of correlation between alkaline phosphatase and RNA is interesting in view of the indications obtained by Binkley [6] that this enzymatic activity in mammalian tissues is attributable in part, if not entirely, to polyribonucleotide-amino acid complexes. The feeding of high levels of molybdenum resulted in a lower protein/DNA ratio in kidney (treatment difference significant with $P < 0.02$) and in liver (difference not statistically significant). If one assumes that the normal constancy of DNA per nucleus (Bovin, Vendrely and Vendrely, '48) has not been disturbed in molybdenum toxicosis, these data suggest that either an impairment of protein synthesis or a more rapid protein catabolism has resulted. The level of RNA per cell has not been affected.

Swanson ('50) has demonstrated that the molybdate ion acts as a potent inhibitor of liver microsome glucose 6-phosphatase *in vitro*. Since it was not considered feasible to attempt comparative studies of microsome preparations in this work, the phosphatase activities of unfractionated liver homogenates from control and "high molybdenum" rats were compared at pH values ranging from 5.5 to 7.5 using glucose 6-phosphate as substrate to determine whether an activity peak at pH 6.5 attributable to a specific glucose 6-phosphatase could be demonstrated. As seen in figure 1 no such activity peak was apparent either in control or high-molybdenum liver homogenates. It is of interest, however, that the livers of rats receiving 800 p.p.m. of molybdenum exhibited a higher phosphatase activity over the entire pH range investigated suggesting that the activity of relatively non-specific acid phosphatases may be elevated in a similar manner to alkaline phosphatase during molybdenum toxicity. Despite this increase in the rate of *in vitro* hydrolysis of glucose 6-phosphate, no marked differences in fasting blood glucose levels were noted during a three-day period of starvation of test animals.

[6] Private communication.

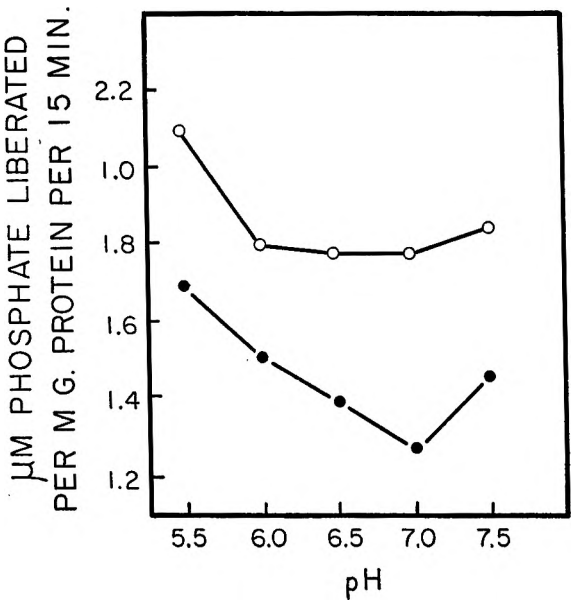

Fig. 1 Glucose 6-phosphate hydrolysis by homogenates of liver from normal (● — ●) and "high Mo" (○ — ○) rats as a function of pH. (For assay system see text.)

DISCUSSION

In these studies the ingestion by the rat of molybdenum in amounts sufficient to retard growth markedly has been found to have no influence on the levels of activity of cysteine desulfhydrase and aryl sulfatase or on the rate of oxidation of cysteine sulfinic acid in tissue homogenates. Whether the adenosine triphosphate (ATP) linked "sulfate activating" system described by De Meio et al. ('53), Bernstein and McGilvery ('52) and Hilz and Lipmann ('55) is influenced by high tissue concentrations of molybdenum is not yet known, but in view of the recent finding of Bandurski et al. ('56) that molybdate activates enzymatic cleavage of ATP *in vitro* by ATP sulfurylase and that pyrophosphate liberation is competitively inhibited by the sulfate ion, studies of the activity of this system during molybdenum toxicity in the rat would be of interest.

The significance of the present finding that liver sulfide oxidase activity is depressed in molybdenum toxicity is not yet clear. Whether this represents the fundamental lesion responsible for growth failure cannot as yet be determined. However, it is of interest that Mills and Murray[7] have recently found that traces of sulfide included in the diet of the black hooded Norwegian rat become markedly toxic if 25 p.p.m. of molybdenum is simultaneously fed. Little is known of factors which govern sulfide liberation in the monogastric animal, but in the ruminant where sulfide forms a normal metabolic product of sulfate reduction in the rumen (Lewis, '54; Anderson, '56) it is possible that a fall in the activity of a system responsible for sulfide detoxification may be of considerable significance. This aspect and its possible implications in influencing trace metal availability are at present under investigation.

No evidence was obtained during present experiments to suggest that the feeding of either molybdate or molybdate plus sulfate supplements had induced a copper deficiency. It is possible that such an effect may perhaps have been obscured by the relatively high levels of copper included in the basal diet (14.2 p.p.m.) but neither in this study nor in the earlier work of Van Reen ('54) was there evidence of the depression of cytochrome oxidase or elevation of isocitric dehydrogenase activities associated with copper deficiency in the rat (Gallagher, Judah and Rees, '56) and no impairment of hemoglobin synthesis was observed.

Thus, in these studies we have failed to detect a dysfunction of sulfur metabolism which could in any way account for the protective effect of sulfate against molybdenum toxicity in the monogastric animal. Systems concerned with the synthesis of adenosine 5′-phophosulfate yet remain to be investigated but if molybdenum has no effect on sulfate activation *in vivo,* it may well be that the observed effects of dietary sulfate are merely the result of an influence of this ion on the permeability of cell membranes to molybdenum. In this re-

[7] To be published.

spect Miller, Price and Engel ('56) and Mills and Murray [8] have found that sulfate ingestion markedly reduces the storage of molybdenum and excessive accumulation of copper in the livers of rats fed high-molybdenum diets.

