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(1893 — 1949)

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(November 3, 1893 — April 23, 1949)

To the wall of the main corridor of the Research Institute and laboratory floor in the new Hospital for Sick Children, Toronto, Canada, is affixed a tablet which, headed by the Aesculapian staff, bears the following inscription:

"FREDERICK FITZGERALD TISDALL
O.B.E., M.D., F.R.C.P.(C.), F.R.C.P.(Lond.).
1893 — 1949

His outstanding achievements in the field of nutritional research brought great good to humanity and world-wide fame to this hospital. Through his efforts these laboratories were created.

Quae molebatur perficiamus."

This last sentence may be translated "As he began let us carry on."

At the time of his death Dr. Tisdall's professional appointments were: Associate Professor of Paediatrics, University of Toronto, Physician and Director of Laboratories, Hospital for Sick Children; Group Captain, Consultant on Nutrition, Royal Canadian Air Force; Chairman, Committee on Nutrition, Canadian Medical Association; Chairman, National Nutrition Committee, Canadian Red Cross Society; Member, Food and Nutrition Board, National Research Council, Washington; Member, Advisory Committee on Nutrition, Food and Agriculture Organization of the United Nations. He was a Fellow of the Royal College of Physicians of Canada and of the Royal College of Physicians of London, England. Among the learned societies of which he was a member or Fellow were the Canadian Paediatric Society, American Pediatric Society, Society for Pediatric Research, American Institute of Nutrition, American Society for Clinical Investigation, Society for Experimental Biology and Medicine and the American Society

of Biological Chemists. In the Index Medicus, from 1920 to 1949, are recorded more than 125 publications of which he was the sole or joint author.

For his World War II services Group Captain Tisdall was created an Officer of the Order of the British Empire by His Majesty King George VI with the following citation:

> *"Group Captain F. F. Tisdall (Royal Canadian Air Force). This officer was full time consultant in nutrition from 1941 until the spring of 1943. Since 1943 he has been honorary consultant and has continued to make available his services for over half of his time.*
>
> *The re-organization of the messing services; the institution of the control of messing through the nutritional laboratory; dairies and mobile milk units for the reconstruction of powdered milk; research into nutritional deficiencies of Royal Canadian Air Force personnel; work with the Canadian Dental Corps on diseases of the mouth — are but a few of the results of this officer's outstanding contribution to the Royal Canadian Air Force which have had great effect not only on the health but also on the morale of the service."*

These formal records need only amplifications to picture the life and achievements of Frederick FitzGerald Tisdall, M.D., paediatrician, teacher, scientist, agriculturist, counselor to governments and, not the least, friend and adviser to the great and lowly in the world of paediatrics and nutrition.

Born in Clinton, Ontario, November 3, 1893, Fred received his preliminary education in that village and in the High Schools of Buffalo, New York. After graduation in medicine from the University of Toronto in 1916 and having served an internship on the infant wards of the Hospital for Sick Children, Toronto, Dr. Tisdall proceeded to Baltimore to join the group of young paediatricians working under Dr. John Howland, men who had been attracted by the opportunity of studying clinical diseases by chemical methods. At that time the Harriet Lane was the only American pediatric department equipped to carry out such chemical studies. Tisdall was particularly fortunate in the group with which he was associated during this formative period of his career. It included Howland, Park, Gamble, Blackfan, Powers, Davison,

Kramer and Ross, brilliant and knowledgeable paediatricians and scientists, all kindly gentlemen, who like Tisdall have done so much to inspire a younger generation of clinical scientific paediatricians. His work during this period had to do with the development and application in infancy of micro methods for the determination of calcium, magnesium, sodium and potassium. Particularly to be noted are the inclusion in the titles of the papers which appeared from 1920 to 1922 of the qualifying statements "clinical method," "simple technique," "clinical significance" and "determination in small amounts of serum." In these earliest publications the pattern for his future research work was becoming established. Conditions were observed by the paediatrician in the course of his examination of both the well and sick infant and child which required clarification. The problems were taken to the laboratory by the observer, a paediatrician with extra training in science, who with or without technical assistance, attacked the problem, scientific help for consultation being available either within or without the department. With elucidation of the problem the paediatrician transferred his results back to the prevention and cure of disease in childhood, the laboratory being simply a tool in his public and private practice.

In 1921, Tisdall's first publications on deficiency diseases, rickets and tetany, appeared. From this time his interest in nutrition expanded from the cure of deficiency diseases in infancy to the prevention of these disorders in the child and in the adult, and broadened by cases observed in his home hospital and city to a consideration of the national and finally the international food situation. Particularly applicable to infant nutrition was his work in 1930 on the incorporation of vitamins and minerals in biscuits and in a cereal mixture. From this latter the precooked infant food Pablum developed. In 1934 the antirachitic value of various vitamin D-containing materials, fish liver oils, irradiated ergosterol, cholesterol and milk was studied on well infants under home conditions.

From 1941, in addition to his nutritional work in the Royal Canadian Air Force, studies were conducted on nutrition in

pregnancy, in school children and in the inhabitants of Northern Canada and Newfoundland. Reports of the above, only a few of his many undertakings, were interspersed with papers on clinical problems, rickets, resorcin poisoning, sensitization to cow's milk, pyloric stenosis, Meckel's diverticulum, acrodynia, etc. He was concerned not only with the diagnosis, the pathological and biochemical changes and cure of disease due to nutrition deficiencies, but also with subclinical deficiencies and especially with prevention through diet. In nutrition his interest spread to food production, distribution and preparation for consumption. In all his nutrition work he was practical to the highest degree. Estimations of the nutritional value of foods which he carried out did not consist solely of long series of analyses on raw samples of material but included values on the food as prepared for consumption in family quantities under home conditions, not only under optimum conditions of preparation but also subjected to all the culinary mistreatments which so frequently occur. He was concerned always with taste and eye appeal and the admonition "consistent with best possible flavour" was always stressed.

From the beginning of his scientific work Tisdall recognized the value and necessity of cooperation with other departments in his own University and with other individuals and groups both within and outside Canada. At all times his proposals of cooperative efforts were accepted with pleasure. His offers of the use of the clinical facilities of the Hospital to University departments which had no patient contact were also gratefully received. The caliber of the workers who were happy to associate with him in joint undertakings may be judged from the author list of the "Medical Survey of Nutrition in Newfoundland," Canadian Medical Association Journal, 1945, by F. F. Tisdall, J. D. Adamson, N. Jolliffe, H. D. Kruse, O. H. Lowry, P. E. Moore, B. S. Platt, W. H. Sebrell, J. W. Tice, R. M. Wilder and P. C. Zamecnick.

Laboratories and research. In 1929, upon the resignation of Miss Angela M. Courtney, Dr. Tisdall was appointed Director of the Nutritional Research Laboratories of the

Hospital. At that time the research staff consisted of one other paediatrician, Dr. Gladys Boyd, one technician and himself. Dr. Tisdall's and Dr. Boyd's duties were many. In the mornings they taught medical students and attended indoor and outdoor hospital patients. In the course of these patient contacts they recommended various biochemical analyses. In the afternoons they repaired to the laboratories, changed their coats and did analyses which they had requested as clinicians, performed any routine chemical examinations which had been requested by other clinicians and carried on with their research problems. After four o'clock they were supposedly free to see private patients in their outside offices. The care of their outside patients did not consume too much time; the day on which one turned up was, for a number of years, a red letter one. In Dr. Tisdall's mind the private practice of paediatrics, to which he devoted an increasingly smaller portion of his time, even as late as 1941, was extremely valuable, especially as back-ground material in teaching medical students the general practice of medicine.

Soon the need for further research workers, both paediatricians and technicians, was recognized. Neither trained professional nor technical help was available. Dr. Tisdall arranged for young paediatricians to receive training in special skills, immunology, biochemistry or physiology, etc. in other university departments and hospitals in Toronto and in the United States. These were then appointed to the University and Hospital staffs. They, like himself, were clinicians, teachers, and research workers and most of them devoted a small portion of their time to the private practice of paediatrics outside the Hospital. Neither University nor Hospital funds were available for the maintenance of research workers whether professional, technical or clerical, nor for equipment. In the predicament Tisdall turned to his patients, private individuals and business and financial institutions. Leaders of industry, Life Insurance executives recognized the clarity of his thinking, his organizing ability, enthusiasm and the practicability of his ideas. In addition to the philanthropic side he readily convinced

them that support of paediatric research indirectly paid good monetary dividends to their organizations. Gradually research workers specializing in activities other than biochemistry and nutrition were drawn into the organization. Among others, paediatricians with special training in immunology, bacteriology, pathology, physiology, neurology and cardiology became associated with the Research Department. So successful was Dr. Tisdall in attracting scientific personnel and funds that at the time of his death the personnel of the Research Laboratories had increased five-fold, the annual budget had increased ten-fold and this without further calls on the funds of the University or Hospital.

