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HOWARD BISHOP LEWIS

(1887 – 1954)

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(November 8, 1887 – March 17, 1954)

Howard Bishop Lewis was born on a farm near Southington, Connecticut on the 8th of November, 1887, the son of Frederick A. and Charlotte R. (Parmalee) Lewis. He completed his high school course in Southington in 1903 and since he was not old enough to meet the entrance-age requirement of Yale College, he worked at home on the farm for a year. During this time he taught himself the equivalent of two years of high school Greek. He often remarked that at the end of the year he was sent to college because he had demonstrated clearly that an awkward lefthanded boy could be of little use on the farm. This, without doubt, was a modest statement on his part, since evidence of his brilliant mind was already at hand. At Yale College, the award of the Chamberlain prize for the best entrance examination in Greek was followed by prizes in chemistry, calculus, Latin composition, as well as the philosophical oration at graduation in 1908. Although he financed most of his study at Yale by waiting on tables and tutoring, time was always found for tennis, swimming, hiking and bridge, a game in which he became quite an expert. Howard Lewis, according to a classmate of those undergraduate days, was in great demand as a tutor, not only in the courses he had taken or was taking, but in others that he attended for the sheer pleasure of learning. Some of the extra money obtained in this way provided trips for himself and roommates to New York to enjoy the opera at the Metropolitan. The songs of these operas were remembered and many of his graduate students will recall that suddenly in the quiet of the laboratory we would hear the professor singing one of his favorite arias.

The year following his graduation from Yale with a Bachelor of Arts Degree was spent in teaching at Hampton Institute in

Virginia. He then entered George Washington University for graduate study, majoring in chemistry. At the end of the first semester he withdrew to teach at the Centenary Collegiate Institute at Hackettstown, New Jersey. In the fall of 1910, he registered in the graduate school at Yale University, where he did his thesis work with Lafayette B. Mendel, who was recognized as a leader in the field of physiological chemistry in this country. Here he also came under the influence of Russell H. Chittenden, who in 1884 was named the first professor of physiological chemistry in the United States. As the administrative head of the Sheffield Scientific School, Chittenden was still interested in the problems of nutrition, particularly those of protein requirements. T. B. Osborne, working in the Connecticut Agricultural Experiment Station at New Haven was actively engaged in a study of protein structure and composition. This work must have been an inspiration to the young graduate student who was later to specialize in studies on the metabolism of amino acids and protein. The cooperation of Treat B. Johnson, a prominent organic chemist on the Yale campus is acknowledged in some of the early papers of Lewis. After obtaining his doctoral degree in 1913, he spent two years at the University of Pennsylvania, as an instructor in physiological chemistry. Here he had as colleagues, A. I. Ringer and A. O. Taylor, who were physiological chemists in the true sense of the word.

In 1915 Lewis joined the staff of the chemistry department at the University of Illinois. Although premedical students often took the introductory course in biochemistry at Urbana, the department was not connected with the medical school at Chicago. There was, however, a strong department of organic chemistry at Urbana, whose members were sympathetic to the only physiological chemist on the staff. The close cooperation with the chemistry department for 7 years emphasized to Howard Lewis the value of good training in both physical and organic chemistry. In future years all candidates for the doctoral degree in his department at the University of Michigan were well trained in both of these fields.

In 1922, at the age of 34, he was called to Michigan to be the Chairman of the department of Physiological Chemistry in the Medical School. He held this position until his death in 1954. For 14 years (1933 to 1947) he was also the director of the College of Pharmacy. A strong graduate program was established in the department at Michigan and during the period 1922 to 1954, 84 men and women earned the doctoral degree and many others the masters degree in biological chemistry.

If anyone who knew Howard Lewis, even though casually, was asked to name some of his outstanding characteristics, the reply would probably include some statement concerning his apparently inexhaustible energy. It was a familiar sight at the University of Michigan to see him move across campus at a speed just short of running. To him, time was always considered a precious commodity which was not to be squandered. He enjoyed competitive sports and played them with the full expenditure of energy that he applied to his professional work. This was often disconcerting to many of his younger opponents and is well illustrated by the remark of one of his graduate students, who was resting after 18 holes of golf with the Chief. "Until today," he groaned, "I thought the purpose of this game was to get the lowest score. Now I find that, in addition, after one hits the ball he is supposed to run and catch it before it drops." Those of us who were closely associated with him were continually amazed at the amount of work which he could accomplish. Although everything which he did appeared to be done in "posthaste" fashion, a more careful appraisal revealed that meticulous planning had preceded the actual performance.

His intense interest in everything in the world about him was apparent to everyone. As one of his former students expressed it, "He had the avid and far-reaching curiosity of a child combined with the intelligence of an adult." His constant, enthusiastic interest in people, particularly young biochemists, seemed to be without bounds. He knew and was genuinely interested in the background, capabilities and needs of all the young scientists in biochemistry and nutrition. For many

years, using his hotel room as an office, he singlehandedly ran the Federation Placement Service. He took each individual's problems very seriously to heart and was able to help many young scientists establish themselves. His files reveal that many of these young people continued to correspond with him for many years.

His outstanding characteristic, however, was the ability to stimulate students to join with him in learning as much as possible about the field of biochemistry. Many of his earlier graduate students will recall how he literally burst into the laboratories early in the morning to enquire about the progress that had been made on an experiment during the past 24 hours. It was soon understood by the graduate students that the laboratory work-day started at 8 or earlier regardless of whether we had worked late in the night.

Students were encouraged to assume considerable independence in the development of their thesis problems, but help was always available if it was required. Although the problems under investigation by the candidates for the masters or doctors degrees covered a wide range of topics, the Chief followed the literature very closely for any new developments in the field. Many of us remember that when we arrived at the laboratory in the morning, a note was often found under the door with the query, "Have you seen this paper?" and a reference to the curent literature. Many times it was found that this number of the journal had arrived at the library on the preceding day. After this happened once or twice, the student tried to see if he could not be the first to report on a new article. Through this somewhat playful competition, students were encouraged early in their careers to make a systematic survey of the current literature. I am sure that all of his graduate students will recall his frequent use of a quotation, "Chance favors the mind that is prepared." To be properly prepared meant a thorough knowledge of what had been done and what was now being done in his field of work.

Although in later years more and more of his time was required for administrative work and committee assignments at

the university, state and national levels, Dr. Lewis still held firmly to the belief that his main responsibility at the university was that of a teacher. During a period of 30 years at the University of Michigan, he was rated by the students as one of the most effective teachers of the medical faculty. This was due not only to the excellent organization and presentation of his lectures, but to his extraordinary gift of arousing the interest of students beyond that of classroom requirements. His lectures were not crammed with facts but because of his great enthusiasm, students were stimulated to explore for themselves some of the fascinating aspects of biochemistry. Dr. Vincent du Vigneaud, who took his beginning course in biochemistry with Dr. Lewis at the University of Illinois, on receiving the Nobel Prize in Chemistry in 1955 for his work on biologically important sulfur compounds stated: "Now where did the sulfur trail start? I think it started at the University of Illinois where my first teacher in biochemistry was the late Professor H. B. Lewis, who was extremely enthusiastic about sulfur. It was his enthusiasm that undoubtedly aroused my interest in the biochemistry of sulfur compounds." Many of his former students who have become prominent teachers and investigators would undoubtedly like to pay H. B. Lewis a similar tribute. This ability to arouse the scientific curiosity of his students was indeed one of his most remarkable attributes.

For many years the lectures in the introductory course in biological chemistry given during the summer school session at the University of Michigan were held at 7 A.M., 6 days per week. His popularity as a lecturer was such that there was always a large group of visitors at these lectures, not for one or two, but for the entire series over an 8-week period. Included in this group were internes and residents from the university hospital, members of the clinical faculty of the medical school, nurses and workers in the field of nutrition. Although a course in nutrition was not given in the Department of Biological Chemistry at Michigan, its importance was stressed at all levels of training. At least one-fourth of

the graduate seminars were devoted to papers dealing with nutrition and many of the thesis problems at both the master and doctoral levels were in this field.

His teaching activities were not confined to the campus. For many years, in spite of an already hopelessly overcrowded schedule, he spent a week as a lecturer and consultant at the Army Medical Service Graduate School at Walter Reed Hospital. Following his death, a letter from the Assistant Commandant in charge of the teaching program stated: "His visits never failed to develop a high degree of enthusiasm in the class and at the end of each year, he was invariably rated as one of the outstanding teachers of a group of internationally known visitors. To a very broad background of information, he added to his presentations a sense of personal enthusiasm and interest which I do not believe I have ever seen equalled."

Requests to speak to medical and dental societies and other groups interested in problems of nutrition were frequent and seldom refused. Members of the American Dietetic Association recall with gratitude that he was always willing to meet with them for a discussion of their problems. His talk before this group in Cleveland in 1951 on the subject, "Fifty years of Study of the Role of Protein in Nutrition" gives an excellent summary of the historical background and the newer developments in this field. The encouragement and inspiration that he gave to workers at the "grass roots" level represented one of his most important contributions to the field of nutrition.

The broad interest of H. B. Lewis in nutrition are evident from his formal commitments at the state and national levels. From 1936 until his illness in 1953, he was a member of the Council on Foods and Nutrition of the American Medical Association. In 1941 and 1942 he served on the Council of the American Institute of Nutrition, as Vice-President of the Institute in 1941 and 1942, and as its President in 1943 and 1944. From 1935 to 1945 he was a member of the editorial board of the Journal of Nutrition. From 1945 to 1948, he served in the Division of Medical Sciences of the National

Research Council and from 1947 to 1952 as Chairman of the Michigan Nutrition Council. The latter group was the continuation of the State Nutrition Committee appointed during the war as an emergency measure.

The research papers and review articles written by H. B. Lewis indicate a wide range of interests. His first paper published in the Journal of the American Medical Association in 1912, while still a graduate student at Yale, was entitled, "The Value of Inulin as a Foodstuff." This was a decade before the isolation of insulin and biochemists and physiologists were searching for a carbohydrate that could be utilized by the diabetic individual. Although this was his first introduction to research, very little work was done by Lewis and his students in the field of carbohydrate chemistry or metabolism. In later years a few papers from his laboratory dealt with the availability of inulin and various pentoses in the diet as a source of calories for the white rat.

Apparently the research on inulin did not occupy all of his time as a graduate student since in 1913 two papers were published in the Journal of Biological Chemistry on the metabolism of hydantoin compounds, one of which was thiohydantoin. Did the interest in this compound provide the spark which eventually led to his recognition as an authority in the field of sulfur metabolism? In the paper on thiohydantoin reference is made to the recent identification of the chemical nature of ergothioneine, the sulfur-containing basic compound of ergot. Nearly 35 years later, one of the last of his graduate students investigated the role of diet on the level of this compound in the blood of the rabbit.

While at the University of Pennsylvania, Lewis initiated his work on the synthesis of hippuric acid which was continued at intervals over a period of 25 years yielding 11 publications. The third paper in the series showed that during the period of high hippuric acid excretion, which followed ingestion of sodium benzoate by man, there was a decreased excretion of uric acid. This led to a study of the factors which influenced the excretion of uric acid by man. A paper from the laboratory

at the University of Illinois with M. S. Dunn and E. A. Doisy demonstrated that the ingestion of a diet high in proteins or amino acids (glycine, alanine, glutamic acid, aspartic acid) but low in purines was followed by an increased excretion of uric acid. In accord with the theory of Graham Lusk on the specific dynamic action of amino acids and proteins, the increased output of uric acid was explained as a stimulation of uric acid production rather than a more rapid excretion. In his later years however, Lewis believed the effect was due to a decreased tubular reabsorption of uric acid in the presence of high concentrations of amino acids, although in the light of modern work it was tempting to consider the action of glycine as one of increased synthesis of purines.

Although the important advances in knowledge of the vitamins occurred during his most productive years, only three papers on vitamins (and those dealt with the scorbutic guinea pig) came from the work of Lewis and his students. This was not due to a lack of interest, since progress in this field of research was closely followed and enthusiastically discussed with his colleagues and students. Failure to work in this field might be explained by the fact that the early work on vitamins was of a physiological rather than biochemical nature. By the time the chemical phase of vitamin research had been reached, his interests were fully occupied with other probelms.

From 1920 to 1953 most of the papers by Lewis and his students were concerned with various aspects of the chemistry and metabolism of the proteins and amino acids with special emphasis on the sulfur-containing amino acids. A series of 30 papers on the metabolism of sulfur was published in the Journal of Biological Chemistry, the first in 1916 and the last in 1941. Fourteen additional papers on sulfur metabolism, 5 of which were excellent review articles, appeared during this period in other journals. The first papers in this series dealt with a comparative study of nitrogen and sulfur excretion by dogs, followed by studies on the oxidation of cystine and some of its derivatives by the rabbit. Later studies were concerned with the value of some cystine derivatives in replacing cystine for the growth of the white rat. Some of these latter papers

may need to be reevaluated since it was not recognized before 1937 that methionine and not cystine was the essential sulfur-containing amino acid. Papers on the growth and composition of the hair of the white rat as affected by the level of methionine and cystine of the diet were also included in the series of papers on sulfur metabolism. It was inevitable that his interest in sulfur metabolism would lead to a study of cystinuria. Although his well planned experiments conducted over a period of 10 years did not yield an explanation as to the underlying defect in cystinuria, many basic facts were established which were helpful to later workers in the interpretation of their results. The work of Dent and others who, a decade after Lewis had completed his work, were able by chromatographic techniques to obtain data suggesting a logical explanation of the cause of cystinuria, was enthusiastically received by Lewis. Before his illness, he had undertaken a reinvestigation of some of his earlier subjects with cystinuria, employing the newer analytical techniques.

Although the research work on sulfur metabolism predominated in his laboratory over a period of years, interest in other phases of protein chemistry and metabolism was maintained. A series of 9 papers was published in the Journal of Biological Chemistry under the general title of "Comparative Studies of the Metabolism of Amino Acids." The rate of absorption of amino acids, changes in the non-protein constituents of the blood and glycogen formation after the administration of various amino acids, oxidation of phenylalanine and tyrosine in the animal body and the production of experimental alcaptonuria in the rat were reported in these papers.

From the University of Illinois in 1921, two papers dealing with the composition and properties of deaminized casein were published by Lewis and his first doctoral degree candidate, Max S. Dunn. In 1923 and 1930 additional work on this subject was reported by Lewis and his students. These papers and 4 others which were studies on the amino acid content of hair, the tyrosine content of cocoons, the products of partial hydrolysis of silk fibroin and the amino acids of Bence-Jones

protein represent the strictly chemical studies on protein made in his laboratory. Most of his papers on proteins and amino acids, many of which have not been mentioned in this article, are concerned with metabolic or nutritional studies.

In 1944 Lewis contributed an article to Nutrition Reviews on the subject of natural toxicants and nutrition, a field in which he had become increasingly interested in his later years. Two of the subjects discussed in this review, selenium poisoning and lathyrism were studied in his laboratory over a period of years. The last graduate student in his department to work on the problem of lathyrism had succeeded in concentrating the toxic material from sweet pea meal by forty-fold. These results were released when it was evident that there was no immediate prospect for the continuation of the research. The death of Dr. Lewis came before the publication of the report on the identification of the toxic compound which is responsible for the characteristic bone changes observed in experimental lathyrism. Nevertheless, before his illness, Dr. Lewis had the satisfaction of knowing that there was a renewed interest in this field, due largely to the work of an orthopedic surgeon, Dr. Ignacio Ponseti, who saw a relationship between the bone changes in experimental lathyrism and some disorders in bone metabolism seen in the clinic.

Other papers equally as important as those which are briefly discussed, were published during his 40 years at the Universities of Pennsylvania, Illinois and Michigan. Although none of his papers report epoch making discoveries, they all contain sound, basic material, which served as a starting point for further progress by younger workers, who were acquiring better tools to do research. His skill in presenting his research material was of the highest order. Meticulous care in giving due credit for work previously done and an extremely conservative interpretation of the data were characteristic of his papers.

Some of his best writing is found in his review articles in which his unusual ability of bringing all of the important facts in a field together in a logical fashion is demonstrated. He

demanded the same excellence in writing from his students in the preparation of a thesis or an article to be submitted for publication. I am sure that all of his graduate students are grateful for this training under his guidance, however galling it may have been when our first efforts at writing were returned with comments exceeding the original length of the papers. As a member of the editorial boards of several journals, he was able to help many young authors improve the quality of their writing.

His memberships in professional and learned societies were too numerous to mention in detail. His services to the American Institute of Nutrition have already been mentioned. Elected to the American Society of Biological Chemists in 1914, he served as its Secretary (1929 to 1933), Vice-President (1933 to 1935), President (1935 to 1937) and as a member of the editorial board of the Journal of Biological Chemistry from 1938 until his death. In 1947 in recognition of his high scholastic standing he was appointed to a distinguished professorship at the University of Michigan, designated as the John Jacob Abel Professorship in Biological Chemistry. He was named the Henry Russel lecturer for the year 1948 to 1949. This honor is awarded yearly to a faculty member at the University of Michigan who is judged by a group of his colleagues to have achieved the highest distinction in his chosen field of scholarship. In 1949 he was elected to the National Academy of Sciences. This was a source of great gratification to his colleagues at the University of Michigan and his friends everywhere.

In 1915 he married Mildred Lois Eaton, who with their two daughters, Charlotte and Elisabeth, survive him. Many of the social activities of the family were based on their common love of music. The faculty and graduate students recall many pleasant evenings around their fireside. The Lewis family was also noted for its impromptu picnics to which faculty members and graduate students were often invited. The annual departmental picnic with the Chief as the first chef frying eggs never failed to draw a full attendance. As the umpire of the baseball game, his decisions were not distinguished for their accuracy,

but they did tend to equalize the score. The day usually ended with "H. B." serving as an auctioneer to dispose of any surplus supplies.

Although in his later years his daily schedule necessitated long hours of work, he still found some time for his hobby of philately in which he established himself as an authority. He also took great pride in his garden, where the newest varieties of plants were found. As the pressure of his campus and national commitments became greater he looked forward with great anticipation to his short vacation periods when he and his family could tramp the mountains near their summer home in New Hampshire.

Howard B. Lewis, besides being a distinguished scientist and a scholar was also a warm human being. Those of us who were privileged to know him regarded him highly as an educator and investigator. To his students he was not only a biochemist with high standards and accomplishments, but a man who had a great appreciation of everything that was going on in the world about him. His personal relations with his students were of the highest quality and provided a model which many sought to reproduce in themselves. In the Library of the Department of Biological Chemistry at the University of Michigan is a plaque which reads:

> In appreciation of Howard Bishop Lewis, Professor and Chairman, Department of Biological Chemistry, 1922–1954, Beloved Teacher and Colleague.

It is fitting to close with a line from a resolution read at the executive faculty meeting of the Medical School following his death:

> "He taught the value of ideals and high standards of accomplishment and gave to his pupils many guiding principles which have contributed to their enduring happiness and success in the practice of medicine and allied fields of science."

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THE URINARY EXCRETION OF FIVE ESSENTIAL AMINO ACIDS BY YOUNG WOMEN [1,2]

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(Received for publication July 11, 1958)

The increasing interest in the subject of amino aciduria in disease emphasizes the need for figures on the excretion of amino acids by normal subjects. Values in the literature prior to 1953 for the urinary excretion of amino acids by human subjects have been summarized by Stein ('53) and recently Evered ('56) summarized the range in values reported for normal adults and for those with specific diseases. Stein ('53), Dustin et al. ('55) and Fowler et al. ('57) determined the amino acid excretion of subjects for a 24-hour period on self-chosen diets. In general, the amino acid intakes of the subjects have not been reported. Wharton and Patton ('53) Steele et al. ('47, '50), and Frazier ('54) have reported the urinary excretion of certain amino acids by subjects fed controlled diets which supplied generous amounts of the amino acids from protein foods.

[1] Published with the approval of the Director as paper no. *898* Journal Series Nebraska Agricultural Experiment Station. A portion of the analytical work was done at Oklahoma State University, Stillwater.

[2] Supported in part by a U. S. Department of Agriculture contract sponsored by the Human Nutrition Research Division, Agricultural Research Service.

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Studies of the quantitative requirements of normal young women for threonine, valine, tryptophan, phenylalanine (with and without tyrosine) and leucine which were reported by Leverton et al. ('56 a–e) included the determination of the urinary excretion of these amino acids under experimental conditions which differed markedly from those of other studies from which values have been reported. In these studies of requirement, the amino acids were fed in crystalline form as part of a rigidly controlled semipurified diet, each subject was studied on several different levels of intake of the same amino acid, and was on each level for several days.

PROCEDURE

The general plan of study, the description of the subjects, the levels of amino acid intake, and the nitrogen intakes and balances have been reported previously (Leverton and co-workers, '56 a–e). The semi-purified diet included sugar, corn-starch, butterfat, corn oil, corn syrup, and jelly, plus mineral and vitamin concentrates and small amounts of a few fruits and vegetables. Crystalline amino acids and diammonium citrate were the chief sources of nitrogen. The essential amino acids, except the one being studied, were fed in the same amounts as they occur in 20 gm of egg protein (for the threonine study the amounts as in 15 gm egg protein were used). The amount of each amino acid being studied was reduced stepwise until the subject was in negative balance, then increased until the total nitrogen excretion was less than the intake or within $\pm$ 5% of the intake.

During the study of each amino acid 24-hour urine collections were made and acidified, except that during the tryptophan study the urine was preserved under toluene. Urine composites were made for each subject for each level of intake on which she was studied.

The total L-threonine content of the urine was determined by the method described by Sheffner et al. ('48) and Steele ('49) using the organism *Leuconostoc citrovorum* 8081 on acid hydrolyzed urine. Only the "free" form of L-tryptophan was

determined and that on the untreated urine using *Leuconostoc mesenteroides* P-60 as described by Frankl and Dunn ('47). This same organism was used for determining total L-valine, L-phenylalanine, L-leucine on the acid hydrolyzed urine, and "free" L-tryosine on untreated urine using the methods described by Steele ('49), and Steele et al. ('50). Under rigidly controlled conditions with experienced technicians the methods gave reproducible results.

RESULTS

The mean daily excretions of all subjects on the different levels of intake of the 5 essential amino acids studied, representing a total of 683 subject-days for 35 subjects, are given in table 1. The results are expressed as total milligrams per 24 hours, as milligrams per gram of creatinine, and as milligrams per gram of urinary nitrogen. For the three amino acids, threonine, tryptophan, and leucine for which the most data were collected, the mean daily urinary amino acid excretions in milligrams per 24 hours for the individual subjects on different levels of intake are shown in figures 1, 2, and 3 respectively. In these figures, the excretion values for each subject may be followed, by following her code number, as her intake changed from one level to another. A line joins the mean values for the subjects studied on each intake except between the highest and next highest levels. Here the horizontal scale is foreshortened and this would exaggerate the slope of a line.

Application of Student's "t" test showed a significant difference only between (1) the mean daily urinary excretion of threonine on the daily intake of 214 mg and of 397 mg (significant at the 1% level of probability) and (2) the mean daily excretion of tryptophan on the daily intakes of 157 mg and 307 mg of L-tryptophan (significant at the 5% level).

Expressing the amino acid excretion on the basis of an individual's creatinine or nitrogen excretion did not alter significantly the variablility or the relationship of changes in intake to changes in excretion.

TABLE 1

Intakes and urinary excretions of five essential amino acids

AMINO ACID (L-FORM)	NUMBER OF SUBJECTS	TOTAL SUBJECT-DAYS	INTAKE	MEAN URINARY EXCRETION PER 24 HR.			
				Total	S.D.[1]	Per 1 gm nitrogen excreted	Per 1 gm creatinine excreted
			mg/day	*mg*		*mg*	*mg*
Threonine, total	5	16	765	32.6	15.1	3.66	24.0
	8	31	397	30.5	6.1	5.49 [3]	28.8
	1	3	*305* [2]	19.8	—	2.19	13.0
	10	31	214	21.3	5.5	4.41 [3]	19.7
	8	25	103	25.2	4.8	4.20 [3]	23.6
	8	48	0	23.6	2.5	3.83 [3]	22.9
Valine, total	7	63	*650*	8.7	2.0	1.08	8.1
	7	51	465	8.5	2.0	.98	7.8
	6	29	375	9.2	2.4	1.06	8.4
Tryptophan, free	8	49	307	9.0	1.1	0.96	7.0
	5	38	*157*	4.3	1.1	.43	3.3
	8	73	120	3.2	0.7	.34	2.5
	8	51	82	3.3	0.9	.31	2.6
	3	11	63	2.9	0.5	.28	2.3
Phenylalanine, total,	8	31	*220*	6.0	3.2	0.64	4.6
with 900 mg tyrosine	7	27	120	4.7	1.8	.49	3.7
Leucine, total	7	22	1860	5.1	0.7	0.57	4.2
	8	24	*620*	8.0	2.9	.85	6.3
	8	24	480	7.6	3.4	.83	5.8
	6	18	370	7.9	3.6	.87	7.1
	3	9	270	8.2	3.7	.87	7.5
	2	6	170	3.5	—	.40	3.2
	1	3	95	3.2	—	.33	3.2

[1] Standard deviation.
[2] Italic figures show amount which has been suggested as a minimum requirement for young women (Leverton et al., '56).
[3] The nitrogen intake of these subjects approximated 6 gm and their excretion was correspondingly lower than for the other subjects.

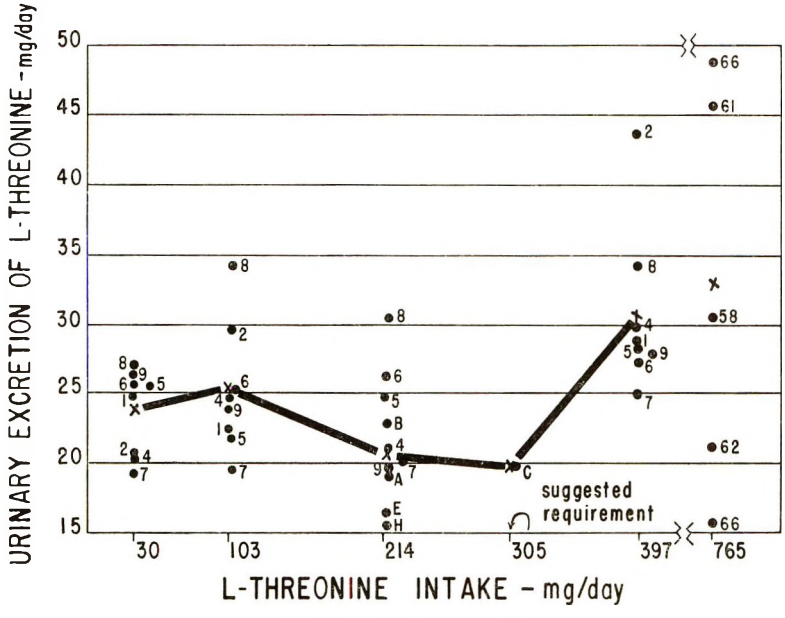

Fig. 1 Urinary excretion of threonine at different levels of intake.

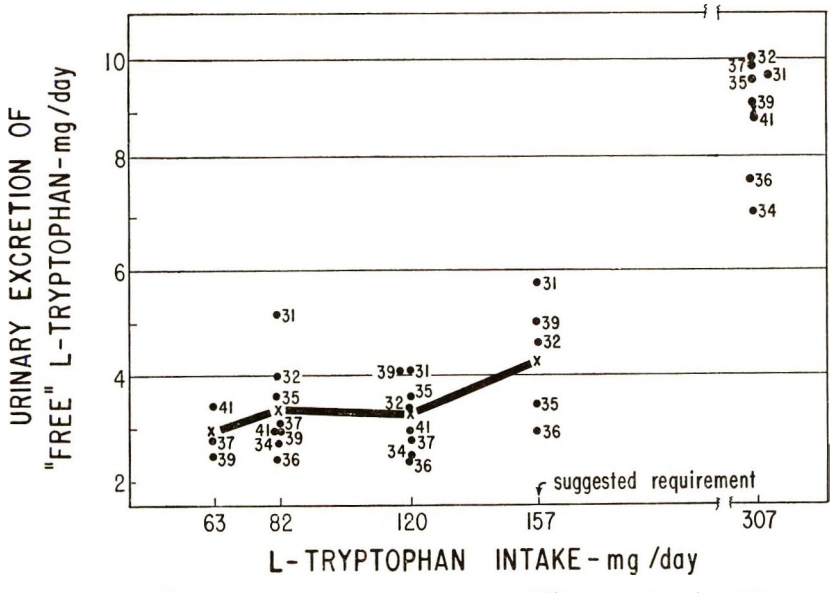

Fig. 2 Urinary excretion of tryptophan at different levels of intake.

During the study of phenylalanine requirement the intake of tyrosine was varied. The figures for the urinary excretion of "free" tyrosine at various levels of intake are given in table 2 and show little variation even with sharp reductions in tyrosine or phenylalanine intakes or both.

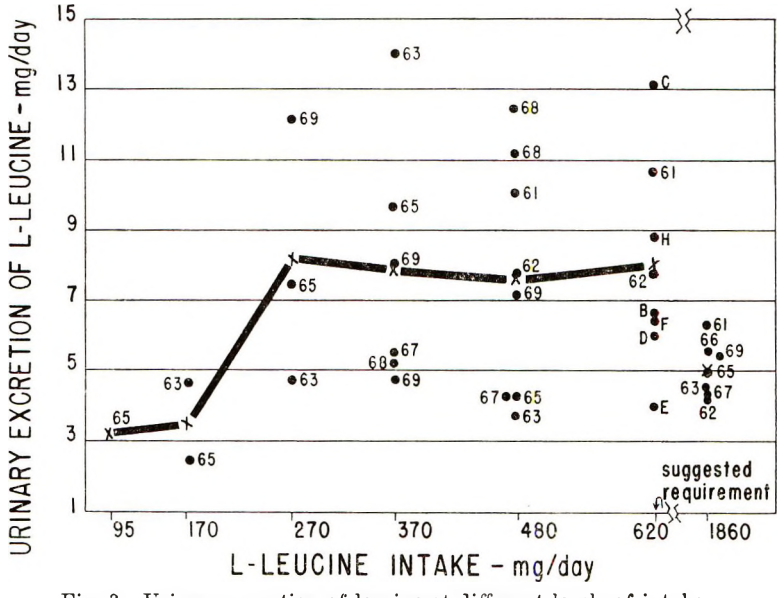

Fig. 3 Urinary excretion of leucine at different levels of intake.

Following these studies of amino acid requirement it was possible to determine the amino acid excretion of an auxilary group of 6 other college girls on the same semi-purified diet and with intakes of all of the essential amino acids which were equivalent to the amounts present in 20 gm of egg protein.[5] The subjects were on this intake for 9 days, and the urinary excretion of the 5 amino acids being reported here was determined for each subject for the last 6 days. The intake and the mean excretion values for the 6 subjects are given in table 3.

The urinary values for threonine and tryptophan are similar to those found for the main group of subjects on similar intakes. The mean excretion of valine on an intake of 1,540 mg

[5] These subjects were being studied by Dr. Hellen Linkswiler at the University of Nebraska in the fall of 1954.

TABLE 2

Urinary excretion of "free" L-tyrosine

TYROSINE INTAKE	PHENYLALANINE INTAKE	NUMBER OF SUBJECTS	URINARY EXCRETION OF FREE TYROSINE	
			Mean	S.D.
mg/day	*mg/day*		*mg/day*	
900	1280	6	7.5	1.8
900	220	8	9.5	1.9
900	120	6	6.5	3.0
450	420	2	5.8	—
450	220	2	4.3	—
200	220	3	6.2	2.2
0	420	2	5.7	—
0	220	5	4.8	1.9

TABLE 3

Intakes and urinary excretions of five essential amino acids — auxiliary group of subjects

AMINO ACID (L-FORM)	INTAKE	EXCRETION		PER 1 GM NITROGEN EXCRETED	PER 1 GM CREATININE EXCRETED
		Mean	S.D.[1]		
	mg/day	*mg/day*		*mg*	*mg*
Threonine, total	890	34.4	3.7	3.90	24.5
Valine, total	1540	10.0	2.6	1.21	7.6
Tryptophan, free	307	6.3	1.9	0.76	4.8
Phenylalanine, total	1280	14.0	3.6	1.68	10.5
Leucine, total	1860	16.5	1.9	1.99	12.5

[1] Standard deviation.

was similar to the excretion of the major group of subjects who had an intake of only 650 mg. The mean excretion of leucine was approximately three times higher for the auxiliary group than for the other group of subjects on the same intake. The auxiliary group had a 6-fold greater phenylalanine intake but only a two-fold greater urinary excretion than the main group. The subjects in the auxiliary group were younger, 17 to 19 years old, than the subjects in the studies of requirement, 19 to 26 years.

DISCUSSION

Within the limits of the amino acid intakes studied, relatively large changes in intake effected only small changes in the

urinary excretion. As the intake of threonine increased from 0 mg to 765 mg, the mean excretion of threonine of the subjects changed only from 23.6 ± 2.5 mg/24 hr. to 32.6 ± 15.1 mg/24 hr. In the case of tryptophan the mean daily excretion of the subjects rose from 2.9 mg on the lowest daily intake of 63 mg to 9.0 mg on the highest intake of 307 mg.

There were individuals who showed an initial excretion above the average of the other individuals of the group, and tended to remain among those who had a higher than average excretion at all levels of intake. There were also subjects who had an initial excretion of an amino acid which was below the average and tended to have low urinary excretions at other levels of intake. Thus it would appear that for some individuals there may be certain patterns of urinary excretion of amino acids, and this observation is in line with the findings of Steele et al. ('50) and Frazier ('54) and Evered ('56). There were a few subjects in the present study, however, who showed no consistency in the relation of excretion to intake of an amino acid.