SUMMARY

The influence of high dietary intakes of molybdenum on certain enzyme systems of rat liver and kidney has been studied with particular reference to systems known to be concerned with the metabolism of sulfur compounds.

The activity of liver sulfide oxidase was found to be markedly depressed in molybdenum toxicity. High molybdenum intakes had apparently no effect on the activity of liver cysteine desulfhydrase, on kidney aryl sulfatase or on the oxidation of L-cysteine sulfinate by liver homogenates. Indications that molybdenum toxicosis may be accompanied by a derangement of protein metabolism were obtained during these studies.

Under the conditions of these experiments in which a relatively high copper intake was maintained, no evidence was obtained to suggest that the limitations to growth imposed by molybdenum toxicosis in the rat could be directly attributed to changes in the activity of enzyme systems normally sensitive to restrictions in the supply of physiologically available copper.

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BIOASSAY OF VITAMIN E BY THE DIALURIC ACID HEMOLYSIS METHOD

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The effect of alloxan and certain of its derivatives, particularly dialuric acid, in causing hemolysis of the red blood cells in vitamin E-deficient rats, was reported by Rose and György ('49, '50a,b and '52) and György and Rose ('49). In rats that received adequate amounts of tocopherol the red cells were completely resistant to this hemolyzing action. The dialuric acid effect can be produced *in vitro,* with washed red cells from vitamin E-deficient animals, but not with cells from animals on an adequate vitamin E intake (György and Rose, '49; and Rose and György, '50a,b). The possibilities of utilizing this phenomenon in the biological assay for vitamin E were pointed out and procedures described (Rose and György, '50a,b and '52; Bliss and György, '51). The response of vitamin E-depleted rats, as measured by the degree to which the red blood cells withstood dialuric acid hemolysis *in vitro,* was shown to be specifically related to the feeding of tocopherols. The relative quantitative effects of the alpha, beta, gamma and delta forms approximated closely the values reported earlier by the rat fertility test (Rose and György, '52). Bliss and György ('51) have pointed to the need for further intensive study to establish reliability of methods based upon this reaction.

We have studied the hemolysis test extensively and have noted a number of precautions that must be observed to obtain good precision in the procedure. This report also summarizes

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studies on the development and the evaluation of precision and specificity, of a new single-dose biossay procedure for vitamin E based on the measurement of hemolysis produced *in vitro* by the action of dialuric acid.

PROCEDURE FOR THE DIALURIC ACID HEMOLYSIS TEST

The hemolysis test used in these studies is essentially that described by Rose and György ('50a,b '52); and Bliss and György ('51), but modified to increase sensitivity and utility by a change in details of procedure, and use of spectrophotometric absorption at 415 mμ. The details of the test follow:

Reagents

Phosphate buffer, pH 7.4. Dissolve 14.2 gm anhydrous Na_2HPO_4 (or an equivalent weight of the hydrated salt) in distilled H_2O, add 20.0 ml of 1.0 N HCl, and add distilled water to make 1,000 ml.

Saline. Dissolve 8.90 gm NaCl in water and add water to make 1,000 ml.

Saline-phosphate solution. Equal parts of saline and phosphate buffer.

Dialuric acid solution. In a dry Erlenmeyer flask grind 10 mg dialuric acid to a fine powder with the end of a stirring rod, then dissolve in 100 ml of saline-phosphate buffer solution.

Procedure

Add 20 to 30 mm^3 of blood (obtained from a fresh cut at the tip of the tail) with a hemoglobin pipette directly to 5 ml of saline-phosphate solution in a 15-ml glass centrifuge tube, mix and centrifuge 10 min. at 2,000 rpm (approx. 500 g). Draw off the supernatant, and resuspend the cells in 4.5 to 5.5 ml of saline-phosphate solution. Add a 1.0-ml aliquot of the suspension, accurately measured, to each of three test tubes. Additional tubes may be used if replication is desired. To each of tubes 1 and 2 add 1 ml of dialuric acid solution, to tube 3 add 1 ml of saline-phosphate solution, and incubate the tubes for 1 hr. at 37°C, then let them stand at room temperature for 1 hr.

It is desirable to set up tube 1 in duplicate. To each of tubes 1 and 3 add 5 ml of saline-phosphate solution, and to tube 2 add 5 ml of water. Mix the contents of each tube *gently* by inverting the tube. Centrifuge the tubes 10 min. at 2,000 rpm and pour off the supernatant liquid into optical glassware suitable for use in the spectrophotometer. Matched pyrex test tubes 12 × 100 mm have been found suitable for use in the Model B Beckman Spectrophotometer. With a tube containing saline-phosphate solution used as a blank, set the meter reading at 100% transmittancy. Read the % transmittancy of tubes 1 and 2 and 3. Convert the transmittancy value to optical densities ($2 - \log_{10} T$). The hemolysis in per cent is calculated by the following formula, using opical density values determined for each tube.

$$\% \text{ hemolysis} = \frac{\text{tube 1} - \text{tube 3}}{\text{tube 2} - \text{tube 3}} \times 100$$

Precautions

1. All glassware must be scrupulously clean and rinsed with alcohol and dried before using, to avoid high and variable blanks.
2. Avoid violent agitation of the red blood cells, especially after the incubation period.
3. The dialuric acid hemolysis will be inhibited if the red blood cell suspension is too dilute. In the above procedure the density of the total hemolysis tube (tube 2) should be not less than 0.7 (about 20% transmittancy), and preferably about 1.0 (10% transmittancy).
4. In manipulative steps of the procedure, through the incubation step, contact of any of the solutions with any material other than glass may lead to difficulty; for example, the plastic-covered magnet of a stirring device, or rubber connections of a pipetting machine may produce complete protection against the hemolytic effect of dialuric acid.

With the above procedure the blood from 100 rats can be handled conveniently in one day by two people working together.