In practical results nutritional deficiencies in the Canadian infant and child have practically disappeared; rickets has become a medical curiosity. As the result of work here and elsewhere the whole picture of disease in childhood has changed. No longer are hospital wards filled with cases of severe malnutrition; infectious diseases and pneumonia, the chief killers in childhood have changed to congenital malformations of the heart, malignancies and accidents, against which assaults had already been started even in the old Hospital.

Teacher and clinician. As a teacher Tisdall was at his best, whether he was conducting a seminar for a group of scientists, giving a clinic for medical students or speaking to a home and school club. Much time was always spent on preparation and his delivery could not be improved upon. Before the scientific audience his presentation was not merely a compilation of work done by others but a report of work which had been performed by himself or under his active direction. The medical student was not left bogged down after a lecture consisting solely of intricate organic formulas previously copied on a blackboard; if he remembered little of the lecture on rickets and scurvy he retained at least the picture of the orange and the bottle of fish-liver oil prominently displayed during the lecture. The housewife carried away the necessity for consumption of well-prepared and well-served meals of milk, meat, eggs, vegetables and fruits. Nor was the

education of the laity through the printed word neglected. As Chairman of the Committee on Nutrition of the Canadian Medical Association he took an active part in disseminating information on the importance of nutrition to both the individual and the nation and on how the present knowledge of nutrition could be applied to everyday life. In this work the Committee was ably assisted from the financial and other standpoints by the Life Insurance Companies in Canada. With the assistance of the Life Insurance Companies many prominent workers in nutrition were brought from both Great Britain and the United States to give addresses throughout Canada. This Committee produced and distributed 4½ million copies of the booklets "What to Eat to be Healthy," "Food for Health in Peace and War" and "What They Eat to be Fit." The Committe sponsored a series of articles on nutrition from the standpoint of the medical practitioner. These appeared monthly for 18 months in the Canadian Medical Association Journal. They were collected and published in book form and distributed and used in the teaching of nutrition in schools. Many panel discussions and seminars were conducted or participated in before national and international scientific and lay organizations. The number of conferences with groups, medical, nutritional, agricultural, industrial and welfare were innumerable, at from international to local level.

With the start of World War II Dr. Tisdall was appointed Adviser on nutrition to the Canadian Department of National Defense in April 1940 with the rank of Major. From October 1941 until the end of the war he was consultant on nutrition to the Royal Canadian Air Force with the rank of Group Captain, a rank equivalent to Colonel in the army, serving in Canada and Great Britain. By and under his advice the Royal Canadian Air Force set up the Directorate of Food Administration under the supervision of dietitians. He also advised in regard to the organization of the Nutrition Division of the Medical branch of the Royal Canadian Air Force. As part of this organization, 4 laboratories were set up across Canada for the assay of foods as served to personnel and for

the assay and investigation of problems concerning food. The procedures employed in these laboratories were set up by the Research Laboratories of the Hospital for Sick Children and the Department of Paediatrics, University of Toronto, and the work of the 4 laboratories was coordinated by the Hospital Research Laboratories. In addition, various investigative procedures were carried out by the Medical Branch of the Air Force with the cooperation of the Hospital Research Laboratories.

As nutritional adviser to the Canadian Red Cross Society the composition of prisoner of war food parcels was placed in his hands. The articles consituting the 11-pound food parcel, the weight being fixed by international convention, were selected not only on account of nutritional value but with great attention to palatability. From January 1941 on the Canadian Red Cross packed and shipped to prisoners of war in Europe and the far East nearly 16½ million of these parcels. At the end of the war data were collected from 5,170 of the British, American and Canadian prisoners of war who had received, in prison camps in Germany and Italy, British, American and Canadian food parcels. Of the men questioned, 82% of the Royal Canadian Air Force and of the Canadian Army and 71% of the British Army gave first preference to the Canadian Red Cross parcel — good evidence of another undertaking well done by Dr. Tisdall.

Building the new hospital. The Hospital for Sick Children, located at 67 College Street, Toronto, was constructed in 1891. As early as 1924 the accommodation was very inadequate. However, due to circumstances beyond local control, the depression, World War II, etc., it was not until 1945 that it became feasible to proceed with the final stages of the financing and planning of the new Hospital for Sick Children at 555 University Avenue. Dr. Tisdall, due to his enthusiasm and willingness to undertake the arduous duties connected with the position and his personal contact and friendship with certain key individuals, was chosen as the representative of the professional staff on the building committee. The first

step in the campaign was that of education, put on by the professional staff of the Hospital. In groups of a dozen or less, financiers, Government and Municipal authorities, representatives from business and industry both executive and workmen, the press, service and Women's clubs, etc. were escorted over the Hospital by members of the professional visiting staff. Not the slightest difficulty was encountered in convincing these individuals, who reported back to their own organizations, of the need for a new hospital. In the course of these tours much goodwill was generated, public relations were so greatly improved that if these had been the sole result of the effort it would have been well worth-while. The most recent member of the professional staff became personally acquainted with the most august member of the Board of Trustees, who in turn saw byways in the Hospital into which he had never penetrated before. Each group learned of the problems facing the other. The trustees were made more aware of the decrease in suffering and mortality which the moneys donated and procured by them had brought about. The working man was informed that the benefits of the Hospital extended far beyond its walls, consisted of more than operating on and administering medicine to patients confined to the hospital; that through preventive paediatrics having its inception in the Hospital there was much less chance of their children falling ill; if it were necessary to admit a child to hospital the length of stay would be shorter than previously and the chance of complete recovery much greater. Business men become cognizant that contributions to the Hospital paid dividends in dollars and cents, that through contributions to the Hospital a larger pool of fit workers was being formed, that the percentage of handicapped individuals was reduced. Especially intriguing to them was the fact that, though a larger hospital was necessary, yet if the incidence of disease and length of hospital stay had not been decreased through work which had been carried out in the hospital, they, as corporations and wealthy individuals would have had to, directly or indirectly, bear a high percentage of the cost of building and maintaining a

children's hospital twice as large as the prospective one to keep pace with the growth of population. Of course there was no need to point out to Insurance Companies the value of a further decrease in mortality. Individuals and private and public corporations throughout the whole of Canada were made aware by clinical demonstrations, the spoken and printed word and through radio that the Hospital for Sick Children was not an institution ministering merely to the City of Toronto but to the whole Dominion.

Previously in Toronto no building appeal for funds anywhere approaching this magnitude, the collection of 14 million dollars, had ever been attempted without the employment of a professional fund raising organization. Let it be recorded, that without too much difficulty the total amount was rapidly subscribed, without the employment of a professional fund raising organization and with an extremely low cost for overhead. Knowledge of the part played by the work done in the Research Laboratories by Dr. Tisdall and under his direction in bettering the health and welfare of the Canadian child, and an appreciation of the necessity for extending research facilities were great factors in the procurement of the large donations from Government bodies, financial institutions, etc. throughout the whole Dominion.

For years before his death Dr. Tisdall was aware that he was suffering from cardio-vascular disease which was apt to result in sudden fatal termination. Up to him was the decision as to whether he was to rust out or wear out and he decided upon the latter course. Only a very few of his closest friends knew of the seriousness of his condition. To the outside world he was still a powerhouse of energy. At all times he was available for consultation. He was as active as ever in the planning of new research projects and in the raising of the funds. He was available at all times to the Hospital clinicians for consultations on patients, to Government authorities on matters concerning local, national and international nutrition. To the outside world he was a paediatrician and scientist of international reputation, a planner and executor at the height

of his career both mentally and physically. Only his intimates knew that he was under a physician's care, only to them would he admit that he was becoming progressively a little more tired, that to keep from slackening his working pace it was necessary to spend more of his few private hours resting. From day to day he kept his personal financial affairs in perfect order. On April 22nd, 1949, Dr. Tisdall assisted in the laying of the cornerstone of the new Hospital and that evening accompanied by his wife and two youngest sons went out to his farm to spend the weekend. Next morning he arose, ate his breakfast and accompanied by one of his sons went out for a short stroll about the estate. Suddenly, without previous exertion and without evidence of pain, he fell to the ground and expired.

Dr. Tisdall is survived by his four sons and his wife Mary Ferguson McTaggart of Clinton, Ontario, to whom he was married in 1934. As he began let us carry on.

T. G. H. DRAKE

ADRENAL GLYCOGEN IN THE GUINEA PIG AND IN THE WHITE RAT[1,2]

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(Received for publication July 12, 1954)

Although the glycogen of many tissues has been investigated under various conditions of nutrition and metabolism, the study of this polysaccharide in the adrenal gland has been almost totally neglected, and this in spite of the intense interest now current in adrenal biochemistry. This report presents a quantitative study of adrenal glycogen in fasted, normal guinea pigs on different levels of ascorbic acid intake, in non-fasted normal and ascorbic acid-depleted guinea pigs, and in normal, white rats in fasted and fed states.

The glycogen concentration in the guinea pig adrenal was not appreciably influenced by any of the nutritional states employed, and was found to be of the order of 25 mg/100 gm of fresh tissue. In the fed rat, however, the concentration of adrenal glycogen is 4 to 5 times as great as in the guinea pig, and this value is almost doubled by fasting.