The values for the excretion of "total" amino acids given in table 1 for the subjects in the present study are similar to the values for "free" amino acids determined by chromatographic methods by Stein ('53), Dustin ('55), and Fowler et al. ('57). The values in the present study are considerably lower than those reported by Wharton and Patton ('53), Steele et al. ('47, '50), and Frazier ('54), but their subjects had intakes which with the exception of tryptophan, were often as much as 10 times the amounts fed in these studies of amino acid requirement. They also reported that the levels of amino acid excretion appeared to be independent of the levels of the intakes.

SUMMARY

Data have been presented for the urinary excretion of threonine, valine, tryptophan, leucine, phenylalanine, and tyrosine by young women who were subjects for studies of the requirements of the 5 essential amino acids mentioned. Several

different levels of each amino acid in crystalline from were fed to each subject.

There was considerable variation in the excretion of all of the amino acids studied among individuals at the same and at different levels of intake. The highest mean excretion occurred on the highest intake for the amino acids threonine, tryptophan, and phenylalanine, and the lowest excretion occurred on the lowest intakes for tryptophan, phenylalanine, and leucine. Differences between mean excretion values on different intakes were seldom significant.

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SOME EFFECTS RELATED TO THE POTASSIUM AND LYSINE INTAKE OF RATS [1]

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Eckel et al. ('54) and Iacobellis et al. ('56) demonstrated that in the rat a loss in muscle potassium is at least partially compensated for by a gain in free lysine, acting as a cation. The partial replacement of potassium with lysine suggests the possibility that the converse might occur. Accordingly, the effect of altering potassium levels in diets containing varying quantities of lysine was studied.

EXPERIMENTAL

In these studies groups of 8 male weanling Charles River CD[3] rats housed in group cages were used. The diets fed were composed primarily of a commercial dry breakfast cereal. The cereal, which contains 3.12% N, mostly from rice and wheat gluten and from small amounts of skim milk, wheat germ and yeast is relatively deficient in lysine as a result of processing

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[3] The CD line of rats was started with Caesarian-delivered rats and is housed in a specially constructed animal house designed to protect the colony against disease transmission.

techniques used in its manufacture. Although microbiological assay of the cereal for lysine gave values of 0.58%, the available lysine as determined by the method of Bruno and Carpenter ('57) was only 0.31%. When fed in diets supplemented with lysine, rat growth equal to that obtained with isonitrogenous casein-containing diets was obtained. The diets fed consisted of cereal 89.5%, corn oil 4%, cod liver oil 1% and choline 0.3%. Diets II, IV and VI contained 4% Salts IV (Hegsted et al., '41), and diets I, III and V contained 1.2% $Na_2HPO_4 \cdot 2H_2O$ and 2.8% Salts IV minus its potassium salts. No lysine was added to diets I and II, 0.25% of L-lysine·HCl, to diets III and IV and 1.0% of L-lysine·HCl to diets V and VI. Starch was added where necessary to bring the percentages of ingredients to 100. Four milligrams thiamine·HCl, 8 mg riboflavin, 4 mg pyridoxine·HCl, 40 mg niacin, 20 mg Ca pantothenate, 1 mg folic acid, 0.2 mg biotin, 1 mg menadione and 0.1 mg of vitamin B_{12} were added to each kilo of diet. On analysis for available lysine and K, diet I was found to contain 0.28% lysine and 0.14% K, diet II, 0.28% lysine and 0.72% K, diet III, 0.53% lysine and 0.14% K, diet IV, 0.53% lysine and 0.72% K, diet V, 1.28% lysine and 0.14% K and diet VI, 1.28% lysine and 0.72% K. The Na content of the odd numbered diets was 1.27% and for the even numbered ones, 0.96%. The rats were fed the experimental diets and distilled water ad libitum.

The experiment was terminated after 6 weeks, the animals being killed by decapitation. Samples of liver, shaved skin and muscle (quadriceps), dissected of gross fat, were obtained for analyses of potassium and sodium by flame photometry, nitrogen by Kjeldahl determination, and amino acid content by paper chromatography. Blocks of heart, liver, kidney, testes, long bone (radius), eye, and skin were fixed in 10% formalin. Blocks of muscle (biceps and triceps) were fixed in Zenker's solution for 12 hours following initial fixation in unacidified Zenker's stock solution. Paraffin embedded sections were stained with Hematoxylin and Eosin, the bones being decalci-

fied with 5% nitric acid. Frozen sections of livers were stained with Sudan IV and hematoxylin.

RESULTS

Differences in growth in these studies were related to the lysine, but not to the potassium content of the diets. At the end of 6 weeks weight gain was 19 gm in group I, 20 gm in

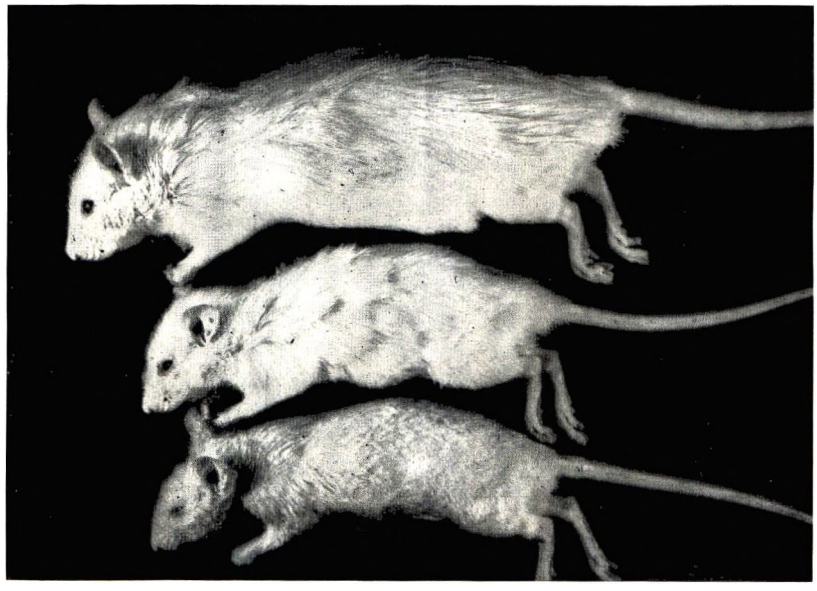

Fig. 1 The effect of dietary potassium and lysine on the hair coats of rats. Top rat, 1.28% lysine, 0.72% potassium. Middle rat, 0.28% lysine, 0.72% potassium. Bottom rat, 0.28% lysine, 0.14% potassium.

group II, 101 gm in group III, 108 gm in group IV, 190 gm in group V and 205 gm in group VI. During the third week the rats in group I started losing their hair and by the end of the experiment showed marked alopecia, particularly on the shoulders, backs and hind quarters. Although some of the rats in group II lost some hair during the 6 week, at the conclusion of the experiment the least involved rat in group I had lost considerably more hair than the most involved animal in group II, (fig. 1). This was clearly a protective effect

of increased potassium in diets deficient in lysine. Minimal hair loss was seen in a few animals in group III and IV and none in groups V and VI. Microscopically the skins of rats in groups I and II showed generalized atrophy of both epidermis and cutaneous appendages. This aspect was not consistent and no difference was found between the two potassium levels.

The histologic changes most suggestive of a protective effect of excessive dietary potassium in the presence of lysine deficiency involved the liver. The amount of Sudanophilic material in the liver cells, generally periportal in location, could be correlated with the dietary lysine levels. Thus, the livers of groups I and II demonstrated moderate amounts of lipid and those of groups III and IV minimal amounts, while the livers of the two highest lysine groups showed only the minute traces of lipid seen in normal rats. A potassium effect was apparent only at the 0.28% lysine level, where it was associated with a reduction in the amount of liver cell lipid. Similarly the quantitative data relevant to the epiphyseal plates of the long bones were suggestive of a protective effect of dietary potassium though less clearly defined than the liver lipid data. The thickness of the epiphyseal cartilage of the distal end of the radius was measured with the aid of a calibrated ocular grid. At the lowest lysine level, a distinct thinning of the epiphyseal plate could be seen. At each lysine level, it was observed that increasing the potassium level of the diet was accompanied by an increased thickness of the epiphyseal plates, though these differences were not statistically significant.

Various other histologic changes were seen that could not be related to dietary potassium levels. The testes of groups I and II showed a distinct though non-specific hypoplasia and failure of maturation of spermia. Other non-specific changes were sporadic interstitial lymphocytic infiltrates in myocardium and renal parenchyma and infrequent foci of degeneration or inflamation in skeletal muscle. These changes were most prominent in group V, with normal levels of both lysine and potas-

sium. The amount of corneal vascularization appeared within normal limits both grossly and microscopically in all groups.

The results of nitrogen, potassium and sodium analyses of liver, muscle and skin are shown in table 1. It can be seen from these data that at the various lysine levels fed, the potassium content of the diets had no significant effect on the tissue concentrations of nitrogen, sodium or potassium. The lysine content of the diet did not affect the sodium and potassium concentrations of muscle and liver but had a marked effect on the sodium and potassium content of the skin. Highest values for both cations were observed in rats fed the diets most deficient in lysine. The skins of rats fed 1.28% lysine contained more sodium and to a limited extent more potassium than those receiving 0.53% lysine. There also appeared to be a slight effect of the high lysine diets in increasing the nitrogen concentrations of the tissues examined.

To obtain information about the pattern of amino acids in the tissues studied, they were examined by one dimensional paper chromatography following hydrolysis in 6 N HCl overnight in an autoclave at 9 lbs. pressure. The solvent used was obtained by shaking 250 ml of *n*-butanol with 250 ml of water, then adding 60 ml of glacial acetic acid and after further shaking, discarding the aqueous layer. The amino acids were stained with ninhydrin and the densities of the 10 spots obtained were measured on a Spinco Analytrol. In general, differences in the amino acid patterns of the various groups were not marked and table 2 does not include individual group values unless they are statistically significant at a 5% level. It can be seen from this table that the amino acid patterns of liver and muscle are quite similar, but differ considerably from those of skin. A decrease in dietary lysine was associated with increases in muscle lysine and histidine and in the spot representing liver phenylalanine, leucine and isoleucine. On many of the chromatograms phenylalanine could be separted from leucine and isoleucine and these strips indicated that the changes were in the leucine-isoleucine component of the spot. A decrease in the density of the spot representing liver glutamic acid and threonine was also associated with lysine defi-

TABLE 1

Nitrogen, sodium and potassium content of rat tissue[1]

DIETS	0.28% LYSINE 0.14% K	0.28% LYSINE 0.72% K	0.53% LYSINE 0.14% K	0.53% LYSINE 0.72% K	1.28% LYSINE 0.14% K	1.28% LYSINE 0.72% K
K, millimole %						
Liver	8.0 ± 0.3	8.6 ± 0.3	8.3 ± 0.1	7.8 ± 0.3	8.1 ± 0.2	8.4 ± 0.2
Muscle	10.9 ± 0.2	10.9 ± 0.2	10.7 ± 0.2	10.1 ± 0.3	10.1 ± 0.2	10.7 ± 0.5
Skin	4.2 ± 0.3	4.4 ± 0.1	3.1 ± 0.3	3.1 ± 0.2	3.5 ± 0.2	3.5 ± 0.2
Na, millimole %						
Liver	3.5 ± 0.2	3.4 ± 0.1	3.1 ± 0.1	3.4 ± 0.3	4.0 ± 0.2	3.3 ± 0.2
Muscle	2.5 ± 0.1	2.3 ± 0.1	2.4 ± 0.1	2.3 ± 0.1	2.8 ± 0.2	2.1 ± 0.1
Skin	6.3 ± 0.2	6.3 ± 0.2	4.6 ± 0.1	4.6 ± 0.1	5.5 ± 0.1	5.6 ± 0.1
N, %						
Liver	2.6 ± 0.1	2.4 ± 0.1	2.5 ± 0.1	2.5 ± 0.1	2.9 ± 0.1	2.8 ± 0.1
Muscle	3.1 ± 0.1	3.2 ± 0.1	3.2 ± 0.1	3.0 ± 0.1	3.3 ± 0.1	3.5 ± 0.1
Skin	3.1 ± 0.5	3.2 ± 0.2	3.0 ± 0.2	2.6 ± 0.1	3.7 ± 0.1	3.4 ± 0.1

[1] All values are on a wet weight basis and include the standard error of the means.

TABLE 2

Amino acid patterns of rat tissues [1]

	SKIN	LIVER	MUSCLE
Cystine	2.5	1.6	1.1
Lysine	6.4	9.1	*
Histidine	7.9	6.8	**
Arginine	6.7	9.2	8.7
Glycine, serine, aspartic acid	21.1	11.8	11.4
Glutamic acid, threonine	18.1	***	23.0
Alanine	14.5	12.1	11.4
Tyrosine	3.2	3.0	2.8
Valine, methionine	7.3	9.2	8.3
Phenylalanine, leucine, isoleucine	12.0	****	16.2

	GROUP					
	I	II	III	IV	V	VI
* Muscle lysine	9.6 ± 0.3	9.8 ± 0.4	8.9 ± 0.4	8.6 ± 0.6	8.6 ± 0.6	8.1 ± 0.6
** Muscle histidine	8.6 ± 1.2	8.2 ± 0.6	7.6 ± 0.3	7.6 ± 0.1	7.2 ± 0.3	6.5 ± 0.5
*** Liver glutamic acid, threonine	18.4 ± 0.5	19.1 ± 0.4	20.0 ± 0.3	21.7 ± 0.8	21.0 ± 0.7	21.5 ± 0.6
**** Liver phenylalanine, leucine, isoleucine	19.2 ± 0.9	19.6 ± 1.5	18.9 ± 0.6	15.7 ± 0.7	15.4 ± 0.9	15.9 ± 1.2

[1] Values are expressed as % of total color. Individual group values with standard errors of the mean are presented where group values are significantly different at $p < 0.05$.

ciency. These amino acid differences were not related to the potassium content of the diet.

DISCUSSION

Grunert et al. ('50) have established the potassium requirement for rats as 0.15% when 1% sodium was present in the diet. When less than 0.09% potassium was present potassium deficiency resulted. The diets used in these studies contained either 0.14% potassium, the rats usual potassium requirement, or 0.72% potassium, approximately 5 times the normal requirement. Under some of the experimental conditions studied the rats' need for potassium appeared to be raised. This was shown by the partial protection against hair loss and a decrease in liver fat observed in group II, receiving the diet lowest in lysine. Grunert et al. ('50) found that the growth requirement of the rat for sodium was 0.05%. The diets used in this work contained 1.27 and 0.96% sodium, approximately 20 times the normal requirement. It seems unlikely that the observations made in these experiments were significantly affected by the relatively small differences in the sodium content of the diets.

It is difficult to interpret the effect of the diets on the chemical composition of the tissues studied. Changes in the lysine but not the potassium content of the diet resulted in marked alterations in the potassium and sodium content of skin but not of liver or muscle. The amino acid pattern of skin hydrolysates, however, was not particularly affected by lysine deficiency although some changes in the amino acid patterns of muscle and liver were observed. Changes in the potassium and sodium of skin cannot be correlated with changes in skin amino acid concentrations. It is surprising that the histidine and particularly the lysine content of muscle would increase in lysine-deficient animals. The effects of lysine deficiency on the testes and long bones essentially confirm the work of Harris et al. ('43) and Gillespie et al. ('45). It should be pointed out that both of these changes may represent the effects of relative inanition or failure of growth rather than a specific attribute

of lysine deficiency. The effects on the liver are similar to though apparently milder than those reported by Sós and Kemény ('56). Abnormal corneal vascularization such as reported by Hock et al. ('45) was not seen.

Because of its abundance in plant and animal tissues, potassium is not usually given consideration as a dietary adjunct in human nutrition (Food and Nutrition Board, '58). It seems hardly likely that potassium deprivation is a nutritional problem under most ordinary dietary conditions. Potassium requirements are greatest when tissue proteins are being increased. Although primary potassium deficiency in humans may not exist, problems involving potassium nutrition may occur as a result of disease or aberrant food supplies. Protein deficiency in people eating diets consisting primarily of plant products has been reported in many parts of the world. Santaló ('55) pointed out that cereals, legumes and vegetables, but not fruits exhibit a positive linear correlation between nitrogen and potassium content. Hansen and Brock ('54) found that infants suffering from nutritional edema, some with kwashiorkor, had an increased need for potassium as indicated by measurements of potassium retention and alleviation of edema by solutions of potassium and sodium salts and glucose before milk adminstration. The need for K in postoperative surgical cases as a protection against nitrogen loss has been stressed by Eliel et al. ('52) and Frost and Smith ('53). The experiments of Cannon et al. ('52), Frost and Sandy ('53) and Muntwyler et al. ('53) on rats have reemphasized the need for potassium in the utilization of amino acids, particularly following protein depletion. The increased need for potassium by rapidly growing guinea pigs has also been pointed out by Roine et al. ('49) and Heincke et al. ('56). The present studies are further evidence of the importance of potassium in protein metabolism and support the view that special consideration of potassium intake should be made during periods of protein deprivation and repletion.

SUMMARY

The effect of feeding diets containing 0.14 and 0.72% of potassium and varying levels of lysine to rats has been studied. Partial protection against hair loss and increased liver fat were observed when the higher potassium level was fed to lysine-deficient rats, indicating an increased potassium requirement in these animals. Variations in the lysine but not the potassium content of the diets resulted in marked changes in the potassium and sodium content of skin but not of liver or muscle. The results of amino acid analyses and histologic studies of tissues from these animals have also been reported.

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THE EFFECTS IN RATS OF VITAMIN B_{12}, WITH AND WITHOUT ETHYL ALCOHOL, ON NITROGEN BALANCE, SERUM ALBUMIN, LIVER NITROGEN AND FAT [1]

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In addition to its role in stimulating the regeneration of red blood cells, vitamin B_{12} has been implicated in a wide variety of metabolic processes. Particularly in animals on low-protein intakes, vitamin B_{12} has been reported to increase growth and improve protein utilization (Bosshardt et al., '46; Ershoff, '47; Hartman et al., '49; Luecke, '49; Emerson, '49; Cunha et al., '50). Many investigators have noted that the administration of vitamin B_{12} is usually associated with an increased appetite and food intake and that this effect may play an important role in the growth response observed (Ershoff, '47; Luecke, '49; Wetzel et al., '50; Anderson and Hogan, '50; Bosshardt et al., '50; Rupp et al., '51). Vitamin B_{12} is also involved in some manner in the synthesis of methyl groups (Bennett, '50; Stekol and Weiss, '50) and has a lipotropic effect in animals on diets deficient in choline and methionine (Drill and McCormick, '49; McCormick and Drill, '50; György and Rose, '50; Dumm et al., '52). In the following experiments, animals were placed on a diet deficient in vitamin B_{12} and the effects of the vitamin and of ethyl alcohol on growth, nitrogen retention, the nitrogen and fat content of the liver and the electrophoretic pattern of the plasma pro-

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teins were studied. Alcohol was added in certain experiments because of its association with liver damage and to note the effect of vitamin B_{12} under these circumstances.

PROCEDURES

Female rats bred in the laboratory were maintained on a dog chow [2] diet from weaning until the start of the experiment. They were started on a diet deficient in vitamin B_{12} at 42 days of age.[3] After 14 days on the deficient diet, the animals were divided at random into two groups. One group (control) was continued on the diet alone. The other group was given daily subcutaneous injections of 7.5 μg of vitamin B_{12}. After 21 days of vitamin B_{12} administration, each group of animals was again divided at random into two subgroups, one of which was given a drinking solution of 15% ethyl alcohol instead of tap water for the remainder of the experiment. This regimen was continued for 28 days, at which time the animals were sacrificed. A total of 42 animals was studied in these experiments.

At approximately weekly intervals throughout the experiment, food and fluid intake and urinary nitrogen were determined for 48-hour periods. At the start of the experiment, and before each change in regime, blood samples were taken from the tail for the determination of the distribution of the plasma proteins. At the termination of the experiments, the animals were sacrificed and blood specimens were taken both from the tail and from the abdominal aorta. The livers were removed, weighed and samples taken for microscopic sections and for

[2] Gaines Dog Chow is used in our breeding and stock rat colony.

[3] Composition of vitamin B_{12}-deficient diet (per 100 gm diet): 25 gm heated soybean meal, 30 gm sucrose, 37 gm "Primex" (hydrogenated cottonseed oil), 2 ml cod liver oil, 6 gm mineral mixture, 0.15 gm thiamine, 0.15 mg pyridoxine, 0.25 mg riboflavin, 0.05 mg folic acid, 0.025 mg biotin, 0.5 mg Ca pantothenate, 0.5 mg niacin, 2.5 mg vitamin K ("Synkavit"). By analysis the diet contained 12% protein. Composition of mineral mixture: 17% NaCl, 2% ferric citrate ($FeC_6H_5O_7 \cdot 3H_2O$) 36% calcium phosphate ($CaHPO_4 \cdot 2H_2O$), 35% potassium citrate ($K_3C_6H_5O_7 \cdot H_2O$), 10% magnesium citrate [$Mg_3(C_6H_5O_7)_2$], 0.08% $CuSO_4$, 0.004% KI. All chemicals were "reagent" grade.

determinations of nitrogen and fat. The adrenals were weighed and their cholesterol content determined.

In the experiments just described, the animals were allowed to eat the diet ad libitum. In addition, a paired-feeding experiment was done. In this experiment rats were started on the deficient diet at 42 days of age and allowed to eat ad libitum. During this period, their food intake was recorded and after 14 days they were divided into pairs on the basis of body weight and ad libitum food intake. One rat in each pair, selected at random, was continued on the deficient diet alone; the other rat in each pair was injected with 7.5 μg of vitamin B_{12} daily and its food intake limited to that of the deficient rat. This experiment was continued for 28 days and was completed on 9 pairs of rats. At that time the food intake of the deficient rats had decreased to the point where it was necessary to discontinue the experiment. The animals were sacrificed and the same determinations on blood, liver and adrenals were performed as in the ad libitum experiments.

The total protein in the blood and in the liver was determined by a micro Kjeldahl method. The distribution of the plasma proteins was determined by paper electrophoresis (barbiturate buffer, pH 8.6; $\mu = 0.1$). The papers were stained with bromphenol blue and the density of the stain read in a Spinco Analytrol. The fat in the liver was determined gravimetrically and the adrenal cholesterol by a modification of the Schoenheimer-Sperry method (Clarke and Marney, '45).

RESULTS

The administration of vitamin B_{12}, which was started at 56 days of age, was associated with an increased rate of growth which was very marked by the end of the experiment compared to that of the control rats (60 gm total gain for vitamin B_{12}-treated, 25 gm total gain for control rats; period of time, 40 days). The administration of 15% alcohol, which was started at 77 days of age, was associated with a decreased rate of growth in the rats receiving vitamin B_{12} and a complete cessation of growth in the deficient rats not receiving vitamin

TABLE 1

Effect of vitamin B_{12} and alcohol on food intake and nitrogen balance

AGE OF RATS	DAYS ON DEFICIENT DIET	FOOD INTAKE							
		Control rats	B_{12}-treated rats		Alcohol-treated rats		B_{12} + Alcohol-treated rats		
		Food intake	Days on B_{12}	Food intake	Days on alcohol	Food intake	Days on B_{12}	Days on alcohol	Food intake
days		*gm/day*		*gm/day*		*gm/day*			*gm/day*
63	21	8.10 ± 0.29 [1]	7	7.90 ± 1.15					
71	29	7.96 ± 0.36	15	14.0 ± 1.36					
84	42	6.50 ± 1.10	28	9.50 ± 1.05	8	3.90 ± 1.16	28	8	6.60 ± 0.97
		NITROGEN BALANCE							
		mg N retained/day		mg N retained/day		mg N retained/day			mg N retained/day
63	21	98.9 ± 7.1	7	97.9 ± 18.3					
71	29	91.7 ± 5.4	15	179.2 ± 26.8					
84	42	77.8 ± 7.6	28	105.4 ± 27.1	8	35.4 ± 20.8	28	8	84.4 ± 16.6

[1] Mean ± standard error.

TABLE 2

Nitrogen balance of pair-fed rats treated with vitamin B_{12}
(7.5 μg injected daily)

AGE	DAYS ON B_{12}-DEFICIENT DIET	DAYS ON VITAMIN B_{12}	CONTROL RATS		VITAMIN B_{12}-TREATED RATS	
			Food intake	Nitrogen balance	Food intake	Nitrogen balance
			gm/day	*mg/day*	*gm/day*	*mg/day*
63	21	7	7.16	74.7 ± 7.23	6.99	86.8 ± 9.37
71	29	15	8.36	85.4 ± 11.6	8.49	91.8 ± 15.45
77	35	21	6.70	66.3 ± 5.47	6.70	67.0 ± 5.57
84	42	28	6.35	54.9 ± 9.53	6.73	70.8 ± 10.10
63-84	21-42	7-28	7.14	69.9 ± 4.52 [1]	7.23	78.7 ± 5.26 [1]

[1] (Fisher's "t" = 1.143; ρ = 0.1 to 0.2).
Data are given as mean ± standard error.
Nine pairs of rats in this experiment.

B_{12}. The rats on alcohol plus vitamin B_{12} continued to grow at a rate about the same as that of the vitamin B_{12}-deficient rats receiving tap water.

In the pair-fed rats, vitamin B_{12} was associated with a small increase in the rate of growth, which was not statistically significant ($p = 0.1 - 0.2$).

The food, caloric intake and nitrogen balance of the ad libitum fed rats are summarized in table 1. Vitamin B_{12} administration was associated with a marked increase in food intake, as has been reported by other investigators (Ershoff, '47; Luecke, '49; Anderson and Hogan, '50). This was also true when the rats received alcohol. The total caloric intake was about the same for the alcohol-treated rats (33.3 ± 7.06 Cal./day) as for the deficient rats not receiving alcohol (36.6 ± 6.19 Cal./day) when the calories from alcohol are included. In the rats treated with vitamin B_{12}, the total caloric intake also was about the same whether or not they received alcohol (53.5 ± 5.91 and 54.8 ± 7.60 Cal./day). The nitrogen balance in the rats fed ad libitum was greater after vitamin B_{12} administration both for the rats on tap water compared to their controls and for the rats on alcohol compared to their controls. Alcohol administration was associated with decreased nitrogen retention both in animals treated with vitamin B_{12} compared to vitamin B_{12}-treated rats on tap water and in deficient rats compared to deficient rats on tap water.

In table 2, the data on nitrogen balance of the pair-fed rats treated with vitamin B_{12} are summarized. Throughout the experiment, the nitrogen balance of the rats treated with vitamin B_{12} was slightly greater than for their pair-fed controls. However, the differences are small and do not meet the usual criteria of statistical significance, even when the data for the whole period of the experiment are combined.

In figure 1, the distribution of the plasma proteins is shown for the pair-feeding and for the ad libitum experiments. Since the determinations during the course of the experiment were done on small volumes of tail blood, the albumin is presented as percentage of the total protein. At the time of the sacrifice,

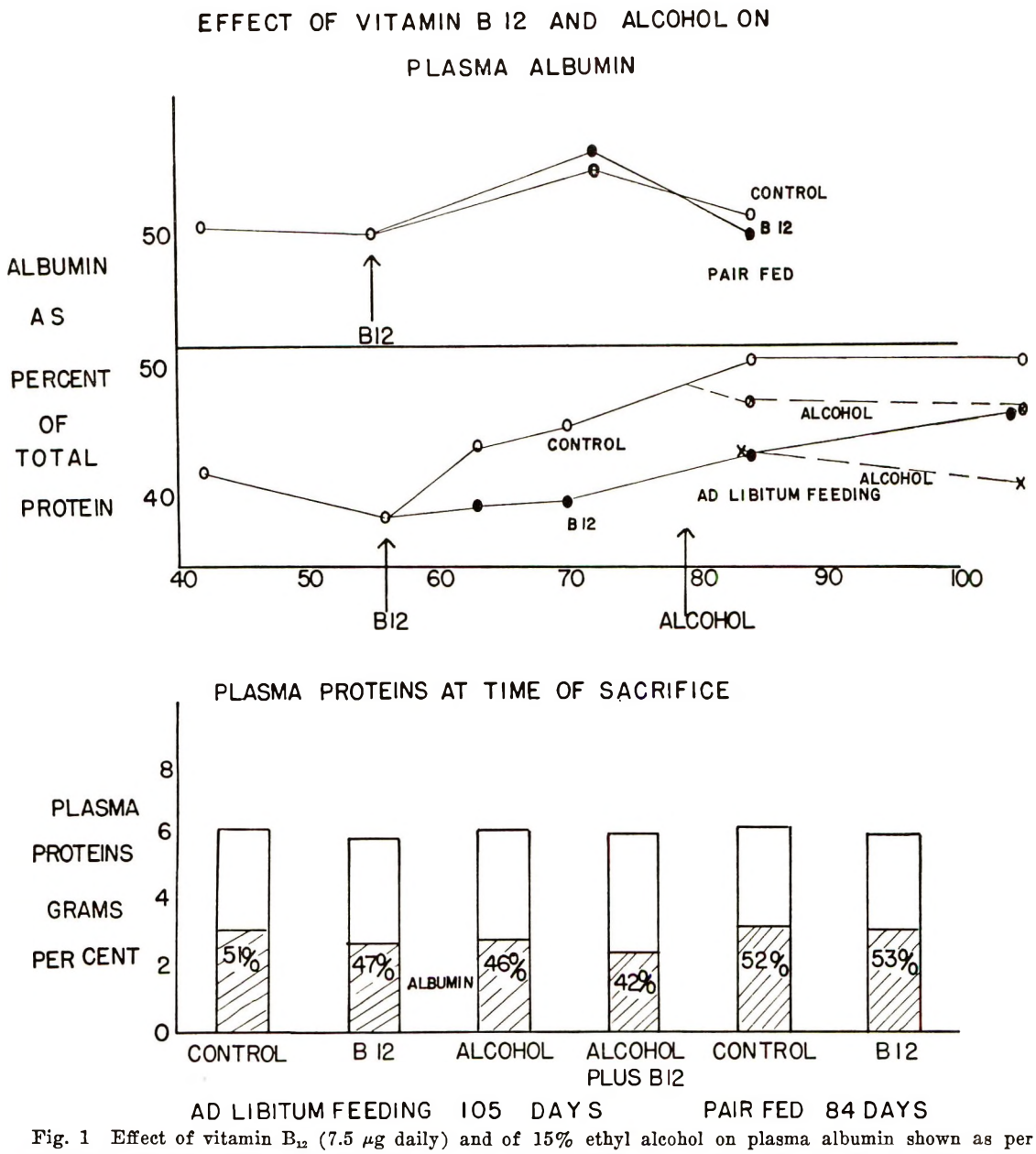

Fig. 1 Effect of vitamin B_{12} (7.5 μg daily) and of 15% ethyl alcohol on plasma albumin shown as per cent of total plasma protein. From top to bottom; top — pair-fed rats treated with vitamin B_{12} (●——●), on diet alone (○——○); middle — ad libitum-fed rats on diet alone (○——○), on diet plus 15% alcohol (⊗——⊗), treated with vitamin B_{12} (●——●), treated with vitamin B_{12} plus 15% ethyl alcohol (×——×); bottom — total plasma proteins in grams per cent at sacrifice and albumin as per cent of total.

the total proteins were determined on the aorta blood and are shown at the bottom of the figure. In the paired-feeding experiment, the administration of vitamin B_{12} had no significant effect on the percentage of plasma albumin. However, in the ad libitum-feeding experiment, vitamin B_{12} administration was associated with a consistent decrease in the percentage of albumin. The administration of alcohol was also associated with a further decrease in the percentage of albumin. For any given point, the difference in the plasma albumin associated with the administration of vitamin B_{12} is not large,

TABLE 3

Effect of vitamin B_{12} and of alcohol on the nitrogen and fat content of the liver
(All values based on weight of fresh tissue)

NUMBER OF RATS	INJECTED SUPPLEMENT	DRINKING SOLUTION	NITROGEN	FAT
			gm %	*gm %*
		Ad libitum experiments		
12	None	Water	2.11 ± 0.081 [1]	30.1 ± 3.46
8	Vitamin B_{12}	Water	2.76 ± 0.098	13.6 ± 4.92
10	None	15% alcohol	2.21 ± 0.012	30.8 ± 1.35
11	Vitamin B_{12}	15% alcohol	2.85 ± 0.062	11.8 ± 1.62
		Pair-fed experiments		
9	None	Water	2.39 ± 0.186	19.1 ± 3.06
9	Vitamin B_{12}	Water	3.37 ± 0.195	7.6 ± 1.64

[1] Mean ± standard error.

but the fact that this change was observed consistently, suggests that this effect of vitamin B_{12} administration on the percentage of albumin is probably a real one. The concentration of total protein was not affected by any of the dietary regimes employed, as is shown in the bottom of the figure.

In table 3, the data on the effect of vitamin B_{12} and of alcohol on the nitrogen and fat content of the liver are summarized for the ad libitum and pair-fed rats. In the ad libitum experiments, the deficient diet was associated with low liver nitrogen (2.11 gm%) and elevated liver fat (30.1 gm%). The administration of vitamin B_{12} resulted in a significant increase in liver nitrogen (2.76 gm%, $p < 0.001$) and decrease in liver fat (13.6 gm%, $p < 0.01$). The composition of the livers of

the rats receiving alcohol was not significantly different from the rats on water (2.21% nitrogen, 30.8% fat). Vitamin B_{12} was associated with a significant increase in liver nitrogen (2.85%, $p < 0.001$) and decrease in liver fat (11.8%, $p < 0.001$) in the rats receiving alcohol.