STUDIES OF THE RESPONSE IN RATS TO A SINGLE DOSE OF *dl*-ALPHA TOCOPHEROL

The work of Rose and György and preliminary experiments of our own have demonstrated that protection of the red blood cells of vitamin E-depleted rats against dialuric acid hemolysis is obtained by feeding a single dose of alpha-tocopherol. This protection is apparent within 5 hours after administration of the dose and continues to be effective for a period ranging from one day to two weeks depending upon the size of the dose and the characteristic response of the individual animal. In order to determine whether estimation of degree of protection against hemolysis at any given time after dosing, or measurement of duration of the protective action would best be correlated with the dose of alpha-tocopherol, the following experiment was carried out.

A conventional vitamin E-deficient diet, composed of crude casein 20, sucrose 56, USP XIII salt mixture no. 1 4, dried brewers' yeast 10, olive oil 10, vitamin A 700 units, and vitamin D 70 units added to the olive oil was used. Thirty-nine female rats, of the Osborne-Mendel strain, selected at radom from our stock colony, with an average weight of 146 gm (range 131 to 159) were maintained on this diet in individual cages on raised screen bottoms, for a period of 119 days. Their average weight at the termination of the experiment was 278 gm (range 213 to 373). With the exception of one rat that was discarded in the early part of the study because of a severe respiratory infection, all of the animals were in excellent condition at the end of the experiment. After two weeks on the vitamin E-deficient diet, the red blood cells of all rats showed 100% hemolysis in the dialuric acid test.

At the 5th week the animals were randomly distributed into 5 groups. Each rat received a single dose of *dl*-alpha-tocopherol in 0.5 ml of olive oil, by stomach tube, and additional doses after each three-week interval until each animal had been given a total of 5 doses. Daily after each dose a sample of blood was taken from the tail of each rat and examined by the dialuric acid test. The examination was repeated until the

degree of hemolysis had returned to 100%, which usually occurred within 10 days after the dose was administered. An occasional rat on the highest dose continued for as long as 18 days before complete hemolysis was observed.

The design of the experiment and an example of the results obtained are shown in table 1. Five graded doses, spaced at equal logarithmic intervals between 0.50 and 2.53 mg of *dl*-tocopherol were fed on each of the 5 dosing days. The doses were arranged according to a Latin-square design so that no animal received the same size dose more than once, and each group received a different dose on each dosing day. The average degree and the range of hemolysis on the second day after administration of the dose is shown in the table for each group. An example of the data obtained on each of the 5 days following a single dose of alpha-tocopherol is shown graphically in figure 1 in the form of a dose response curve for each day. It is apparent that there is a good correlation between the average percent hemolysis and the dose. Portions of each of the dose-response curves are suitable as a reference curve in a biossay.

The relationship of the size of the dose to the duration of the response is also evident from this chart. Twenty-four hours after administration of the 0.50-mg dose, the average hemolysis for the group had dropped from 100 to 58%. On the second day after the dose it had increased to 90%. If we consider an average hemolysis of 80% or more to mark the end of the response period, then the response period for the 0.50-mg dose may be regarded as two days. The durations of response to the 0.75 mg and 1.12-mg doses were 3 and 5 days, respectively, and those of the two highest doses were obviously much longer.

Study of the data in table 1 reveals a number of interesting aspects. If the average hemolysis data for each dosing day, i.e., the data in each column, were plotted against the log of the dose a family of overlapping lines would be obtained similar in shape and position to the line designated 2 in figure 1. The same treatment of the hemolysis data for each group, i.e., the data in each row, would result in a similar group of dose

TABLE 1

Design of experiment and average hemolysis data on second day after indicated dose of dl-alpha-tocopherol

DOSE SEQUENCE [1]		1	2	3	4	5
Group I, rats 1–8	Dose, mg	0.50	0.75	1.12	1.69	2.53
	% Hemolysis	75.5	75.2	32.8	19.1	7.4
	Range	(54–90)	(24–100)	(1–73)	(0–56)	(0–34)
Group II, rats 9–16	Dose, mg	0.75	1.12	0.50	2.53	1.69
	% Hemolysis	59.5	45.6	92.1	7.6	26.3
	Range	(30–90)	(3–86)	(73–100)	(0–21)	(0–80)
Group III,[2] rats 17–24	Dose, mg	1.12	2.53	1.69	0.50	0.75
	% Hemolysis	41.7	1.0	17.0	96.3	79.1
	Range	(4–91)	(0–5)	(0–62)	(74–100)	(13–100)
Group IV, rats 25–32	Dose, mg	1.69	0.50	2.53	0.75	1.12
	% Hemolysis	19.0	90.0	19.5	78.1	68.3
	Range	(0–84)	(66–100)	(0–48)	(23–100)	(5–100)
Group V, rats 33–39	Dose, mg	2.53	1.69	0.75	1.12	0.50
	% Hemolysis	9.4	5.8	54.3	48.7	91.6
	Range	(1–19)	(0–12)	(5–96)	(0–92)	(62–100)

[1] Interval between doses is approximately 21 days.

[2] Rat 19 discarded after first dose.

response lines. It is apparent that a bioassay procedure could utilize either the responses of different groups fed graded doses at the same time, or the response of one group of animals fed the different doses in successive experimental periods.