[1] Presented before the Division of Biological Chemistry at the 122nd meeting of the American Chemical Society at Atlantic City, New Jersey, September 1952. Some of this work was also included in a report made at the meetings of the Federation of the American Societies for Experimental Biology, Chicago, Illinois, April, 1953 (Fed. Proc., *12:* 251).

[2] Taken in part from a portion of a dissertation submitted by Nancy Lee Noble in partial fulfilment of the requirements for the degree of Doctor of Philosophy at Emory University, March 1953.

[3] Predoctorate Research Fellow of the National Cancer Institute, United States Public Health Service. Present address: Miami Heart Institute, University of Miami School of Medicine, Coral Gables 34, Florida.

EXPERIMENTAL

Only male animals were used. Control and experimental groups were studied in parallel and included young adults of comparable age and weight. All animals were kept in the colony for at least one week before use and any showing signs of illness or other abnormality during the investigation were discarded. Except for the first two groups of guinea pigs, the animals were housed in a constant temperature room at 24 to 25°C. A commercial chow [4] supplemented weekly with Oleum Percomorphum provided the basal diet for the guinea pigs, and adequate ascorbic acid intake was achieved by daily supplements of Lilly's Cevalin [5] (groups 1 and 2) or of cabbage (groups 3 and 4). A standard chow ration [6] furnished an adequate diet for the rats, all of which were of the Sprague-Dawley or of the Holtzman strain. Food was allowed ad libitum except during terminal fasting periods, and free access to water was permitted until induction of anesthesia by intraperitoneal injection of Nembutal (9 mg/100 gm guinea pig and 6 mg/100 gm rat) prior to withdrawal of blood and subsequent removal of tissues for analysis.

Blood was collected by heart puncture and oxalated for ascorbic acid determination according to Roe and Kuether ('43) as a check on vitamin C nutritional status (Kuether, Telford and Roe, '44) in the three groups of guinea pigs (groups 1, 2 and 5) whose daily food intakes were recorded. The same method was employed in analysis of the chow for calculation of total daily ascorbic acid intake. The livers and adrenals were analyzed for total glycogen in all the animals. Trichloracetic acid-soluble glycogen (Bloom, Lewis, Schumpert and Shen, '51) of the adrenal was also determined in one group of 24-hour-fasted rats and in one group of non-fasted rats.

[4] Purina Rabbit Chow Checkers, "complete ration."

[5] We wish to thank Eli Lilly and Company for supplying the Cevalin *gratis* for these experiments.

[6] Purina Laboratory Chow Checkers.

Total glycogen

The term "total glycogen" applies to that found following the usual digestion of tissue with strong alkali, in contrast to "TCA-soluble glycogen," which refers to the polysaccharide isolated from a trichloracetic acid extract. In the first two groups of guinea pigs, the total glycogen of adrenal and liver was determined essentially according to Good, Kramer and Somogyi ('33) and Nelson's method ('44) was employed for colorimetric assay of the acid-hydrolyzed glycogen. In the last three groups of guinea pigs and in all the rats, the isolated glycogen was assayed without previous hydrolysis by use of Dreywood's anthrone reagent (Dreywood, '46; Morris, '48). Since the procedure for liver glycogen did not differ essentially from that for the adrenal, details will be given only for the latter tissue, for which the technique was especially adapted and tested to determine its reliability for the small amounts of glycogen involved. The whole liver was always used and, generally, the two glands of an adrenal pair, except that in one group of 24-hour-fasted rats (series A), the left and right glands were analyzed separately for total glycogen.

The dissected glands were weighed quickly on a micro torsion balance, placed immediately in graduated centrifuge tubes containing hot 30% KOH (0.9 ml for rat and 1.5 ml for guinea pig adrenals) and digested for one hour. Two volumes of 95% ethanol and 0.1 to 0.2 ml of 6% Na_2SO_4 were then added, and after the contents had been brought carefully to a boil, the tubes were placed in the refrigerator overnight before centrifugation. If re-precipitation was to be carried out (as in analysis of guinea pig but not of rat tissues), the glycogen was dissolved in 1 ml of water. Following centrifugation and draining of the tube, the isolated glycogen was washed with a lipid solvent (3 ml of re-distilled ether for guinea pig adrenal, and 2 ml of a 3:1 ethanol-ether mixture for rat adrenal) and then dissolved in distilled water. The volume was brought to 11 ml where both glands were analyzed, or to 5.5 ml for single gland analysis. Before treatment of the aqueous polysaccharide solution from adrenal of the rat (but not of guinea

pig) with anthrone, centrifugation (20 minutes at 2,000 r.p.m.) was necessary to remove interfering water-insoluble particles. For color development, a 5-ml aliquot of the clear solution was treated with 10 ml of freshly prepared anthrone [7] reagent and assayed as recommended by Durham et al. ('50). Glycogen values were calculated as milligrams of glucose per 100 gm of fresh adrenal (or grams of glucose per 100 gm of liver).

Assay of glycogen samples isolated from the same livers directly by anthrone and after acid hydrolysis by the Nelson method gave similar values. Comparison of guinea pig adrenal glycogen values found by the two colorimetric techniques (see table 1 below) also indicates good agreement. As a further test of the reliability of our procedure for the small amounts of glycogen (about 40 μg, as glucose) involved in adrenal analysis, the entire procedure for a gland pair was applied to the determination of polysaccharide in 1- and 2-ml aliquots of a solution prepared from a commercial sample so as to contain 40 μg of glycogen per milliliter. The mean values found for the 40- and 80- μg samples respectively were 107.4 ± 1.2 [8] and 99.7 ± 0.6 [8]% of the theoretical figures.

TCA-soluble glycogen

The procedure described by Bloom et al. ('51) was somewhat modified for determination of the TCA-soluble fraction, and adrenals from two rats were sometimes pooled for a single analysis. The dissected and weighed glands were placed immediately in a centrifuge tube containing 1.5 ml of 10% trichloracetic acid and ground with a glass rod at room temperature. Following centrifugation for 10 minutes, 1 ml of clear supernatant fluid was removed to a clean, dry centrifuge tube and treated with 2 ml of ethanol and 0.1 ml of 6% Na_2SO_4. After standing overnight in the refrigerator, it was centrifuged, drained, the glycogen dissolved and brought to 5.5 ml

[7] The anthrone was purchased from Law and Company, Consulting and Analytical Chemists, Atlanta, Ga.

[8] Standard error (standard deviation of the mean): $S.E. = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$

with water, centrifuged again to remove any insoluble material, and finally assayed with anthrone as above. This technique was tested with small portions of rat livers containing amounts of total glycogen comparable to those in an adrenal pair. The mean TCA-soluble fraction was found to be 87.4% of the total glycogen in the liver of non-fasted rats, and 28.5% of the total in the liver of 24-hour-fasted rats. These values agree with those reported by Bloom and his associates for non-fasted rat liver ('51) and by those found by Russell [9] after 24 hour of fasting.

TABLE 1

Total adrenal glycogen of fasted and non-fasted guinea pigs on various levels of ascorbic acid intake

GROUP NO.	NO. OF ANIMALS	ASCORBIC ACID INTAKE	TERMINAL FAST	MEAN BLOOD ASCORBIC ACID	ADRENAL GLYCOGEN MEAN ± S.E. (AS GLUCOSE)	P FROM "T" TABLE
		mg/100 gm [1]	*hours*	*mg %*	*mg %*	
1	9	1.48	24	0.30	27 ± 2 [2]	
2	8	2.84	24	0.45	24 ± 3 [2]	
3	13	(adequate)	24	...	27.2 ± 1.5 [3]	<0.05
4	11	(adequate)	0	...	22.3 ± 1.4 [3]	
5	10	0.57	0	0.08	25.3 ± 2.1 [3]	

[1] Mean total ascorbic acid intake (chow + vitamin supplement) per 100 gm of body weight/day for three weeks: groups 1 and 2 on Cevalin supplement; groups 3 and 4 on cabbage supplement ad libitum for three weeks but intake not measured; group 5 on chow only.

[2] Glycogen assayed by Nelson method after acid hydrolysis.

[3] Glycogen assayed by anthrone without previous hydrolysis.

RESULTS AND DISCUSSION

Mean values and standard errors of the analytical results are presented in the tabulated data. Fisher's "t" table ('50) was used in appraising significance of differences between means.