In the pair-fed rats treated with vitamin B_{12}, the liver nitrogen averaged 3.37 gm% compared to 2.39 gm% for the deficient rats not receiving vitamin B_{12} ($p < 0.01$). The fat content of the livers of the vitamin B_{12}-treated rats averaged 7.6 gm% compared to 19.1 gm% for the deficient rats, ($p < 0.01$).

The data on adrenal weight and cholesterol are summarized in table 4. For comparison, values for adrenal weight and cholesterol in a group of rats receiving a 12% casein diet are also included.[4] The adrenal weight in rats on the soy bean diet expressed per 100 gm of body weight did not differ significantly in any group. However, in the ad libitum-fed rats, vitamin B_{12} was associated with lower adrenal cholesterol levels expressed either as percentage of adrenal tissue or as milligrams per 100 gm body weight of the rats. No significant changes in the level of adrenal cholesterol were observed, either in the pair-fed rats on the soy bean diet or in the ad libitum-fed rats on the casein diet.

The effects of the various dietary situations on the microscopic appearance of the liver are shown in figures 2, 3 and 4. Rats on tap water and the deficient diet ad libitum showed profound fatty infiltration of the liver at the time of sacrifice (fig. 2a). In some areas, early fibrosis of the liver tissue was also seen. The staining capacity of the cells was reduced. In the rats receiving vitamin B_{12} and eating the diet ad libitum, fatty infiltration was at a minimum and the staining capacity of the cells was within normal limits (fig. 2b). In figure 3 are sections from the livers of rats on the deficient and supplemented diet fed ad libitum and with 15% ethanol as drinking solution. As the data in table 4 also show, the lipotropic effect of vitamin B_{12} was demonstrated in spite of alcohol administration. In figure 4 are sections from the livers of

[4] Ralli, E. P., and M. E. Dumm, unpublished data.

TABLE 4

Adrenal weight and cholesterol

DRINKING SOLUTION	SUPPLEMENT	ADRENAL WEIGHT		ADRENAL CHOLESTEROL	
		mg	*mg/100 gm*	*gm %*	*mg/100 gm*
		(Soybean meal diet — ad libitum)			
Water	None	31.9 ± 1.9 [1]	21.6 ± 1.2	5.99 ± 0.36 [2]	1.32 ± 0.14
Water	Vitamin B_{12}	38.5 ± 1.6	20.4 ± 1.0	4.77 ± 0.42 [2]	0.99 ± 0.13 [2]
Alcohol	None	26.4 ± 2.0	21.7 ± 1.5	6.20 ± 0.47 [2]	1.36 ± 0.15 [2]
Alcohol	Vitamin B_{12}	30.6 ± 2.0	20.2 ± 0.2	4.99 ± 0.10 [2]	1.01 ± 0.08 [2]
		(Soybean meal diet — pair-fed)			
Water	None	32.8 ± 2.5	21.4 ± 1.8	5.66 ± 0.56	1.23 ± 0.19
Water	Vitamin B_{12}	36.2 ± 2.8	22.6 ± 1.3	5.11 ± 0.71	1.11 ± 0.15
		(12% casein diet — ad libitum)			
Water	None	28.8 ± 2.0	22.1 ± 2.0	5.10 ± 0.27	1.12 ± 0.11
Water	Vitamin B_{12}	25.5 ± 1.5	19.9 ± 1.3	5.75 ± 0.45	1.11 ± 0.08

[1] Mean ± standard error.

[2] Vitamin B_{12}-treated compared to controls. Differences significant at 0.05 level.

pair-fed rats. In spite of the limited food intake, vitamin B_{12} still exerted a lipotropic effect (fig. 4b) and prevented the fatty infiltration which occurred in the deficient rats (fig. 4a).

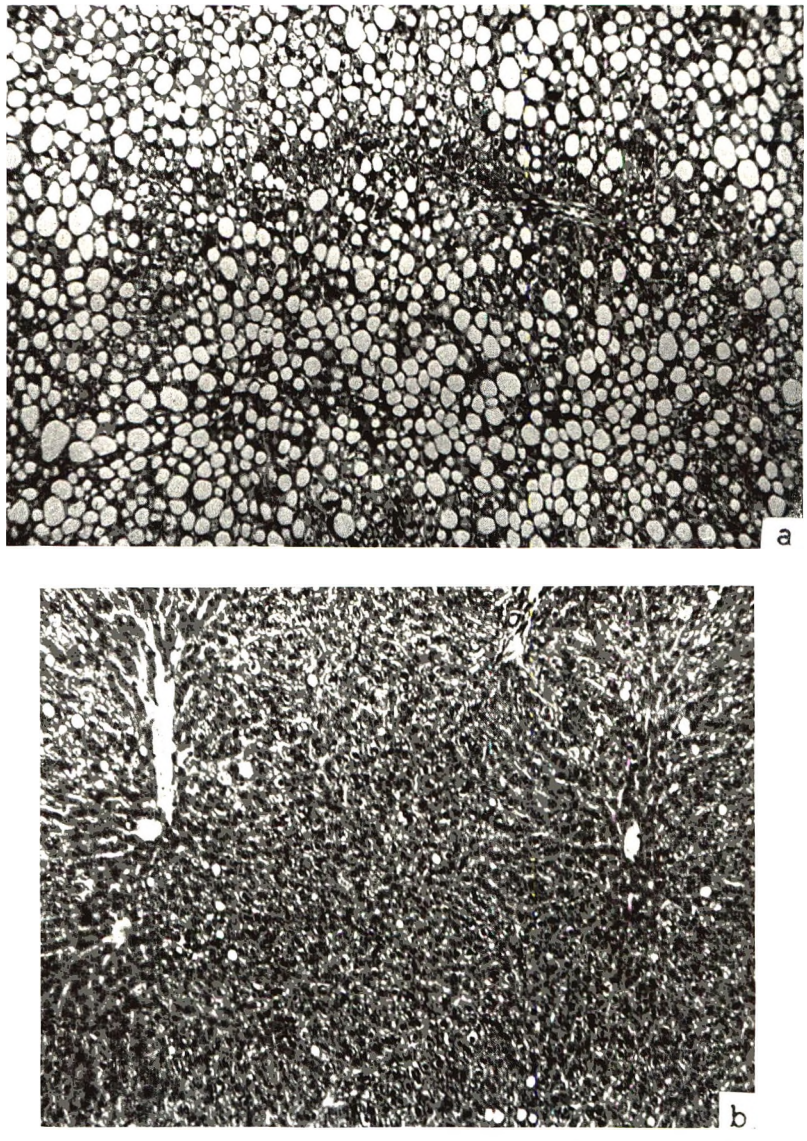

Fig. 2 Microscopic sections of livers of rats fed soybean meal diet ad libitum with tap water as drinking solution; a, Control; b, 7.5 μg vitamin B_{12} daily.

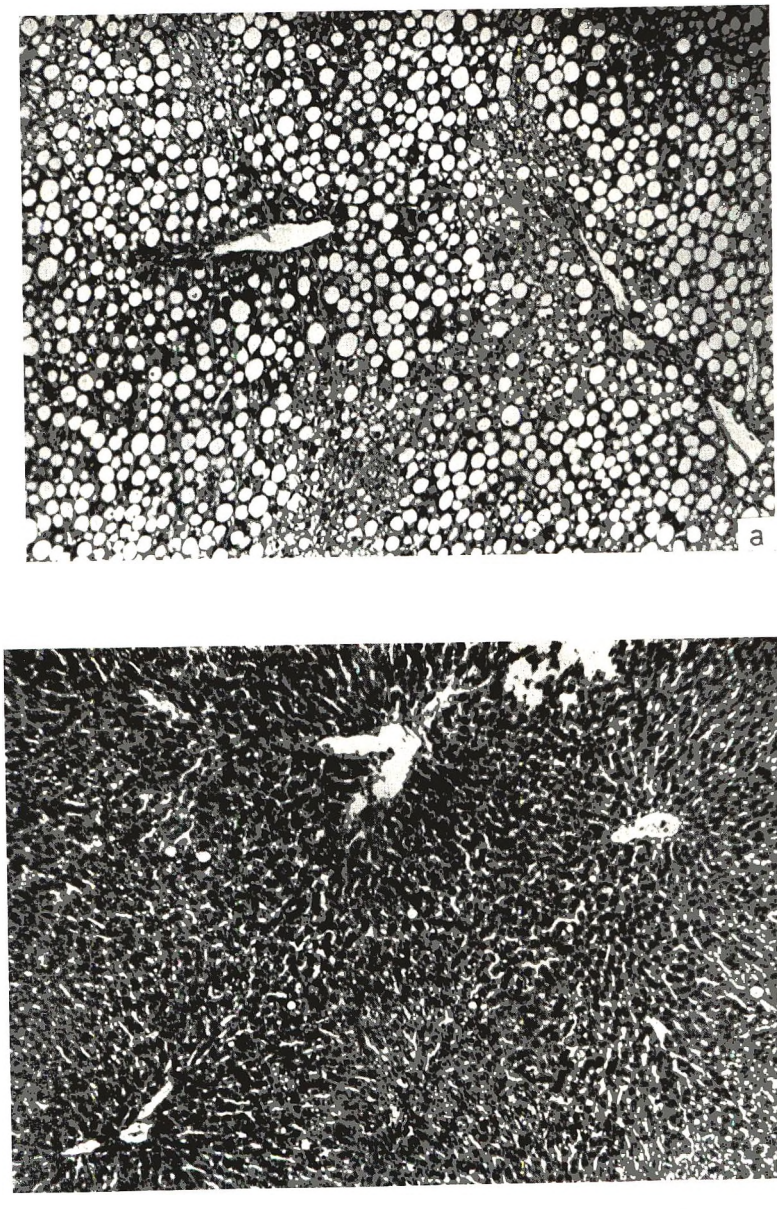

Fig. 3 Microscopic sections of livers of rats fed soybean meal diet ad libitum with 15% ethyl alcohol as only drinking solution; a, Control; b, 7.5 μg vitamin B_{12} daily.

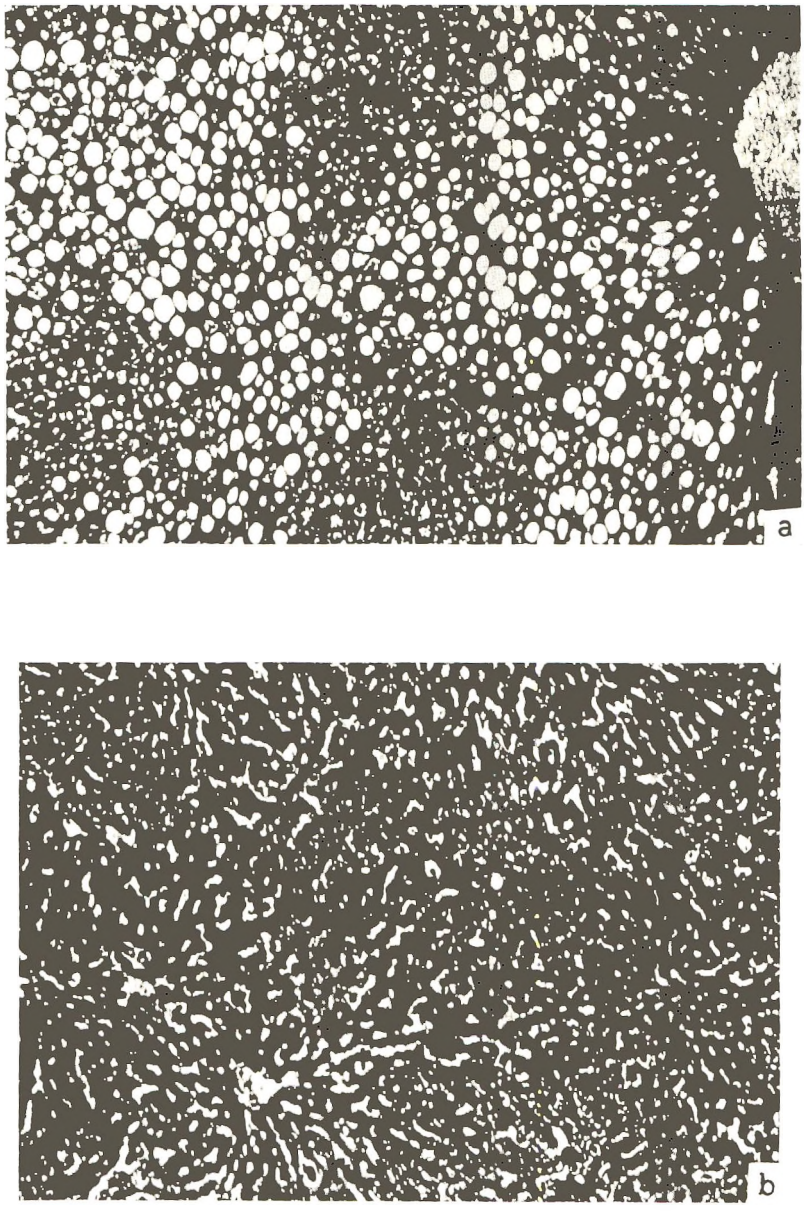

Fig. 4 Microscopic sections of livers of pair-fed rats on soybean meal diet; a, Control; b, vitamin B_{12} treated.

DISCUSSION

In earlier unpublished experiments from this laboratory, the effect of vitamin B_{12} on the liver, adrenals and plasma proteins was studied in young female rats on a 12% casein, 72% carbohydrate, 11% fat diet to which no choline or vitamin B_{12} was added.[3] When this diet was fed ad libitum, the injection of 5 μg of vitamin B_{12} daily was without significant effect on growth, liver nitrogen or fat, on the distribution of the plasma proteins or on adrenal weight or cholesterol, as was shown in table 4.

In the present experiments on rats fed a soy meal diet containing 12% protein and 39% fat, the growth-promoting effect of vitamin B_{12} was obvious when the rats were allowed to eat ad libitum. However, in the pair-fed rats on this diet, vitamin B_{12} had little or no effect on growth or on nitrogen balance. These observations confirm the results of other observers, that the growth-promoting effect of vitamin B_{12} depends on food intake and does not occur in pair-fed animals (Bosshardt et al., '50; Rupp et al., '51). The effect of vitamin B_{12} in rats on a 12% protein soybean meal diet and the lack of effect in animals on a 12% casein diet is presumably related both to the presence of vitamin B_{12} in the casein diet and also to the higher methionine content of casein (3.0 to 3.3 gm/100 gm) compared to soybean meal protein (1.6 to 2.0 gm). Block and Bolling ('45).

The administration of ethyl alcohol decreased food intake, growth and nitrogen retention, in spite of the fact that the total caloric intake was unaltered. When vitamin B_{12} was given along with alcohol, food intake, growth and nitrogen retention were improved and were about equal to the values observed on deficient rats not receiving alcohol.

There is no evidence from either the nitrogen or fat content of the liver or in the histological appearance of the tissue that the ingestion of alcohol was associated with any increase in liver damage beyond that attributable to the diet alone. When

[3] See footnote 3, page 42.

rats receiving alcohol were treated with vitamin B_{12}, liver nitrogen increased and liver fat decreased to the same extent as in rats not receiving alcohol.

The "lipotropic" effect of vitamin B_{12} occurred in both the paired-feeding and the ad libitum experiments and apparently does not have any relation to increased food intake or growth. The fact that this effect was not observed in the casein experiments in which the total protein content of the diet was the same although the methionine content was higher, suggests that the critical factor was the role of vitamin B_{12} in the synthesis of methyl groups (Bennett, '50; Stekol and Weiss, '50).

One of the more intriguing effects of vitamin B_{12} in the ad libitum experiments was the consistent tendency for a decrease in the albumin concentration of the plasma. The fact that this did not occur in the paired-feeding experiment or in experiments where a 12% casein diet was used, suggests that this is related to the increased food intake and growth and is probably associated with an increased deposition of protein in other body tissues.

Adrenal weight was not affected by the dietary situation in any group of animals. However, adrenal cholesterol was lower in the ad libitum-fed rats treated with vitamin B_{12} than in their controls. This effect was not observed in the pair-fed rats on the soybean meal diet or in the ad libitum fed rats on the casein diet, suggesting that the lower adrenal cholesterol is related in some way to increased growth and food intake. Apparently it is not simply a result of the manipulations associated with the daily injection of vitamin B_{12}, since it did not occur in the pair-fed or casein-fed rats. The data suggest that the adrenals of the ad libitum-fed rats on the soybean meal diet with no vitamin B_{12} supplementation either were less intensively stimulated to release adrenal steroids or were somewhat refractory to stimulation compared to the animals treated with vitamin B_{12}. We have previously observed that rats on low protein diets have elevated levels of adrenal cholesterol which are less affected by stress than are the

adrenal cholesterol levels of normal animals. It was suggested that the secretion of protein hormones by the pituitary may be decreased in animals suffering from a general protein deficiency (Gershberg and Ralli, '52). In the present experiments, the synthesis of the protein hormones of the anterior pituitary may have been increased in the vitamin B_{12}-treated rats. If so, this increase was probably associated with the increased food intake, growth and protein synthesis of these animals.

SUMMARY

Female rats were maintained on a soybean meal diet containing 12% of protein. The effect of supplementing the diet by injecting 7.5 μg of vitamin B_{12} daily was studied in both ad libitum and paired-feeding experiments. A 15% solution of ethyl alcohol was the only drinking solution for sub-groups of both the vitamin B_{12}-treated and the control rats fed ad libitum.

The rats receiving vitamin B_{12} and eating the deficient diet ad libitum grew more rapidly, ate more of the diet and retained more nitrogen than their controls. Vitamin B_{12} was also associated with increased growth, food intake and nitrogen balance in the alcohol treated rats. In the pair-fed rats, vitamin B_{12} did not significantly increase either growth or nitrogen retention. Caloric intake was essentially the same for the ad libitum rats on the diet alone as for rats on diet plus alcohol. In the rats receiving vitamin B_{12}, the total caloric intake was considerably greater than in the deficient rats and did not differ significantly between the group on vitamin B_{12} plus water and the group on vitamin B_{12} plus alcohol. Alcohol *per se* was associated with decreased growth, food intake and nitrogen balance. In both the paired feeding and the ad libitum experiments, the administration of vitamin B_{12} was associated with lower levels of liver fat and higher levels of liver nitrogen. Drinking 15% ethanol for the period of the experiment did not further alter the liver composition with respect to total fat and nitrogen. Administration of vitamin B_{12} led to a decrease in the plasma albumin expressed

as percentage of the total plasma proteins in the ad libitum-fed rats. Alcohol further decreased plasma albumin. Total plasma proteins at sacrifice were not affected by any regimen tried; vitamin B_{12} had no effect on plasma albumin levels of the pair-fed rats.

Adrenal weight per 100 gm of body weight was not affected by the administration of vitamin B_{12} or alcohol. Adrenal cholesterol was decreased in the ad libitum-fed rats on vitamin B_{12} regardless of whether or not they had ingested ethyl alcohol. Vitamin B_{12} did not affect the adrenal cholesterol concentration of the pair-fed rats.

Microscopic sections of the livers from animals treated with vitamin B_{12} showed much less fat than did the sections from deficient rats. This was also the case when alcohol and vitamin B_{12} were given.

ACKNOWLEDGMENT

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DEPOSITION OF FLUORIDE, CALCIUM AND PHOSPHORUS IN EXPERIMENTAL LOW-PHOSPHORUS RICKETS

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It has been shown that 95% of the fluoride present in an animal carcass is found in the bones (Lawrenz et al., '40) and thus it is of interest to observe the deposition of fluoride in these tissues in various types of bone dyscrasias. Perhaps the most widely studied disease affecting calcification is rickets. The deposition of fluoride in rachitic animals has been reported in two studies both of which pertained to elevated levels of fluoride (Shultz, '36; Kempf and Nelson, '41). No data were presented in one study (Shultz, '36) and the deposition of fluoride was determined in the other (Kempf and Nelson, '41) only during a short period of healing. It has been noted also that 300 p.p.m. of fluoride reduced the severity of developing rickets by increasing bone density (Morgareidge and Finn, '40) and, in addition, the life span of rachitic rats appeared to be increased (Finn and Kramer, '40).

The purpose of the present experiments, therefore, was to study the deposition of fluoride in rachitic rats receiving a low level of fluoride in the drinking water. In addition, data are presented on the fat, ash, calcium and phosphorus content of the femurs, mandibles, and teeth. The design of the study permits the separation of the effects of rickets *per se* and inanition.

EXPERIMENTAL

A diet adequate in calcium and vitamins and extremely limited in phosphorus (0.40% Ca and 0.017% P) was first proposed by Day and McCollum (Day and McCollum, '39; Follis et al., '40) and later modified (Coleman et al., '50, '53; Cramer et al., '56) as a simplified approach to the production of experimental rickets. The diet as modified by Coleman et al. ('50) was employed in this study and contained 0.442% Ca, 0.018% P and 2.8 p.p.m. F. The control diet was supplemented with Na_2HPO_4 to provide adequate phosphorus and an adequate Ca/P ratio and on analysis was found to contain 0.437% Ca, 0.559% P and 3.0 p.p.m. F.

Ten litters of three female weanling, Sprague-Dawley rats each were divided into three equal groups as follows: group I received the rachitogenic diet and groups II and III received the rachitogenic diet supplemented with Na_2HPO_4. Group II was pair-fed to group I, and group III was fed ad libitum. The fluoride intake which was provided in the drinking water was approximately equalized for all groups. This was accomplished by periodic changes in the fluoride content of the water. Most of the time the water contained 20 p.p.m. of fluoride. The rats were individually housed in screen-bottom cages and the diet intakes were recorded. The rats were roentgenographed after three weeks and sacrificed at 6 weeks. The mandibles and femurs were dissected free of soft tissue, and molars and incisors removed. All samples were dried overnight at 105°C, ashed for three hours at 550°C, dissolved in 2 ml of 1.0 N HCl per 100 mg of ash and diluted to an appropriate volume. Suitable aliquots were removed and analyzed for fluoride (Willard and Winter, '33, as modified by Armstrong, '36 and McClure, '39), calcium and phosphorus (A.O.A.C., '55, p. 102 and 115). Representative samples of the dry bones and teeth were extracted for 8 hours with alcohol and for 4 hours with ether for the determination of fat.

RESULTS AND DISCUSSION

A. General. The mean weight gains, diet and fluoride intakes and dry weights of the femurs, mandibles, molars and incisors are presented in table 1. The three groups of rats had a mean fluoride intake of 12.3 to 13.2 mg. Although receiving equal quantities of diet, the pair-fed non-rachitic rats gained significantly more than their rachitic pair mates. The rats fed ad libitum and receiving the phosphorus-supplemented diet showed adequate weight gains of 2.3 gm/day and food utilization at the rate of 0.27 gm weight gain per gram of diet.

TABLE 1

Weight gains, diet and fluoride intakes, and weights of skeletal and dental tissues of rats receiving a low phosphorus rachitogenic diet supplemented with Na_2HPO_4

	RATS RECEIVING RACHITOGENIC DIET [1]	RATS RECEIVING RACHITOGENIC DIET + Na_2HPO_4, PAIR-FED	RATS RECEIVING RACHITOGENIC DIET + Na_2HPO_4, AD LIBITUM-FED
Weight gain, (gm/day)[2]	0.5 ± 0.02 [3]	0.7 ± 0.04	2.3 ± 0.1
Diet intake, (gm/day)	4.6 ± 0.1	4.4 ± 0.1	8.3 ± 0.2
F intake, (mg)	12.8 ± 0.5	12.3 ± 0.4	13.2 ± 0.3
Oven-dry weights of skeletal and dental tissues			
	gm	*gm*	*gm*
Femurs	0.239 ± 0.012	0.430 ± 0.008	0.589 ± 0.015
Mandibles	0.091 ± 0.004	0.212 ± 0.005	0.250 ± 0.009
Molar teeth	0.104 ± 0.001	0.116 ± 0.002	0.124 ± 0.005
Incisor teeth	0.149 ± 0.004	0.177 ± 0.002	0.191 ± 0.003

[1] Initial mean weight of each of the three groups of 10 rats was 37 gm.
[2] Duration of study was 42 days.
[3] Standard errors calculated according to Mantel ('51).

It was evident from the roentgenographs that the rats receiving the low-phosphorus diet had developed rickets as previously described, whereas rats given the phosphorus-supplemented diet were normal. Characteristic of classical rickets, the femurs and mandibles of the rachitic rats weighed markedly less than those of their non-rachitic litter mates. Only small weight differences occurred in the teeth.

The mean concentration of fat of the femurs of 5 randomly selected rats from each group was: rachitic, 17.7 ± 1.4% [1]; pair-fed, 9.3 ± 1.5%; and ad libitum-fed, 5.1 ± 0.6%. Similarly the mandibles contained 8.8 ± 0.4%, 3.8 ± 0.4% and 2.9 ± 0.6% respectively. The molars contained 1.7 ± 0.2%, 1.6 ± 0.4%, 1.9 ± 0.2%, and the incisors 1.3 ± 0.3%, 1.0 ± 0.1% and 1.3 ± 0.1% fat respectively. The femurs and mandibles of the rachitic rats thus contained markedly higher concentrations of fat than the same bones of the non-rachitic rats, whereas no change was found in the molars or incisors.

As shown in table 2, the ash concentration of the bones of the rachitic rats was markedly less than that of their non-rachitic pair-fed litter mates ($P < 0.001$) whereas smaller reductions were seen in the ash concentration of the molars and incisors ($P < 0.05$).

B. Deposition of fluoride in the bones and teeth. Although the bone ash of the rachitic rats showed a two and one-half-fold increase in fluoride concentration when compared with their non-rachitic pair-fed mates (table 2), only about half as much total fluoride was found. No differences in deposition of fluoride were apparent in the molars and incisors of the rachitic and their non-rachitic pair-fed litter mates.

Differences between the rachitic and the non-rachitic pair-fed litter mates represent the effect of rickets since the diet intakes were equalized. On the other hand, variations between the pair-fed and ad libitum-fed non-rachitic rats are attributed to inanition. No differences were found in the concentration of ash and fluoride in either the mandibles or the molars of the two groups of non-rachitic rats and only small differences were seen in the femurs and incisors (table 2). Differences in ash and deposition of fluoride in the present study are therefore due to the rachitogenic diet rather than to inanition.

The markedly higher concentration of fluoride but lower total fluoride in rachitic bones as compared to bones of non-rachitic rats is explained largely by the smaller amount of bone ash present in rachitic bones. This situation would

[1] Standard error.

TABLE 2

Deposition of fluoride in the ash of the bones and teeth

	RACHITIC (R)	PAIR-FED (PF)	AD. LIB. FED (ALF)	P VALUES [2]	
				R vs PF	PF vs ALF
Femurs					
% Ash, oven dry	18.28 ± 0.75 [1]	48.20 ± 0.49	51.43 ± 0.82	< 0.001	< 0.001
% F, ash	0.435 ± 0.015	0.179 ± 0.007	0.150 ± 0.006	< 0.001	> 0.05
Total F, mg	0.187 ± 0.009	0.370 ± 0.019	0.442 ± 0.029	< 0.001	< 0.05
Mandibles					
% Ash, oven dry	31.77 ± 1.31	57.84 ± 0.55	57.11 ± 1.07	< 0.001	> 0.05
% F, ash	0.410 ± 0.024	0.163 ± 0.008	0.131 ± 0.004	< 0.001	> 0.05
Total F, mg	0.116 ± 0.005	0.199 ± 0.011	0.181 ± 0.010	< 0.001	> 0.05
Molars					
% Ash, oven dry	74.32 ± 0.65	76.28 ± 0.38	76.17 ± 0.37	< 0.05	> 0.05
% F, ash	0.069 ± 0.007	0.060 ± 0.003	0.055 ± 0.003	> 0.05	> 0.05
Total F, mg	0.055 ± 0.004	0.053 ± 0.004	0.052 ± 0.004	> 0.05	> 0.05
Incisors					
% Ash, oven dry	73.53 ± 0.58	75.45 ± 0.69	76.42 ± 0.31	< 0.05	> 0.05
% F, ash	0.103 ± 0.007	0.090 ± 0.005	0.067 ± 0.003	> 0.05	< 0.01
Total F, mg	0.117 ± 0.007	0.121 ± 0.007	0.098 ± 0.005	> 0.05	< 0.05

[1] Standard error.

[2] Calculated by analysis of variance for 10 litter-mate trios.

furnish fewer exchangeable sites for fluoride per unit volume of rachitic bone. It is likewise possible that differences in composition of the bone mineral itself, as evident in the Ca/P ratio (table 3) could play some part in skeletal fluoride deposition. Other mechanisms such as fluoride incorporation in forming bone and recrystallization (Neuman and Neuman, '50) may also contribute to differences in fluoride retained by calcified tissues.

No data on the deposition of fluoride in severe phosphorus deficiency appear to be available. The deposition of fluoride in rats receiving diets containing less drastic changes in the Ca/P ratio and also containing low levels of fluoride has been reported by Lawrenz and Mitchell ('41). They found that increasing the phosphorus content from 0.14 to 0.71% in a diet containing 0.71% of Ca plus adequate amounts of vitamins A and D increased the weight of the bones but decreased the concentration of fluoride, so that the total fluoride content was unchanged. The rats received 13 and 32 p.p.m. of fluoride in the diet. The phosphorus deficiency of their diet evidently was not sufficient to produce the marked changes in fluoride deposition observed in the present study where the diet contained only 0.018% of P. The effects of simple inanition in the present study parallel those of Lawrenz and Mitchell ('41) on the low-phosphorus diet in that the total F deposited in the bones and teeth was similar in the rats on either the pair-fed or ad libitum regimen.

C. Calcium and phosphorus in the bones and teeth. The ash of the femurs and mandibles of the rachitic rats was lower in calcium and higher in phosphorus, so that a markedly lower Ca/P weight ratio was found than in the same bones of the non-rachitic pair-fed rats. No changes were observed in either the bones of the two non-rachitic groups or in the molar and incisor teeth of all three groups.

Although a large body of data is available on the ash, calcium and phosphorus content of the bones and teeth of rachitic rats and requires no additional review (Karshan, '33, Karshan and Rosebury, '33, Shohl et al., '33, Sobel and Hanok,

TABLE 3

Concentration of calcium and phosphorus in the ash of the bones and teeth

	RACHITIC (R)	PAIR-FED (PF)	AD. LIB. FED (ALF)	P VALUES [2]	
				R vs PF	PF vs ALF
Femurs					
Ca	33.11 ± 0.47 [1]	36.79 ± 0.13	36.91 ± 0.35	< 0.001	> 0.05
P	20.37 ± 0.29	18.57 ± 0.24	18.22 ± 0.18	< 0.001	> 0.05
Ca/P	1.63 ± 0.02	1.98 ± 0.02	2.03 ± 0.02	< 0.001	> 0.05
Mandibles					
Ca	35.05 ± 0.73	37.47 ± 0.46	37.10 ± 0.26	< 0.01	> 0.05
P	19.34 ± 0.17	18.80 ± 0.32	18.94 ± 0.16	> 0.05	> 0.05
Ca/P	1.82 ± 0.05	2.00 ± 0.02	1.96 ± 0.02	< 0.001	> 0.05
Molars					
Ca	38.14 ± 0.21	37.70 ± 0.27	37.94 ± 0.28	> 0.05	> 0.05
P	19.37 ± 0.20	19.53 ± 0.27	19.50 ± 0.36	> 0.05	> 0.05
Ca/P	1.97 ± 0.03	1.93 ± 0.03	1.95 ± 0.03	> 0.05	> 0.05
Incisors					
Ca	35.85 ± 0.15	35.65 ± 0.11	35.28 ± 0.25	> 0.05	> 0.05
P	20.12 ± 0.28	21.01 ± 0.29	21.80 ± 0.63	> 0.05	> 0.05
Ca/P	1.79 ± 0.03	1.70 ± 0.03	1.63 ± 0.03	< 0.05	> 0.05

[1] Standard error.

[2] Calculated by analysis of variance for 10 litter-mate trios.

'48), it cannot be compared unequivocably with the present data because of the imposed ingestion of fluoride in the present study. The marked resistance to change in the calcium and phosphorus concentration of the molars and incisors in this study has been observed with other stress diets (Karshan, '33; Karshan and Rosebury, '33; Wynn et al., '57; McClure, '58). Fluoride deposition in the teeth also was not affected in the present study by the rachitic state of the animal.

Rachitic rats as compared to their non-rachitic, pair-fed mates showed the greatest changes in the femurs, followed by the mandibles. Minor or no differences were observed in the various constituents between the non-rachitic pair-fed and ad libitum fed rats. Changes in the rachitic rat were due, therefore, to the extreme phosphorus deficiency of the diet, rather than to simple inanition.

SUMMARY

1. Rats were rendered rachitic on a diet adequate in vitamins A and D and in calcium but markedly deficient in phosphorus. Litter-mate rats received the same diet supplemented with Na_2HPO_4. An equalized intake of fluoride was provided by the drinking water. Ash, fat, fluoride, calcium and phosphorus were determined in femurs, mandibles, molar and incisor teeth.

2. The bones of the rachitic rats showed a marked reduction in ash, whereas the teeth were essentially normal. The bones of the rachitic rats also contained less calcium but more phosphorus, resulting in a highly significant reduction in the Ca/P ratio. There was no change in the calcium or phosphorus concentration of the teeth of rachitic rats, nor in the Ca, P or Ca/P ratio in the bones and teeth of the non-rachitic pair-fed and ad libitum-fed rats.