Data were available for the degree of hemolysis on each of the 5 days following each of the 5 different doses. An analysis of variance was carried out after all the hemolysis data had

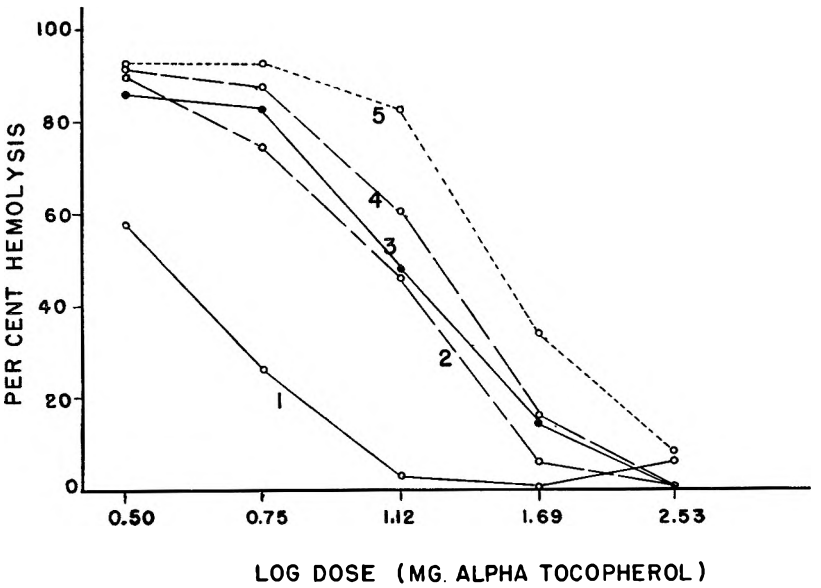

Fig. 1 Average hemolysis in per cent in the dialuric acid test on each of the 5 days following a single dose of alpha-tocopherol. The response curve for each day is indicated by the appropriate number. Each point is the average of 8 rats. The dose in milligrams of alpha-tocopherol is plotted on a log scale.

been subjected to an inverse sine transformation (Snedecor, '40). This step was desirable since the within-group variation was not uniform, the groups at the extremes of the response scale showing less variability than the others. The greater homogeneity of the transformed data increased the validity of the analysis of variance.

The purpose of the statistical treatment was to determine by which of the alternate procedures and on which day after dosage greatest precision was obtained.

It is apparent from table 2 that the variation of the assay is reduced by the use of several animal groups at the same time, rather than by administering different doses to the same group at 3-week intervals. There is no significant effect of animal grouping in the data for the second, third, and 4th days and for the other two days the effect is significant at the 5% level, whereas, the variation resulting from different times of dosing is significant at the 0.1% level on every day but the second.

As shown in table 3, the very large effect of dose level is related to a highly significant linear regression. This table gives the analysis of variance for the second day data in each of the 5 experiments where several groups were fed graded levels of alpha-tocopherol at the same time. The second day data were selected because, as can be seen in figure 1, they show the most useful dose response relationship, and because they showed the smallest variability (table 2). The highest dose was eliminated since it fell outside the linear range. The tabulation shows the breakdown of the dose effect into the regression effect and departure from linear regression. In each experiment the regression effect is highly significant, with no significant departure from linearity.

On the basis of these data an estimate was obtained of the standard error of an assay in which an equal number of doses of both standard and unknown are administered, and upon which the potency of the unknown is assumed to be 100%. With 8 animals per group and with three levels of both standard and unknown the estimated standard error of the assay is 15%, decreasing to 11% if the number of dose groups is increased to 5.

VARIABILITY OF RESPONSE TO ALPHA-TOCOPHEROL AS MEASURED BY THE DIALURIC ACID HEMOLYSIS TEST

The response of vitamin E-depleted rats to single doses of alpha-tocopherol is characterized by a large degree of variability among individual animals. For example, the minimum dose required to protect the blood of an animal against

TABLE 2

Response to alpha-tocopherol as measured by the dialuric acid hemolysis test in rats: analysis of variance of data of five consecutive experiments for each of the first five days after dose

SOURCE OF VARIATION	DEGREES OF FREEDOM	DAY AFTER DOSE									
		1		2		3		4		5	
		M.S.[1]	F	M.S.	F	M.S.	F	M.S.	F	M.S.	F
Animal groups	4	1756	3.4[2]	714	1.5	838	1.8	926	2.4	1057	3.1[2]
Time of dose	4	3260	6.2[2]	899	1.9	6244	13.5[3]	5782	15.0[3]	5076	15.3[3]
Dose level	4	21354	40.8[3]	28381	60.6[3]	18057	39.1[3]	18812	48.9[3]	11870	35.6[3]
Interaction	12	483	1	398	1	720	1.6	994	2.6[4]	882	2.6[4]
Within groups	95	523		468		462		385		333	

[1] M.S. = Mean square.
[2] Significant at the 5% level.
[3] Significant at the 0.1% level.
[4] Significant at the 1% level.

TABLE 3

Response to alpha-tocopherol as measured by the dialuric acid hemolysis test in rats: analysis of variance of second day data for each of five consecutive experiments

SOURCE OF VARIATION	DEGREES OF FREEDOM	EXPERIMENT									
		1		2		3		4		5	
		M.S.[1]	F	M.S.	F	M.S.	F	M.S.	F	M.S.	F
Regression	1	6674	24[2]	18297	62[2]	15288	39[2]	18898	45[2]	11705	21[2]
Deviation from regression	2	58	1	306	1	262	1	4	1	720	1
Within groups	25	275		299		397		419		568	

[1] M.S. = Mean square.
[2] Significant at the 0.1% level.

dialuric acid hemolysis, for a period of one to two days, varied from 0.5 to 2.50 mg. The large degree of variability among individual animals is again illustrated by the range of response shown in table 1 for each group for each dose. In table 4 are shown the results of an experiment in which each of a total of 402 female rats, after approximately two weeks' depletion on a vitamin E- deficient diet and showing 100% hemolysis in

TABLE 4

Variability of response to alpha-tocopherol as measured by the dialuric acid hemolysis test in rats

% HEMOLYSIS	NO. OF RATS	PERCENT OF ANIMALS	
0	52	12.9	
1–10	70	17.4	47.0
11–20	67	16.7	
21–30	37	9.2	
31–40	39	9.7	
41–50	17	4.2	
51–60	24	6.0	39.3
61–70	19	4.7	
71–80	22	5.5	
81–90	19	4.7	
91–99	22	5.5	13.7
100	14	3.5	
Total	402		

[1] All animals received a single dose of 1.2 mg of alpha-tocopherol. Hemolysis was measured on the second day after dose.

the dialuric acid test, was fed a single dose of 1.2 mg of alpha-tocopherol. The degree of hemolysis was determined on the second day following the dose; and it can seen that the observed responses covered the entire range of possibilities. It appeared that in order to have a more uniform animal population for an experimental group, one should include only those animals the responses of which fall within the central part of the response range. For example, it has been of value in this regard to select for assay use only those rats

that show a response of more than 20% and less than 80% hemolysis on the second day after a 1.2 mg dose of alpha-tocopherol.