Table 1 summarizes the findings in the guinea pig experiments except for liver glycogen, which was found in the concentrations expected for the nutritional state involved without any correlation to adrenal polysaccharide. The data offer

[9] Unpublished data, Jane A. Russell, Dept. of Biochemistry, Emory University.

no evidence that ascorbic acid nutritional status influences the level of adrenal glycogen in the guinea pig. The only statistically significant difference in adrenal glycogen concentration was that between the 24-hour-fasted guinea pigs of group 3 and the non-fasted animals of group 4, both of which were allowed cabbage supplements daily. However, the "t" value indicates only borderline significance ($P < 0.05$). Furthermore, while the mean adrenal glycogen of the 24-hour-fasted group 1 (given Cevalin daily) is very close to that of group 3, the adrenal glycogen concentration of 24-hour-fasted group 2 (on twice as much Cevalin as group 1) is not statistically different from that of any other group. Thus, our data suggest that adrenal glycogen tends to be higher in the fasted than in the non-fasted guinea pig, but the evidence is by no menas clear-cut.

Table 2, however, shows clearly that in the rat, in which adrenal glycogen concentration is much higher than in the guinea pig, the levels of adrenal polysaccharide are markedly higher in the fasted than in the fed state. Mean values of the untreated, fasted groups in series A range from 174 to 199 mg% of total glycogen, without significant difference between any of the series A groups. Two 16-hour-fasted rats, not presented in table 2, had glycogen concentrations of 220 and 245 mg%, respectively. In the course of various experiments, some to be reported later, glycogen was determined in the adrenals of 45 rats after a 24-hour-fast. In some, water was given by stomach tube (as to the controls in series C) or saline was injected (as into the controls of series D). The adrenal glycogen of this composite 24-hour-fasted group was 183.8 ± 4.6 mg% and statistical analysis showed no significant difference between any of the sub-groups. Analysis of individual glands in 7 untreated, 24-hour-fasted rats (table 2, series A), shows also that glycogen concentration does not differ significantly in the two glands of an adrenal pair, although the right adrenal (usually the smaller of the pair) often has a slightly higher concentration.

The difference in adrenal glycogen levels between fasted rats and non-fasted or glucose-fed rats is marked. Comparison of non-fasted series B with the 24-hour-fasted group of series A, or with the composite 24-hour-fasted group of 45

TABLE 2

Total glycogen of adrenal and liver in fasted rats before and after administration of glucose or fructose and in non-fasted rats

	NO. OF RATS	HRS. AFTER BEGINNING FAST		MEAN GLYCOGEN AND S.E.	
				ADRENAL	LIVER
				mg %	%
Series A: Fasted without further treatment					
	7	8		174 ± 16	2.21 ± 0.30
	8	24		195 ± 11	0.12 ± 0.02
	6	48		174 ± 16	0.57 ± 0.17
	7	24	L.A.[1]	194 ± 13	0.15 ± 0.03
			R.A.[2]	199 ± 13	
Series B: Unfasted (usual uninterrupted dietary regimen)					
	11	0		100 ± 7	6.03 ± 0.33
Series C: 5 ml of water (controls) or of 30% sugar solution (1.5 gm) by stomach tube					
Water at 21 hrs.	7	24		170 ± 16	0.11 ± 0.02
Glucose at 16 hrs.	2	17; 19		126 ± 4.5	1.09
Glucose at 21 hrs.	9	24		109 ± 8	2.93 ± 0.12
Glucose at 48 hrs.	3	52		86 ± 14	3.22
Fructose at 21 hrs.	4	24		226 ± 30	1.09 ± 0.08
Series D: 0.9% NaCl (controls) or 10% sugar solution I.P. at 21 hrs. of fast					
2 ml saline	3	22–23		187 ± 14	0.07 ± 0.03
4 ml glucose (0.4 gm) [3]	2	23		124 ± 15	0.94
5 ml fructose (0.4 gm) [3]	2	23		128 ± 28	1.52
2 ml glucose (0.2 gm)	6	22–23		152 ± 11	1.11 ± 0.28
2 ml fructose (0.2 gm)	6	22–23		120 ± 17	1.59 ± 0.08

[1] Left adrenal of each pair analyzed separately.

[2] Right adrenal of each pair analyzed separately.

[3] Considerable fluid in peritoneal cavity two hours later.

rats gives "t" values which indicate a highly significant difference ($P < 0.001$). The difference in adrenal glycogen between the glucose-fed rats of series C and their controls given water is likewise highly significant ($P < 0.001$). The failure of an effect after oral administration of fructose was

probably due to poor absorption of the sugar, since all 4 animals developed marked diarrhea and, also, had liver glycogen levels significantly lower than the 21-hour-fasted rats given glucose by stomach tube ($P < 0.001$).

Intraperitoneal injection of the hexoses produced less marked lowering of the fasting adrenal glycogen level, probably because of the smaller dose of sugar employed and also, perhaps, because the time allowed after injection was not of optimum duration for clearer manifestation of the effect.

TABLE 3

Adrenal glycogen fractions in fasted and non-fasted rats

	NO. OF VALUES	MEAN GLYCOGEN AND STANDARD ERROR				
		TOTAL	TCA-SOLUBLE FRACTION		RESIDUAL (TOTAL — TCA)	
		mg %	*mg %*	*% of total*	*mg %*	*% of total*
24-hr. fast	45	184 ± 5				
24-hr. fast	9 [1]		104 ± 7	57 ± 4	79 ± 11	43
No fast	11	100 ± 7				
No fast	9 [2]		48 ± 5	48 ± 6	52 ± 9	52
Difference (fasted minus non-fasted)		84 ± 10	56 ± 9 (67 ± 13% of difference)		28 (33% of difference)	

[1] Represents glands from 12 rats.

[2] Represents glands from 15 rats.

The "t" values between the three-saline-injected controls and the rats given 0.2 gm of hexose I.P indicate $P < 0.10$ for glucose and $P < 0.05$ for fructose; however, "t" values found by comparison of these sugar-injected groups with the composite 24-hour-fasted group of 45 rats show $P < 0.001$ for both differences. In general, then, the results indicate that administration of either glucose or fructose to the fasted rat, by proper route and in suitable dosage, can lower the fasting level of adrenal polysaccharide to that found in animals on a regular, uninterrupted dietary regimen.

Table 3 presents a comparison of total and TCA-soluble glycogen of adrenal in 24-hour-fasted and in non-fasted rats.

The difference in levels of the acid-soluble fraction in the two nutritional states is highly significant ($P < 0.001$). This fraction accounts for about one-half of the total adrenal polysaccharide and the data suggest that it is more readily altered than is the "residual" fraction (total — TCA-soluble). If further data should show this to be true, it would indicate that the behavior of rat adrenal TCA-soluble glycogen is similar to that reported for this fraction in the liver (Bloom et al., '51), gastrocnemius muscle (Bloom et al., '51; Bloom and Russell, '52; Bloom and Knowlton, '53), diaphragm and heart of the rat (Bloom and Russell, '52).

SUMMARY

1. The total glycogen concentration in the guinea pig adrenal is of the order of 25 mg% on a wet tissue basis, and is not appreciably altered by fasting or by inadequate ascorbic acid intake.

2. The total glycogen concentration in the rat adrenal after fasting periods of 8 to 48 hours ranges generally from 175 to 200 mg%. It does not differ significantly in the two glands of a pair.

3. The non-fasted rat on a regular dietary regimen, or the fasted rat given glucose or fructose has considerably less adrenal glycogen than the fasted animal.

4. The adrenal of the rat, like the liver, gastrocnemius muscle, diaphragm and heart contains a glycogen fraction which can be readily extracted from the fresh tissue with 10% trichloracetic acid. This fraction accounts for about one-half of the total glycogen which can be isolated from the adrenal of fed or fasted rats after the usual procedure of alkaline digestion of the tissue.

ACKNOWLEDGMENT

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EFFECTS OF MALNUTRITION IN EARLY LIFE ON SUBSEQUENT GROWTH AND REPRODUCTION OF RATS [1]

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Many experiments are on record concerning the realimentation of rats whose weight gains had been temporarily reduced or arrested through dietary restrictions in the intake of the whole ration (Osborne and Mendel, '14), of calories (McCay et al., '39; Jackson, '36; Clark and Smith, '38), of proteins (Jackson, '36), of inorganic elements (Clark and Smith, '38) or of some of the B vitamins (Clemensen, '33). The pertinent literature has been fully reviewed at various times (Osborne and Mendel, '14; Jackson and Stewart, '20; Jackson, '25; McCay, '42). There is general agreement that the capacity to grow is not related to chronological age (Osborne and Mendel, '14). Realimentation with an adequate ration leads to rapid resumption of growth of previously stunted rats which may ultimately attain the body weight of unrestricted littermates and exceed the latter in life span (Osborne and Mendel, '17; McCay et al., '35). Osborne and Mendel ('15) emphasized that "the size or age at which inhibition of growth is effected does not alter the capacity to resume growth" and that stunted rats upon realimentation subsequently reach the full size characteristic of the species. Others, however, observed that prolonged malnutrition may prevent complete recovery upon realimentation (Clark and

[1] Paper no. 3239, Scientific Journal Series, Minnesota Agricultural Experiment Station.

Smith, '38; Jackson and Stewart, '20; McCay et al., '39) and that this effect was more pronounced in male rats (Jackson and Stewart, '20, Jackson, '37). Female rats retained the ability to reproduce successfully after realimentation from early stunting (Osborne and Mendel, '17; Jackson and Stewart, '20).