3. The femur and mandible ash of the rachitic rats was increased two and one-half times in fluoride content as compared with the bone ash of non-rachitic litter mates. However, markedly less total fluoride was present in the rachitic bones. Fluoride in the teeth was not altered by the rachitic state of the animals.

4. The data permitted the conclusion that the observed changes in the rachitic rats were due to the extremely low phosphorus content of the diet rather than to inanition.

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GROWTH OF GERM-FREE AND CONVENTIONAL CHICKS: EFFECT OF DIET, DIETARY PENICILLIN AND BACTERIAL ENVIRONMENT [1]

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This report deals with the rearing of germ-free chicks and with a study of the growth response to dietary penicillin of germ-free and conventional chicks. Formulation of new diets for the rearing of germ-free chicks became necessary when in preliminary experiments diet L–165 described by Reyniers et al. ('50) failed to give satisfactory growth under our experimental conditions.

The study of the growth response to dietary penicillin was undertaken to answer the basic question if, as is the present consensus of opinion (Jukes, '55), antibiotics exert their effect by action on the intestinal microflora. Luckey ('52) reported that no stimulation of growth occurred when germ-free chicks and turkeys were fed antibiotics at a level of 50 mg/kg of diet. In subsequent studies Luckey et al. ('55) reported that lower levels of antibiotics (oxytetracycline 25 mg/kg, procaine penicillin 11 mg/kg) caused slight growth

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increments in germ-free chicks. These conclusions were, however, based on observations on a very limited number of birds. In more extensive studies with germ-free and conventional turkeys it was found that dietary penicillin (45 mg/kg) or oleandomycin (30 mg/kg) had a growth-promoting effect in conventional but not in germ-free turkeys. (Forbes, Supplee and Combs, '58).

The results reported in this paper were obtained in the past two and one half years and are based on experiments with over 350 germ-free chicks.[4]

EXPERIMENTAL

Chickens. White Leghorn chickens were used in the experiments. The method used for rearing germ-free chicks was patterned after that of Reyniers et al. ('49). Eighteen-day-old embryonated eggs were immersed for two minutes at 37°C in a 0.15% (W/V) detergent solution,[5] then for 10 minutes in a 2% (W/V) mercuric chloride solution maintained at 37°C. From this bath the eggs were then transferred through a tube without exposure to the non-sterile air into a previously steam sterilized Reyniers germ-free unit. All eggs, whether used to produce germ-free or conventional chicks, were subjected to the same treatment. Incubation was then completed either inside the germ-free units or in the incubator. As a rule 24 eggs were incubated in each tank and yielded from 18 to 22 chicks. The germ-free and conventional chicks were weighed and subdivided into two groups of 7 to 10 chicks of approximately equal average weight. In each germ-free unit the two groups of chicks were kept in wire cages (10 11/16″ wide, 22 1/8″ long, 12 3/4″ high and of 3/8″ × 3/8″ mesh size), one group serving as control, while the other received the diet supplemented with penicillin. Chickens were weighed weekly.

[4] In this report the word "germ-free" is intended to mean free of bacteria and fungi.

[5] PN-700 conditioner, Service Industries, Philadelphia, Pa.

Rearing conditions. The weight gain of chicks was studied under 4 environmental conditions. Chicks were reared: (1) in the Reyniers Units and maintained free of bacteria and fungi, (2) in the Reyniers Units but not maintained under sterile conditions, (3) in the animal room "pre-infection" from January 1956 to January 1958 and (4) in the animal room "post infection," i.e., after January 1958 when the animal room was intentionally contaminated with the lyophilized intestinal contents of chicks reared at the National Institute for Research in Dairying, Shinfield, Reading, England.

In the germ-free units the temperature during the hatching of the chicks was 37°C; it was gradually reduced to room temperature from the first day until the 14th day of life of the chicks. In the animal room the chicks were reared on wire in standard electrically-heated brooders. The lights were kept on continuously both in the germ-free units and in the animal room.

Chicks from one batch of eggs were distributed in two or three groups and the growth and the growth response to dietary penicillin was studied simultaneously under two or three of the environmental conditions described.

Diets. One casein-starch diet (C-1) with two modifications (C-1R and C-7), and one soybean-corn meal diet (C-8) were used. The composition of these diets is shown in table 1. The diets were made up in accordance with the requirements for chicks as reported in Publication 301 of the National Research Council ('54). Vitamin supplements were adjusted so that after the autoclaving processes a 5- to 10-fold excess of each vitamin still would be available according to the reported lability of vitamins to steam sterilization (Reyniers et al., '50).

The main difference between diets C-1 and C-7 is that in the C-1 diet most of inorganic phosphate is provided as potassium monophosphate (K_2HPO_4) while in the C-7 diet it is in the form of calcium phosphate ($Ca_3(PO_4)_2$). The main difference between C-1 and C-1R is that in the C-1 diet after autoclaving the thiamine level was occasionally found to be

TABLE 1

Composition of diets

CONSTITUENT	AMOUNTS PER 100 GM OF DIET			
	C-1	C-1R	C-7	C-8
Soybean oil meal, gm	—	—	—	35.00
Yellow corn, gm	—	—	—	58.20
Cornstarch, gm	57.0	57.0	58.25	1.6 [1]
Casein (purified), gm	25.0	25.0	25.0	—
Corn oil, gm	5.0	5.0	5.0	0.5
Alphacel, gm	3.0	3.0	3.0	—
Glycine, gm	1.5	1.5	1.5	—
L-Arginine-HCl, gm	1.0	1.0	1.0	—
DL-Methionine, gm	0.5	0.5	0.5	—
Choline·HCl, gm	0.22	0.27	0.27	0.27
Thiamine·HCl, gm	0.01	0.1	0.025	0.1
Ca Pantothenate, mg	10.0	10.0	10.0	10.0
Nicotinic acid, mg	10.0	10.0	10.0	10.0
Riboflavin, mg	2.0	4.0	4.0	4.0
Pyridoxine·HCl, mg	2.0	2.0	2.0	2.0
Folic acid, mg	1.0	1.0	1.0	1.0
Vitamin B_{12}, mg	0.005	0.005	0.005	0.005
Biotin, mg	0.1	0.1	0.1	0.1
Menadione, mg	0.8	0.8	0.8	0.8
Vitamin A, I.U.	2,000	2,600	2,600	2,600
Vitamin D_3, ICU	100	100	100	100
a-Tocopherol, mg	5	5	5	5
$CaCO_3$, gm	2.50	2.5	—	1.6
K_2HPO_4, gm	1.72	1.72	—	—
Na_2HPO_4, gm	1.40	1.40	0.5	—
$Ca_3(PO_4)_2$, gm	—	—	3.16	—
KCl, gm	—	—	0.7	—
NaCl, gm	0.5	0.5	0.6	0.5
$MgSO_4 \cdot 7H_2O$, gm	0.5	0.45	0.4	—
$MnSO_4 \cdot H_2O$, mg	—	30	40	30
$MnCl_2 \cdot 4H_2O$, mg	25	—	—	—
$FeSO_4$, mg	12	12	12	8
$CuSO_4$, mg	1	1	1	0.8
$CoCO_3$, mg	1	1	1	0.5
$ZnSO_4 \cdot 7H_2O$, mg	1	1	1	1
KI, mg	0.3	0.3	0.3	0.15
$CaHPO_4 \cdot 7H_2O$, gm	—	—	—	2.2

[1] Used as mix for B vitamins.

suboptimal while in the autoclaved C-1R diet it exceeds the requirement of the chick 5- to 10-fold. Thiamine levels in the diets were determined by the fluorimetric method described in the Official Methods of Analysis of the Association of Official Agricultural Chemists ('55).

Feed was made up and mixed in 10- or 20-kg batches, distributed in 1-kg portions in gauze bags, sterilized and then either transferred into the tanks or made available for the conventional chicks in the animal rooms. The feed was sterilized by placing a 1-kg portion in a 9″ × 17″ gauze bag in a layer not exceeding one inch in thickness in the individual clave of each germ-free unit. The sterilization process was started by creating and maintaining a vacuum of 26 inches of mercury for 5 minutes, the vacuum was broken with saturated steam which was allowed to flow through the clave for 10 minutes, whereupon steam pressure was allowed to rise to 17 pounds per square inch (252 to 255° F.) and was maintained for 25 minutes, the materials inside the clave were then dried by creating and maintaining a vacuum for 20 minutes, after which the vacuum was broken with filtered sterile air.

In the tanks, whether under germ-free or under contaminated conditions, the chickens were given canned sterilized water.[6] In the animal room however, tap water was offered. Food for 7 to 10 chicks was offered in covered trays (19 7/8″ long, 2 1/2″ wide) provided with 9 holes (1 3/4″ × 1 1/8″). Food and water were offered ad libitum. Food intake was measured by difference with no allowance for spillage.

Weighed quantities of penicillin in starch were sealed in ampuls, sterilized by irradiation and introduced into the germ-free units via the germicidal trap. Each ampul contained either 25 mg of potassium penicillin or 45 mg of procaine penicillin mixed with 1 gm of starch. Irradiation[7] (1,800,000

[6] MacDonald-Bernier Co., Boston, Mass.

[7] Irradiation of the various samples was performed by Dr. Howard Andrews, Radiation Branch, National Cancer Institute, and Mr. John Hickey, Sanitary Engineering Branch, National Institutes of Health, Bethesda, Md. to whom we are greatly indebted for this service.

rep from a high voltage electron source) did not affect the antibacterial activity of the penicillin as measured by the tube dilution method using *Micrococcus pyogenes var. aureus* H as test organism. The content of each ampul was mixed with 1 kg of sterilized food inside the germ-free unit. Random samples of the supplemented food were taken from the food trays and assayed for penicillin content. Procaine penicillin was always found to be present at the expected level; potassium penicillin, however, had a half life of about three days when the food which contained potassium penicillin stood in the tank at 35°C. As a rule fresh food was offered about twice a week.

Sterility tests. At least once a week swabs in test tubes, fresh thioglycollate medium and trypticase soy broth were placed in the tanks. Moistened swabs were used to take anal samples or fresh fecal specimens from all animals. Samples of food, water and waste material were also collected. Some of the anal swabs were immediately inoculated in the thioglycollate and in the broth tubes. Other samples of fecal material and anal swabs were put in cotton-stoppered test tubes and were taken out of the tank with the inoculated media. Gram stains were made of smears from the anal swabs or from the fecal specimens. The remaining samples were inoculated in cooked meat medium, in trypticase soy broth, on Sabouraud's agar, as well as on tomato juice agar and on brain-heart infusion agar containing 5% blood. Replicate cultures were incubated at room temperature and at 37°C. Some of the blood agar plates were also incubated under anaerobic conditions. Cultures were examined periodically over a period of two weeks. Gram stains were made of samples of the broth cultures. Selected blood plates were examined for tiny colonies under the stereoscopic microscope. In some but not all experiments one animal was sacrificed at the termination of the experiment and samples of intestinal contents were cultured in the various media.

Statistical analysis. The Fisher F test was used, data from successive replicate experiments being combined. Since it was not possible to obtain equal numbers of males and of females

in each group, the numbers of animals in the various groups were equalized by selection using a table of random numbers. Thus where in some tables groups of 7, 10 or 11 chicks were compared, in actuality these represent a random choice from a much larger number of chicks in most groups. No discrepancy exists between the total data as compared to the randomly selected data.

TABLE 2

Growth of germ-free and conventional chicks on a semisynthetic diet (C-1) with and without supplements of potassium penicillin

ADDITION TO DIET	SEX	WT. ON 28TH DAY (11 CHICKS/GROUP)		
		Germ-free	Conventional in R.U.[1]	Conventional in A.R.[1]
		gm	*gm*	*gm*
None	M	383	293	299
	F	332	292	281
Potassium penicillin, 25 mg/kg	M	353	311	282
	F	346	296	283

[1] R.U. = Reyniers Units; A.R. = Animal Room.

Analysis of variance

SOURCE OF VARIATION	DF	MS [2]	F	p
Germ-free vs. conventional in R.U. and A.R.	1	108,743	71.1	< 0.001
Conventional in R.U. vs. A.R.	1	2,910	1.9	Not signif.
Penicillin vs. no penicillin	1	67	1	Not signif.
Male vs. female	1	7,276	4.8	< 0.05
All other interactions	7	12,633		Not signif.
Error	120	1,529		

[2] Mean square.

RESULTS

Effect of diets on the growth of germ-free chicks

Growth of germ-free and conventional chicks (table 2) appeared adequate on the C-1 diet. However, in occasional experiments growth was somewhat depressed and the weights varied widely. This suggested a border line deficiency. Since thiamine is the only vitamin that is rapidly destroyed during steam sterilization (Reyniers et al., '50) it was most likely to be the critical factor. Analyses of the diet from several ex-

periments indicated a thiamine content of 0.4 to 2.1 mg per kilogram of food with an average of 1.3 mg. Thus the level of thiamine found in the autoclaved food was such that 99% destruction had occurred in contrast to 85 or 90% found by Reyniers et al. ('50) using a slightly milder sterilization procedure. The minimum requirement of conventional chicks is 1.7 mg/kg, and preliminary experiments have indicated that the requirement of the germ-free chick for thiamine is essentially the same.

Since a small extension in sterilization time resulted in considerable additional destruction of thiamine, a simple method to improve the heat stability of the thiamine was sought. It had been shown (Waibel et al., '54) that K_2HPO_4 accelerated the destruction of thiamine. Hence diet C-7 was devised by altering the salt mixtures as shown in table 1. Only 87% of the thiamine in diet C-7 was destroyed during autoclaving so that sterilization conditions were not quite as critical as with diet C-1. However, growth of the germ-free chicks on the C-7 diet occasionally appeared to be slower than on diet C-1 (table 3 vs table 2). The reason for slower growth may be the unavailability of the calcium and the phosphate fed as calcium phosphate.

The toes of some of the chicks fed the C-7 diet curled outwards. It was not determined which nutritional factors were involved, but the condition was not observed in chicks fed the other diets.

The suboptimal thiamine level sometimes found in the C-1 diet caused by small variations in the steam sterilization process could also be prevented by increasing the thiamine content 10-fold. Thus diet C-1R, containing adequate thiamine, was found to give a significantly better growth than diet C-7 (table 4). Adequate growth was also obtained with the C-8 diet, a soybean-corn meal diet with high thiamine supplement (table 5).

Effect of the intestinal flora on the growth of chicks

As shown in tables 2 and 3, conditions in the Reyniers Units *per se* have no effect on the growth of chicks. Chicks reared

in units which intentionally were not maintained sterile grew at the same rate as chicks reared in batteries in the animal room.

TABLE 3

Growth of germ-free and conventional chicks on a semisynthetic diet (C-7) with and without supplements of procaine penicillin

ADDITION TO DIET	SEX	WT. ON 28TH DAY (10 CHICKS/GROUP)		
		Germ-free	Conventional in R.U.	Conventional in A.R.
		gm	*gm*	*gm*
None	M	330	322	301
	F	321	299	303
Procaine penicillin, 45 mg/kg	M	361	315	321
	F	298	303	314

Analysis of variance

SOURCE OF VARIATION	DF	MS	F	p
Main effects				
Germ-free vs. conventional in R.U. and A.R.	1	8,143	5.9	< 0.05
Conventional in R.U. vs. conventional in A.R.	1	1	1	Not signif.
Penicillin vs. no penicillin	1	1,166	1	Not signif.
Male vs. female	1	10,268	7.4	< 0.01
Interactions				
All other interactions	7	14,488	—	Not signif.
Error	108	1,381		

Germ-free chicks on the C-1 diet gained 14 to 30% more weight than chicks fed the same autoclaved diet but reared under contaminated conditions (table 2). On the C-7 diet the difference in growth between the germ-free and conventional chicks was not as marked, yet was statistically significant (table 3). On the "natural" diet (C-8) growth of the germ-free chicks was 18 to 25% better than that of chicks from the same batch reared in the animal room (table 5). Again on the C-1R diet the germ-free chicks showed increased weight gain compared to the conventional chicks (table 8). These results demonstrate that one or more components of the intestinal flora have a growth-depressing effect on the chicks.

The germ-free chicks which grew at a faster rate consumed about the same amounts of food per gram of body weight as the contaminated chicks reared in tanks or in the animal room. Thus average food efficiency, measured as grams of food consumed per gram of weight gain, was the same for the germ-free birds as for the conventional birds (table 6). Food efficiency on the casein-starch diet was 2.7 and was 4.4 on the soybean-corn meal diet. These ratios can only be considered as approximations due to the uncontrolled spillage of food.

TABLE 4

Comparison of the growth of germ-free chicks on semi-synthetic diets C-1R and C-7 [1]

SEX	AV. WT. ON 28TH DAY (7 CHICKS/GROUP)	
	C-1R DIET	C-7 DIET
	gm	
Male	403	362
Female	362	303

Analysis of variance

SOURCE OF VARIATION	DF	MS	F	p
C-1R vs. C-7 diet	1	17,500	9.7	$< .01$
Male vs. female	1	17,701	9.8	$< .01$
Interaction	1	514	0.3	Not signif.
Error	24	1,801		

[1] The main difference between diets C-1R and C-7 is that in C-1R calcium and phosphate are provided mainly as potassium phosphate (K_2HPO_4) and calcium carbonate ($CaCO_3$) while in the C-7 diet they are offered mainly as calcium phosphate ($Ca_3(PO_4)_2$). The C-1R diet contained 0.1 gm and the C-7 diet 0.025 gm of thiamine·HCl per 100 gm of diet.

Effect of dietary penicillin in a clean environment

In repeated experiments extending over a period from January 1956 to January 1958 potassium penicillin (25 mg/kg) or procaine penicillin (45 mg/kg) added to the C-7, C-8 or the C-1R diet failed to elicit a growth response in the germ-free or conventional chicks (tables 2, 3, and 5). The possibility that autoclaving of the diet prevented the occurrence of the growth-promoting effect was investigated. Experiments with

conventional chicks from the same batch reared in the animal room fed autoclaved and non-autoclaved C-8 diet showed that chicks grew better on the non-autoclaved diet than on the autoclaved diet. However, supplements of penicillin had no effect on the growth of the chicks on either the autoclaved or the non-autoclaved diet.

TABLE 5

Growth of germ-free and conventional chicks on a soybean meal-corn diet (C-8) with and without supplements of procaine penicillin

ADDITION TO DIET	SEX	AV. WT. ON 28TH DAY (11 CHICKS/GROUP)	
		Germ-free	Animal room
		gm	*gm*
None	M	363	288
	F	332	280
Procaine penicillin, 45 mg/kg	M	354	301
	F	323	281

Analysis of variance

SOURCE OF VARIATION	DF	MS	F	p
Germ-free vs. animal room	1	66,056	53.6	< 0.001
Penicillin vs. no penicillin	1	10	1	Not signif.
Male vs. female	1	10,847	8.8	< 0.01
All other interactions	4	3,289		Not signif.
Error	80	1,233		

Effect of dietary penicillin in an "infected" environment

Since chicks had not previously been raised in our animal room and since only chicks issuing from decontaminated eggs were reared in this room, the flora presumably was not the one encountered in the usual chick laboratory. In order to test this possibility lyophilized intestinal contents[8] were obtained from chicks reared in the animal rooms of the National Institute for Research in Dairying, Shinfield, England, where a growth response to penicillin was regularly obtained. One or two grams of this lyophilized material was mixed with 2 kg of C-1R diet, after which the penicillin supplement was

[8] Kindly prepared and furnished by Dr. M. Lev, National Institute for Dairying, Shinfield, Reading, England.

TABLE 6

Food efficiency of germ-free and conventional chicks

STATUS	DIET	NO. OF EXPTS.	NO. OF CHICKS	AV. GAIN PER CHICK [1]	AV. FOOD USED/CHICK	AV. FOOD USED PER GRAM GAIN
				gm	*gm*	*gm*
Germ-free	C-1	5	40	307 ± 12 [2]	772 ± 55	2.7 ± 0.2
Conventional in Reyniers Units	C-1	3	27	240 ± 9	663 ± 50	2.7 ± 0.2
Conventional in Animal Room	C-1	3	34	249 ± 14	706 ± 113	2.7 ± 0.4
Germ-free	C-8	5	41	310 ± 7	1326 ± 182	4.1 ± 0.2
Conventional in Animal Room	C-8	6	62	241 ± 6	1076 ± 106	4.4 ± 0.2

[1] Growth for 28 days.

[2] Standard error of the mean.

added to half of the ration. In this experiment and in all successive experiments a growth response to dietary penicillin occurred. After three successive experiments the growth response to penicillin supplements occurred even when the lyophilized intestinal material was no longer mixed with the diet. Comparison of the weights of the chicks in these experiments with those performed immediately before the animal room was "infected" indicates that after the "infection" the growth of the chicks was depressed and that supplements of penicillin reversed this growth inhibition (table 7). Penicillin restored the growth rate of the chicks to the "pre-infection" level. Except for a decreased growth rate no abnormalities were noted in the chicks raised in the "infected" quarters. A comparison of the growth response to penicillin of germ-free chicks with that of chicks raised in the "infected" animal room indicated that penicillin mixed with the diet stimulates the growth of the conventional but not that of the germ-free birds (table 8). Penicillin did not accelerate the growth rate of the conventional chicks to a level equal to that of the germ-free chicks.

TABLE 7

Growth of conventional female chicks on diet C-1R with and without procaine penicillin before and after deliberate "infection" of the animal room [1]

STATUS	AV. WT. ON 28TH DAY (21 CHICKS/GROUP)	
	No supplement	Penicillin (45 mg/kg)
	gm	*gm*
Before contamination	315	300
After contamination	279	313

Analysis of variance

SOURCE OF VARIATION	DF	MS	F	p
Main effects				
Before vs. after contamination	1	2,851	1	Not signif.
Penicillin vs. no supplement	1	1,890	1	Not signif.
Interaction				
Status vs. treatment	1	12,463	10.9	< 0.001
Error	80	1,139		

[1] "Infection" with intestinal contents of chicks reared at the National Institute for Research in Dairying, Shinfield, England.

TABLE 8

Growth and growth response to dietary procaine penicillin (45 mg/kg diet) of germ-free chicks and chicks reared in a deliberately "infected" animal room [1]

STATUS	SEX	AV. WT. ON 28TH DAY (7 CHICKS/GROUP)	
		No supplement	Penicillin
		gm	*gm*
Germ-free	M	368	385
	F	344	324
Conventional	M	312	345
	F	276	314

Analysis of variance

SOURCE OF VARIATION	DF	MS	F	p
Main effects				
Status: Germ-free vs. conventional	1	26,535	5.52	Not signif.[2]
Treatm: Penicillin vs. no supplement	1	4,097	< 1	Not signif.
Sex: M vs. F	1	19,800	4.12	Not signif.
Interactions				
Status vs. treatment	1	4,810	5.77	< 0.05
All other interactions	3	2,781	3.33	Not signif.
Error	48	834		

[1] "Infected" with intestinal contents of chicks raised at the National Institute for Research in Dairying, Shinfield, England.

[2] Not significant over and above the difference caused by status and treatment interaction.

DISCUSSION

The results support the thesis of Coates et al. ('52) that on an adequate diet penicillin can only "stimulate" the growth of chicks whose growth is retarded by infection with a penicillin-sensitive organism. In the absence of bacteria, or in the absence of infection by a penicillin-sensitive organism which reduces the growth rate, penicillin has no effect.

Preliminary experiments in which germ-free chicks were infected with pure cultures of *Escherichia coli* isolated from the intestines of normal chicks have shown that these do not depress the growth of the chicks free of all other bacteria. Lev, Briggs and Coates ('57) have reported that *Clostridia welchii* type A was found in chicks from "infected premises

one day after feeding, but not in those from the "clean" environment. A study of the growth response to penicillin of germ-free chicks infected with this strain of *Clostridia* and other intestinal organisms is reported elsewhere (Lev and Forbes, '59).

Inhibition of growth-depressing organisms is but one of the mechanisms of the growth-promoting effect of antibiotics. Other mechanisms are not excluded by the present findings. Thus Wiseman et al. ('56) have shown a growth-stimulating effect of penicillin and bacitracin in chicks on a diet low in folic acid. Such a sparing effect did not play a part in the results reported here.

SUMMARY

Chicks hatched and reared in the absence of bacteria and fungi grow 15 to 25% faster than conventional chicks on the same autoclaved casein-starch or soybean meal-corn diet. The increased growth rate is not due to improved feed efficiency but is related to the fact that the germ-free chicks eat more than the conventional chicks.

Better growth was obtained when potassium and calcium were provided in the diet in the form of potassium monophosphate and calcium carbonate rather than as calcium phosphate.

In repeated experiments over a two-year period using two different diets no growth response to dietary penicillin supplements was observed either in the germ-free or in the conventional chicks which were kept in an animal room where chicks had not been raised previously. A growth response to penicillin was obtained only after the animal room was deliberately "infected" with intestinal contents from chicks reared in premises where a growth response to antibiotics occurred regularly. The growth-promoting effect of dietary penicillin is shown to be due to a reversal of a transmissible growth-depression condition.

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SOME METABOLIC EFFECTS OF A HIGH-FAT, HIGH-PROTEIN DIET DURING SEMISTARVATION UNDER WINTER FIELD CONDITIONS [1]

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INTRODUCTION

Relatively little is known about the metabolic effects of a calorically deficient diet which is high in fat and protein and low in carbohydrate. Involuntary semistarvation, as in famines, usually involves a diet low in fat and protein. In particular, there is much confusion over the extent and duration of ketosis during semistarvation (Keys et al., '50). This seems to be largely a result of the laborious and inefficient methods in use, until very recently, for the estimation of ketones. Because of these technical difficulties, much of the extensive older literature was based on data too limited and unreliable to reveal the whole pattern of response to ketogenic diets.

The observations reported below were made during a field study of a survival ration composed almost entirely of fat and protein. Because of strict weight and space limitations, a primary consideration in the formulation of survival rations is maximum caloric density and a ration with the highest practicable fat content would seem to be the obvious answer. Serious consideration of this solution has been prevented, however, by a fear of ketosis.

In the course of preliminary studies in this laboratory (Drury, '56), it had become apparent that the usual reliance

[1] This paper received a security clearance dated July 28, 1958. The views expressed are those of the authors and do not necessarily represent official Air Force policy.

on qualitative spot tests for ketone bodies or a "strong odor of acetone on the breath" during field trials of high-fat rations had resulted in a very misleading impression of the magnitude of the ketosis problem. We had also been struck with the similarity of certain unpleasant symptoms, commonly attributed to ketosis, to those of hypoglycemia. These symptoms had rendered otherwise highly satisfactory rations extremely unpopular during short term field tests (Kark et al., '45). Our exploratory experiments had indicated that these symptoms were transitory, and, in addition, it seemed reasonable to suppose that they might be relieved to a great extent by the use of quite small doses of sugar given at strategic times in the course of the trials.

Accordingly, the experiments reported in this paper were planned to determine whether men can function adequately for a reasonable length of time under realistic survival conditions on a calorically restricted diet consisting almost entirely of pemmican — a dehydrated, high-fat, high-protein meat product with a long history of use in trail rations (Stefansson, '44). A further purpose was to measure some of the effects of a small sugar supplement to the basic pemmican diet.

EXPERIMENTAL

Ten healthy adult males, 21 to 40 years of age, were used throughout the 9 days study. Seven of the subjects were Air Force volunteers. The remaining three were civilian field investigators. The subjects' weights ranged from 140 to 220 pounds. During the study, outdoor temperatures varied from +14°F. to −55°F.

The subjects were restricted to an undeveloped and deserted area, in the center of which was a large unused but heated building where blood samples were drawn, nude weights obtained, and in which the subjects were allowed to remain under supervision for a total of not more than two hours per day.

Unheated snow caves were used as shelters throughout the study. The subjects slept in double sleeping bags on spruce bough beds. Due to the excellent thermal properties of the

caves (morning inside temperatures averaged +20°F.), little, if any, cold stress was experienced at night. The subjects wore standard Air Force arctic clothing during the day and were required to remain active outdoors for at least 9 hours per day. The men built a large community gathering place and their own snow caves, which were occupied in pairs as sleeping quarters. In order to generate heat, a great many snowshoe and ski trips were made over deep, soft snow for distances ranging up to a mile, as well as short hikes along cleared roads.

Each man received a ration containing 1000 Cal. of food energy per day. The ration was issued in the form of 5 ounces of pemmican [2] and a pillbox full of opaque gelatin capsules. The capsules of half of the men (the pemmican group) contained a total of one additional ounce of pemmican while those of the remaining subjects (the pemmican + sugar group) contained a total of 40 gm of sugar. The men were assigned to the two groups by the double blind method. This was done in an attempt to confine the study to objectively measurable physiological changes. In particular, it was desired to avoid dividing the subjects into privileged and underprivileged groups. For this same reason, the three field investigators shared the life of the rest of the subjects in every detail.

Since the primary purpose of the sugar supplement was to maintain the blood sugar level within normal limits with the smallest possible dilution of the high caloric density of pemmican, it was decided to spread the sugar intake over the whole day. Therefore, a rigid schedule was set up for the ingestion of the capsules. Each man divided his day's supply into equal doses and took one of these doses on-the-hour, every waking hour of the day. It was felt that this procedure would have the added advantage of preventing large fluctuations in level which might have been expected had the subjects, while

[2] Quartermaster Meat Food Product Bar, composed of equal amounts of cooked, dehydrated pork and beef with enough beef fat added to bring the final concentration up to 42 to 47%. The pemmican used in this investigation had the following average composition: protein, 46%; fat, 46%; water, 6%; salt, 2%.

in a semistarving condition, taken large doses of sugar at widely separated intervals.

No schedule was prescribed for the eating of the pemmican. Most of the men ate it at regular meal times, but there was some tendency to postpone or do without breakfast. Some of the men, while saving a sizeable portion for supper, which was usually cooked, found it desirable to nibble at the cold bar throughout the day.

A control period of two days, immediately preceding the field trial, established the levels of fasting blood sugar and urinary ketone body excretions and accustomed the men to the experimental procedures. During this time the men lived in a room set aside as a barracks in the laboratory and subsisted at the hospital mess. Throughout the entire study, 24-hour collections of urine were made and fasting blood sugars were determined daily by a modification of the method of Folin-Wu. Twenty-four-hour excretions of nitrogen were determined by the micro Kjeldahl method. Fecal nitrogen was ignored, chiefly because the diet had such a low residue that practically no feces, in several cases none at all, were produced. Under these conditions, the carmine marking technique is very unsatisfactory. Ketone body excretion was determined by converting the ketones to acetone by autoclaving, according to the method of Michaels et al. ('51), followed by steam distillation and subsequent determination of acetone by a modification of Frommer's test.

RESULTS AND DISCUSSION

All subjects completed the field study without difficulty. Although the laboratory studies brought out several significant differences between the two regimens on the physiological level, there were no easily discernible differences in the performance and subjective feelings of the two groups.

Consumption of the ration. The ration of the subjects in the pemmican group was 168 gm per day, totaling 1512 gm for the 9-day study. The pemmican allowance of the pemmican

TABLE 1

Food consumption and weight changes of the experimental subjects

SUBJECT NUMBER	DAILY CONSUMPTION OF PEMMICAN [1] DAY OF STUDY									TOTAL FOOD CONSUMPTION			MEAN DAILY CALORIC INTAKE	TOTAL WEIGHT CHANGE
	1	2	3	4	5	6	7	8	9	Pemmican	Sugar	Calories		
	gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*			*lb*
1	168	168	168	168	168	168	168	168	168	1512	0	8845	983	— 10.25
2	168	168	*156*	168	*84*	168	168	168	168	1416	0	8284	920	— 12.25
3	168	*142*	168	168	168	168	168	168	168	1486	0	8693	966	— 11.50
4	168	*126*	168	168	168	168	168	168	168	1470	0	8600	956	— 6.00
7	168	168	168	168	168	168	140 [2]	168	168	1484	0	8447	966	— 12.50
5	140	140	*72*	140	140	140	140	140	140	1192	360	8773	875	— 9.50
6	140	140	140	140	140	140	140	140	140	1260	360	9171	1019	— 11.25
8	140	140	140	140	140	140	140	140	140	1260	360	9171	1019	— 12.50
9	140	140	*65*	*56*	*56*	140	140	140	140	1017	360	7749	861	— 9.25
10	140	140	140	140	140	140	140	140	140	1260	360	9171	1019	— 9.75

[1] Reduced intakes due to refusals are italicised.

[2] The reduction in this case was due to loss of the capsules and does not represent a refusal of pemmican as such.

+ sugar subjects was 140 gm per day or 1260 gm for the whole study. The actual intakes are shown in table 1. It is readily apparent that there was no serious failure on the part of the subjects to eat approximately as much ration as was offered, although several were unable to eat all of their daily ration for a short period due to mild sensations of nausea. This difficulty was experienced only in the early part of the study. After the 5th day nausea disappeared and food refusals ceased. This is especially important in view of the fact that the most vigorous opposition to the use of pemmican (Kark et al., '45) was based on experiments which lasted only three days. Momentary faintness or dizziness upon standing up suddenly also occurred during the first few days of the study. This, of course, is a common symptom of starvation (Keys et al., '50).

As expected, the subjects grew weaker and tired more easily as the study progressed. We have no reason to believe that this slowly increasing weakness was due to anything other than general starvation, such as would occur on any 1000 Cal. diet.

Fluid balance. Weight changes are given in table 1. It is apparent that a substantial part of these losses must be ascribed to dehydration, since the average total decrease of 10.5 pounds, or over one pound per day, is much too great to be accounted for by the caloric deficit. The pattern of the weight losses is very suggestive. Thus, there was a large initial loss in weight when the men were placed on the restricted diet, following which the daily losses stabilized. The recovery period after termination of the study was characterized by a sharp initial gain in weight, tapering off after the first two or three days. A universal and striking accompaniment of the recovery period was a pronounced thirst during the first day or two following resumption of normal eating habits. Although this is indirect evidence of changes in water balance, it seems to confirm the observation of Consolazio and Forbes ('46), who reported a high retention of water and a sharp increase in body weight during early recovery.