In an attempt further to decrease the variability among individual rats, the effect of fasting prior to administration of the test dose was investigated. Two groups of female rats were used. Group E consisted of 96 and group F of 80 animals. The animals were housed in group cages, 8 to a cage, on raised screen bottoms. The same size dose of alpha-tocopherol was administered to each rat throughout the experiment. The animals were tested twice with a 3-week interval between tests, with the basal diet fed ad libitum. After a 2-week period the procedure was repeated, but modified to include a 24-hour period of fasting before feeding the alpha-tocopherol dose. Table 5 summarizes the average responses under the two conditions of dosing. Although the two groups differed somewhat in their average response to the test dose, the results were combined for statistical analysis since each group was tested under similar conditions. When the differences in response of the groups at the different times and conditions of feeding were tested against the consistency in response of the individual animals, it was concluded that there is no appreciable trend for the responses to change with time, and that fasting has a significant effect on the response to vitamin E. However, there is no gain in precision when the animals are fasted before testing. The standard deviation in terms of percent hemolysis for the duplicate determinations for each animal under ad libitum feeding is 16.7, and when fasted, 17.6. It should be noted that these studies involve only female rats. It was our impression that females gave more consistent results than males, but the variation of response between sexes has not been evaluated.

BIOLOGICAL ASSAY OF VITAMIN E

The assay data for various tocopherols shown in table 7 were obtained by the following procedure. Female rats weighing between 125 and 175 gm were maintained on the vitamin

TABLE 5

Effect of fasting on the response of rats to administration of a single dose of alpha-tocopherol in the dialuric acid hemolysis test

	DOSE	AVERAGE PERCENT HEMOLYSIS								
		N [1]	Group E		N	Group F		N	Combined Groups E and F	
Non-fasting	First	96	57.2		80	64.3		176	60.4	
				58.2			65.1			61.3
	Second	96	59.2		80	65.8		176	62.2	
Fasting	Third	96	54.0		80	61.0		176	57.2	
				53.8			62.5			57.8
	Fourth	96	53.6		80	64.0		176	58.3	

[1] N = Number of rats in each group.

E-free ration for two weeks. At the end of this depletion period a sample of blood from each rat was checked by the dialuric acid hemolysis test. The animals from our colony have invariably shown 100% hemolysis after two weeks' depletion. A dose of 1.0 mg of *dl*-alpha-tocopherol acetate in 0.5 ml of an olive oil solution freshly prepared was fed directly into the esophagus with a syringe with blunted hypodermic needle. Two days later the dialuric acid hemolysis test was performed again. All animals showing hemolysis of 80% or more and 20% or less were discarded. The remaining animals were housed in group cages, 8 to a cage. Enough animals were processed by this preliminary standardizing dose so that there were available at least 100 rats for assay purposes. After an interval of two to three weeks the animals were ready for an assay. Animals were used repeatedly for 6 to 12 months allowing a 2- to 3-week interval between doses. After 12 to 14 months on the vitamin E-deficient diet, the animals had become obese, and many had developed granulomas of the hind feet and occasionally tail infections. The apparent vitamin E requirement shows a tendency to increase with age. When the rats failed to respond normally to the usual doses the entire lot was replaced with younger animals.

In the assay the standard of reference was a sample of *dl*-alpha-tocopheryl acetate of known purity.[2] Three doses were selected so that the average responses would fall in the central portion of the dose response curve. The doses were graded logarithmically, the highest dose being twice the lowest and the middle dose equal to the lowest multiplied by the square root of 2. The reference standard and each of the materials tested were fed at these levels. In the case of samples of unknown potency, a potency was assigned on the basis of

[2] We are indebted to Hoffmann-La Roche, Merck and Company and Distillation Products Industries for samples of the tocopherols used in these studies and for analytical data regarding their purity. The samples ranged in purity from 96 to 100% except for the alpha-tocopheryl acetate used as the reference standard. It assayed 93% by gold chloride titration and the weighted average of 4 comparisons with International Standard for vitamin E by the hemolysis assay was 94%.

preliminary tests and the three test doses calculated on the basis of the assumed potency. The test materials and the reference standard were prepared by dilution in olive oil so that each dose was contained in a 0.5 ml volume. The doses were assigned to the test animals at random. On the second day following the dose, the blood of each rat was tested by the dialuric acid hemolysis procedure. The results of a typical assay are shown in table 6.

The individual hemolysis values shown here have been used to calculate the potency of the test compound by the standard procedures described by Bliss ('51). In addition to

TABLE 6

Data of an assay of d-alpha-tocopherol against the standard dl-alpha-tocopherol acetate

These data were treated by standard procedures (Bliss, Statistical Methods in Vitamin Research, p. 448 of VITAMIN METHODS, Vol. II, edited by György, 1951), after application of the arcsine transformation (Snedecor, Statistical Methods, 3rd Edition, 1940, p. 382).