Only two experiments have been noted in which an attempt was made to restrict the growth of rats during very early infancy (Brüning, '14; Jackson and Stewart, '20). In both instances, young rats were intermittently removed from their mothers for a total of as much as one-fourth of the whole nursing period. These animals weighed as little as 10 to 12 gm at three weeks of age (Stewart, '18, Jackson and Stewart, '20) or 16 gm on the 25th day of life (Brüuing, '14) compared to 25 gm for unrestricted littermates of the corresponding age. When these stunted rats were subsequently fed presumably adequate rations ad libitum their growth rate and maximum weight was somewhat less than that of unrestricted littermates. Some females thus treated produced litters (Jackson and Stewart, '20).

McKenzie ('28) reported that a litter of pigs whose preweaning growth had been severely restricted gained weight, after weaning, at a rate similar to that achieved by pigs normally on the same ration.

During studies on reproduction of rats fed protein-free, amino acid-containing rations (Schultze, '55), young rats became available that were severely stunted during the nursing period, presumably due to inadequate lactation of their mothers. This paper presents a summary of observations on the growth and reproductive performance of such rats when they were fed adequate rations after weaning. Included also are some observations made with rats that were fed amino acid rations for longer periods of time.

EXPERIMENTAL

Animals. The rats used for these experiments were offspring from mothers that had been fed the protein-free amino

acid rations described in a preceding paper (Schultze, '55). They had been weaned at 4 weeks of age when some individuals weighed as little as 11 gm. At that time they were transferred, for realimentation, in groups of 4 to 5 to cages with raised screen bottoms and fed a ration which supports adequate growth of this strain of rats. Their weight increments were recorded for periods of 6 to 12 weeks after they had reached the weight of 35 to 40 gm, which is the weaning weight (at three weks of age) of our rats raised on normal rations. The females were bred when they weighed about 150 gm and they were handled thereafter as previously described (Schultze, '54). Some male rats were not transferred from the amino acid-containing ration to the natural ration until they had consumed the former for about 16 weeks after weaning.

Rations. The preceding papers (Gander and Schultze, '55; Schultze, '55) described the rations used. The stunting of the young rats was accomplished by feeding to their mothers throughout pregnancy and lactation rations which contained either 8% (rations AA_2 and AA_3) or 16% (ration AA_4) of a mixture of the "essential amino acids" plus 5.1% of ammonium citrate or 12.2% of a mixture of 16 amino acids (ration AA_{11}). These rations were accessible to the young until they were 4 weeks of age and, in the case of some male rats, until they were about 20 weeks old. For realimentation of the stunted rats, they were fed ration OC_1 containing 84% of rolled oats and 6.5% of casein, 3% of salt mixture, 2% wheat germ oil, 1% of corn oil, a vitamin mixture and 0.33% of DL-methionine (Schultze, '54).

RESULTS AND DISCUSSION

The results of observations on the realimentation of rats stunted during infancy (groups 1–5) are summarized in table 1. For comparison, this table also includes a record of the weight gains of the rats whose growth was not stunted (groups 6 and 7). The 6-week weight increment, starting at 35 to 40 gm, during the realimentation period accomplished

TABLE 1

Weight increments of young rats[1]

	GROUP						
	1	2	3	4	5	6	7
Maternal ration	$AA_{2 \text{ or } 3}$	$AA_{2 \text{ or } 3}$	AA_4	AA_{11}	AA_{11}	OC_1	OC_1
Sex	M	F	F	M	F	M	F
Number of rats	40	23	12	11	5	22	80
Mean weaning weight, gm	20[2] ± 0.8	20[2] ± 0.8	30[2] ± 1.3	22[2] ± 1.4	22[2] ± 1.1	43[3] ± 1.0	40[3] ± 0.6
Mean 6-weeks weight gain,[4] gm	147 ± 1.5	100 ± 4.3	112 ± 3.4	158 ± 3.0	109 ± 5.7	190 ± 7.8	120 ± 1.3
Mean 12-weeks weight gain,[4] gm	191 ± 4.3	..	..	..	..	303 ± 10.2	..

[1] Weights given as the nearest whole number ± standard error of the mean.

[2] At 4 weeks of age.

[3] At three weeks of age.

[4] After reaching weight of 35 to 40 gm.

by the stunted male rats, was only about 80% of that of rats whose growth during the pre-weaning period followed the "normal pattern" observed in this colony (group 6). (In this connection it is well to bear in mind that "normal growth" does not necessarily represent the maximum growth potential of a strain of a given species (Cox et al., '54.) During this period of realimentation, the depression of the growth rate of the males was somewhat more pronounced than was observed with the females. The effect of early stunting on subsequent growth of males became particularly noticeable in later stages of realimentation (during the second 6-week period of measurement). Some of these animals attained a stationary weight of about 250 gm only. While these experiments were not continued for longer periods, these animals gave no indication that they would have resumed growth at a later date. Our results indicate, therefore, that stunting as a result of malnutrition during infancy can lead not only to a reduced growth rate during subsequent periods of adequate feeding but that it may also prevent the attainment of the maximum weight usually accomplished by the particular strain of this species (mean of about 450 gm for males of our colony). In this respect, these results are in accord with those of Jackson and Stewart ('20) and Brüning ('14) who accomplished stunting of the young by a different technique.

The post-weaning weight increments of both male and female offspring from rats fed a diet containing the 10 essential amino acids and ammonium citrate (groups 1 and 2) were smaller than those achieved by weanlings of similar weight whose mothers were fed a ration containing 16 amino acids (groups 4 and 5).

A similar failure of male rats to achieve complete realimentation is illustrated in table 2. In this instance feeding of the amino acid rations AA_3 or AA_4 (see Schultze, '55) to the stunted rats was continued for 12 to 16 weeks. When they were then fed the rolled oats-casein ration OC_1 they gained an average of about 24 gm during the first week but there-

after, their weight increments were very small until they reached and maintained during 4 weeks a stationary weight of about 230 gm. Under these conditions, the early stunting and subsequent growth retardation likewise seemed to inflict permanent damage on male rats.

In connection with our studies on the reproduction of rats fed amino acid diets (Schultze, '55), it was of particular importance to determine if stunting of rats as a result of infantile malnutrition would affect their reproductive performance and the survival of the young. Many of the female rats

TABLE 2

Weight increments of stunted males transferred to adequate ration at 16 to 20 weeks of age

Number of rats	19
Mean weight at 4 weeks of age, gm	18 ± 0.5 [1]
Mean weight increment 5th to 10th week, fed AA ration, gm	51 ± 3.8
Mean weight increment 5th to 16th week, fed AA ration, gm	101 ± 6.4
Mean weight when transferred to OC_1 ration, gm	153 ± 2.2
Mean weight increment 1st week while fed OC_1 ration, gm	24 ± 0.7
Mean weight increment 6 weeks while fed OC_1 ration, gm	54 ± 1.2
Mean weight increment 12 weeks while fed OC_1 ration, gm	64 [2] ± 4.9
Mean stationary weight [3] while fed OC_1 ration, gm	231 [2] ± 4.3

[1] Standard error of the mean.
[2] Mean of 9 rats.
[3] For 4 weeks.

whose growth performance during realimentation is recorded in table 1 were, therefore, bred when they weighed about 150 gm with normal males fed the same ration. These animals readily conceived and a summary of their reproductive performance and the survival of their young is recorded in table 3. It is evident that severe stunting of female rats during early infancy did not reflect itself in impaired reproductive performance after realimentation. The size and weight of the litters were about the same as that obtained from similar rats that had developed normally while fed the same ration (Gander and Schultze, '55). Judged by visual observations and by the weight increments of the young

during the nursing period, the lactation of the mothers was not impaired. As is usually the case, the rats gained weight while they reared their first litters. Their weight at the end of the first reproductive and nursing period, however, was lower than that of similar rats of the same age that had not been stunted during infancy. These experiments were not continued beyond the production of the first litter and it can, therefore, not be stated whether or not these animals would eventually have reached full size.

TABLE 3

Reproductive performance of rats stunted in infancy

	GROUP 1	GROUP 2
Maternal ration	AA_2, AA_3, or AA_5 [1]	AA_4 [1]
Ration consumed after 4 weeks of age	OC_1 [2]	OC_1 [2]
Number of rats	32	12
Weight of rats at 4 weeks of age, gm	19.4	29.9
Number of litters born alive	31	12
Mean number of young born per litter	6.4	8.4
Young born dead, %	2.9	8.6
Mortality of young in three weeks, %	20.2	22.6
Mean weight gain of mothers during lactation, gm	22.6	17.5
Mean weight of young at three weeks of age, gm	38.4	38.1

[1] Protein-free amino acid rations containing 8% (group 1) or 16% of mixture of 10 amino acids (see Schultze, '55).

[2] Ration containing 84% rolled oats and 6.4% casein, as main components.

No attempt was made to study the fertility of the realimented male rats.