The causes of these changes in fluid balance are unknown, but they appear to be related to the caloric intake and not to the specific composition of the diet (Drury, '56). If the weight curves of the subjects in Sargent's monumental study (Sargent et al., '53) are plotted, it will be seen that this same pattern of weight loss was obtained with all the diets tested at the 1000 Cal. level. We can see no reason why this type of dehydration, *per se,* should be detrimental to health or performance.

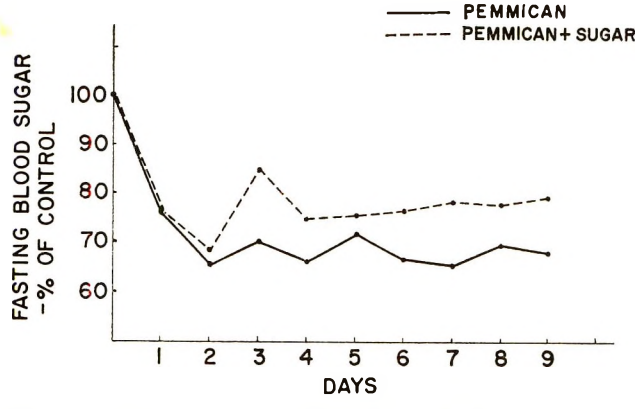

Fig. 1 Fasting blood sugar levels during the 9-day field study.

Fasting blood sugar. Figure 1 shows the daily fasting blood sugar values for all subjects expressed as percentage of control levels. An analysis of variance of the blood sugars, wherein these values were adjusted with regard to the regression of the post-treatment levels on the control levels revealed that during the field study, the fasting blood sugar values for the subjects who received the dietary sugar were significantly higher (at the 0.05 level of confidence) than those of men receiving pemmican only. No subjective differences were noted between the two groups which could be attributed to this difference in blood sugar levels.

Nitrogen balance. Figure 2 shows the mean daily nitrogen balances of the subjects. Considering the 9-day period as a

whole, there are no significant differences between the total, overall nitrogen balances of the subjects receiving sugar with their pemmican as against those receiving no sugar.

Ketosis. Daily ketone body excretions, measured as acetone, are shown in figure 3. A cursory examination of the data revealed that the values for subject no. 8 were exceptionally high. Without subject 8, the difference between the two groups is significant at a level between 0.05 and 0.10; with subject 8, there is no significant difference between the groups. When subject 8 was retested on the same diet several months

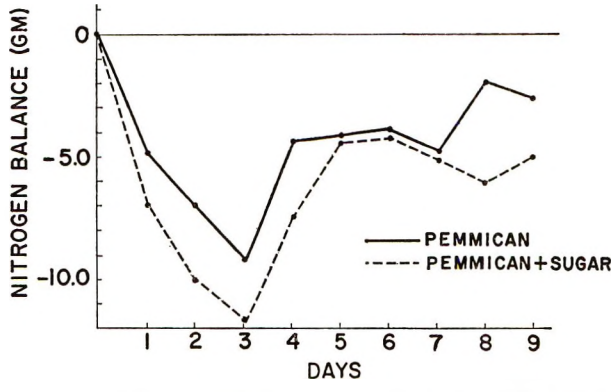

Fig. 2 Nitrogen balances during the 9-day field study.

later, his acetone production was within the "normal" range of the diet. The only possible clue to his behavior in the experiment was the attack of diarrhea which he suffered on the third day, and we have no proof whatever that the two phenomena were causally connected. Nevertheless, we have concluded that carbohydrate, at the low level employed in this experiment, does significantly depress ketosis in most individuals on a high-fat, low-calorie diet. Apparently it is possible, under certain as yet unknown circumstances, for normal individuals to show a temporarily elevated response to ketogenic stresses.

Two other conclusions can be drawn from the ketone body data. First, the excretion of ketone bodies in the most extreme

case (7 gm on the third day by subject 8) is still too small to produce deleterious effects of any appreciable magnitude. Second, there is a marked adaptation to the ketogenic diet. In most cases, the excretion of ketones was negligible by the last day. It should be noted in passing that our control values (mean = 121.2 mg per 24 hours) are somewhat higher than the figures usually reported. This is due to our use of an improved, quantitative method which accounts for β-hydroxybutyrate as well as acetoacetate and acetone. In the past,

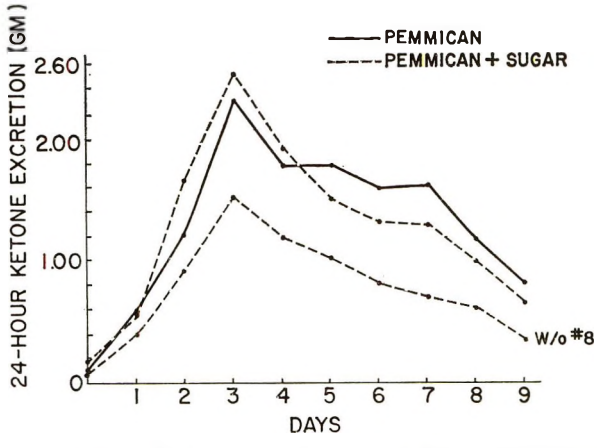

Fig. 3 Urinary ketone excretions during the 9-day field study.

most investigators have employed the Rothera reaction, which is far more sensitive to acetoacetate than to acetone, and which misses the β-hydroxybutyrate completely.

Metabolic adaptation. The sequential changes which have been mentioned briefly in the foregoing paragraphs indicate that there is an adaptation to a high-fat, high-protein, low carbohydrate, low-calorie diet. Thus, while the fasting blood sugar levels (percentage of controls) dropped sharply at the beginning of the period of restricted carbohydrate intake, they quickly leveled off and perhaps even showed some tendency to rise again (fig. 1), even though there was no increase in the availability of carbohydrate. The initial drop can be

plained as an indication of the depletion of liver glycogen, but why is this fall arrested? It is true that some of the amino acids and the glycerol derived from fat metabolism are glycogenic, but these potential sources of carbohydrate intermediates would not be capable of supplying the quantity of glucose metabolized by the normal, well-fed man. Apparently, the requirement is much reduced during semistarvation — a possible explanation of the significant increase in blood sugar levels brought about by the ingestion of a mere 40 gm per day.

The curve of daily nitrogen balances (fig. 2) follows a similar pattern. On the third day of the study, the nitrogen metabolism reached a maximum. Following this, it dropped rapidly until the 5th day. From then until the end of the study, nitrogen metabolism followed no well-defined pattern, although there was a tendency for the mean daily balances of the men receiving sugar to be somewhat lower than those of the men receiving pemmican alone.

The excretion of ketones, as shown in figure 3, reached a peak on about the third day of the experiment, after which it fell steadily, with the values approaching the range of normal urine by the 9th day. This phenomenon shows some similarity to the results cited by Sargent ('54), i.e., a transitory rise in ketone excretion. In our studies, however, there was a caloric deficit throughout the experiment. The curve which we obtained cannot be explained on the basis of changes in the level of physical activity of the subjects, since we have repeatedly obtained the same picture in subjects engaged in sedentary activity within the laboratory.

It is evident from the foregoing that the mechanism of intermediary metabolism must undergo modifications during the first few days of subsistence on a low carbohydrate diet. It has been observed that a diet high in fat and low in carbohydrate results in depressed utilization of glucose in man (Sweeney, '57; Himsworth, '34) and animals (Garner and Roberts, '55) as reflected in reduced tolerance to oral or intravenous glucose. A diminished carbohydrate utilization following a fast has also been reported in humans (Lundbaek et

al., '50). The fact that, in spite of the low sugar intake, the blood glucose levels of the men receiving sugar in the current study were elevated above those of the men receiving pemmican alone, seems to bear out these observations. The percentage of the total calories which was supplied by sugar (endogenous as well as exogenous) was probably too small to interfere materially with the trend toward adaptation to a fat and protein diet.

There is also some evidence that animals may become adapted to a high-fat diet and to caloric restriction. The nitrogen balance shows a tendency to approach equilibrium (Strang et al., '31), and animals are able to maintain their weight with fewer calories if given a high-fat diet (Kaunitz et al., '56). Substitution of fat for carbohydrate has been shown to cause only a transitory increase in nitrogen excretion (Thomson and Munro, '55). Turning to *in vitro* studies, Whitney and Roberts ('55) have observed depressed glycogenesis and fatty acid synthesis in animals fed a high-fat diet, while Tepperman et al. ('56) observed increased oxidation of palmitic acid in rats fed high-fat diets. Roberts and Samuels ('49) observed an upswing in the blood sugar curve in animals after a three-day fast. Their experiments also indicated that the feeding of a high-fat diet prior to fasting was followed by a sparing of carbohydrate and protein during the fasting period.

SUMMARY

1. The performance of 10 subjects, living outside in a severely cold environment and subsisting on a daily intake of 1000 Cal. of either pemmican or pemmican + sugar was deemed adequate for most survival situations faced by aircrews in the Arctic.

2. The fasting blood sugar levels of the subjects receiving sugar were significantly higher than those of subjects receiving pemmican only.

3. The nitrogen balances of the subjects were not significantly affected by the isocaloric supplement of sugar.

4. The 24-hour ketone body excretions of the subjects receiving sugar were somewhat less than those of subjects receiving no sugar.

5. Sequential changes in the negative nitrogen balances and ketone body excretions were interpreted to mean that the experimental subjects were becoming adapted to a carbohydrate-free diet and caloric restriction.

ACKNOWLEDGMENT

The authors are indebted to Lt. Colonel R. B. Payne for his valuable assistance with the statistical analyses and to Mr. Channing Murray for his assistance as a subject-observer.

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SOME BIOCHEMICAL EFFECTS OF RESTRICTED DIETS DURING SUCCESSIVE FIELD TRIALS IN WINTER [1]

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INTRODUCTION

The first paper in this series (Drury et al., '58) dealt with the effects of a supplement of 40 gm of sugar on physiological reactions to a high-fat, high-protein diet fed at the 1000 Cal. level. The study which forms the subject of the current paper was originally designed to confirm the results of the first study and to extend the observations to the effects of somewhat larger sugar supplements. In an effort to minimize the effects of individual variation, as well as to obtain the maximum amount of data from the available subjects, each subject was exposed to two different diets with an intervening "recovery" period of one week. This, of course, is a common procedure in ration studies and our only thought in employing it was expediency. The results, however, were so unexpected and, in our opinion, of such theoretical interest that they overshadowed the original aim of the study. The primary purpose of this paper, then, is to present evidence for the persistence of adaptation to a low-carbohydrate, low-calorie diet over a period of one week of ad libitum feeding.

EXPERIMENTAL

Twelve adult men, including 10 military personnel and two civilians, were used as subjects for the experimental field

[1] This paper received a security clearance dated July 28, 1958. The views expressed are those of the authors and do not necessarily represent official Air Force policy.

studies. Following a control period to establish levels of fasting blood sugar and urinary nitrogen and acetone production, and to accustom the men to the experimental procedures, all subjects were taken to an isolated site to begin the field study. Period I of the dietary regimen began at this time and continued for 5 days. Unlimited water intake was permitted. The subjects were divided into three groups of 4 men each, receiving rations of the following composition:

GROUP	PEMMICAN [1]	SUGAR	CALORIES
	gm	*gm*	
"0"	168	0	1000
"40"	140	40	993
"80"	112	80	990

[1] Quartermaster Meat Food Bar: protein, 46%; fat, 46%; salt, 2%; water, 6%.

Environmental temperatures during this period ranged from −10° to −48°F.

At the end of period I, the subjects were returned to the laboratory and allowed to eat a mixed diet ad libitum. This interim "recovery" period lasted one week.

At the end of this week, the subjects were again taken to the isolated site to begin period II. For this study, every man received either more or less sugar than he had received in period I, in the following manner:

1. Two of the 4 men who had received diet "0" now received diet "40"; the remaining two received diet "80".
2. Two of the 4 men who had received diet "40" now received diet "0"; the remaining two received diet "80".
3. Two of the 4 men who had received diet "80" now received diet "40"; the remaining two received diet "0".

During the second 5-day period, the environmental temperature ranged from +10° to −10°F.

The environmental conditions, living arrangements, scope of physical activities, and the experimental procedures employed were exactly the same as reported previously, with the addition of physical fitness measurements administered by the procedure of Johnson et al. ('42) before and after each experimental period.

RESULTS

The results of the successive field studies are presented in figures 1–5. The interval of 7 days which separated period I from period II was originally intended as a recovery period. A cursory examination of the data, however, made it quite evident that the subjects' responses to the dietary treatments during period II were modified in such a way as to preclude consideration of the latter as a simple replication of period I. The period II responses differed both quantitatively and qualitatively and will be discussed individually in the following sections.

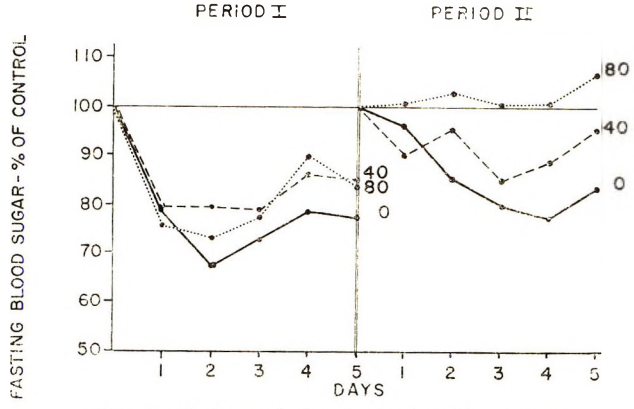

Fig. 1 Daily variation in fasting blood sugar.

During period I, the fasting blood sugar levels responded to the dietary treatments in much the same way as noted previously, i.e., a large initial drop followed by a slight rise (fig. 1). An analysis of the adjusted variance in period I values, using the pre-period I control values as the independent variable, showed that the response of fasting blood sugar to stepwise increases in dietary sugar was curvilinear and apparently followed the law of diminishing returns (table 1). There is a significant ($p < 0.05$) difference between the "0" and "40" diet groups, i.e., an increased level in the "40" group, while there is no significant difference between the "40" and "80" diet groups.

This pattern of fasting blood sugar values persisted throughout the "recovery" period, for an examination of the pre-period II control values (obtained a week after the end of period I) revealed a significant ($p < 0.05$) difference between the groups which had previously received zero and 40 gm of sugar, while there was no difference between the "40" and "80" groups (table 1).

TABLE 1

Comparison of period I and pre-period II fasting blood sugars

	FASTING BLOOD SUGAR				
	Diet [1] "0"	p [2]	Diet [1] "40"	p [2]	Diet [1] "80"
	mg %		*mg %*		*mg %*
Period I	63	< 0.05	77	ns	74
Pre-period II	80	< 0.05	94	ns	91

[1] Diet during period I.
[2] Significance of differences between periods at probability level.

TABLE 2

Average data for subjects undergoing field trials

	PERIOD I	PERIOD II	p [1]
Fasting blood sugar, mg %	71.7	84.3	< 0.001
Total nitrogen balance, gm	— 23.0	— 12.0	< 0.01
Total ketone excretion, gm	4.3	1.7	< 0.05
Total weight loss, lb	7.50	9.00	ns
(adjusted)	(5.00)	(6.00)	(ns)
Physical fitness decrements	— 9	— 7	ns

[1] Significance of differences between periods at probability level.

That the caloric restriction *per se* during period I may have had a carryover effect into period II is indicated by the generally higher fasting blood sugar values during period II (table 2). The mean values in period II are significantly ($p < 0.001$) greater, regardless of dietary treatment during period II. Figure 2 shows the effect of the dietary treatments upon the nitrogen balances during both periods.

Subjects receiving sugar (at the expense of some of the dietary protein) apparently lost more tissue nitrogen in both

periods, but these differences could have occurred by chance alone, as determined by an analysis of variance. The tendency for an initially large negative nitrogen balance to occur, followed by an upswing of the curve, is shown by the graphs in figure 2. What is perhaps more striking is the difference in response during period II, when compared with period I. Each subject catabolized less tissue protein during period II than

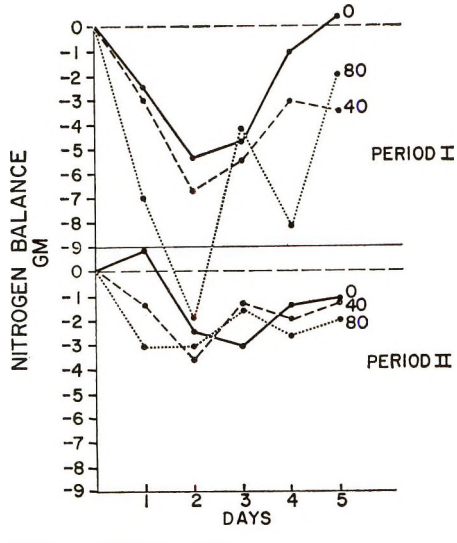

Fig. 2 Daily variation in nitrogen balance.

he had in period I. When the mean nitrogen balances of both periods were analyzed statistically, it was found that, as a group and regardless of diet, the total nitrogen losses during period II were significantly less ($p < 0.01$) than during period I (table 1).

In addition to this general change from period I to period II, there is some evidence that the amount of sugar ingested during period I may have had a specific effect on the nitrogen balances during period II (figure 3). When the subjects were grouped so that the only variable was the period I sugar intake, it was noted that men who had received the most sugar in period I lost more tissue nitrogen in period II.

The pattern of daily ketone excretion demonstrates the same dissimilarity between period I and period II (fig. 4). In period I, the subjects' responses were much the same as in the previous study, i.e., a sharp initial rise in ketone excretion, followed by an apparent adjustment to lower levels. During period II, however, the pattern was completely different. The men receiving the sugar supplement did not exhibit

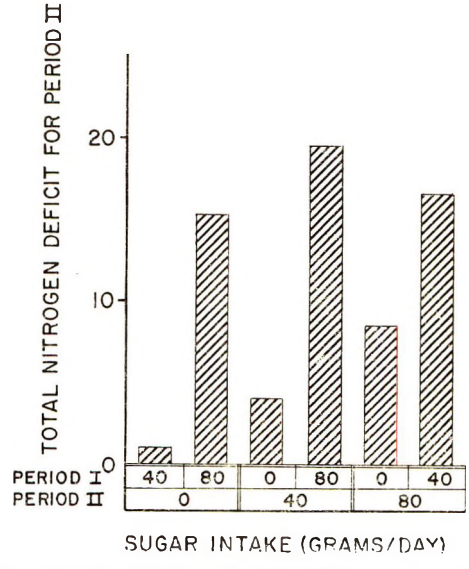

Fig. 3 Negative nitrogen balance during period II. Each bar represents the mean of two subjects.

ketosis at all during the field phase, while those receiving no sugar exhibited an increase in ketone excretion which reached a peak on the last day of the field phase. Furthermore, the total ketone excretion of all the men, regardless of dietary regimen in either period, was significantly ($p < 0.01$) lower during period II.

In figure 5, the total 5-day excretions of ketones in period II are grouped so that the effect of the previous diet can be studied. Here the residual effect of the previous sugar intake is apparent only in the group receiving no sugar in period II — the other groups did not develop ketosis during period II.

Since the subjects' metabolic responses to the stress of simulated survival improved, according to the indices used in this study, the question of whether the imposed stress during period II was not as severe as in period I naturally arose. Due to the unexpected nature of these results, the only measures of total stress that could be used were weight loss and physical fitness.

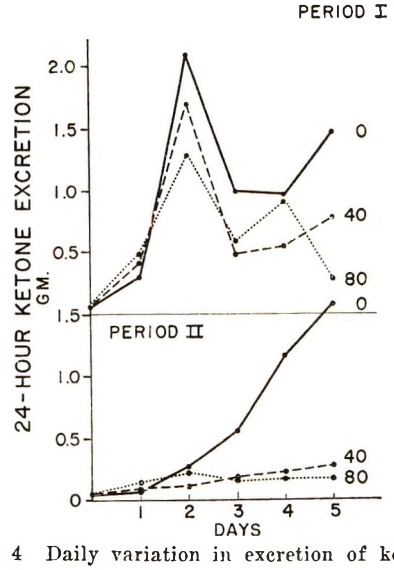

Fig. 4 Daily variation in excretion of ketones.

On a severely restricted diet, body weight decreases rapidly during the first day or two, then stabilizes to a fairly constant rate of loss. To correct for this initial loss (which we believe is largely water loss), the line connecting the points which represent constant weight loss was extrapolated to zero days and the difference between this point and the measured initial weight was subtracted from total weight loss. Despite the trend for the adjusted weight losses to be greater in period II, the differences are not significant (table 1). By this criterion, then, stress during period II was of the same magnitude as in period I.

As in the previous study (Drury et al., '58), increasing weakness and aversion to heavy work were noted as the study progressed. As a further indication of the extent of stress during the experimental periods, physical fitness tests were run. Since the change in the physical fitness index should give some indication of the magnitude of the stress involved, a

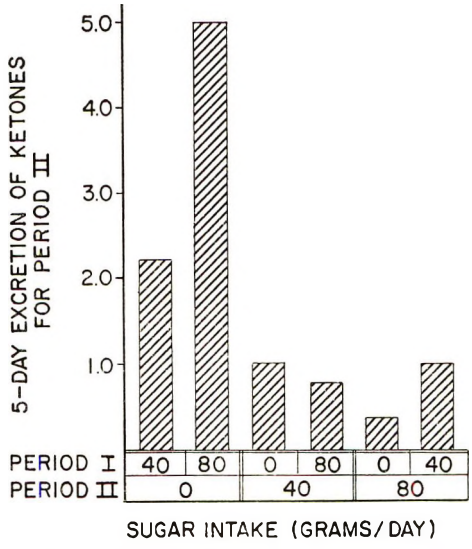

Fig. 5 Ketone excretion during period II. Each bar represents the mean of two subjects.

comparison of the changes induced by period I versus period II was made. The decrements in the physical fitness indices induced by the period II treatment are essentially the same as those induced by period I (table 1).

DISCUSSION

The behavior of the fasting blood sugar levels, nitrogen balances and ketone excretion during period I suggest that adaptive changes have taken place in the direction of the establishment of new levels compatible with increased fat catabolism. As indicated in the introduction, the dissimilarity between the results of period I and period II was unexpected

and our experimental design proved inadequate to secure the data necessary to arrive at a final decision between several statistically tenable hypotheses. Since both experimental trials were conducted in the field under rigorous environmental conditions, the increased ambient temperatures, differences in physical exercise, familiarity with the survival site, and increased experience in coping with the problems of simulated survival might have had the net result of imposing less stress on the subjects during period II. However, since the caloric intake was identical and since there was no significant difference in the weight losses, it seems reasonable to assume that the energy expenditure must have been substantially the same. Furthermore, the deterioration in physical fitness which generally accompanies caloric restriction during environmental stress, was not appreciably changed, as estimated by the decrements in the indices of physical fitness.

We consider it probable, although admittedly not proven, that a metabolic adjustment or adaptation to restricted caloric intake and possibly even to the slight differences in composition of the various diets, persisted throughout the intervening 7-day period. The marked improvement in nitrogen balance and fasting blood sugar levels observed in period II, as well as the increased efficiency of fat catabolism, as estimated by the lower ketone excretion during period II, indicate that the effects of caloric restriction can still be detected after a period of subsequent ad libitum food intake. The similarity between the pre-period II control and period I fasting blood sugars suggests that small differences in diet during caloric restriction may also have a persistent effect. It is common knowledge that dietary adaptations do occur. Observations in the literature indicate that the influence of fasting, at least, may persist for an appreciable period. Folin and Denis ('15) observed that a trend toward lower ketone and nitrogen excretion in fasting obese individuals began in the second fasting period. Taylor et al. ('45) noted that men undergoing successive fasts separated by 5- to 6-week intervals, maintained their blood sugar at higher levels, lost less nitrogen,

and excreted less ketone bodies during the 5th week than during the initial fast.

Even though a simple explanation of the dissimilarities between the two experimental periods is not possible, it is quite evident that the occurrence of such dissimilarities necessitates caution in the interpretation of field studies which are separated by short recovery periods. It is not unreasonable to suppose that individuals who have apparently recovered from a stress retain a latent capacity for the effective management of a subsequent stress of the same type.

SUMMARY

1. The adequacy of pemmican as an emergency ration for short-term survival has been confirmed.

2. The isocaloric substitution of pemmican with sugar in amounts over 40 gm was found to have little, if any, effect on fasting blood sugar, nitrogen balance, and ketonuria.

3. Evidence is presented that caloric restriction *per se* and the composition of the diet during caloric restriction have effects which last well beyond the end of the period of dietary stress. Even after an interval of a week of ad libitum dietary intake, these effects may modify responses to a second stress period, manifesting themselves in higher fasting blood sugars, lower nitrogen excretion and a decreased production of ketone bodies.

ACKNOWLEDGMENT

The authors are indebted to Lt. Colonel R. B. Payne for his valuable assistance with the statistical analyses.

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SEQUENCE IN WHICH THE AMINO ACIDS OF CASEIN BECOME LIMITING FOR THE GROWTH OF THE RAT [1]

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Casein is the protein most commonly used in laboratory diets and as early as 1915 Osborne and Mendel ('15) established that the adequacy of casein as a source of dietary protein for the rat was determined by its content of the sulfur-containing amino acids. This has been confirmed repeatedly. It is also well known that casein is low in both tryptophan (Krehl et al., '46) and threonine (Griffith and Nawrocki, '48). Nevertheless, despite the immense number of experiments in which rats have been fed diets containing casein supplemented with various amino acid mixtures, it is difficult to find information about the sequence in which amino acids other than methionine, threonine and tryptophan become limiting for the growth of rats fed a low level of this protein. There is even a measure of doubt as to whether tryptophan or threonine is the second most limiting amino acid because a tryptophan-deficient protein such as gelatin is usually fed with casein in order to create a more severe tryptophan deficiency and because tryptophan is usually added to casein-containing diets designed for the study of threonine deficiency (Harper et al., '53).

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It is a simple matter to calculate from a knowledge of the amino acid composition of casein and of the amino acid requirements of the rat, the sequence in which the indispensable amino acids in a diet containing a low level of casein as the only source of protein should become limiting for the growth of the rat. The reliability of the sequence so calculated can be checked by conducting growth experiments using diets low in casein and supplemented with amino acids in the order calculated. However, if the results of the growth experiments deviate from those expected on theoretical grounds, the order in which the amino acids become limiting for growth can only be determined empirically, i.e., by measuring growth responses to the various possible combinations of indispensable amino acids, even though this is laborius, uninspiring and time-consuming.

As changes in liver fat deposition, in organ and cell structure, in enzyme activities and in various metabolic processes are known to occur when animals fed on low-protein diets are given amino acid supplements, it should be of value to know what deficiencies are being studied under such conditions. Also, it is necessary to know the sequence in which the amino acids in a protein become limiting for growth in order to study in detail amino acid balance and imbalance (Harper, '58). Therefore, the experiments reported below were undertaken in order to provide such information about casein.

EXPERIMENTAL

Male weaning rats of the Sprague-Dawley strain, 21 days old and weighing from 40 to 50 gm were used throughout this investigation. Each group consisted of 5 rats maintained in individual, suspended cages with ½ in. mesh screen bottoms. The average initial weights for the groups within each experiment did not differ by more than 1 gm. The rats were fed ad libitum and were weighed twice weekly. The experiments were of two weeks duration but a few were continued for three to 5 weeks in order to determine whether the two-week period was

adequate. In no case in which the longer experimental period was used were the relative values altered.

The percentage composition of the basal diet was as follows: casein, 6.0; carbohydrate 84.6; corn oil, 5.0; salts (Hegsted et al., '41) 4.0; choline chloride 0.15; and vitamin mixture in sucrose, 0.25. The vitamin mixture provided in milligrams per 100 gm of diet: thiamine·HCl, 0.5; riboflavin, 0.5; nicotinic acid, 2.5; calcium pantothenate, 2.0 pyridoxine·HCl, 0.25; biotin, 0.01; folic acid, 0.02; vitamin B_{12} 0.002; and inositol 10.0. Each rat was given orally at the beginning of the experiment and each week thereafter two drops of halibut liver oil diluted with corn oil and fortified to provide α-tocopherol 4.0 mg; 2-methyl-1, 4-napthoquinone, 0.04 mg; vitamin A 400 I.U.; and vitamin D, 4 I.U. Ascorbic acid (50 mg/kg) was added to minimize the destruction of thiamine (Kandutsch and Baumann, '53) and all rations were refrigerated. All additions of amino acids, as indicated in the tables of results, were compensated by adjusting the percentage of carbohydrate.

In many of the experiments two series of diets were used, one containing sucrose, the other containing dextrin, as the carbohydrate. This provided a simple method of obtaining measurements with two levels of protein intake (Harper and Spivey, '58).

RESULTS

The results of some 14 experiments involving approximately 600 rats have been condensed and are presented in tables 2 to 5. The average weight gains with their standard errors have been reported to the nearest whole number. The calculated percentage deficit of each of the indispensable amino acids in a diet containing 6% of casein is shown in table 1 and the results of the initial experiments based on these calculations are presented in table 2. As was to be expected, methionine proved to be the most limiting amino acid in casein. Threonine, not tryptophan, was the second amino acid to become limiting for the growth of rats fed this diet. The combination of methionine and threonine caused a substantial growth response

regardless of the type of dietary carbohydrate and further supplementation with tryptophan was without effect. Supplementation of the diet containing methionine and threonine with various combinations of histidine, lysine, and tryptophan (the amino acids calculated to be most limiting after methionine and threonine) gave no further growth response.

TABLE 1

Calculation of sequence in which amino acids become limiting for the growth of the rat when 6% of casein is the entire source of dietary protein.

	AMINO ACID REQUIREMENTS	AMINO ACIDS IN 6 GM CASEIN [1]	PERCENTAGE DEFICIT
	gm/100 gm diet	*gm*	
Methionine	0.6	0.19	65
Cystine		0.02	
Threonine	0.5	0.24	50
Isoleucine	0.5	0.40	20
Tryptophan	0.2(0.15)	0.07	65(53)
Leucine	0.8	0.54	30
Valine	0.7	0.41	40
Histidine	0.4	0.18	55
Phenylalanine	0.9	0.34	25
Tyrosine		0.34	
Lysine	1.0	0.46	55
Arginine	0.2	0.22	0

[1] Casein = 14.4% N.

TABLE 2

Growth of rats fed on diets containing sucrose or dextrin and 6% of casein supplemented with amino acids

GROUP NO.	AMINO ACID SUPPLEMENTS			AVERAGE WEIGHT GAIN	
	DL-methionine	DL-threonine	DL-tryptophan	Sucrose	Dextrin
	%	%	%	*gm/2 wk.*	*gm/2 wk.*
1	—	—	—	12 ± 1	22 ± 1
2	0.3	—	—	16 ± 1	34 ± 3
3	—	0.4	—	12 ± 1	
4	—	—	0.1	10 ± 1	
5	0.3	0.4	—	30 ± 2	41 ± 3
6	—	0.4	0.1	12 ± 1	
7	0.3	—	0.1	16 ± 1	32 ± 2
8	0.3	0.4	0.1	30 ± 2	39 ± 2
9	As for gp. 8 plus L-histidine · HCl, 0.15%; L-lysine · HCl, 0.2% or histidine and lysine			26–30 ± 2	

The sequence of amino acid deficiencies determined experimentally obviously did not correspond with the calculated sequence so a series of mixtures of amino acids each lacking only arginine and one of the other indispensable amino acids was prepared and these were tested as supplements to both the sucrose and the dextrin diets containing 6% of casein plus

TABLE 3

Growth of rats fed on diets containing sucrose or dextrin and 6% of casein supplemented with methionine and tryptophan and various amino acid mixtures

GROUP NO.	AMINO ACID MIXTURES [1]	AVERAGE WEIGHT GAIN	
		Sucrose	Dextrin
		gm/2 wk.	*gm/2 wk.*
1	None	27 ± 2	40 ± 3
2	Complete	36 ± 2	50 ± 3
3	Minus tryptophan	16 ± 2	21 ± 1
4	Minus isoleucine	13 ± 2	19 ± 1
5	Minus leucine	20 ± 1	38 ± 2
6	Minus leucine and isoleucine	—	38 ± 2
7	Minus histidine	23 ± 2	33 ± 2
8	Minus valine	23 ± 2	24 ± 1
9	Minus phenylalanine	24 ± 2	38 ± 2
10	Minus lysine	31 ± 1	43 ± 5

[1] Complete mixture:

	Sucrose	Dextrin		Sucrose	Dextrin
	%	%		%	%
DL-Tryptophan	0.15	0.15	L-Lysine·HCl	0.5	0.58
DL-Isoleucine	0.3	0.8	L-Cystine	—	0.2
L-Leucine	0.3	0.54	L-Tyrosine	—	0.34
L-Histidine·HCl	0.25	0.22	DL-Methionine	0.4	0.4
DL-Valine	0.6	0.82	DL-Threonine	0.54	0.54
DL-Phenylalanine	0.2	0.34			

0.4% of DL-methionine and 0.5% of DL-threonine. The results presented in table 3 show that amino acid mixtures lacking tryptophan, isoleucine, leucine, histidine, valine or phenylalanine failed to stimulate growth. Only when all 6 of these amino acids were added to the basal diet (group 10) was the rate of gain greater than that of the negative control group, and only after further supplementation with lysine (group 2) did it significantly exceed that of the basal group. Even when

the 7 amino acids were included at these levels (designed for the dextrin-containing diet to make the levels of essential amino acids at least equivalent to those of a diet containing 12% of casein supplemented with 0.2% of L-cystine) the best growth rate was only 25 gm/wk, well below that obtained with a complete purified diet. In several cases the procedure used in these experiments produced severe growth retardations notably with the omission of tryptophan and isoleucine. The rate of gain after the omission of leucine with isoleucine was greater than that obtained when only isoleucine was omitted. From this experiment it appeared that tryptophan, isoleucine, leucine, histidine, valine, and phenylalanine were approximately equally limiting after methionine and threonine, and that lysine was one slightly less limiting then the other 6.