COMPOUND FED	STANDARD			TEST COMPOUND		
	dl-alpha-tocopheryl acetate			*d*-alpha-tocopherol		
DOSE IN MG [1]	0.75	1.06	1.50	0.75	1.06	1.50
	96	25	39	90	5	29
	60	33	11	86	13	0
	39	92	8	11	17	3
Percent hemolysis in di-	100	93	25	51	10	9
aluric acid test, for	90	14	13	91	3	7
each rat on 2nd day	65	63	90	2	64	5
after dose	92	76	10	17	85	2
	100	39	23	81	19	1
	100	75	45	81	27	2
	89	35	18	70	9	0
Average				34		
	83.1	54.5	28.2	55.8	25.2	5.8

[1] Dose based upon content of alpha-tocopherol.

Standard deviation (s)	17.9	
Combined slope (b)	— 125.6	
λ (s/b)	0.1425	
Log potency	0.1665	(146.7%)
Standard error of log potency	0.0442	(15.7%)

the relative potency and the standard error of the potency are shown the standard deviation of the effect metameter (s) and the value for the combined slope (b). The ratio of s/b usually called λ, is a convenient index of the precision of an assay which is independent of the units in which doses are measured and of the experimental design. It varies from about 0.50 for a relatively inaccurate assay to 0.03 for an accurate one (Gaddum, '53). In order to facilitate comparison of the

TABLE 7

Relative potency of various sources of vitamin E activity

FORM OF VITAMIN E	NO. OF ASSAYS	RELATIVE POTENCY [1]	95% CONFIDENCE LIMITS
dl-alpha-Tocopheryl acetate	Used as standard in each assay	100	
dl-alpha-Tocopherol	9	101.6 (6.1) [2]	90.6–113.9
d-alpha-Tocopherol	8	132.8 (6.4)	121.1–145.7
d-alpha-Tocopheryl acetate	10	147.4 (8.5)	132.1–164.5
d-alpha-Tocopheryl succinate	9	113.5 (5.7)	103.2–124.8
d-gamma-Tocopherol	2 [3]	21.7 (2.4)	17.5– 26.1
d-gamma-Tocopheryl acetate	5	18.9 (1.2)	16.7– 21.3

[1] Relative potency based on tocopherol content only.

[2] Numbers within parentheses indicate standard error of relative potency.

[3] In a third assay the assumed potency was too low, resulting in an almost horizontal slope. This assay was not calculated, although the estimate of potency by inspection of the results was about 20%.

accuracy obtainable with this test it is noted that the values for λ in the 43 assays reported in table 7 ranged from 0.13 to 0.29 for 40 assays, with the distribution centering about a value of 0.20; the λ values for the three assays that fell out of line were 0.410, 0.435, and 0.448.

In table 7 are summarized the results of 43 assays, wherein the biological potencies of various tocopherols were compared with that of *dl*-alpha-tocopheryl acetate. Each of the compounds was fed on the basis of the tocopherol content. It is noteworthy that the natural *d*-forms are approximately 35

to 50% more potent than the synthetic racemic compounds, that the gamma-tocopherol has about one-fifth the potency of alpha-tocopherol and that the tocopheryl esters are approximately equivalent to the free tocopherols.

DISCUSSION

The continuing interest in vitamin E, in its function in metabolism, and in its possible benefits in human nutrition justifies the effort expended in establishing a biological assay procedure that has ready utility as well as an acceptable degree of precision. There is no need to dwell upon the difficulties incumbent in the classical method based upon failure of reproduction. With the tocopherols, biological potency undoubtedly has been an important variable in the many attempts to learn of some of the value of vitamin E in the prevention and treatment of disease. The facility of the assay method described here will allow easy application to the variety of products that claim vitamin E potency, and may help to bring some order out of the present conflicting reports of benefit.

The suggestions of György and Rose have been modified and extended, and thoroughly tested to provide a basis for the method described. A study of the limitations of the single dose assay provided means of improving precision to an important degree, principally by standardization of the test animals. The use of a standardized lot of animals for a series of assays greatly reduces the cost of the assay.

Although chemical methods for the determination of the tocopherols separately are in use, the difference in the rate of absorption through the intestinal wall gives rise to the problem of biological specificity. Biological potencies for the different tocopherols and their esters have been measured by Joffe and Harris ('43), Harris et al. ('44), Harris and Ludwig ('49a,b) and Rose and György ('52). Results reported here vary somewhat from the previously published values, but are of the same order. The principal difference is in the values for *d*-gamma-tocopherol and its acetate, the values reported here

being appreciably higher than those of Joffe and Harris ('43). Also, we have failed to find any striking difference in biological potency in favor of the ester forms of the tocopherols.

Large single oral doses of methylene blue (20 mg) diphenyl-paraphenylenediamine (DPPD, 10 mg) or of selenium dioxide (0.1 mg) produce no measurable effect in the hemolysis test.

It may be of interest that the hemolysis test is affected by the addition of tocopherol to blood of deficient animals *in vitro*. Under these conditions, however, the biological specificity is lost, and the several tocopherols exert the same quantitative effect. This observation is demonstrative of the selectivity of the intestinal wall with respect to the various isomers and homologues of the vitamin.

The precision of the assay is found to fall well within the range expected of the bioassay methods for vitamins.

SUMMARY

A biossay procedure that utilizes the protective effect of a single dose of vitamin E in rats against the *in vitro* hemolytic action of dialuric acid upon vitamin E-deficient erythrocytes has been described, and its precision, specificity, and dependability studied. A number of precautions that must be observed to obtain good precision in the hemolysis test are set forth. A new single-dose bioassay is described that may be completed in two days and is so designed that the test animals may be used for a number of assay periods. The method will allow easy application to a variety of products that contain vitamin E. Biological potencies determined by this method are reported for several tocopherols and their esters. The results differ from earlier reports in the literature in that the values reported here for *d*-gamma tocopherol and its acetate are higher and that there is no significant difference between the ester and alcohol forms of the various tocopherols. The *d*-alpha-tocopherol, in accordance with earlier work, is about 33% more potent than the racemic form.