SUMMARY

1. Rats whose early growth had been severely inhibited by malnutrition during infancy were realimented after weaning by ad libitum feeding of a ration which supports good growth.

2. The weight increment of stunted young rats during the first 6 weeks of realimentation was about 80% of that attained by animals of the same strain whose early growth had not been retarded.

3. During the second 6-week period of realimentation, the growth of male rats was severely retarded.

4. Many of the male rats reached a subnormal stationary weight and were apparently permanently stunted as a result of malnutrition during the first 4 weeks of life.

5. Male rats whose early growth had been restricted for 20 weeks made only an incomplete recovery during realimentation.

6. After realimentation female rats whose growth had been retarded during infancy showed no impairment in their reproductive performance. The survival and preweaning growth of their young was normal.

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THE EFFECT OF HYPOTHYROIDISM ON THE METABOLISM OF CAROTENE IN DOGS

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TWO FIGURES

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Conflicting reports on the utilization of carotene in hypothyroidism led to a study of the problem in rats which has been reported earlier (Arnrich and Morgan, '54). The investigation was extended in the present study with dogs as the experimental animals.

Dogs occupy a unique position among species ordinarily studied with respect to vitamin A metabolism. Surprisingly few data can be found on the subject of vitamin A metabolism in the dog. These animals may normally excrete vitamin A in the urine (Catel, '38) and this excretion is accompanied by considerably higher levels of vitamin A in blood and tissues than are usually found in other species. Though the dog, like other animals, is capable of developing a deficiency at low levels of dietary vitamin A intake, there is some indication that the growth requirement for vitamin A is higher in dogs than in other animals (Frohring, '35). In view of these species differences it seemed of interest to determine whether thyroid hypofunction might lead to changes in carotene utilization such as had been found in rats.

PROCEDURE

Experimental

Six mature, purebred cocker spaniels produced in the laboratory colony had been reared and maintained on a purified,

vitamin A-free diet of the following composition: vitamin test casein [1] 18.0, salt mix [2] 2.5, $CaCO_3$ 1.5, hydrogenated fat [3] 10.0 and granulated sugar 68.0%. Each 100 gm of diet supplied the following amounts of vitamins in milligrams: thiamine, riboflavin, pyridoxine and folic acid, 0.2 each, pantothenic acid and para-aminobenzoic acid 6.0 each, niacin 4.0, choline 100.0, inositol 50.0 and biotin 0.01. Each dog received daily 1 ml of vitamin D and E supplement furnishing 210 I.U. of vitamin D and 25 mg of mixed tocopherols.[4] Adult animals received 25 gm of diet per kilogram of body weight daily.

After the animals had been depleted of their initial vitamin A stores, at three months of age as indicated by a vitamin A analysis of liver biopsy samples, they were given 12.9 mg of carotene [5] daily as the sole source of vitamin A. At that time the vitamin-test casein was replaced by raw casein since the amount of vitamin A present in raw casein is negligible in the presence of the large amounts of carotene administered. The amount of vitamin A in a sample of raw casein was too small for chemical analysis but the biological assay method indicated the presence of 12.8 μg%. This regime was continued until the dogs were mature enough to withstand the thiouracil treatment. Attempts at administration of thiouracil to younger dogs had resulted in the death of the animals.

The experimental period under consideration extended over 8 months and was begun when the dogs were 19 months old. At this time the casein level in the diet was increased to 40% for 4 of the 6 animals. No effect was noted from the change in protein level and the data will therefore be discussed together. A liver biopsy was performed to determine the level of liver vitamin A at the onset of the observation period. The operation was repeated after 4 months at which

[1] Vitamin test casein General Biochemicals Inc.

[2] Axelrod, Morgan and Lepkovsky ('45).

[3] Primex.

[4] Concentrate of mixed tocopherols. Each gram contains 340 mg of mixed tocopherols. Distillation Products Industries.

[5] Carotene in oil. General Biochemicals Inc. Potency 50,000 I.U. per gram.

time three of the dogs were given thiouracil in the diet at a level of 0.74% until the end of the experiment. This amount had been shown to produce thyroid deficiency with hyperplasia of the gland in dogs (Danowski et al., '46). The animals were autopsied 4 months later.

Fasting blood samples were taken from the femoral artery 48 hours after the last carotene dose both in the pre-experimental period and at regular intervals during the 8 months period under consideration. Serum analyses for vitamin A, carotene and cholesterol were made.

The liver biopsy procedure involved the removal of a peripheral liver sample of approximately 2 gm under sodium nembutal anesthesia. Aseptic conditions were observed. The dogs received 100,000 units of penicillin [6] postoperatively for three days. Recovery from the anesthesia occurred in a few hours and activity and appetite appeared normal.

The liver biopsy samples were considered representative of the whole organ, since samples taken from the several lobes of one dog liver had been shown on analysis to contain vitamin A within the variability found between aliquots from the homogenate of the rest of the organ. These results confirmed the earlier work of Rouir ('47).

At autopsy a lethal dose of sodium nembutal was given and as soon as anesthesia had set in, the animals were sacrificed by exsanguination from the carotid artery. The organs were removed rapidly, weighed and prepared for further analyses.

Analytical

Serum and tissues were analyzed for vitamin A and carotene by the same methods used in the previous study (Arnrich and Morgan, '54).

The determinations for serum cholesterol were made using the method described by Schoenheimer and Sperry ('34).

The urine for vitamin A determinations was collected in metabolism cages with stainless steel bottoms. The samples

[6] Hypercillin. Procaine Penicillin G in sesame oil. Cutter Laboratories, Berkeley, California.

were received in dark bottles in 5 ml of 50% KOH solution and were removed to the refrigerator as soon as they were voided. Under these conditions they were found to be stable for 24 hours. All analyses were therefore made on the same day and no attempts were made to interpret results from overnight collections. The KOH concentration was adjusted to 5% and 10 ml aliquots of the final solution were used for analysis by the usual procedure.

RESULTS AND DISCUSSION

Serum levels of vitamin A, carotene and cholesterol

The administration of large doses of carotene caused a gradual rise in vitamin A serum levels during the pre-experimental period. At the onset when the young animals' liver stores had been depleted to the low level of 10.3 μg per gram the corresponding serum vitamin A levels averaged 108 μg%. During the next few months the blood levels rose to an approximate average value of 300 μg%, and remained there with little change, though carotene supplementation was continued (fig. 1).

A considerable rise in serum vitamin A was observed in dogs 701F, 706M and 708M following the administration of thiouracil during the experimental period. This was evident in 701F immediately, but occurred more slowly in 706M, so that a definite increase was established in all three animals only during the last two months. The serum concentration at autopsy averaged 333 μg% in controls versus 881 μg% in the experimental animals.

A 100% increase in serum vitamin A was observed by Williamson ('47) in rabbits following thyroidectomy. In normal and thyroid-deficient rats previously studied in this laboratory no statistically significant difference was found in serum vitamin A between the groups and results were not reported at that time. According to Lewis and co-workers ('42) the normal equilibrium level of vitamin A in rat serum is close to 33 μg%, and the group averages of the rats studied

by us approximated this value. At this level a regulatory mechanism seems to operate in rats which prevents further significant increases in serum vitamin A in the presence of ever increasing liver stores. Comparison of the average

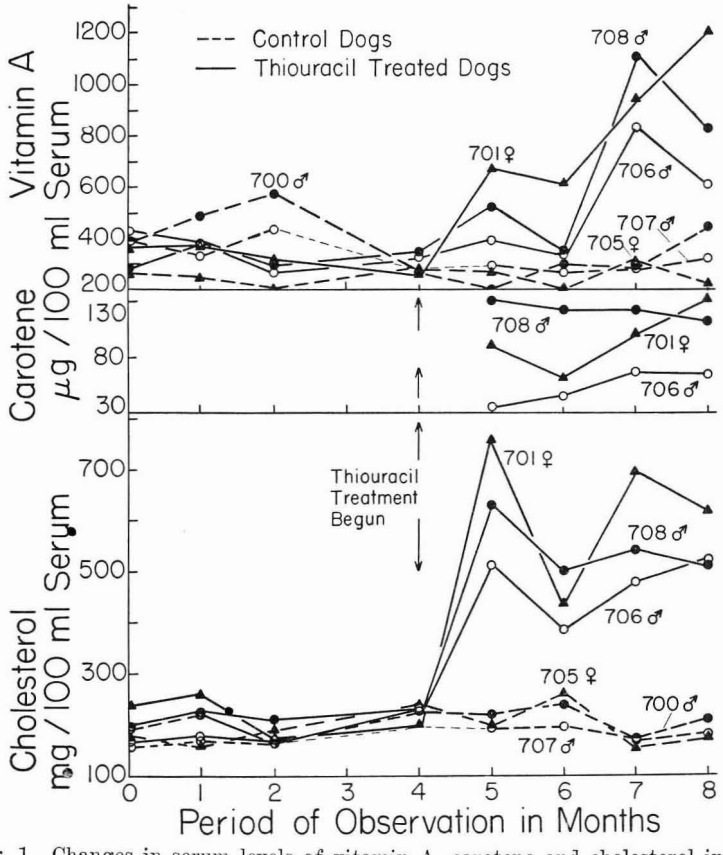

Fig. 1 Changes in serum levels of vitamin A, carotene and cholesterol in dogs due to thiouracil feeding.

serum vitamin A value of 326 μg% for normal dogs reported in the present experiment with results obtained for stock dogs and with data found in the literature indicate that the level encountered here is low. One might infer that the dogs on this experiment, not having reached the equilibrium level which may lie in the vicinity of 1094 μg% (the average for the

stock dogs) could respond with a rise in serum concentration while rats, having reached the optimum level for that species, resisted the change.