Because of the possibility that amino acid imbalances created by omitting one amino acid at a time from a complex mixture might influence the results (Harper, '58), the opposite procedure of adding amino acids singly or in small groups was also used. The results of such studies are shown in table 4. Again the rate of gain did not exceed that of the basal group unless tryptophan, isoleucine, leucine, histidine, phenylalanine and valine were all added to the diet. Again a somewhat greater growth rate was obtained when lysine was also included. As was to be expected growth was inferior when the diet contained sucrose rather than dextrin, therefore, in an effort to obtain normal growth rates, a number of the trials were run with diets containing only the latter carbohydrate.

Comparable, but somewhat higher values, were obtained in one experiment in which the diets contained 9% of casein supplemented with various amino acid mixtures.

In previous studies, also, (Benton et al., '56; Deshpande et al., '55, '57) the rate of gain had been suboptimal for rats fed on diets containing a low level of protein supplemented with what were considered to be adequate levels of essential amino acids. In order to determine whether the dispensable amino acids might be required under these conditions, group 17 (table 4) was fed a diet containing 6% of casein plus sufficient

TABLE 4

Growth of rats fed on diets containing sucrose or dextrin and 6% of casein supplemented with various amino acids [1]

GROUP NO.	AMINO ACID SUPPLEMENTS							AVERAGE WEIGHT GAIN	
	DL-trypto-phan	DL-iso-leucine	L-leucine	L-histi-dine	DL-valine	DL-phenyl-alanine	L-lysine	Sucrose	Dextrin
								gm/2 wk.	*gm/2 wk.*
1	—	—	—	—	—	—	—	27 ± 1	42 ± 3
2	—	—	—	—	—	—	+		40 ± 2
3	—	+	—	—	—	—	—	26 ± 2	37 ± 2
4	—	—	+	—	—	—	—		40 ± 3
5	+	+	—	—	—	—	—	26 ± 2	41 ± 3
6	+	—	+	—	—	—	—		37 ± 3
7	—	+	+	—	—	—	—	25 ± 3	40 ± 2
8	+	+	+	—	—	—	—	22 ± 2	42 ± 3
9	+	+	+	+	—	—	—		43 ± 2
10	+	+	+	—	+	—	—		42 ± 4
11	+	+	+	—	—	+	—		37 ± 4
12	+	+	+	+	+	—	—		43 ± 2
13	+	+	+	—	+	+	—		35 ± 3
14	+	+	+	+	—	+	—		38 ± 4
15	+	+	+	+	+	+	—	32 ± 2	47 ± 3
16	+	+	+	+	+	+	+	36 ± 2	52 ± 3
17	As for 16 plus L-arginine HCl, 0.28%, L-glutamic acid, 1.9%.								71 ± 4
18	12% casein + 0.2% L-cystine or 25% casein								80 ± 5

[1] All diets except that for group 18 contained DL-methionine, 0.4%; and DL-threonine, 0.54%. Levels of other amino acids are shown in footnote 1, table 3.

of the indispensable amino acids to give levels of the L-amino acids equal to those in a diet containing 12% of casein supplemented with 0.2% of L-cystine, together with sufficient glutamic acid to equalize the nitrogen intake. The growth rate of this group was higher than that of any previous group and was very nearly equal to that of a group receiving an adequate quantity of intact casein.

TABLE 5

Growth of rats fed on diets containing dextrin and 6% of casein supplemented with amino acids

GROUP NO.	CASEIN	AMINO ACID SUPPLEMENT	GAIN
	%		*gm/2 wk.*
1	12	L-Cystine, 0.2%	81 ± 2
2	6	Amino acid mixture [1]	49 ± 3
3	6	Amino acid mixture + glutamic acid (arginine and lysine lacking)	48 ± 3
4	6	Amino acid mixture + lysine (lacking arginine and glutamic acid	58 ± 2
5	6	Amino acid mixture + lysine + glutamic acid (lacking arginine)	63 ± 3
6	6	Amino acid mixture + arginine + glutamic acid (lacking lysine)	42 ± 1
7	6	Amino acid mixture + arginine + lysine (lacking glutamic acid)	64 ± 2
8	6	Complete amino acid mixture [2]	79 ± 3

[1] Amino acid mixture as for group 15, table 4. This mixture lacked lysine, arginine and glutamic acid. Levels of supplements as indicated in table 4.

[2] As for group 17, table 4.

This observation served as the basis for the final experiments. The basal diet was identical with that fed to group 15 (table 4). The results presented in table 5 show that additional glutamic acid did not stimulate growth unless lysine or lysine and argine were also provided and, in agreement with the results of the previous experiments, that lysine stimulated growth when no glutamic acid was added. When arginine, lysine and glutamic acid were all provided, growth was comparable to that obtained with an adequate purified diet but the omission of any one of the three caused a reduction in the

growth rate. A mixture of DL-aspartic acid, DL-alanine and glycine was as effective as glutamic acid and an increase in the level of the dispensable amino acid mixture from 1.9 to 4% of the diet was without effect.

DISCUSSION

The conclusion from these experiments is that the indispensable amino acids in a diet containing a low level of casein as the only source of protein became limiting for the growth of the rat in the following order: first, sulfur-containing amino acids; second, threonine; third, tryptophan, isoleucine, leucine, histidine, valine and phenylalanine, all about equally limiting; fourth, lysine; and finally, arginine. The response obtained with a supplement of glutamic acid is a complicating factor. Since a supplement of glutamic acid did not stimulate the growth of rats fed the diet containing a mixture of all of the essential amino acids except lysine and arginine, whereas a supplement of lysine did stimulate growth when the diet lacked arginine and glutamic acid, it would appear that, when the diet contains only 6% of casein and dextrin and as much as 1.5% of the D-forms of some of the indispensable amino acids, the dispensable amino acids are just slightly less limiting than lysine and must be provided before the full response to supplements of arginine and lysine can be obtained.

A response to nitrogenous compounds other than indispensable amino acids has been observed in rats fed on diets containing only the indispensable amino acids but not in rats fed on diets containing protein (Rose et al., '49; Lardy and Feldott, '50; Frost and Sandy, '51). It is conceivable that the effect of glutamic acid is not strictly nutritional. It could be an indirect effect in which an excess of glutamic acid reduces the amounts of the essential amino acids entering into catabolic reactions. This effect of glutamic acid poses a problem worthy of further study.

The relative responses to methionine and threonine were different with the two types of diets and this is presumably related to the effect of the type of dietary carbohydrate on

protein intake (Harper and Katayama, '53; Harper and Spivey, '58). When the diet contains sucrose the protein intake, and therefore the threonine intake, is low. Thus the response to methionine may be small (Treadwell, '48; Harper et al., '54), and that to threonine greater, because additional threonine is needed for full utilization of the methionine supplement. When sucrose is replaced with dextrin the total protein intake, and therefore the threonine intake, is higher; and, in this case, as might be expected, the response to methionine was greater and that to threonine less.

The observation that threonine is the second most limiting amino acid for the growth of rats fed a diet containing a low level of casein is in agreement with previous work (Griffith and Nawrocki, '48), as is that indicating that lysine is one of the least limiting amino acids in casein for the growth of the rat (Benton et al., '56). It may also be worth noting that in previous studies (Harper et al., '53), in which the diets contained only 10 mg instead of 25 mg of niacin/kg., tryptophan appeared to be more limiting than threonine for growth. The tryptophan requirement of the rat increases when the niacin content of the diet is low (Krehl et al., '46; Oesterling and Rose, '52; Salmon, '54) so it would appear that by adjusting the level of niacin in diets containing low levels of casein, either tryptophan or threonine can be made the second most limiting amino acid for the growth of the rat.

The difference between the sequence of amino acid deficiencies determined experimentally and that calculated is striking. Three factors could be responsible for the observed deviations (a) the amino acid analyses for casein may be in error, (b) all of the amino acids in casein may not be readily available, (c) the estimated requirements for the rat may be high. Although there may be errors in the amino acid analyses, the values for casein are probably among the most dependable. It has frequently been analyzed in a large number of laboratories using different methods (Block and Bolling, '51). There is some evidence that the isoleucine in casein is not completely available (Deshpande et al., '57) but more observations will

be needed before the significance of this factor can be estimated. Finally, and perhaps the most likely source of error, is the estimated requirements of the rat. It is difficult to determine accurately the minimum requirement for maximum growth because of the nature of the growth curve (Almquist, '56). Also, the requirement, expressed as a percentage of the diet, is not constant but may be influenced by factors which affect food consumption, such as the type of dietary carbohydrate (Harper and Spivey, '58). The figures used for most of the requirements are those of Rose ('38). These have been extremely valuable estimates and have permitted almost without exception prediction of the most limiting amino acid in dietary proteins. However, the requirements of the rat have not been frequently reinvestigated and now that purified laboratory diets have been greatly improved and much more is known about dietary interrelationships, a good case can be made for reinvestigation of the requirements. This is particularly desirable in view of the need for greater accuracy in the further study of dietary interrelationships.

Severe growth retardations were produced in several cases when mixtures of all of the essential amino acids but one were added to the low-protein diet (table 3). This procedure has been shown to cause amino acid imbalances under other dietary conditions (Deshpande et al., '55; Sauberlich, '56; Deshpande et al., '57) and appears to provide a reliable method for producing them. The effects were most severe when tryptophan, isoleucine, leucine or valine were the limiting amino acids. This suggests that, despite the failure to obtain a growth response with a combination of these 4, they may be slightly more limiting than histidine or phenylalanine. The known antagonisms among isoleucine, leucine, and valine (Harper et al., '55; Benton et al., '56) are complicating factors. Rose ('57) reported that the effect of isoleucine deficiency was particularly severe in subjects receiving an amino acid mixture lacking or low in this amino acid. The same was true in these experiments with rats. However, when leucine was also omitted from the amino acid mixture (table

3), the omission of isoleucine was less detrimental. This is in accord with previous observations on these relationships (Benton et al., '56) and indicates that in studying deficiencies of leucine, isoleucine, and valine, the severity of the deficiency of each one may be influenced by the relative levels of the others.

SUMMARY

In an effort to determine the sequence in which the amino acids of casein become limiting for the growth of young rats, feeding experiments were conducted using diets containing sucrose or dextrin and 6% of casein supplemented with various amino acid mixtures. From the results of these experiments it was concluded that the sulfur-containing amino acids are most limiting; threonine next; then tryptophan, isoleucine, leucine, histidine, valine and phenylalanine all about equally limiting; lysine slightly less so than the preceding 6; and finally arginine, least limiting. A mixture of all of these together with glutamic acid was required with 6% of casein to support a rate of gain of young rats equivalent to that obtained with comparable rats fed an adequate purified diet. A number of the amino acid mixtures caused imbalances or antagonisms which resulted in growth retardation.

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SALT MIXTURES FOR PURIFIED-TYPE DIETS

I. EFFECT OF SALTS IN ACCELERATING OXIDATIVE RANCIDITY [1]

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The importance of protecting experimental diets against oxidative rancidity has been recognized for many years. The destruction of essential fatty acids, fat-soluble vitamins, and certain water-soluble vitamins associated with the development of rancidity has been reviewed by Burr and Barnes ('43) and by Holman ('54). Recently the detrimental effect of rancid diets on the growth of rats in certain nutritional studies was again emphasized when our laboratory was temporarily without cold storage space. A comparison of the odor of a variety of purified diets suggested that the type of salt mixture present in the diet had a marked influence on the rate at which the diets became rancid.

The effects of the salt mixtures could not be related to the kind or concentration of the cations that are recognized as catalyzing the development of rancidity. This report presents studies on the influence of several salt mixtures, their individual components, and the particle size of the carbohydrate on the speed with which purified diets became rancid.

[1] A preliminary report of some of these data was given at the meeting of the American Chemical Society at Minneapolis, Minnesota, September 11–16, 1955.

EXPERIMENTAL PROCEDURE

In the early experiments the salt mixtures most frequently used in this laboratory were each incorporated into chick diet C2 (Fox, Ortiz and Briggs, '55). This was a purified-type diet which contained casein, gelatin, corn oil, glucose, all vitamins required by the chick, and salts at a level of 6%. It was found that the same effect of salts in accelerating rancidity could be demonstrated in a simplified diet mixture consisting of casein,[2] fat, carbohydrate, and salts in proportions similar to those in diet C2; therefore, the simpler mixes were used for most of this work.

The Chick Salts A (Briggs et al., '52) was mixed in this laboratory from U.S.P. or C.P. grades of chemicals. Many different lots of Chick Salts A, HMW Salts [2] (Hubbell, Mendel and Wakeman, '37; Nutritional Biochemicals Corp., '57), and Wesson Salts [2] (Wesson, '32; Nutritional Biochemicals Corp., '57) were used with similar results. Two lots of Jones-Foster Salts [2] (Jones and Foster, '42; Nutritional Biochemicals Corp., '57) were used.

These experiments were carried out with the same ingredients as are used for animal diets in this laboratory. Insofar as possible all constituents except the salts were combined in a premix and the salts were then added to aliquots of the premix. The final mixtures were similar to our animal diets with respect to homogeneity and particle size, except where indicated. In a few experiments 1 mg of β-carotene was dissolved per gram of oil so that disappearance of the color of the carotene could be followed. When the effect of particle size of the carbohydrate was studied, some of the coarse crystalline sugars were ground to a fine powder in a porcelain mortar. The particle size of the carbohydrate samples was

[2] Purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio.

assessed by determining the percentage of the sample which passed through each of a series of screens.[3]

The individual diets or mixes were made up in 50 gm quantities and stored in 75 ml clear glass bottles with tightly fitting screw caps. Each bottle was filled approximately three-fourths full of diet mix and stored in a dark incubator at 37°C. When peroxide determinations were to be made, larger amounts of diet mixes were prepared.

The diet mixes were checked by the same person daily for development of a rancid odor and for color changes. Consistent agreement was obtained when several individuals evaluated the diet mixes for a rancid odor. In some experiments peroxide values were determined by the Lingenfelter ('45) modification (described by Volz and Gortner, '47) of the Kokatnur and Jelling method ('41). All experiments were repeated several times with good agreement in the relative effects of the salts from test to test. Since the different batches of oil used over the three-year period varied, there were differences from experiment to experiment in the absolute time periods required for the initiation of rancidity. For this reason most of the data presented are from single typical experiments.

RESULTS

The compositions of Wesson Salts, HMW Salts, and Chick Salts A are presented in table 1. Results with diet C2 containing each of the three salt mixtures and either cottonseed oil or a hydrogenated vegetable oil are presented in table 2.

[3] Particle size distribution of carbohydrates, measured by the percentage which passed through the sieves:

U.S. SIEVE NO.[1]	SUCROSE				GLUCOSE		CORN STARCH
	Technical, fine	Technical, coarse	Technical, ground[2]	Reagent, coarse	Technical, fine	Reagent, coarse	Technical, fine
80 (177)	100	16	82		58	9	100
35 (500)		74	18		41	87	
20 (840)		9		17	1	4	
10 (2000)		1		83			

[1] Size of sieve openings in microns given within parentheses.
[2] This is the technical, coarse sucrose after grinding in a mortar. Coarse reagent sucrose and glucose were also ground; their particle size distribution was similar to that for sucrose, technical, ground. For this reason, the values for the reagent sucrose and glucose after grinding are not given.

Based on the development of a rancid smell, Chick Salts A and Wesson Salts behaved similarly and were much more active than the HMW Salts in producing rancidity. The hydrogenated vegetable oil was very resistant to oxidation with each of the salts.

TABLE 1

Composition of salt mixtures (grams per 100 gm mixture)

CONSTITUENT	CHICK SALT A	WESSON	HMW
$CaCO_3$	25.000	21.000	54.300
$Ca_3(PO_4)_2$	23.333	14.900	
K_2HPO_4	15.000		
KH_2PO_4		31.000	21.200
KCl		12.000	11.200
Na_2HPO_4	12.166		
NaCl	14.666	10.500	6.900
$MgSO_4$	4.070	9.000	
$MgSO_4 \cdot 7H_2O$			1.600
$MgCO_3$			2.500
$MnSO_4 \cdot H_2O$	0.700	0.020	0.035
$FeC_6H_5O_7 \cdot 3H_2O$ (16.7% Fe)	0.666		
$FePO_4 \cdot 4H_2O$		1.470	2.050
$CuSO_4 \cdot 5H_2O$	0.033	0.039	0.090
KI	0.066	0.005	0.008
NaF		0.057	0.100
$ZnCO_3$ (56% Zn)	0.033		
$K_2Al_2(SO_4)_4 \cdot 24H_2O$		0.009	0.017
Glucose [1]	4.267		
Cu	0.0084	0.0097	0.0229
Fe	0.111	0.368	0.514
Mn	0.228	0.065	0.0114

[1] Chick Salts A originally contained $MgSO_4 \cdot 7H_2O$; when the change was made to anhydrous $MgSO_4$, glucose was added so that the percentage composition of the salt mixture remained unchanged.

The validity of the rancid odor as an index of oxidative rancidity was confirmed by means of peroxide determinations. These tests indicated that in each case the rancid odor was detected within one or two days of the initiation of rapid peroxide formation (fig. 1). Once a rancid odor was detected, it grew stronger very rapidly and persisted even after the decline in the peroxide value. There was an almost imperceptible rise in the peroxide value of the mixture containing

no salts and this mixture also had no rancid odor. The diet mix containing Chick Salts A showed a 4-day induction period, as evidenced by a low peroxide value during this interval. The induction period for the diet containing the Wesson Salts was much shorter than that for the preceding diet, otherwise the curves were essentially the same.

Simultaneously with development of a rancid odor, small yellow spots could be seen in most of the mixes. These grew in number and size as the experiment proceeded. The yellow-colored material could be easily extracted with fat solvents and it was found in the initial stages to have a higher peroxide content than fat from adjacent areas of the mix.

TABLE 2

Effect of types of fat and salt mixture upon the development of rancidity in purified "diet C2" [1] *(Stored at 37°C)*

FAT	NO. OF DAYS TO DEVELOP RANCIDITY [2]		
	Chick Salts A	HMW Salts	Wesson Salts
Cottonseed oil [3]	5	21	5
Hydrogenated oil [4]	> 42	> 42	> 42

[1] Each salt mixture incorporated at a level of 6%.

[2] Experiment terminated after 42 days.

[3] Wesson oil, from Wesson Oil and Snowdrift Sales Co., New Orleans, Louisiana.

[4] Crisco, from Procter and Gamble, Cincinnati, Ohio.

Mixes that had carotene added to the oil were quite yellow; however, the carotene color disappeared completely within one to two days after the detection of a rancid odor. Sealing the diet mixes in an atmosphere of nitrogen did not delay the loss of the carotene color, so apparently only small amounts of oxygen were required for the initial oxidative destruction. Thus, the detection of a rancid odor was closely associated with a rise in peroxide value and with destruction of added carotene; the initial odor rapidly became stronger and was associated with the appearance of yellow spots in the mixes. The validity of the olfactory test was further substantiated by the consistency in the relationship between Chick Salts A, Wesson Salts, and HMW Salts in accelerating the develop-

ment of rancidity that has been observed in more than 30 experiments carried out over a period of three years. For these reasons it is believed that the olfactory test was an adequate means of assessing oxidative rancidity for the purposes of this study.

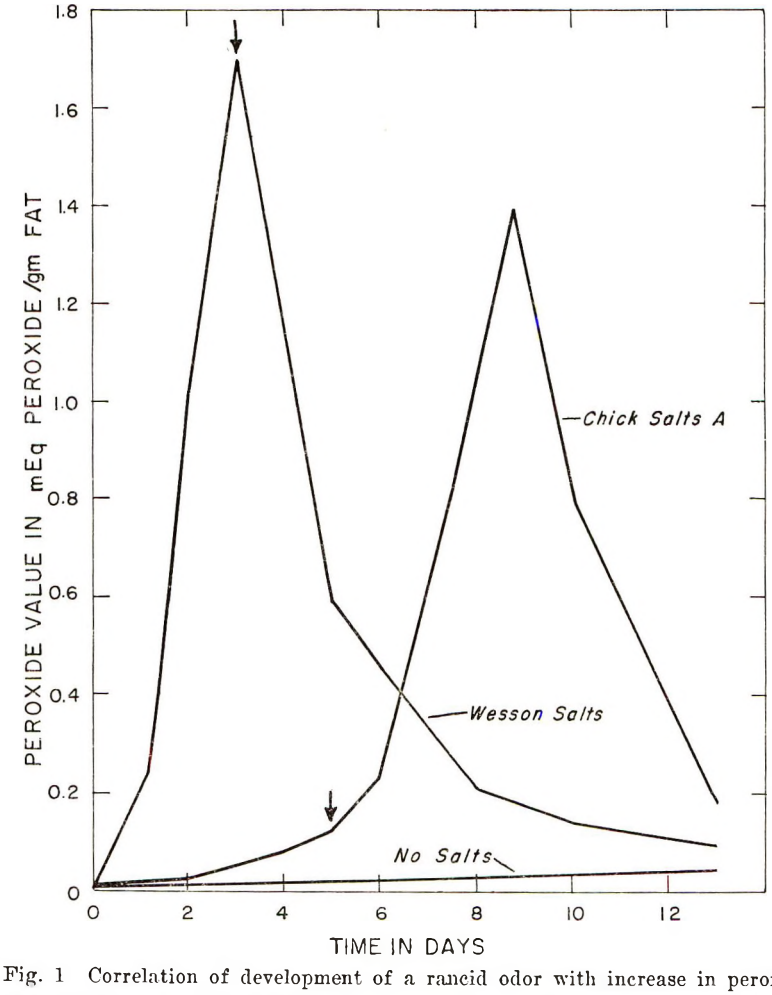

Fig. 1 Correlation of development of a rancid odor with increase in peroxide content of diet mixes stored at 37°C. Arrows indicate time when rancid odor was first detected. Diet mixes contained 70% sucrose, 20% casein, 4% cottonseed oil, 6% salts.

Attempts were then made to determine which constituents in the HMW, Wesson, and Chick Salts A were primarily responsible for accelerating the rancidity. Each individual constituent was added to a diet mix at a level equivalent to the largest amount contributed by one of the three salt mixtures when that mixture was incorporated at a level of 6%. The individual constituents were also tested at 10 times higher

TABLE 3

Effect of individual constituents of Chick Salts, A, HMW Salts and Wesson Salts upon development of rancidity in diet mix [1] *(stored at 37°C)*

SALTS	WEIGHT/100 GM MIX	NO. DAYS TO DEVELOP RANCIDITY [2]
	gm	
None	0	> 78
Chick Salts A	6	1
HMW Salts	6	11
Wesson Salts	6	5
	mg [3]	
$CuSO_4 \cdot 5H_2O$	5	> 78
	50	5
$FePO_4 \cdot 4H_2O$	123	11
	1230	1
$MnSO_4 \cdot H_2O$	42	26
	420	11

[1] Twenty per cent casein, 4% cottonseed oil, salts as indicated, and sucrose to make a total of 100%.

[2] Experiment was terminated after 78 days.

[3] The smaller weight of each individual constituent was equal to the largest amount of that substance which would be present if one of the three complete salt mixtures were used in a diet at a level of 6%.

levels. To conserve space, only the data (table 3) are presented for the individual constituents which accelerated the development of rancidity. Even at the higher levels, rancid odors were detected prior to 78 days only with $FePO_4 \cdot 4H_2O$, $CuSO_4 \cdot 5H_2O$, and $MnSO_4 \cdot H_2O$. Ferric citrate, which is present in Chick Salts A, did not promote rancidity in the diet mix.

In further experiments the rancidity-accelerating effects of mixtures containing some of the salt constituents were tested

(table 4). For HMW and Wesson Salts the net effect of the complete salt mixture was duplicated, insofar as the rate of development of rancidity was concerned, by the combination of $MnSO_4 \cdot H_2O$, $FePO_4 \cdot 4H_2O$, and $CuSO_4 \cdot 5H_2O$ in amounts equivalent to those contributed by the original salt mixture. The results with Chick Salts A are less clear-cut since mixture A2 contained components other than copper, iron, and manganese salts. The interaction of the substances present in this mixture may have affected the acceleration in the rate of development of rancidity. The indictment of copper sulfate, ferric phosphate, and manganese sulfate is further substantiated by the fact that none of the other submixes produced rancidity during the 50-day test period.

TABLE 4

Development of rancidity with certain parts of the salt mixtures in diet mixes[1] *(stored at 37°C)*

COMPLETE OR PARTIAL SALT MIXTURES	GM/100 GM MIX	NO. DAYS TO DEVELOP RANCIDITY[2]
None	0	> 50
HMW[3]	6	15
HMW (A, B, C, and D)	6	5
A ($CaCO_3$, $MgCO_3$, NaCl)	3.822	> 50
B (KH_2PO_4, KCl, $MgSO_4 \cdot 7H_2O$)	2.040	> 50
C ($MnSO_4 \cdot H_2O$, $FePO_4 \cdot 4H_2O$, $CuSO_4 \cdot 5H_2O$)	0.1305	5
D (KI, NaF, $K_2Al_2(SO_4)_4 \cdot 24H_2O$)	0.0075	> 50
Wesson[3]	6	3
Wesson (A, B, C, and D)	6	5
A ($CaCO_3$, $Ca_3(PO_4)_2$, NaCl)	2.784	> 50
B (KH_2PO_4, KCl, $MgSO_4$)	3.12	> 50
C ($MnSO_4 \cdot H_2O$, $FePO_4 \cdot 4H_2O$, $CuSO_4 \cdot 5H_2O$)	0.0917	6
D (KI, NaF, $K_2Al_2(SO_4)_4 \cdot 24H_2O$)	0.0043	> 50
Chick Salts A (A1 + A2)	6	5
A1 ($CaCO_3$, K_2HPO_4, Na_2HPO_4, $Ca_3(PO_4)_2$, $MgSO_4$, KI, glucose)	5	> 50
A2[4] ($CaCO_3$, NaCl, $FeC_6H_5O_7 \cdot 3H_2O$, $MnSO_4 \cdot H_2O$, $ZnCO_3$, $CuSO_4 \cdot 5H_2O$)	1	2

[1] 20% casein, 4% cottonseed oil, salts as indicated, and sucrose to make a total of 100%.

[2] Experiment was terminated after 50 days.

[3] From Nutrition Biochemicals Corporation, Cleveland, Ohio.

[4] Salts A2 contained 0.034 gm $CaCO_3$, so that this mixture (Salts A2) was one-sixth of the weight of the complete Salts A.

Data are presented in table 5 on the effect of purity and particle size of the carbohydrate upon the rate of rancidity development with three different salt mixtures. In tests with Chick Salts A, the development of rancidity was delayed with carbohydrate of very fine particle size. The purity of the

TABLE 5

Effect of purity and particle size of sucrose, glucose, and corn starch upon the development of rancidity in diet mixes[1] *(No. days for development of rancidity at 37°C)*

CARBOHYDRATE	CHICK SALTS A	WESSON SALTS	JONES-FOSTER SALTS
Sucrose			
Technical,[2] fine	8		10
Technical,[3] coarse	5	3	7
Technical,[4] ground	12	6	8
Reagent, coarse	4	3	4
Reagent,[4] ground	11	8	7
Glucose			
Technical,[5] fine	14	8	8
Reagent, coarse	7	6	7
Reagent,[4] ground	12	6	7
Cornstarch			
Technical,[6] fine	10		10

[1] 20% casein, 4% cottonseed oil, 6% salts, and 70% carbohydrate. Data for Chick Salts A are the average of three experiments; data for Wesson and Jones-Foster Salts are each from one experiment. For screen size of carbohydrate, see footnote 3 of text.

[2] Domino 10X confectioners sugar, from American Sugar Refining Company, Boston, Massachusetts; contained 3% corn starch.

[3] Commercial table sugar.

[4] The immediately preceding carbohydrate finely ground in a porcelain mortar.

[5] Cerelose, from Corn Products Refining Company, Argo, Illinois.

[6] Argo cornstarch, from Corn Products Refining Company, Argo, Illinois.

carbohydrates (reagent vs. technical) tested had no effect upon rate of rancidification and there was no essential difference between sucrose and glucose. The same trends were observed with Wesson Salts and Jones-Foster Salts; however, the results were less clear-cut.

DISCUSSION

These experiments indicate how complex the factors are which influence the development of rancidity in experimental diets. It is likely that conditions other than minerals and particle size may change the rate of and degree of rancidification. For example, the HMW Salts mixed in this laboratory caused rancidity to develop at a faster rate than the commercially prepared salts; however, Wesson Salts from the two sources behaved similarly. No information was obtained which offers a reasonable explanation for these discrepancies.

The difficulties inherent in this problem are emphasized by the fact that the study of the isolated components of the mineral mixture does not always provide answers which can be totaled for an evaluation of the complete mixture. This is illustrated by ferric phosphate and copper sulfate, both of which accelerate the development of rancidity. Although the Wesson Salts mixture contains less of these two minerals than the HMW Salts (table 1), the Wesson Salt mixture catalyzes the development of rancidity at a faster rate when incorporated into purified diets. Manganese also illustrates this point. There is 20 to 30 times as much manganese sulfate in the Chick Salts A as in the HMW or Wesson Salts. The activity of the Chick Salts A might be attributed to the high manganese content since this mixture contains ferric citrate in place of the phosphate. Other work (unpublished) also shows that the citrates are much less active than the inorganic salts in promoting the development of rancidity. This provides at best only a partial answer since the diet mixture containing the manganese sulfate as the only inorganic constituent showed no traces of rancidity until the 26th day of storage, whereas the diet containing the complete Chick Salts A became rancid after only one day (table 3).

The difference between the HMW and Wesson Salts in hastening rancidity cannot be resolved at this point. The HMW Salts mixture, which is associated with slower development of

rancidity than the Wesson Salts, contains higher amounts of $MnSO_4 \cdot H_2O$, $FePO_4 \cdot 4H_2O$, and $CuSO_4 \cdot 5H_2O$ than the Wesson Salts. These differences suggest the possibility that some constituents of the HMW Salts suppressed the rancidity-accelerating effects of other components, or that some constituent(s) of the Wesson Salts acted synergistically with the manganese, iron, copper, or other metals to accelerate rancidity.

The HMW Salts and Wesson Salts are usually incorporated into rat diets at a level of 4%; however, comparisons were made in these experiments at a level of 6% to provide a constant basis of comparison with the Chick Salts A. In other experiments rancidity has been observed to occur very quickly in rat diets containing 4% Wesson Salts and more slowly with the same amount of HMW Salts.[4]

The results from the present experiments on the influence of carbohydrates and their particle size on rancidity do not completely agree with those of Thomson and Hegsted ('56). They found with the Jones-Foster Salts mixture that rancidity appeared about three to 4 days faster in complete diets with glucose than with sucrose and that particle size of the carbohydrate had no effect on this phenomenon. In the present experiments all the mixes containing Jones-Foster Salts became rancid at approximately the same time. The diet mix containing the coarse reagent-grade glucose became rancid much more rapidly than any of the other mixes; this was probably due to the extreme coarseness of this sucrose (see footnote 3). Our experiments suggest that when Wesson Salts and Chick Salts A were used, the development of rancidity was accelerated by increasing the particle size of the carbohydrate and that similar results were obtained with glucose and sucrose.

These experiments indicate the need for improving the salt mixtures currently used in experimental diets. Although the present study was carried out at 37°C, other work showed that

[4] The HMW salts was not tested at the lower levels of 2.0 or 2.5%, recommended in the original paper.

purified diets stored at room temperature became rancid very rapidly. The rate may be fast enough so that food in an animal's cage may have to be changed every few days in order to obviate the complications resulting from a rancid diet.

In modifying any salt mixture it may be well to use amounts of manganese, copper, and iron that are only slightly greater than the animal's actual requirement. Where possible, it would be advisable to use these salts in the form of citrates since preliminary work suggests that they produce rancidity more slowly than do equivalent amounts of the inorganic salts. The final basis for determining whether any proposed change in a salt mixture is beneficial can come only from animal feeding experiments. Slight changes in the composition of a salt mixture, which from a theoretical standpoint should have no influence on the growth of animals, may have a profound effect.

SUMMARY

The rancidity-accelerating activities of Chick Salts A, the mixtures of Hubbell, Mendel and Wakeman and of Wesson have been compared in complete purified experimental diets and in simplified diet mixtures stored at 37°C. The principal findings are:

1. Rancidity developed much more slowly with HMW Salts than with either Chick Salts A or Wesson Salts. Similar differences between these salts were seen in both complete diets and simplified diet mixes.

2. The principal constituents of these salts that accelerated oxidative rancidity were $CuSO_4 \cdot 5H_2O$, $FePO_4 \cdot 4H_2O$, and $MnSO_4 \cdot H_2O$. Ferric citrate did not promote the development of rancidity. A study of the rate of development of rancidity when individual salt constituents were used in the diet mixtures did not correlate quantitatively with the behavior of the complete salt mixture.