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THE EFFECT OF PENICILLIN ON THE INTESTINAL SYNTHESIS OF THIAMINE IN THE RAT

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One of the many mechanisms that have been suggested to explain the effect of antibiotics in improving the growth of rats on B vitamin-deficient rations has been that they increase the microbial synthesis of those vitamins in the intestine. Antibiotics have been reported to alter the intestinal microflora of rats (Guzman-Garcia, Sarles and Baumann, '53) and chicks (Monson et al., '54). Schendel ('54) reported that in two groups of pair-fed rats which were maintained for three weeks on a limiting level of C^{14}-labeled thiamine in the diet, the specific activity of the carcass thiamine was lower in the penicillin supplemented group. He considered this to support the theory of increased synthesis.

In the experiments reported herein, an attempt was made to estimate the effect of penicillin on the amount of thiamine synthesized by the intestinal flora and available to the rat.

EXPERIMENTAL

Weanling male albino rats of the Sprague-Dawley strain were kept individually in wire-bottom cages in a ventilated hood. Food and water were given ad libitum. The experimental diet is described in table 1. For the rats that received the antibiotic, 50 mg of crystalline sodium penicillin G were mixed with each kilogram of the diet. The diets were stored under refrigeration.

Thiamine labeled with C^{14} in the 2 position of the thiazole ring with a specific activity of 6 μc/mg was the only thiamine in the diet. The radiothiamine was administered by pipetting an aqueous solution of the vitamin onto the daily feed. The concentration of each solution was such that 0.5 ml/gm of diet gave the desired level. The radiothiamine was administered in this manner in order to reduce its loss in the diet (Lyman and Elvehjem, '51; Waibel, Bird and Baumann, '54).

Three rats were sacrificed at the beginning of the experiment and the intestinal contents caudad to the ileocecal sphincter were removed. The carcasses were frozen, passed 4 times

TABLE 1

Composition of the basal diet

CONSTITUENTS		CONSTITUENTS	
	%		*mg/kg*
Sucrose	73.0	Water-soluble vitamins:	
"Vitamin free" casein (Labco)	18.0	Folic acid	4.0
Mineral mixture 446	4.0	Biotin	0.6
Corn oil	4.0	Calcium pantothenate	40.0
Choline dry mix (25% choline)	0.4	Vitamin B_{12}	0.05
Vitamin premixes	0.6		*per 100 gm*
	mg/kg	Fat-soluble vitamins:	
Water-soluble vitamins:		Vitamin A	2000 I.U.
Nicotinic acid	100.0	Vitamin D	200 I.U.
Riboflavin	16.0	alpha-Tocopheryl acetate	12 mg
Pyridoxine hydrochloride	6.0	2-Methyl-1,4-naphthoquinone	1 mg

Composition of mineral mixture 446

CONSTITUENTS		CONSTITUENTS	
	gm		*gm*
NaCl	243.198	$CuSO_4 \cdot 5H_2O$	0.4
$K_3C_6H_5O_7 \cdot H_2O$	533.0	$MnSO_4$	2.8
K_2HPO_4	174.0	$K_2Al_2(SO_4)_4 \cdot 24H_2O$	0.2
$CaHPO_4 \cdot 2H_2O$	800.0	KI	0.1
$CaCO_3$	368.0	$CoCl_2 \cdot 6H_2O$	0.2
$MgCO_3$	92.0	$ZnCO_3$	0.1
$FeC_6H_5O_7 \cdot 3H_2O$	36.0	NaF	0.002
		Total	2250.000

through a meat grinder, homogenized in a Waring blendor and assayed for total thiamine by the method of Sarret and Cheldelin ('44). It was found that per gram of initial weight the rats contained an average of 0.85 μg of thiamine.

Two groups of rats were given radiothiamine at the level of 0.5 μg/gm of diet for a period of 21 days and another two groups a level of 5.0 μg/mg for a period of 14 days. One group of rats on each level of radiothiamine was given penicillin while the other group served as control. At the end of the feeding period the rats were killed with ether. The livers were homogenized in a Potter-Elvehjem homogenizer and aliquots were taken for the assay of thiamine (Sarret and Cheldelin, '44) and for the determination of the radioactivity.

In order to measure the radioactivity, aliquots of the liver homogenate were extracted with 0.1 N sulfuric acid, enzyme-digested with clarase and papain and filtered. Aliquots of this filtrate were pipetted into wet combustion tubes and dried in a vacuum desiccator over P_2O_5. The radioactivity was measured by combustion of the samples with Van Slyke combustion liquid prepared by dissolving 30 mg of CrO_3 in 167 ml of H_3PO_4 and 333 ml of fuming H_2SO_4. The liberated $C^{14}O_2$ was quantitatively collected in an ionization chamber which had been evacuated under 10^{-5} mm Hg pressure. Counts were then made on a Vibrating Reed Electrometer.

RESULTS AND DISCUSSIONS

The data from these experiments are given in tables 2 and 3. From table 2, which shows the results from the rats on the sub-optimal thiamine intake, it can be seen that the animals which received penicillin had more thiamine in their livers than did the control rats. This is in agreement with previous reports (Schendel, '54; Guggenheim et al., '53; Scott and Griffith, '57). However, the specific activity of the liver thiamine was lower in the penicillin-supplemented than in the control rats. It can be seen from tables 2 and 3 that penicillin exerted its effects on the level and specific activity of liver thiamine at both levels of

TABLE 2

Effect of penicillin on available microbial thiamine in weanling rats receiving suboptimal amounts of thiamine
(Radiothiamine level 0.5 μg/gm of diet, feeding period 21 days)