With the rise in serum vitamin A small amounts of carotene were observed in the blood of the hypothyroid dogs. This was the only incident throughout the investigation where carotene was noted in the petroleum ether extract of the serum samples. In analyzing the data it should be kept in mind that only values exceeding 30 μg% are significant of circulating carotene. Serum values below this level are due to a faint yellowish pigment found in the petroleum ether extract of the sera of all dogs irrespective of carotene supplementation and should be designated "apparent carotene."

The maximum carotene concentrations found in the experimental animals were 68, 132 and 134 μg%. Dog 701F was the only one showing a correlation in individual blood samples between carotene and vitamin A (fig. 1).

Hypothyroidism is accompanied by an increase in serum cholesterol. Though this change is not as reliable a criterion as is the depression of the basal metabolic rate, it is probably safe to conclude that an increase in serum cholesterol following the administration of goitrogenic substances indicates that the animal is responding to the treatment.

The three dogs receiving thiouracil showed an increase in serum concentrations of 174% over their respective controls in the terminal blood samples (fig. 1). The greatest overall response to thiouracil in both serum vitamin A and cholesterol concentrations was found in dog 701F, and dog 708M had the lowest range for both substances.

Since the rise in serum vitamin A was concurrent with the appearance of carotene and the elevation of the serum cholesterol it is possible that we are dealing with a basic change due to hypothyroidism affecting lipids and lipid-soluble fractions of the blood. Elucidation of the underlying causes for the rise in cholesterol may also shed light on the rise in vitamin A and carotene.

Serum vitamin A levels after carotene absorption

Preliminary tests had shown that in normal dogs the absorption of 36.9 mg of carotene was accompanied by a temporary rise of vitamin A in the serum. The peak of the curve

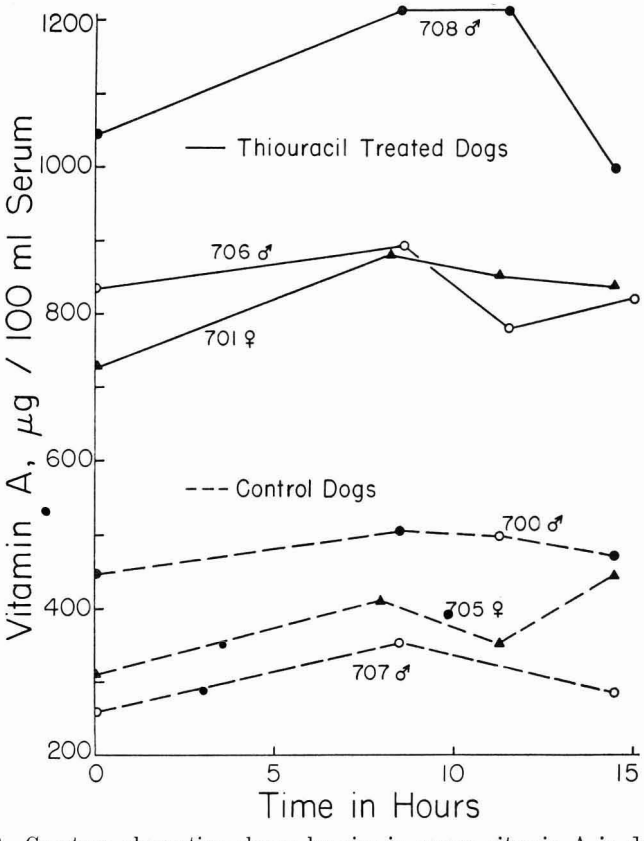

Fig. 2 Carotene absorption shown by rise in serum vitamin A in dogs following ingestion of 36.9 mg of carotene.

fell between the 8th and 11th hour after the administration of the dose. Data for such a test made in the 7th month of the experimental period are given in figure 2.

The absolute values of the maximum serum levels were higher in the hypothyroid dogs than in their controls, just as the post-absorptive serum levels were higher, but the rela-

tive increase as indicated by the slope of the curves is rather similar. The average increase in serum vitamin A level after 8 hours amounted to 87 μg% in the normal dogs and to 128 μg% in the thyroid-deficient animals.

The similarity in absorption rate in normal and deficient dogs might lead to the conclusion that absorption and conversion rates are similar in the two types of animals and that the increase observed in the post-absorptive serum values reflects a block in the uptake of the vitamin by the liver. If this were true the temporary rise in the serum level following a dose of carotene would be expected to be of greater magnitude and longer duration than was found here. Nor is this concept supported by data for liver vitamin A which will be discussed presently. It must therefore be concluded that the elevated serum levels are phenomena independent of carotene-vitamin A utilization.

Liver vitamin A

The accumulation of vitamin A from dietary carotene in the liver was followed by analysis for vitamin A of liver biopsy samples. After the 4th month of observation before thiouracil administration was begun 5 of the 6 dogs had an average liver vitamin A concentration of 224 μg per gram with a range of 172 to 264 (table 1).[7] The average for the 5 animals had increased to 330 μg per gram at the time of autopsy 4 months later with a range of 297 to 403. All dogs, regardless of treatment, had accumulated additional vitamin A in their livers. The degree of increase in the two normal animals was similar, 85 and 93 μg per gram. Greater variability was found in the deficient dogs, 708M showing the smallest increase, 38 μg per gram. 701F somewhat exceeded the control animals with an increase of 129 μg per gram, while 706M almost doubled its liver vitamin A concentrations with an increase of 185 μg per gram. The corresponding control

[7] Biopsy data for dog 707M are not available since this animal had responded unfavorably during a previous operation.

TABLE 1

The vitamin A content of the organs and serum of mature normal and hypothyroid dogs fed constant amounts of carotene

DOG NO.	PERIOD IN MONTHS	BODY WT.	THYROID WT.	LIVER		KIDNEY	ADRENALS		OVARIES OR TESTES	SERUM			DIET
				Vitamin A	Carotene	Vitamin A	Vitamin A	Carotene	Vitamin A	Vitamin A	Carotene	Cholesterol	
		kg	*gm*	*μg/gm*	*μg/gm*	*μg/gm*	*μg/gm*	*μg/gm*	*μg/gm*	*μg/100 ml*	*μg/100 ml*	*mg/100 ml*	
707M	4	8.8											18% casein
	8	9.2	0.52	488	48	31	43	12	18	326	6	180	18% casein
700M	4	8.6		212 [1]						268		228	40% casein
	8	8.8	0.82	297	24	48	65	38	18	448	32	210	40% casein
705F	4	7.6		256 [1]						284		240	40% casein
	8	7.9	0.46	349	12	11	27	8	3	228	20	175	40% casein
706M	4	9.5		218 [1]						330		233	18% casein
	8	8.4	2.54	403	47	10	54	24	18	606	66	524	18% casein + 0.74% thiouracil
708M	4	9.6		264 [1]						350			40% casein
	8	10.6	2.11	302	21	60	51	60	26	834	114	510	40% casein + 0.74% thiouracil
701F	4	7.3		172 [1]						264		200	40% casein
	8	8.2	3.07	301	9	7	98	75	4	1204	134	618	40% casein + 0.74% thiouracil
679F		13.0	0.59	3005	0	3562	55	0	10	1128	0		Stock
618F		10.3	0.51	1896	0	524	93	0	11	1060	0		Stock

[1] Obtained by biopsy.

dog 707M had the highest concentration of all dogs at autopsy with 488 μg per gram. No biopsy figure is available for comparison for this animal. It appeared from analysis of these autopsy data that no notable differences in liver vitamin A storage were caused by hypothyroidism in spite of considerable changes in the level of circulating vitamin A.