3. In general, rancidity developed more slowly when the carbohydrate was finely powdered than when it was in larger particle sizes. With particle size constant, no differences were

observed between reagent and technical grades of either glucose or sucrose.

4. The rancid odor was detectable within a day or so of the initial rapid rise in peroxide value and destruction of added carotene.

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THE INFLUENCE OF THE PROTEIN LEVEL OF THE DIET ON SERUM GLYCOPROTEIN CONCENTRATIONS IN THE RAT [1,2]

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Clinical studies on the influence of diet on the carbohydrate-containing proteins of serum have been concerned primarily with the effects of fasting and the feeding of carbohydrate, protein, or fat test meals to normal individuals. The reported results were negative and have been reviewed by Südhof and Kellner ('57) and by Stary ('57). Starvation (Shetlar and Shetlar, '55; Weimer and Nishihara, '57) and protein depletion (Weimer and Nishihara, '57) have been found to cause a decrease in the serum glycoproteins of rats.

Inasmuch as protein depletion produced significant decreases in the protein-bound carbohydrates of serum, it seemed possible that the depletion-repletion technique of Wissler and associates ('46) might offer certain advantages in evaluating the effects of dietary protein levels on the concentration of the serum glycoproteins. In the present study, a modification of the rat repletion method has been utilized to investigate the effects of the protein level of the diet on the concentration and distribution of the protein-bound carbohydrates of serum.

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[2] A preliminary report was presented at the 42nd annual meeting of the Federation of American Societies for Experimental Biology, Philadelphia, Pa., April, 1958.

MATERIALS AND METHODS

Animals. Adult, male Sprague-Dawley rats weighing approximately 400 gm were housed singly in suspended type wire cages with wire bottoms. They were maintained on a commercial chow [3] and tap water until the initiation of the study. The use of larger animals was dictated by the difficulties in obtaining adequate blood samples from smaller rats, especially when depleted.

Diets.[4] Due to limitations in space, it was not possible to feed all groups simultaneously. The Normal, Protein-free, 8% Protein and 27% Protein groups were fed first. The 17, 40 and 64% Protein groups were tested in a second trial. At the time of the second trial, additional animals in the Normal, Protein-free and 27% Protein groups were included with results in good agreement with those obtained previously. *Depletion.* The animals were fed a protein-free diet previously described (Weimer and Nishihara, '57) until they had lost 25% of their original weight, the depletion requiring approximately 22 days. *Repletion.* The diets contained 10% vegetable oil, 4% salt mixture, (U.S.P. XIV), and the vitamin diet fortification mixture which was incorporated into the protein-free diet. The contents of casein and starch were adjusted to yield the desired protein level. All diets were fed ad libitum and daily food consumption was measured.

Serum Chemistry. Blood samples were obtained by cardiac puncture under light ether anesthesia. To avoid the effects of repeated bleedings (Weimer et al., '57) each animal was bled only once. Total serum glycoprotein and seromucoid polysaccharide were determined by the procedures of Weimer and Moshin ('53). Seromucoid protein was determined in the isolated fraction by the method of Lowry et al. ('51). The globulin fraction was isolated from serum by an adaptation of the Pillemer and Hutchinson method (Weimer et al., '57). Globulin protein and total serum protein were determined by

[3] Purina Laboratory Chow.

[4] Obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

a biuret reaction (Weichselbaum, '46). Globulin polysaccharide was determined by an orcinol reaction (Weimer et al., '57). Albumin polysaccharide was determined by subtracting the globulin polysaccharide value from the amount of total serum glycoprotein. The protein content of the albumin fraction was estimated in a similar manner. All chemical analyses were carried out in duplicate.

The mean, standard error of the mean, t and probability values were determined by standard statistical procedures (Snedecor, '56).

Hematology. Approximately 1.5 ml of each of the blood specimens were added to tubes containing a dried mixture of ammonium and potassium oxalates (Kolmer et al., '51). Hematocrit values were determined in Wintrobe tubes by the procedures outlined by Kolmer et al. ('51). Hemoglobin was determined by an acid-hematin method in a Klett-Summerson photoelectric colorimeter (Kolmer et al., '51).

RESULTS AND DISCUSSION

Table 1 summarizes the alterations in weights of the various groups and the hematologic data. Hemoglobin and hematocrit values were in the normal range following depletion but the hematologic results suggested a pronounced hemodilution during repletion. The observations are in accord with those of Peo et al. ('57) and with the earlier studies reviewed by Allison ('55) which demonstrated that changes in plasma volume may accompany depletion and repletion. The relative concentration changes of the serum components were of such magnitude however, that only in a few cases were the results masked by the fluctuations in plasma volume.

Although the biologic evaluation of casein was not of primary importance in the current study, the nutritional data summarized in table 2 are of interest due to the wide range in the protein content of the diets and the employment of larger rats. The 40% protein diet was the best balanced for weight repletion as shown by the more rapid repletion time and the higher food efficiency ratio.

TABLE 1

General and hematologic data [1]

GROUP	NO. RATS	ORIGINAL WEIGHT [2]	DEPLETED WEIGHT [2]	REPLETED WEIGHT [2]	HEMOGLOBIN [2]	HEMATOCRIT [2]
		gm	*gm*	*gm*	*gm/100 ml*	%
Normal	26	407 ± 4.4			14.4 ± 0.18	45 ± 0.4
Protein-free	22	403 ± 7.2	305 ± 5.6 **		14.4 ± 0.17	46 ± 0.5
8% Protein	19	403 ± 5.8	306 ± 4.5 **	400 ± 6.3	13.1 ± 0.09 **	43 ± 0.5 **
17% Protein	27	399 ± 4.6	303 ± 3.3 **	402 ± 4.2	12.3 ± 0.31 **	42 ± 0.7 **
27% Protein	19	403 ± 6.6	302 ± 4.8 **	406 ± 4.7	13.3 ± 0.20 **	42 ± 0.6 **
40% Protein	18	405 ± 3.6	303 ± 3.3 **	400 ± 3.9	12.1 ± 0.13 **	40 ± 0.4 **
64% Protein	17	406 ± 2.6	309 ± 2.2 **	406 ± 3.3	13.1 ± 0.21 **	43 ± 0.5 **

[1] Statistically significant differences from normal values are indicated by ** $P = < 0.01$.

[2] Including the standard error of the mean.

The data demonstrating the effects of the protein level of the diet on the concentration and distribution of the protein-bound carbohydrates of serum are presented in table 3. Protein depletion caused significant reductions in all of the serum glycoproteins with the exception of the polysaccharide component of the albumin fraction.

Following repletion on the 8% protein diet an increase was noted in the total serum glycoprotein concentration due to the elevation of the globulin polysaccharide. The values however were significantly lower than normal. Decreases were exhibited by the seromucoid and albumin fractions. The 17% protein diet was adequate to restore total glycoprotein, albumin and globulin polysaccharide concentrations to their normal values. This was the only repleted group in which the bound carbohydrate of the albumin fraction was in the normal range. The increased protein content of the diet had no effect on the seromucoid fraction.

Repletion with the 27, 40, and 64% casein diets elicited comparable changes in the serum glycoprotein pattern. Total glycoprotein and globulin polysaccharide values returned to normal. Seromucoid hexose concentrations, although significantly greater than those of the rats fed lower levels of protein, were subnormal. Albumin polysaccharide concentrations were also significantly lower than normal. The changes in the partition of the serum glycoproteins due to diet were reflected in the A/G ratios (protein-bound carbohydrate). The carbohydrate-containing proteins of serum were most sensitive to alterations in the protein content of the diet in the range zero to 27%. Increased dietary protein levels had negligible additional effects on either the protein-bound carbohydrates or proteins of serum.

Certain deductions may also be made with regard to the influence of dietary carbohydrate on the concentrations of the serum glycoproteins. The amount of starch in the repletion diets varied from 22% (64% protein) to 78% (8% protein). It was apparent that, in diets which were adequate for the restoration of carcass weight, the concentration of the pro-

TABLE 2

Summary of nutritional data [1]

GROUP	REPLETION TIME	WEIGHT GAINED	FOOD CONSUMPTION	FOOD EFFICIENCY RATIO [2]	PROTEIN CONSUMPTION	PROTEIN EFFICIENCY RATIO [3]
	days	*gm*	*gm*		*gm*	
8% Protein	16.8	94	396.5	0.24	31.7	3.0
17% Protein	11.2	99	291.6	0.34	49.6	2.0
27% Protein	11.5	104	291.0	0.36	78.6	1.3
40% Protein	8.6	97	191.8	0.51	76.7	1.3
64% Protein	11.9	97	235.6	0.41	150.8	0.6

[1] Average values.

[2] Food efficiency ratio $= \frac{\text{Weight gained}}{\text{Food consumed}}$.

[3] Protein efficiency ratio $= \frac{\text{Weight gained}}{\text{Protein consumed}}$.

TABLE 3

Effects of depletion and repletion on the protein-bound carbohydrates of serum [1]

GROUP	TOTAL SERUM GLYCOPROTEIN [2]	SEROMUCOID POLYSACCHARIDE [2]	ALBUMIN POLYSACCHARIDE [2]	GLOBULIN POLYSACCHARIDE [2]	A/G RATIO [2] (PROTEIN-BOUND CARBOHYDRATE)
	mg %	*mg %*	*mg %*	*mg %*	
Normal	164 ± 2.0	26 ± 0.7	19 ± 0.8	145 ± 2.1	0.13 ± 0.006
Protein-free	127 ± 2.1 **	22 ± 0.6 **	19 ± 1.3	108 ± 2.3 **	0.18 ± 0.015 *
8% Protein	135 ± 1.8 **	16 ± 0.5 **	16 ± 1.0 *	119 ± 2.1 **	0.13 ± 0.011
17% Protein	162 ± 1.9	16 ± 0.5 **	21 ± 1.2	141 ± 2.0	0.15 ± 0.012
27% Protein	162 ± 2.3	23 ± 0.8 **	15 ± 1.6 **	147 ± 1.8	0.10 ± 0.011 *
40% Protein	161 ± 2.7	22 ± 0.6 **	15 ± 1.3 **	146 ± 2.5	0.10 ± 0.009 **
64% Protein	163 ± 2.6	23 ± 0.4 **	14 ± 1.3 **	149 ± 2.3	0.09 ± 0.008 **

[1] Statistically significant differences from normal values are indicated: * $P = < 0.05 > 0.01$; ** $P = < 0.01$.

[2] Including the standard error of the mean.

tein-bound carbohydrates of serum did not parallel the level of starch in the diet.

Significant decreases in the protein fractions of serum occurred during depletion (table 4). With the exception of the 8% level of protein, all other repletion diets were adequate for the restoration of the major components of the serum proteins. A higher level of dietary protein, 27%, was required to restore the protein moiety of the seromucoid fraction to normal. A greater degree of hemodilution (table 1) was apparently responsible for the subnormal seromucoid protein value in the 40% protein group.

In contrast to the response of the total serum glycoproteins, the concentrations of the total serum proteins were not only restored to the normal range but were significantly increased due to the elevation of the globulin fractions. Similar observations following repletion with casein or casein hydrolysates have been reviewed by Allison ('55).

In an attempt to delineate further the differential responses of the proteins and protein-bound carbohydrates of serum to protein repletion a study was made of their relationships (table 5). Following repletion there was a significant decline in the polysaccharide-protein ratio of the globulin fraction in all groups. The corresponding ratio for the albumin fraction was in the normal range irrespective of the nutritional state. Decreased total glycoprotein-total protein ratios occurred in all experimental groups. The ratios for the groups which received the higher levels of protein more closely approximated normal values. Significant differential responses also occurred in the carbohydrate-rich seromucoid fraction during repletion.

The studies reported above demonstrate that the protein level of the diet does affect the concentration and distribution of the serum glycoproteins. One of the most pertinent observations was the occurrence of a differential response of the protein-bound carbohydrates and proteins of serum to repletion which resulted in decreased polysaccharide-protein ratios particularly in the globulin and seromucoid frac-

TABLE 4

Effects of depletion and repletion on serum protein concentrations [1]

GROUP	TOTAL SERUM PROTEIN [2]	SEROMUCOID PROTEIN [2]	ALBUMIN [2]	GLOBULIN [2]	A/G RATIO (PROTEIN) [2]
	gm %	*gm %*	*gm %*	*gm %*	
Normal	5.8 ± 0.06	0.27 ± 0.006	2.7 ± 0.04	3.1 ± 0.08	0.87 ± 0.023
Protein-free	4.9 ± 0.07 **	0.23 ± 0.008 **	2.5 ± 0.04 **	2.4 ± 0.06 **	1.04 ± 0.034 **
8% Protein	5.5 ± 0.07 **	0.19 ± 0.004 **	2.7 ± 0.06	2.8 ± 0.07 **	0.96 ± 0.042
17% Protein	6.4 ± 0.08 **	0.21 ± 0.006 **	2.8 ± 0.07	3.6 ± 0.07 **	0.78 ± 0.027
27% Protein	6.0 ± 0.06 *	0.26 ± 0.005	2.6 ± 0.05	3.4 ± 0.06 **	0.76 ± 0.023 *
40% Protein	6.2 ± 0.08 **	0.24 ± 0.007 **	2.7 ± 0.05	3.5 ± 0.08 **	0.77 ± 0.025 *
64% Protein	6.1 ± 0.07 **	0.26 ± 0.007	2.6 ± 0.05	3.5 ± 0.05 **	0.74 ± 0.018 **

[1] Statistically significant differences from normal values are indicated: * $P = < 0.05 > 0.01$; ** $P = < 0.01$.
[2] Including the standard error of the mean.

TABLE 5

Protein-carbohydrate relationships [1]

GROUP	$\frac{\text{TOTAL GLYCOPROTEIN}}{\text{TOTAL PROTEIN}} \times 100$ [2]	$\frac{\text{SEROMUCOID POLYSACCHARIDE}}{\text{SEROMUCOID PROTEIN}} \times 100$ [2]	$\frac{\text{ALBUMIN POLYSACCHARIDE}}{\text{ALBUMIN PROTEIN}} \times 100$ [2]	$\frac{\text{GLOBULIN POLYSACCHARIDE}}{\text{GLOBULIN PROTEIN}} \times 100$ [2]
	%	%	%	%
Normal	2.8 ± 0.04	9.6 ± 0.25	0.7 ± 0.03	4.7 ± 0.11
Protein-free	2.6 ± 0.04 **	9.6 ± 0.19	0.8 ± 0.05	4.5 ± 0.08
8% Protein	2.5 ± 0.03 **	8.4 ± 0.15 **	0.6 ± 0.04	4.3 ± 0.07 **
17% Protein	2.5 ± 0.02 **	7.7 ± 0.18 **	0.8 ± 0.04	3.9 ± 0.06 **
27% Protein	2.7 ± 0.02	8.8 ± 0.28 *	0.6 ± 0.06	4.3 ± 0.05 **
40% Protein	2.6 ± 0.03 **	9.2 ± 0.30	0.6 ± 0.05	4.2 ± 0.05 **
64% Protein	2.7 ± 0.04	8.8 ± 0.24 *	0.6 ± 0.05	4.3 ± 0.05 **

[1] Statistically significant differences from normal values are indicated: * $P = < 0.05 > 0.01$; ** $P = < 0.01$.
[2] Including the standard error of the mean.

tions. The results also provide some evidence for the hypothesis that different physiologic mechanisms may be involved in the metabolism of the carbohydrate-rich and carbohydrate-poor proteins of serum (Weimer and Redlich-Moshin, '53). Many factors might be responsible for the noted variances: (a) greater utilization of the protein-bound carbohydrates for cellular regeneration (b) sub-optimal amounts of a nutritional factor in purified diets necessary for the synthesis of protein-bound carbohydrates (c) utilization of carbohydrate-rich proteins of serum (e.g., seromucoid fraction) in the synthesis of carbohydrate-poor proteins (d) slower rate of synthesis (e) slower release from the site of synthesis. It should be pointed out that (a) and (c) have been suggested by Shetlar et al. ('49) and by Werner ('49) respectively, to account for the increased concentrations of serum glycoproteins in various pathologic and physiologic states.

The probability of a time factor being involved in the restoration of polysaccharide-protein ratios to normal following repletion is under investigation in a serial study.

SUMMARY

The influence of the protein level of the diet on the concentration and distribution of the serum glycoproteins has been investigated by the rat depletion-repletion method. Male rats weighing approximately 400 gm were fed a protein-free diet until they had lost 25% of their weight. They were then repleted with isocaloric diets containing 8, 17, 27, 40, and 64% of casein. When their original weight was regained, the animals were exsanguinated. The protein-bound carbohydrates and proteins of whole serum, and of the seromucoid, albumin, and globulin fractions, and the hemoglobin and hematocrit values of blood were determined.

Following depletion, significant decreases occurred in all serum constituents with the exception of the protein-bound carbohydrate of the albumin fraction. Diets containing 17% or more of casein were adequate to restore total glycoprotein and globulin polysaccharide levels to their normal range. Sub-

normal values for the polysaccharide components of the seromucoid and albumin fractions occurred on repletion diets. With the exception of the 8% level of casein, all other repletion diets were not only adequate for the restoration of the total serum proteins but elicited marked increases due to the elevation of the serum globulins. The differential response of the protein-bound carbohydrates and proteins of the seromucoid and globulin fractions resulted in decreased polysaccharide-protein ratios for the fractions in all repleted groups.

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EFFECT OF ANTIBIOTICS ON THE WEIGHT OF CHICKS AND RATS FED RAW OR HEATED SOYBEAN MEAL [1]

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INTRODUCTION

In addition to their ability to minimize infections, appropriate antibiotics regularly improve the growth of rats or chicks on diets limiting in one of the B vitamins (Biely and March, '51; Monson et al., '54, Lih and Baumann, '51; Sauberlich, '52; Guggenheim, '53). Preliminary evidence suggests that antibiotics may also "spare" other nutrients (Migicovsky et al., '51; Berry and Schuck, '54). Recently Borchers et al. ('57) fed rats a mixture of 0.1% each of procaine penicillin and streptomycin sulfate in diets containing soybean meal as the only source of protein, and observed that the weight of the rats on raw soybean meal plus antibiotics equalled that on the heated soybean meal. In the present study, the effects of 5 antibiotics were determined in rats and chicks fed raw or heated soybean meal.

EXPERIMENTAL

Day-old New Hampshire × Single Comb White Leghorn crossbred chicks in groups of 10, were housed in conventional batteries with raised screen bottoms. Feed and water were

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supplied ad libitum and the individual birds were weighed weekly for a period of 4 weeks. The basal ration contained the following in grams: raw or heated soybean meal,[3] 450; salts V (Briggs et al., '43), 60; A and D "feeding oil" (1500 I.U. vitamin A and 300 I.U. vitamin D/gm), 5; choline chloride, 2; DL-methionine, 5; soybean oil, 40; and sucrose, 433. The vitamin mix provided in milligrams per kilogram of diet: biotin, 0.2; pyridoxine, 4.0; riboflavin, 6.0; calcium pantothenate, 20.0; niacin, 50.0; α-tocopherol, 3.0; inositol, 1000.0; thiamine·HCl, 6.0; vitamin B_{12}, 0.03; *p*-aminobenzoic acid, 100.0; and folic acid, 4.0. The antibiotics were added at levels of 1000 mg or less per kilogram of diet. All the protein in the diet (20%) was furnished by soybean meal. Methionine was added at a 0.5% level which, with the methionine in the soybean meal, brought the total level in the diet up to an estimated 0.8%.

In the rat experiments, male albino rats of the Holtzman strain were housed in individual cages, fed diets and water ad libitum, and the individual animals were weighed weekly for 4 weeks. Each experimental ration was supplied to 5 rats. The basal ration contained the following in grams: raw or heated soybean meal, 250; DL-methionine, as indicated in tables 3 and 4; Wesson salts, 40; starch, 503; hydrogenated vegetable oil,[4] 200; and choline chloride, 1. The vitamin mix provided in milligrams per kilogram: riboflavin, 3.0; thiamine·HCl, 6.0; pyridoxine, 2.0; niacin, 25.0; calcium pantothenate, 20.0; menadione, 10.0; inositol, 100.0; biotin, 0.1; folic acid, 2.0; vitamin B_{12}, 0.02; and α-tocopherol, 0.03. One drop of Haliver oil per week supplied vitamins A and D. The antibiotics were added at the comparatively high level of 1000 mg per kilogram of ration to correspond with the levels used by Borchers et al. ('57).

[3] The raw meal used throughout these studies was solvent extracted, uncooked meal obtained from Archer-Daniels-Midland Co. The heated meal was commercial 44% protein, solvent-extracted meal.

[4] Crisco.

RESULTS

High levels of novobiocin, procaine penicillin, chlortetracycline, zinc bacitracin or streptomycin resulted in significant increases in the growth of chicks fed raw soybean meal (table 1); the magnitude of the growth response ranged from 31 to 51%. These antibiotics stimulated growth less effectively when heated soybean meal was fed. Low levels of these antibiotics (10 mg per kilogram), stimulated growth only slightly when the raw soybean meal was fed and actually appeared to depress growth slightly with the heated meal. In another experiment in which novobiocin was fed with raw soybean meal at different levels ranging from 10 to 1000 mg per kilogram of diet, growth stimulation was obtained only at the highest level fed.

TABLE 1

Effect of two levels of antibiotics on the weight of 4-week-old chicks fed raw or heated soybean meal

ANTIBIOTIC	RAW SOYBEAN MEAL		HEATED SOYBEAN MEAL	
	Av. weight	% of control weight	Av. weight	% of control weight
mg/kg	*gm*		*gm*	
None	175	100	342	100
Procaine penicillin, 1000	243	139 [1]	376	110 [1]
Chlortetracycline, 1000	230	131 [1]	355	104
Novobiocin, 1000	264	151 [1]	376	110 [1]
Zinc Bacitracin, 1000	244	139 [1]	389	114 [1]
Streptomycin, 1000	260	149 [1]	379	111 [1]
None	198	100	380	100
Procaine penicillin, 10	170	86	328	86
Chlortetracycline, 10	217	110	359	94
Novobiocin, 10	205	104	336	88
Zinc Bacitracin, 10	207	105	338	89
Streptomycin, 10	216	109	356	94

[1] Significant at the 0.01 level.

When sucrose was replaced by a level of sorbitol that produces marked increases in the growth of thiamine-deficient rats (Yudkin and Morgan, '57; Jones, '58), the growth of chicks on the raw soybean meal failed to improve (table 2).

TABLE 2

Effect of antibiotics and carbohydrates on the weight of 4-week-old chicks fed raw or heated soybean meal

SOYBEAN MEAL SOURCE	CARBOHYDRATE	SUPPLEMENT	AVERAGE FINAL WEIGHT	% OF CONTROL WEIGHT
		%	*gm*	
Raw	Sucrose	None	246 ± 25.9 [1]	100
Raw	Sucrose	Procaine penicillin, 0.1	279 ± 14.1	113
Raw	Sucrose	Sorbitol, 10	254 ± 9.9	103
Raw	Sucrose	Sorbitol, 10 + Procaine penicillin, 0.1	272 ± 10.2	111
Raw	Dextrin	None	187 ± 8.6	76
Raw	Dextrin	Procaine penicillin, 0.1	216 ± 14.5	88
Raw	Starch	None	223 ± 22.5	91
Raw	Starch	Procaine penicillin, 0.1	260 ± 24.1	106
Heated	Sucrose	None	364 ± 9.5	100
Heated	Sucrose	Procaine penicillin, 0.1	400 ± 11.3	110

[1] Standard error of the mean.

TABLE 3

Effect of antibiotics on the growth of rats fed raw or heated soybean meal

SUPPLEMENT	0.1% DL-METHIONINE		0.3% DL-METHIONINE [1]		0.6% DL-METHIONINE	
	Av. final weight	% of control weight	Av. final weight	% of control weight	Av. final weight	% of control weight
mg per kg	*gm*		*gm*		*gm*	
Raw soybean meal	142 ± 10.7 [2]	100	147 ± 7.9	100	179 ± 10.5	100
+ Procaine penicillin, 1000	175 ± 14.7	123	174 ± 9.0	118 [3]	187 ± 15.6	104
+ Chlortetracycline, 1000	196 ± 10.7	138 [3]	202 ± 9.2	137 [3]	194 ± 8.9	108
+ Novobiocin, 1000	148 ± 12.9	104	166 ± 6.9	113	153 ± 8.4	85
+ Zinc bacitracin, 1000	177 ± 17.0	125	183 ± 12.0	124 [3]	180 ± 9.6	101
+ Streptomycin, 1000	187 ± 7.5	142 [3]	188 ± 12.2	128 [3]		
Heated soybean meal			190 ± 9.6	100	177 ± 12.3	100
+ Procaine penicillin, 1000			215 ± 3.7	113	207 ± 9.6	117
+ Chlortetracycline, 1000			205 ± 7.4	108	186 ± 15.4	105
+ Novobiocin, 1000			178 ± 6.3	94	191 ± 3.8	108
+ Zinc bacitracin, 1000			213 ± 8.1	112	207 ± 12.9	108
+ Streptomycin, 1000			216 ± 5.7	114		

[1] Results for the 0.3 DL-methionine level with the raw soybean meal are the average of two experiments.
[2] Standard error of the mean.
[3] Significant at the 0.05 level.

The replacement of the sucrose in the raw soybean diet by starch or dextrin actually depressed growth somewhat. In this series, 0.1% of procaine penicillin produced about a 10% increase in weight under all the various conditions tested.

Rats fed soybean meal as the sole source of protein usually grew better when a high level of one of the 5 antibiotics was also present in the diet (table 3). The least effective antibiotic tested was novobiocin. Chlortetracycline, zinc bacitra-

TABLE 4

Effect of antibiotics on the growth of rats fed soybean meal with graded levels of methionine

SOYBEAN MEAL SOURCE	ANTIBIOTIC	ADDED DL-METHIONINE	AVERAGE FINAL WEIGHT	% OF CONTROL WEIGHT
	mg per kg	*gm %*	*gm*	
Raw	None	0.1	138 ± 9.2 [1]	100
Raw	Procaine penicillin, 1000	0.1	165 ± 6.3	120 [2]
Raw	Chlortetracycline, 1000	0.1	169 ± 1.3	122 [2]
Raw	None	0.3	127 ± 5.9	100
Raw	Procaine penicillin, 1000	0.3	162 ± 11.3	128 [2]
Raw	Chlortetracycline, 1000	0.3	161 ± 6.2	127 [2]
Raw	None	0.6	145 ± 6.2	100
Raw	Procaine penicillin, 1000	0.6	166 ± 11.3	114
Raw	Chlortetracycline, 1000	0.6	162 ± 4.7	112
Heated	None	0.6	198 ± 15.8	100
Heated	Procaine penicillin, 1000	0.6	194 ± 12.2	98
Heated	Chlortetracycline, 1000	0.6	217 ± 6.8	110

[1] Standard error of the mean.

[2] Significant at the 0.05 level.

cin, streptomycin, and procaine penicillin all increased growth significantly when raw soybean meal was fed in diets limiting in methionine. The effectiveness of the antibiotics was diminished or disappeared entirely when a high level of synthetic methionine (0.6%) was added to the raw soybean diet, or when a moderate level (0.3%) was added to the diet containing the heated soybean meal (table 3). Three levels of synthetic methionine, with or without antibiotics, were fed in another experiment (table 4). Procaine penicillin and chlortetracycline improved growth significantly when the methio-

nine in the diet was limiting, but when the level of the amino acid was adequate, the response due to antibiotics was about half of that obtained on the methionine-deficient diets, and the differences in weight were no longer statistically significant at the 0.05 level. The addition of methionine alone to the basal diet did not seem to improve growth. In all cases, however, growth on raw soybean meal plus antibiotic was inferior to that in control groups fed heated soybean meal.

DISCUSSION

The present experiments with chicks show that antibiotics added at a high level improve the growth of birds fed raw soybean meal, although not to the level obtained with heated soybean meal. The magnitude of the percentage increases in weight brought about by the addition of antibiotics to the raw meal (31 to 51%) was considerably greater than that which usually results when these drugs are added to diets limiting in one of the B vitamins, and very much greater than the gains in weight resulting when antibiotics are added to nutritionally complete chick diets. Thus, antibiotics would seem to be more effective against the defect(s) in raw soybean meal, than against the nutritional defects usually studied, or against the bacteriological defects ("disease level") frequently present in chickens.

The diets used in these experiments were adequate with respect to all the known required vitamins and minerals, and Borchers and Ackerman ('51) have shown that neither liver, yeast nor casein improved the nutritional quality of ray soybean meal except for the methionine furnished. Thus, it appears unlikely that the antibiotics were sparing some limiting nutrient in our studies; this conclusion is also supported by the normal growth obtained with the heated soybean meal in the absence of antibiotics.

Any interpretation of the present results with antibiotics must necessarily involve the nutritional defect in raw soybean meal, the nature of which is uncertain in spite of much experi-

mental work on the subject. One of the adverse factors in raw soybean meal is the trypsin inhibitor (Read and Haas, '38). However, Borchers et al. ('48) showed that a concentrated trypsin inhibitor did not depress growth when fed to either chicks or rats; a crude trypsin preparation overcame the growth depression caused by raw soybean meal, but the activity of this preparation remained even after destruction of the trypsin activity by autoclaving (Borchers and Ackerman, '51). It is possible that antibiotics, by their action on the intestinal flora, aid in destroying or weakening the trypsin inhibitor in a manner similar to its destruction when raw soybean meal is heated. Another possibility is that antibiotics may be acting toward a better digestibility or absorption of the protein in the raw meal. It has frequently been suggested that a major defect in the raw meal consists of an unavailable linkage that renders methionine resistant to release by digestive enzymes. If this is so, growth stimulation due to high levels of antibiotics would imply denaturation or partial hydrolysis of the protein either directly, since the required level of antibiotic is so high, or indirectly, through altered microbial flora. Jones ('58) observed that lower levels of antibiotics increase utilization of vitamin A acetate, apparently via a more efficient hydrolysis of the vitamin A ester. A similar improvement in the hydrolysis of raw soybean protein might, therefore, be postulated. Still another possibility is an improved absorption of difficultly-absorbable substances (methionine peptides) through the thinner intestinal walls that have been reported in antibiotic-fed chicks.

The present experiments add further examples to the list of differing effects of antibiotics in different species. In the rat experiments, chlortetracycline and streptomycin consistently gave the best responses, while novobiocin gave the lowest response. In the chick experiments, streptomycin and novobiocin consistently gave the best responses. Physiological differences were probably responsible for the different behavior of novobiocin in both species. In previous experiments (Braham, '58; Braham et al., '58), in which riboflavin was

the limiting factor, novobiocin was more effective in stimulating the growth of chicks than of rats; it was partially effective in chick diets limiting in folic acid.

SUMMARY

High levels of procain penicillin, chlortetracycline, novobiocin, zinc bacitracin, or streptomycin improved the growth of chicks fed raw soybean meal by an average of 31 to 51%; when heated soybean meal was fed, the antibiotics caused lesser increases, 4 to 14%. Growth on raw soybean meal plus antibiotics was, however, less than that on the heated meal.

Antibiotics also stimulated the growth of rats fed raw soybean meal as the only protein source in the diet. The effect was most marked when the level of methionine in the diet was marginal. Novobiocin was considerably less effective in the rat than in the chick.

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EFFECT OF DIETARY PROTEIN LEVEL ON SEVERAL OXIDATIVE ENZYMES OF THE HEART, MUSCLE AND LIVER OF CATTLE[1]

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Many workers have studied the effect of dietary protein on enzymes of various tissues of experimental animals. Elson ('47) reported that succinoxidase activity of rat and mouse livers is reduced in protein depletion. Beneditt, Steffee, Hill and Johnston ('49) found that when rats were fed protein-deficient diets, succinoxidase decreased progressively with time and faster than liver protein. Nielands ('54) found that the inhibition of the sulfhydryl group of lactic dehydrogenase by the inhibitor *p*-chloramercurobenzoate was reversed with excess cysteine. Ross and Ely ('51) found that low-protein diets resulted in a decrease in lactic and succinic dehydrogenases in the liver within 30 days with both young and adult rats. Williams et al. ('50), Litwak et al. ('50, '52), Westerfeld and Richert ('49, '50, '54) and Wainio et al. ('53, '54) have done much to relate dietary protein to enzymatic activity in animal tissues.

In the present study succinoxidase and lactic dehydrogenase were studied in the heart ventricle and gracilis muscle, and xanthine oxidase in the liver of two-year old heifers fed 4 levels of dietary protein. In some cases the ruminants did not respond to deprivation of protein in the same manner as the rats, mice and dogs studied by the above investigators.

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METHODS

Twenty Hereford heifers at 26 months of age and averaging 666 lb. each were divided equally into 4 dietary groups and fed the rations shown in table 1, which provided 1.34, 1.06, 0.71 and 0.62 lb. of crude protein per animal per day. It was estimated that the respective rations contained 100, 77, 56 and 46% of the digestible protein recommended by the National Research Council (Bedrak, '58).