RAT NO.	WEIGHTS		THIAMINE ADMINISTERED		THIAMINE IN LIVER		SPECIFIC ACTIVITY OF LIVER THIAMINE	AVAILABLE MICROBIAL THIAMINE
	Initial	Final			Total	Conc.		
	gm	*gm*	*μg*	*μc*	*μg*	*μg/gm*	*μc/mg*	*μg*
1 *	68	124	67.7	0.406	19.5	2.48	1.92	+ 85
2 *	63	111	62.6	0.376	16.6	2.55	2.04	+ 67
3 *	67	115	61.7	0.370	14.5	2.34	1.71	+ 97
Av.	66	117	64.0	0.384	16.9	2.46	1.89	+ 83
4	65	93	52.3	0.314	6.5	1.13	2.38	+ 25
5	66	95	51.4	0.309	9.6	1.77	2.42	+ 21
6	61	90	51.4	0.309	7.8	1.56	2.57	+ 17
Av.	64	93	51.7	0.311	8.0	1.49	2.46	+ 21

* All starred figures represent animals which received 50 mg of penicillin/gm of diet.

TABLE 3

Effect of penicillin on available microbial thiamine in weanling rats receiving adequate amounts of thiamine
(Radiothiamine level 5 μg/gm of diet, feeding period 14 days)

RAT NO.	WEIGHTS		THIAMINE ADMINISTERED		THIAMINE IN LIVER		SPECIFIC ACTIVITY OF LIVER THIAMINE	AVAILABLE MICROBIAL THIAMINE
	Initial	Final			Total	Conc.		
	gm	*gm*	*μg*	*μc*	*μg*	*μg/gm*	*μc/mg*	*μg*
7 *	55	114	679	4.08	61.6	12.0	4.62	+ 157
8 *	53	122	732	4.40	68.0	13.4	5.51	+ 21
9 *	59	106	646	3.88	56.4	13.2	5.03	+ 75
Av.	56	114	686	4.12	62.0	12.9	5.05	+ 84
10	52	98	590	3.54	44.9	9.5	5.97	− 41
11	50	95	542	3.25	46.8	11.7	5.44	+ 14
Av.	51	96	566	3.40	45.8	10.6	5.70	− 14

* All starred figures represent animals which received 50 mg of penicillin/gm of diet.

thiamine intake but the effect was more pronounced in the case of the rats which received the suboptimal level of the vitamin.

From these results, it would seem that penicillin could not have acted by increasing the intestinal absorption of the dietary radiothiamine, since if the antibiotic increased the amount of dietary thiamine (radiothiamine) absorbed, this should result in a higher specific activity of the tissue thiamine in the antibiotic-supplemented rats than in the controls. By the same reasoning, penicillin could not have acted by reducing the utilization or the diversion of the dietary thiamine by the intestinal microbes, since the net effect of this would also be to increase the amount of dietary radiothiamine which goes into the tissues and thus to increase the specific activity of the tissue thiamine in the penicillin-supplemented rats. Therefore, the increased amount of thiamine in the tissues accompanied by the decrease in its specific activity in the rats which received penicillin as compared to rats which did not indicates that more non-labeled thiamine has been introduced into the tissues as a result of feeding the antibiotic.

It is noteworthy that in the case of the rats which received 5 μg of radiothiamine per gram of diet for 14 days (table 3), the specific activity of the liver thiamine was nearly the same as that of the dietary thiamine. This clearly indicates that in this group of rats no thiamine of microbial origin was being absorbed. This is strong evidence that with rats on a sucrose diet containing adequate amounts of all the known vitamins, the microbial synthesis of thiamine was of no physiological significance. This last result is in agreement with earlier reports on germ-free rats (Luckey, Pleasant and Reyniers, '55) and chicks (Luckey et al., '55).

The estimation of the intestinal synthesis of vitamins has been a challenging problem for many years. In these experiments, where only C^{14}-labeled thiamine was supplied in the diet over a prolonged period of time and at physiological levels, it was possible to estimate roughly the amount of the available microbially synthesized thiamine in the rat and the effect of antibiotics thereupon. The isotope dilution calcula-

tion is based on two not unreasonable assumptions. The first assumption was that the labeled thiamine from the diet and the available non-labeled thiamine synthesized by the intestinal microflora were mixed together either in the lumen of the intestines or in the tissues following absorption. The second was that the recently absorbed thiamine rapidly came into equilibrium with tissue thiamine. These two assumptions were supported by the reported presence in the tissues of enzyme systems which are capable of the phosphorylation and dephosphorylation of thiamine and cocarboxylase, respectively (Leuthardt and Nielson, '52; Eich and Cerecedo, '54). The calculation was based on the dilution of the administered radiothiamine with the non-labeled thiamine initially present in the tissues and that supplied by microbial synthesis.

The equation used for the calculation of the amount of the available microbial thiamine and an example of the calculation are as follows: μc radiothiamine administered = S.A. of tissue thiamine × (mg thiamine administered + mg thiamine initially in the rat + mg available microbial thiamine). Using the values from table 2 for rat no. 1: 0.406 μc = 1.92 μc/mg × (0.068 mg + 0.058 mg + mg available microbial thiamine). Thus the available microbial thiamine in this case was 86 μg. Similarly-calculated values for all the animals are recorded in the last columns of tables 2 and 3. These results indicate that in the case of the rats which received the thiamine-limiting diet the available microbial thiamine was estimated at approximately 4 and 1 μg per day with and without penicillin, respectively, while in the rats which were given the adequate diet the values were 6 and 0 μg of thiamine per day in the same order.

SUMMARY

By the use of C^{14}-labeled thiamine it was found that the amount of available thiamine supplied by the intestinal microflora in rats given diets either adequate or limiting in the vitamin was increased in the presence of 50 mg of penicillin per killogram of diet. The amounts of the available microbial thiamine under the different treatments were estimated. In

rats which were fed a sucrose diet adequate in vitamins, no thiamine other than that supplied in the diet could be detected in the liver.

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