TABLE 1

Rations containing four levels of protein

CONSTITUENT	AMOUNT			
	Ration 1	Ration 2	Ration 3	Ration 4
Cottonseed meal, lb. (41% protein)	2.0	1.1	0.4	0.0
Cane molasses, lb. (8% crude protein)	3.5	4.5	5.3	5.8
Pangola hay, lb. (2.8% crude protein, dry wt.)	9.0	9.0	9.0	9.0
Mineral-Vitamin mix [1]	1.0	1.0	1.0	1.0
Crude protein, lb./day offered	1.39	1.12	0.88	0.76
Crude protein, lb./day eaten	1.34	1.06	0.71	0.62
Estimated digestible protein, lb./day eaten	0.96	0.74	0.46	0.38
Estimated % N.R.C. recommendations for digestible protein	100	77	56	46

[1] The salt is sold as the Carey Trace Mineralized salt, by the Carey Salt Company, Hutchinson, Kansas. It contains the following: NaCl, 98; $MnCO_3$, 0.052; FeO, 0.347; $CuCO_3$, 0.006; $CoCO_3$, 0.002; CaI_2, 0.009; and $ZnCO_3$, 0.010%. The vitamins: A and D = 22,000 and 2,000 U.S.P. units/animal/day, respectively; and E = 0.5 gm/animal/day. Each heifer was fed 59 gm of mineral-vitamin mix per day, mixed with 99 gm sterilized bone meal and 294.6 gm of citrus meal. The animals also had (1) plain salt, (2) bone meal and (3) trace salt free choice.

The heifers were fed the rations a total of 160 to 180 days before being slaughtered. They were fed for 112 days and then bred during the next two estrous periods, as needed. Forty-four days later dietary groups 1, 2, 3 and 4 had 3, 4, 1 and 3 normal embryos, respectively. Pregnancy had no effect on enzyme activity and for this reason no data concerning this are presented. When the animals were sacrificed, samples of the heart, liver and gracilis muscle were obtained within 10 minutes, frozen solid immediately and analyzed for enzyme activity within a few days. Samples handled in this

manner gave the same enzyme activities over a period of several days as those analyzed fresh. The tissues were homogenized with glass homogenizers, diluted to appropriate concentrations and analyzed with a Warburg respirometer. The succinoxidase was determined according to Schneider and Potter ('43) at 38°C; the lactic dehydrogenase by the method of Green and Brosteaux ('36) using adrenaline as the electron carrier; and xanthine oxidase either by the method of Axelrod and Elvehjem ('41) or as recommended by Dhungat and Sreenivasan ('54). Nitrogen in the homogenates was determined by the Kjeldahl method, and the enzyme activity calculated as microliters of oxygen uptake per milligram of nitrogen per hour. It was also calculated on the wet weight basis. Lactic acid was determined in the left ventricle of the heart and gracilis muscle by the colorimetric procedure of Barker and Summerson ('41). The statistical significances of the effects of the dietary treatments were evaluated according to Snedecor ('46). Bedrak ('58) has reported on the weight changes, several blood constituents and reproductive performance of these heifers.

RESULTS AND DISCUSSION

In figure 1 data are presented for the effect of dietary protein level on the succinoxidase activity in the heart and gracilis muscle of the heifers. The heart had less ($P < 0.05$) succinoxidase activity in those heifers fed the two lower levels of dietary protein. The significance of the low value for the group receiving 0.71 lb. dietary protein is not known. More work would be justified to confirm this observation. Wainio and co-workers ('54) deprived rats of protein and found no decrease in succinoxidase activity in the heart on the nitrogen basis. The difference between the hearts of cattle and rat may be a species difference in response to dietary protein, or it may be due to the variation in physiological age. The protein decreased ($P < 0.05$) in the heart as the dietary protein was decreased (table 2).

The muscle showed an increase ($P < 0.01$) in succinoxidase activity as the dietary protein was decreased (fig. 1). The

values were multiplied by 10 before being plotted. The increase was also significant when the activity was calculated on the wet weight basis. As shown in table 2 the protein of the muscle decreased ($P < 0.05$) as the dietary protein was decreased. However, this decrease in protein was not of the same magnitude as the increase in succinoxidase activity. As far as the authors know there has been no previous report of the effect of dietary protein on the succinoxidase of muscle.

TABLE 2

Effect of dietary protein level on the percentage of protein in the heart ventricle, gracilis muscle and liver of heifers

CRUDE PROTEIN EATEN PER DAY	NO. HEIFERS	% PROTEIN ON WET WEIGHT BASIS		
		Heart [1]	Muscle [1]	Liver [2]
lb.		%	%	%
1.34	5	17.2	20.7	14.4
1.06	5	16.8	20.7	14.3
0.71	5	16.7	20.2	13.4
0.62	5	16.6	19.2	12.8

[1] $P < 0.05$.
[2] $P < 0.01$.

Data are shown in figure 1 for the effect of dietary protein level on the lactic dehydrogenase activity of the heart and muscle of the heifers. Approximately three times as much activity was present in the heart as in the muscle. Neither tissue was influenced by the dietary protein level. Ross and Ely ('51) found that low-protein diets gave a decrease in the lactic dehydrogenase of the liver of rats. Unfortunately this enzyme was not determined in the liver of these cattle. Heart is well known for its capacity to utilize lactic acid and would be expected to have an active lactic dehydrogenase system. As shown in table 3, lactic acid was found to be about half as concentrated in the hearts of the heifers that ate 0.71 and 0.62 lb. of protein as the ones that consumed 1.34 and 1.06 lb. of protein per day. The difference in lactic acid concentration was not significant. However, the data suggest that a considerable variation may exist in the concentration of a metabolite,

and the activity of a corresponding oxidative enzyme may not be affected. As is evident in table 3 the lactic acid in the muscles of the heifers fed the low-protein diets was greater than in those fed the higher protein diets, but these differences were not significant.

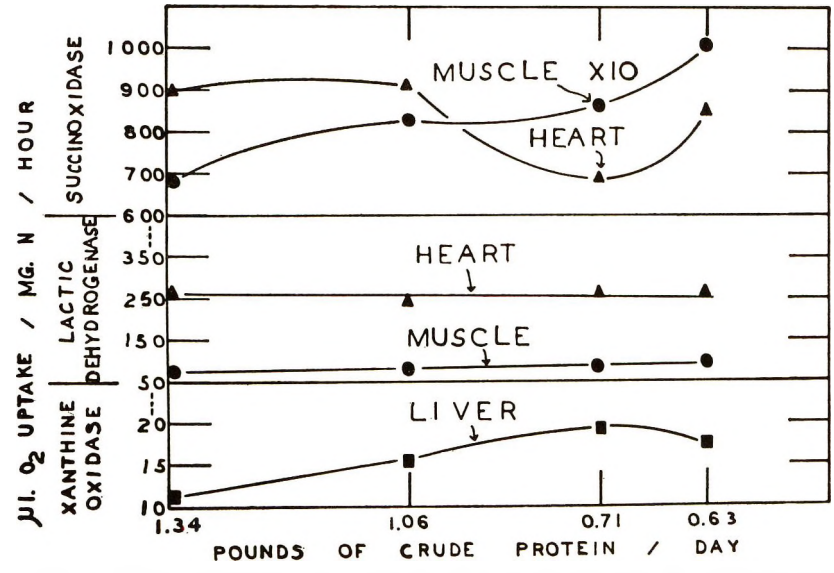

Fig. 1 Effect of pounds of crude protein eaten per day by cattle on the succinoxidase and lactic dehydrogenase of the heart and gracilis muscle, and xanthine oxidase in the liver.

In figure 1 are presented data that were obtained for the effect of level of dietary protein on xanthine oxidase in the liver of heifers. These values were determined using pyrophosphate according to Dhungat and Sreenivasan ('54). The lower-protein dietary groups had more ($P < 0.01$) xanthine oxidase activity. A similar experiment was made a year previous to the present study with yearling heifers and the xanthine oxidase was determined in the livers without using the pyrophosphate method of Axelrod and Elvehjem, ('41). While only about half the activity was observed in these heifers, the same dietary effect occurred. These data are very different

from those of many workers (MacQuarrie and Venosa, '45; Williams and co-workers, '50, '52; Westerfeld and Richert, '49, '50; Wainio et al., '53) who observed in rats, mice and dogs that this enzyme in liver decreases when dietary protein is decreased.

TABLE 3

Effect of dietary protein on the lactic acid of the heart and gracilis muscle of heifers

PROTEIN EATEN PER DAY	NO. HEIFERS	LACTIC ACID/GM WET WEIGHT	
		Heart	muscle
lb.		*mg*	*mg*
1.34	5	6.1 ± 3.0 [1]	2.9 ± 1.2
1.06	5	7.9 ± 5.0	1.8 ± 0.8
0.71	5	3.2 ± 2.2	3.9 ± 2.8
0.68	5	4.2 ± 3.8	3.8 ± 4.2

[1] Mean and standard deviation.

There have been a few reports of factors that increase xanthine oxidase activity. Rabbi et al. ('55) reported that a lack of a water-soluble factor in casein, not vitamin B_{12}, resulted in an increase of the enzyme in the liver of rats. Dhungat and Sreenivasan ('52) found that aerobic incubation of liver slices from protein-deprived rats in serum containing glucose increased xanthine oxidase activity. In the present study the high protein group which was lowest in xanthine oxidase also was fed the lowest amount of molasses (table 1). Is it possible that extra glucose from the molasses was present in the homogenates of the livers from the low protein dietary group and that this caused a stimulation of the xanthine oxidase activity? Dinning ('53) reported that liver from vitamin E-deficient rabbits had very high xanthine oxidase activity. The heifers of the present study were fed vitamin E (table 1), but the low-dietary-protein groups may have failed to utilize the vitamin. Also, protein deprivation may deplete the liver of non-enzyme protein and thereby cause greater activity per milligram of total nitrogen in the liver of the low-protein dietary groups. However, the decrease ($P < 0.01$) in protein in the liver as shown in table 2 is not of sufficient magnitude to account for the increase in enzyme activity on this basis.

SUMMARY

Twenty Hereford heifers 26 months of age were divided equally into 4 dietary groups and fed rations containing 1.34, 1.06, 0.71 and 0.62 lb. of crude protein per day as cottonseed meal, cane molasses, Pangola hay and a mineral-vitamin mixture. The heifers were fed the rations for 160 to 180 days before being slaughtered. They were fed for 112 days and then bred during the next two estrous periods, if needed. Forty-four days later dietary groups 1, 2, 3 and 4 had 3, 4, 1 and 3 normal embryos, respectively. Pregnancy had no effect on enzyme activity.

Succinoxidase decreased ($P < 0.05$) in the heart ventricle, but increased ($P < 0.01$) in the gracilis muscle as the dietary protein decreased.

Approximately three times as much lactic dehydrogenase was found in the heart as in the muscle, but the rations had no effect on the enzyme in either tissue.

Xanthine oxidase activity increased ($P < 0.01$) in the liver of the lower dietary protein groups.

The protein in the heart ($P < 0.05$), gracilis muscle ($P < 0.05$) and liver ($P < 0.01$) decreased as the dietary protein was decreased.

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THE DEVELOPMENT AND CURE OF PYRIDOXINE DEFICIENCY SYMPTOMS IN WEANLING MICE

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The functions of vitamin B_6 in the animal body have been studied for the past quarter of a century. Pyridoxine and its derivatives, pyridoxal and pyridoxamine which possess vitamin B_6 activity, have been shown to be important factors in the transamination and decarboxylation of amino acids as well as in the metabolism of fatty acids.

The role of pyridoxine in the hematological system has also been investigated. Fouts et al. ('38) reported the development of a severe microcytic, hypochromic anemia when vitamin B_6 was eliminated from the diet of puppies. The anemia was cured by the addition to the diet of the missing factor. Later, Fouts et al. ('40) demonstrated the development of a similar type of anemia in adult dogs. Street et al. ('41) treated the vitamin B_6-deficiency anemia in dogs with vitamin B_6 concentrates which produced a rapid increase of erythrocyte and hemoglobin levels. On the other hand, ferrous sulfate produced no hematological response.

Hughes and Squibb ('42) confirmed the need of pyridoxine in the diet of swine since a deficiency of this vitamin resulted in poor appetite, reduced growth, unsteady gait, epileptic-like fits and convulsions, and a microcytic, hypochromic anemia. A cessation of the fits, a return to normal blood hemoglobin, and a resumption of growth and physical well-being occurred upon

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addition of the factor to the diet. Wintrobe et al. ('43) reported that the anemia developed by pyridoxine-deficient swine was characterized by microcytosis, an increase of polychromatophilia, reticulocytes, and nucleated red cells.

Rinehart and Greenberg ('49) and Poppen et al. ('52) agreed in the description of the characteristics of the hypochromic anemia developed in pyridoxine-deficient rhesus monkeys.

Morris et al. ('53) reported a severe anemia in mice after a prolonged pyridoxine depletion. Extremely low hemoglobin, erythrocyte, and leucocyte levels and large, platelike reticulocytes of unusual morphology were found. The injection of gonadotrophin had no direct effect on the blood picture in vitamin B_6 deficiency. Working with adrenalectomized and intact mice, Mueller et al. ('51) and Weir and Mueller ('51) induced a pyridoxine-deficiency anemia which showed the same hematological picture in both the adrenalectomized and intact mice.

The present investigation was designed to ascertain the effect of parenterally administered pyridoxine, folic acid, or vitamin B_{12}, singly and in combination, on the growth, hematologic data, clinical symptoms, and survival of pyridoxine-deficient weanling male and female mice.

EXPERIMENTAL PROCEDURE

In the present study 150 male and 150 female 21-day old weanling mice [2] were used. Each mouse was weighed and placed in an individual compartment of the experimental cage in a constant temperature and constant humidity room. Ten per cent of the mice were randomly selected and placed on diet L. A second group, consisting of an additional 10% of the mice, were fed a pyridoxine-deficient diet, diet L_{19}. The remaining 120 male and 120 female mice were fed a diet which was pyridoxine-deficient and to which 25 mg of deoxypyridox-

[2] CFW strain of pure inbred albino mice obtained from the Carworth Farms, New City, Rockland County, New York.

ine was added per kilogram of ration, diet L_{17}. Deoxypyridoxine possesses antipyridoxine activity; it was added to the pyridoxine-deficient diet in order to effect a more rapid and complete pyridoxine depletion. After 5 days on diet L_{17}, the mice were fed diet L_{19}. The composition of the three diets is shown in table 1. Food and water were given ad libitum. The weight and food consumption for each mouse were recorded three times per week.

TABLE 1

Composition of the synthetic diets

COMPONENTS	DIET L	DIET L_{17}	DIET L_{19}
	%	%	%
Purified casein [1]	30	30	30
Sucrose	48	48	48
Lard	15	15	15
Salt mixture [2]	5	5	5
Alphacel	2	2	2
	mg/kg	*mg/kg*	*mg/kg*
Thiamine hydrochloride	10	10	10
Riboflavin	10	10	10
Pyridoxine	10	0	0
Calcium pantothenate	40	40	40
Choline chloride	500	500	500
Alpha tocopherol	40	40	40
Vitamin A ester concentrate, (67,500 units)	337.5	337.5	337.5
Vitamin D (Viosterol), (5000 units)	12.5	12.5	12.5
Deoxypyridoxine	0	25	0
	μg/kg	*μg/kg*	*μg/kg*
Biotin	200	200	200

[1] Vitamin-free casein. Nutritional Biochemical Corp., Cleveland, Ohio.

[2] Hawk-Oser Salt Mixture. Hawk, P. B., B. L. Oser and W. H. Summerson 1954 Practical Physiological Chemistry, 13th Edition. The Blakiston Company, New York, N.Y., p. 1374.

In order to obtain representative, normal hematologic data on male and female mice complete blood counts were randomly made on 25% of the 21-day old weanlings on the day they were received in the experimental laboratory. The blood count included a hemoglobin, erythrocyte, leucocyte and differential count. Duplicate hemoglobin determinations were made

by the oxyhemoglobin method of Evelyn ('36) using the Evelyn Photoelectric Colorimeter.

The blood smears were stained with Wright's stain and differential counts were made by counting 200 cells. Lymphocytes and monocytes are reported as mononuclear cells; polymorphonuclear neutrophils, eosinophils, and basophils are reported as polynuclear cells, although in the actual count each type of cell was recorded individually.

The mice were observed daily for clinical pyridoxine-deficiency symptoms. Twice weekly complete blood counts were made to ascertain the onset and progress of the anemia. Upon development of the anemia, as evidenced by the blood counts, the mice were randomly placed on vitamin supplementations. The supplementation consisted of daily injections of 50 μg of pyridoxine, 0.4 μg of vitamin B_{12}, or 5 μg of folic acid, singly or in all possible combinations. These amounts of the respective vitamins were dissolved in physiological saline made up to 0.1 ml. For each of the treatments, there were 9 males and 9 females. The treatments were: A, diet L without vitamin supplementation; B, diet L_{19} without vitamin supplementation; C, injection of the saline solution only; D, pyridoxine; E, vitamin B_{12}; F, folic acid; G, pyridoxine and vitamin B_{12}; H, pyridoxine and folic acid; I, vitamin B_{12} and folic acid; J, pyridoxine, vitamin B_{12} and folic acid. The mice were continued on diet L_{19} throughout the 20-day period of vitamin supplementation.

Complete blood counts were made on the day vitamin supplementation was initiated and on the second, 10th, and 20th day of treatment. At the end of the 20th day of vitamin treatment the experiment was terminated.

The hematologic data and weight data were statistically analyzed by the analysis of variance according to techniques given by Cochran and Cox ('50).

RESULTS AND DISCUSSION

Fifty-three males and 53 females of the 120 males and 120 females given deoxypyridoxine succumbed during the 5-day

period on diet L_{17} or within a week after they were changed from diet L_{17} to diet L_{19}. None of the mice placed directly on diet L_{19} or diet L succumbed during this period. Hematologic studies of the mice that succumbed showed no evidence of a severe anemia but the average daily weight loss was greater than that of the survivors which also had received deoxypyridoxine. Apparently, despite the low concentration of deoxypyridoxine (25 mg per kilogram of ration) and the short period (5 days) it was fed, deoxypyridoxine proved too toxic for 21-day-old weanling mice as evidenced by the death of 44% of the mice fed diet L_{17}.

The average, normal hematologic data and the average weight of the weanling mice at 21-days of age and prior to feeding the three experimental diets are shown in table 2. The mean hemoglobin, erythrocyte, leucocyte and differential count values are within accepted normal values.

The average hematologic data and the average weights of the mice fed diet L and the pyridoxine-deficient diets prior to starting the vitamin supplements are summarized in table 3. Analysis of variance of the data demonstrated a highly significant difference ($P < 0.01$) in hemoglobin, erythrocyte, leucocyte and differential count levels between the pyridoxine-deficient mice and the controls. There was a drop of approximately 38, 35 and 52% in the hemoglobin, erythrocyte and leucocyte levels, respectively, of the pyridoxine-deficient mice compared to the controls. The leucopenia was accompanied by lymphopenia and granulocytosis. The mononuclear cells decreased from the normal 70 to 50% and the polynuclear cells increased from the normal 30 to 50%. In the pyridoxine-deficient mice the proportion of mononuclear cells to polynuclear cells was equal.

Rinehart and Greenberg ('49) reported that the 50% decrease in the total leucocyte count of pyridoxine-deficient rhesus monkeys affected granulocytes and mononuclear leucocytes proportionately without selective lymphopenia. In the case of pyridoxine-deficient mice the 52% decrease in the total number of leucocytes was accompanied by lymphopenia.

TABLE 2

The average normal hematologic data and the weights of 21-day old weanling mice

SOURCE	NUMBER OF MICE		MEAN		RANGE	
	Male	Female	Male	Female	Male	Female
Hematologic data						
Hemoglobin, gm/100 ml	38 [1]	38 [1]	12.90	12.90	11.60–14.80	11.20–14.10
Erythrocytes, $mm^3 \times 10^{-6}$	38	38	9.48	9.48	6.73–13.45	8.14–11.11
Leucocytes, $mm^3 \times 10^{-3}$	38	38	6.48	5.80	1.30– 9.85	1.65– 9.20
Mononuclear cells, %	38	38	75.70	70.50	57.00–90.00	58.00–85.00
Polynuclear cells, %	38	38	24.30	29.50	10.00–43.00	15.00–42.00
Initial weight, gm			11.20	11.60	8.00–14.60	9.40–13.40

[1] These animals were selected at random from a total of 150 male and 150 female mice the day they were received in the laboratory.

TABLE 3

Average hematologic data and weight of mice prior to vitamin supplementations

DIET	NUMBER OF MICE		MEAN		RANGE	
	Male	Female	Male	Female	Male	Female
Diet L						
Hemoglobin, gm/100 ml	9	9	12.30	12.00	11.10–13.10	11.20–12.50
Erythrocytes, $mm^3 \times 10^{-6}$	9	9	11.64	11.09	8.99–13.45	10.19–12.60
Leucocytes, $mm^3 \times 10^{-3}$	9	9	8.76	9.23	4.00–10.10	7.40–10.15
Mononuclear cells, %	9	9	71.80	69.10	68.00–75.00	66.00–78.00
Polynuclear cells, %	9	9	28.20	30.90	25.00–32.00	22.00–34.00
Weight, gm	9	9	23.10	22.70	18.70–26.00	17.90–31.40
Diet L_{19}						
Hemoglobin, gm/100 ml	9	9	7.30	7.60	3.10– 9.80	5.70– 9.00
Erythrocytes, $mm^3 \times 10^{-6}$	9	9	6.22	7.91	4.40–10.06	6.52– 9.15
Leucocytes, $mm^3 \times 10^{-3}$	9	9	3.78	5.10	1.90– 5.15	3.20– 8.75
Mononuclear cells, %	9	9	50.30	49.20	48.00–54.00	43.00–53.00
Polynuclear cells, %	9	9	49.70	50.80	46.00–52.00	47.00–57.00
Weight, gm	9	9	13.20	12.10	9.00–19.40	7.00–18.40
Diet L_{17}[1]*–Diet* L_{19}						
Hemoglobin, gm/100 ml	72	72	7.70	7.40	4.10– 9.60	3.10– 9.40
Erythrocytes, $mm^3 \times 10^{-6}$	72	72	7.34	7.33	2.65– 9.21	2.82– 9.11
Leucocytes, $mm^3 \times 10^{-3}$	72	72	4.22	4.24	1.85–10.10	1.70– 7.25
Mononuclear cells, %	72	72	50.00	49.90	41.00–56.00	42.00–58.00
Polynuclear cells, %	72	72	50.00	50.10	44.00–59.00	42.00–58.00
Weight, gm	72	72	11.40	12.40	7.50–16.50	7.20–18.40

[1] Kept on this diet 5 days only.

The blood smears of the pyridoxine-deficient mice disclosed a marked hypochromia, microcytosis, poikilocytosis, anisocytosis and polychromatophilia. A moderate number of "target cells" and a few microblasts were present. Ghost cells were also frequent in the blood smears. The anemia was classified as a hypochromic, microcytic anemia.

Analysis of variance of the weight data prior to treatment disclosed a highly significant ($P < 0.01$) difference between the controls and the mice fed the pyridoxine-deficient diet. The pyridoxine-deficient mice weighed approximately 10 to 12 gm less than the controls fed diet L which contained pyridoxine.

Several clinical symptoms other than the presence of the hypochromic, microcytic anemia and growth inhibition were observed in the pyridoxine-deficient mice. Many of the mice developed a bloody diarrhea. Although the mice did not exhibit baldness, a thinning of the hair was noted on several of the animals. Two of the mice developed a contraction of the neck and the head became drawn to the side. Frequently, an animal would develop a "frog-like" gait and the hind legs became paralyzed. Many of the deficient mice developed tremors and a few exhibited convulsive seizures. All of the deficient animals exhibited diminished vigor and an unkempt appearance.

The hematologic levels and the weight loss of the mice fed deoxypyridoxine for 5 days prior to being fed diet L_{19} were not statistically different from those of mice fed diet L_{19} only. The clinical symptoms associated with pyridoxine deficiency were exhibited by the mice fed the deoxypyridoxine as well as those fed the pyridoxine-deficient diet. This is in agreement with the conclusions of Hawkins and Evans ('52) who suggested that the effects of deoxypyridoxine are probably the same as those of pyridoxine deficiency. However, the pyridoxine-deficiency symptoms appeared about two weeks earlier in the mice fed the deoxypyridoxine than in those not receiving it.

The average weight and the average hematologic data of the male and female mice on the initial, second, 10th, and 20th day of subcutaneous injections of 50 μg of pyridoxine, 0.4 μg of vitamin B_{12}, or 5 μg of folic acid, singly or in combinations are summarized in table 4. Analysis of variance for the rates of weight change during the treatment period disclosed a statistically highly significant ($P < 0.01$) rate of weight increase for mice receiving subcutaneous injections of pyridoxine when compared to the pyridoxine-deficient mice not receiving pyridoxine injections. The mean rate of increase for the mice receiving pyridoxine injections was 0.37 gm per day; the animals receiving no pyridoxine had a mean rate of weight change of −0.25 gm per day. There were no significant differences among any of the remaining treatments for the rate of weight change.

A highly significant ($P < 0.01$) difference was encountered between the hemoglobin levels of the mice receiving pyridoxine injections and those receiving no pyridoxine. The mean daily change in hemoglobin of mice on the pyridoxine treatments was 0.18 gm per 100 ml of blood per day compared to −0.24 gm per 100 ml of blood for mice not receiving pyridoxine.

The erythrocyte and leucocyte counts increased at a highly significant ($P < 0.01$) rate for the mice receiving pyridoxine injections when compared to the mice not on pyridoxine treatment. The erythrocyte and leucocyte counts for the mice given daily injections of 50 μg of pyridoxine increased at a mean daily rate of 170,000 and 290 per cubic millimeter of blood per day, respectively. The erythrocyte and leucocyte counts for mice not receiving pyridoxine injections decreased at a rate of 300,000 and 90 per cubic millimeter of blood per day, respectively.

The application of the analysis of variance for the differential counts demonstrated that 50 μg of pyridoxine daily changed the proportion of mononuclear and polynuclear cells at a highly significant ($P < 0.01$) rate in the pyridoxine-deficient mice. The pyridoxine injections caused an increase in

TABLE 4

Average weight and hematologic data of mice during the treatment period

TREATMENT [1]	MALE							FEMALE						
	No. of mice	Weight	Hemoglobin	Erythrocyte	Leucocyte	Mononuclear cells	Polynuclear cells	No. of mice	Weight	Hemoglobin	Erythrocyte	Leucocyte	Mononuclear cells	Polynuclear cells
		gm	*gm/100 ml*	*mm³ × 10⁻⁶*	*mm³ × 10⁻³*	*%*	*%*		*gm*	*gm/100 ml*	*mm³ × 10⁻⁶*	*mm³ × 10⁻³*	*%*	*%*
							Initial							
A	9	23.1	12.3	11.64	8.76	72	28	9	22.7	12.0	11.09	9.23	69	31
B	9	13.2	7.3	6.72	3.78	50	50	9	12.1	7.6	7.91	5.10	49	51
C	9	11.5	7.1	6.17	3.16	49	51	9	12.7	7.2	7.13	4.13	51	49
D	9	12.2	7.7	7.47	4.17	50	50	9	10.9	7.5	7.76	4.96	49	51
E	9	10.7	8.0	6.83	3.76	51	49	9	11.1	6.5	6.93	4.76	47	53
F	9	10.3	7.8	7.56	3.62	49	51	9	10.6	7.7	7.63	3.55	51	49
G	9	10.9	8.5	7.93	5.41	51	49	9	10.8	8.0	7.74	4.62	51	49
H	9	11.8	7.9	7.89	4.48	49	51	9	11.6	7.6	7.33	3.50	51	49
I	9	10.9	7.5	6.88	4.80	49	51	9	10.4	7.5	7.29	3.92	51	49
J	9	12.6	7.4	7.90	4.32	49	51	9	12.4	7.2	6.88	4.49	49	51
							Second day							
A	9	22.4	12.0	11.23	8.77	69	31	9	22.2	12.0	11.06	9.58	68	32
B	8	11.8	6.5	6.66	4.06	49	51	9	11.8	7.4	7.77	5.12	49	51
C	6	10.9	6.3	6.04	3.71	45	55	8	10.4	6.7	6.90	3.88	50	50
D	9	12.2	8.2	7.20	4.48	50	50	8	10.7	7.5	8.17	4.22	49	51
E	7	10.3	7.4	6.67	3.50	49	51	7	10.6	6.3	6.64	4.96	47	53
F	8	9.4	7.2	6.87	3.59	49	51	9	10.2	7.4	7.23	3.31	47	53
G	9	9.3	7.7	7.80	4.83	52	48	9	10.3	6.6	7.29	5.53	49	51
H	8	12.2	8.4	8.01	4.23	52	48	8	11.6	7.4	7.45	5.08	51	49
I	6	10.8	6.5	7.01	5.86	45	55	6	9.4	7.3	7.14	3.29	48	52
J	9	12.2	6.9	7.56	5.71	50	50	8	11.9	7.2	7.28	5.19	50	50

						Tenth day							
A	9	22.8	11.6	11.26	9.06	70	30	9	23.7	12.3	11.77	9.87	70	30
B	1	10.2	2.4	2.82	1.25	40	60	2	11.1	5.3	6.32	4.47	49	51
C	1	10.4	6.1	5.15	4.50	44	56	1	10.0	4.1	3.94	1.95	43	57
D	9	17.8	10.2	10.01	7.93	58	42	7	13.6	9.8	9.89	6.67	56	44
E	1	8.6	7.5	8.94	5.70	43	57	4	11.0	5.8	7.29	5.41	44	56
F	3	12.3	6.8	7.72	6.40	49	51	3	9.7	5.3	6.12	3.43	44	56
G	8	17.7	10.0	9.57	7.48	59	41	9	16.1	9.8	9.57	7.06	57	43
H	7	19.2	10.4	9.99	6.89	57	43	7	17.7	9.3	9.05	7.28	57	43
I	3	13.2	7.5	7.26	4.80	53	47	1	10.1	5.7	7.92	2.90	56	44
J	9	19.5	9.9	10.05	8.80	58	42	7	18.0	10.4	10.23	8.44	61	39
						Twentieth day							
A	9	22.7	11.2	10.79	8.97	71	29	9	24.5	12.0	11.58	10.14	69	31
B	All died							All died						
C	All died							All died						
D	9	20.2	11.5	11.06	9.29	67	33	7	17.7	11.0	11.07	9.14	67	33
E	All died							1	9.0	5.3	7.89	9.20	43	57
F	1	21.2	8.5	7.92	8.05	56	44	All died						
G	8	20.4	11.6	11.13	9.48	67	33	9	18.2	11.0	10.27	8.62	62	38
H	7	23.2	11.6	11.27	9.45	67	33	6	19.3	11.8	11.19	8.52	66	34
I	2	18.2	8.3	8.75	6.87	55	45	All died						
J	9	24.4	12.2	11.73	9.68	68	32	7	21.3	11.5	11.05	9.28	67	33

[1] See table 2.

the percentage of mononuclear cells from about 50 to 70; and caused a decrease in the percentage of polynuclear cells from about 50 to 30.

Vitamin B_{12} and folic acid alone, in combination, or in combination with pyridoxine had no significant influence on the hemoglobin, erythrocyte, leucocyte and differential counts of the pyridoxine-deficient mice.

The application of Student's "t" test demonstrated that the mice receiving 50 μg of pyridoxine subcutaneously did not vary significantly in weight or in hematologic findings from the control group at the end of 20 days of treatment. None of the variables investigated were significantly affected by sex.

All visual signs of pyridoxine deficiency in the pyridoxine-deficient mice were corrected by subcutaneous injections of pyridoxine. At the end of 20 days of pyridoxine treatment the mice were active, the eyes were bright and clear, the fur was glossy, and the general appearance was one of well-being.

As shown in table 4, the mice receiving injections of pyridoxine, with few exceptions, survived as did the controls fed diet L which contained pyridoxine. All of the mice fed the pyridoxine-deficient diet and given no treatment or receiving saline injections succumbed. Only three males and a female administered vitamin B_{12} or folic acid singly or in combination survived. Although the 4 mice showed some improvement in weight and hematologic findings, in all probability they would have succumbed if the experiment had not been terminated. Generally, the mice succumbed during the first 10 days of treatment.

The growth retardation, the terminal hypochromic, microcytic anemia, and the clinical symptoms resulting from pyridoxine deficiency were alleviated by daily subcutaneous injections of 50 μg of pyridoxine. Vitamin B_{12} and folic acid alone and in combination had no curative effects on the symptoms associated with pyridoxine deficiency. The inability of vitamin B_{12} and folic acid to restore to normal the hematologic values in mice afflicted with a hypochromic, microcytic anemia

is in agreement with the many reports that vitamin B_{12} is effective in pernicious anemia only and folic acid in macrocytic anemias other than pernicious anemia.

Whereas Coursin ('56) reported that most pyridoxine-deficiency symptoms in children were corrected within several days by injections of pyridoxine, in this study approximately 20 days were required to correct the deficiency symptoms induced in mice by a pyridoxine-deficient diet.

CONCLUSION

Normal hemoglobin, erythrocyte, leucocyte and differential count values for 21-day old weanling male and female mice are presented. Mice fed deoxypyridoxine for 5 days prior to continuing on the pyridoxine-deficient diet and mice fed the pyridoxine-deficient diet without deoxypyridoxine developed similar pyridoxine-deficiency symptoms. However, 44% of the animals receiving the deoxypyridoxine died early in the study; also, the deficiency symptoms developed earlier than in the mice not receiving the deoxypyridoxine. Retarded growth, a severe, hypochromic, microcytic anemia with accompanying lymphopenia and granulocytosis and the presence of "target" cells, diarrhea, thinning of the fur, neurological symptoms, diminished vigor and unkempt appearance were symptoms developed by the pyridoxine deficient mice.

Daily injections of 50 μg of pyridoxine for 20 days restored growth and hematologic levels to normal values and cleared all other clinical pyridoxine-deficiency symptoms. Pyridoxine-deficient mice receiving no treatment, injections of saline, or injections of vitamin B_{12} and folic acid, singly or in combination, succumbed during the treatment period.

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