The experiments reported earlier with normal and thyroid-deficient rats had shown that at the low level of carotene intake the deficient animals stored more vitamin A in their livers than did normal controls (Arnrich and Morgan, '54). This was shown to be largely due to a greater utilization of the formed vitamin A by the larger, more rapidly growing normal animals. At higher levels of carotene intake the storage of vitamin A was approximately the same in both normal and deficient rats since the amount necessary to satisfy the maintenance requirement was probably too small by comparison to be reflected in the storage. Hypothyroid dogs, like rats, are capable of utilizing carotene as a source of vitamin A as indicated by the increase in liver vitamin A concentration found here following the administration of thiouracil. An increased storage of vitamin A from carotene could not be demonstrated in the dogs, and in that respect the study on dogs differs from that done on rats. The dose used for the dogs corresponded in terms of requirement to the low carotene dose given to the rats in the previous study. The dogs were given 1,466 μg per kilogram per day or 12 times the amount set tentatively for the minimum requirement by Frohring ('35) at 120 μg per kilogram (200 I.U.). The rats received 56.5 μg per 100 gm daily or almost 10 times the minimum requirement of 6.0 μg (10 I.U.). With a comparable dose in terms of minimum requirement the hypothyroid dogs were expected to show larger liver vitamin A stores than the controls, had they behaved like rats. However the accretion of liver vitamin A for the period of thiouracil treatment was quite similar to that found in the normals. A possible explanation for the different responses in dogs and rats may be found in the fact that the hypothyroid state did not lead

to the definite decrease in body weight in the dogs (table 1) that was found in rats, so that a change in vitamin A utilization due to the deficiency might not have occurred. This may point to the lack of implication of vitamin A in the basal metabolic processes since these deficient dogs presumably had lowered basal energy production.

Urinary excretion of vitamin A

Preliminary experiments on stock dogs fed preformed vitamin A indicated an excretion level ranging from 30 to 100 μg% in the urine. Repeated attempts to establish with significance urinary excretion of vitamin A in both normal and deficient animals met on the whole with negative results. Only dog 708M had measurable amounts of vitamin A in the urine towards the end of the experiment, and he was the only one at that time whose serum vitamin A concentration exceeded 1000 μg% and therefore approached levels usually encountered in vitamin A-fed dogs. Unpublished results from this laboratory had shown earlier that dogs fed carotene as the sole source of vitamin A did not excrete vitamin A in their urine, and this was thought to be characteristic of carotene metabolism. However reconsideration of the data showed that the vitamin A blood levels and liver stores of carotene-fed animals were considerably lower than those of vitamin A-fed dogs excreting the vitamin in their urine. It is more difficult to raise the level of vitamin A in blood and tissues of dogs with carotene than with vitamin A in the ration, and the higher plane of vitamin metabolism found after vitamin A feeding might be essential for urinary excretion. Further work on a possible threshold level for urinary excretion is needed.

Vitamin A and carotene in various organs

A number of tissues were analyzed at autopsy for vitamin A and carotene (table 1). Samples from two females from the stock colony were included for comparison. These ani-

mals had received preformed vitamin A throughout their lives at a level of 13,800 I.U. per day.

All animals receiving carotene had appreciable amounts of the provitamin accumulated in their livers. The organs of the stock dogs were free of carotene. Quantities found in the carotene-fed dogs constituted between three and 10% of the total amount of vitamin A present and animals with high vitamin A stores also had high carotene levels.

Kidney vitamin A stores were variable and were neither directly nor inversely related to liver reserves. The two females had lower kidney concentrations than three of the males, but the 4th male, 706M, showed a low level similar to that of the females. Since he was thiouracil-treated the result seems not surprising in the light of the data obtained in hypothyroid rats, where low kidney vitamin A levels were encountered in all females as well as in hypothyroid males. However, the other thyroid-deficient dog, 708M, had the largest kidney vitamin A level of all 6 animals. Since there was no indication that he was less affected by the drug than dog 706M, as shown by the changes produced in his blood picture and by the increase in thyroid weight (table 1) no conclusion can be drawn as to the effect of thyroid deficiency on kidney vitamin A levels.

All kidney vitamin A values in carotene-fed dogs were insignificantly small when compared with those in stock females. The highest level found in the dogs in this experiment was 60 μg per gram, while one of the stock animals had as much as 3,562 μg per gram of vitamin A in the kidney, exceeding the value of 3,005 μg per gram of vitamin A found in the liver of the same animal.

No traces of carotene were found in any of the kidney samples.

The adrenal glands were analyzed since gross inspection had revealed that the cortical area of the carotene-fed dogs was of a bright orange color not seen in dogs fed vitamin A. This was shown to be due to the presence of carotene which

in some cases nearly equalled in amount the adrenal vitamin A.

Of the tissues studied, liver, kidney, blood and adrenals, the latter was the only one in which comparable amounts of vitamin A were found in both carotene- and vitamin A-fed dogs in spite of the much larger quantities of the vitamin found elsewhere in vitamin A-fed dogs. It may be that the amount of vitamin A in the adrenals is limited by the functional role of the vitamin in that organ and is therefore independent of exogenous vitamin A reflected in other tissues.

The vitamin A concentration in the testes of the 4 males was fairly consistent. The ovaries of the female stock dogs had between two and three times as much vitamin A as the two carotene-fed females. If vitamin A concentration in the ovaries should depend on the functional state, greater fluctuations would be expected with the state of the estrus cycle than would be noticeable in testes.

Thyroid weights

The thyroid weights reflected the hyperplasia caused by the administration of the goitrogenic drug. The change in 708M whose thyroid was twice as heavy as that of the corresponding control 700M, was not as striking as the increase observed in the other two hypothyroid animals which amounted to a 5- or 6-fold augmentation (table 1).

SUMMARY

In normal and thiouracil-treated young mature dogs comparable increases in liver vitamin A concentrations were observed following the ingestion of carotene. Thiouracil administration caused, however, a rise in blood vitamin A concentration as compared with those found in normal controls. The increase was accompanied by similar changes in serum cholesterol levels and by the appearance of significantly increased serum carotene.

Absorption of a single dose of carotene was reflected in normal dogs by a peak in the vitamin A level between the 8th and 11th hour following the dose. The magnitude of the change over the predosing level was not significantly altered in hypothyroidism.

Only traces of vitamin A could be demonstrated in the urine of either normal or hypothyroid dogs fed carotene with the exception of an instance where the blood level had exceeded 1,000 μg% of vitamin A following thiouracil treatment. It is suggested that the circulating level of vitamin A was too low after carotene feeding to cause excretion in the urine.

Following carotene administration both hypothyroid and normal dogs had considerable quantities of carotene in their livers. Carotene and vitamin A were present in the adrenals of carotene-fed dogs in nearly equal amounts. Females on stock diet fed vitamin A did not have more vitamin A in their adrenals than did the carotene-fed dogs in spite of the large differences in vitamin A levels elsewhere.

Absorption and utilization of carotene appeared not to be affected by hypothyroidism in mature dogs but the circulating levels of vitamin A, carotene and cholesterol were significantly raised in this condition. This may be a non-specific effect on lipid levels.

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OBSERVATIONS ON THE PROTECTIVE EFFECT OF LINSEED OIL MEAL AND SOME EXTRACTS AGAINST CHRONIC SELENIUM POISONING IN RATS [1]

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It is generally recognized that protein may minimize the severity of selenium poisoning (Moxon, '37; Smith, '39; Gortner, '40; Smith and Stohlman, '40; Rosenfeld and Beath, '46), although there is less agreement as to the completeness of protection that may result from the various types of supplements investigated (Smith and Stohlman, '40; Moxon, '41). Moxon ('41) found crude casein and linseed oil meal the most effective of several protein supplements tested with rats but whole beef liver and linseed oil meal were the best with dogs. In the present study, continued work with protein supplements with rats has shown linseed oil meal more effective than purified casein in protecting against poisoning by either naturally occurring selenium or sodium selenite. The activity of certain linseed oil meal extracts has also been demonstrated. Evidence is presented which suggests that the active principle of the linseed oil meal probably is not protein in nature, indicating a need for some re-evaluation of protein effects and a consideration of other feed constituents in future studies.

[1] Approved for publication by the Director of the South Dakota Agricultural Experiment Station as paper No. 330 of the Journal Series.

METHODS

Male Sprague-Dawley albino rats were used throughout these experiments. They were housed in individual wire cages and allowed food and water ad libitum. The rats were placed on experiment at a weight of about 70 to 80 gm, the averages for the various groups in each experiment being within a two to 3 gm range. Bi-weekly weights were kept, and the livers were observed for gross damage and weighed at the end of the experiment. The ratio of liver weight per 100 gm of body weight affords a numerical means of expressing the extent of liver damage.

The diets used in these studies all contained salts IV (Phillips and Hart, '35), 2.0%; animal protein factor,[2] 0.1%; dried brewers' yeast, 2.0%; and lard, 3.0%. The remaining 92.9% of the diet was made up of corn, seleniferous corn, and purified casein alone and with or without linseed oil meal or its fractions. The seleniferous corn was used in an amount necessary to give the desired selenium level and non-seleniferous corn was then used to complete the diet. In preparing diets containing Na_2SeO_3, the salt was dissolved in 70% ethanol and sprinkled on the other ingredients. Vitamins A and D were supplied by administration of diluted haliver oil once weekly.

EXPERIMENTAL AND RESULTS

Casein and linseed oil meal diets at various selenium levels

Linseed oil meal or casein was added to the diets at the 21 or 8% level respectively, at which concentrations each supplement furnished the same amount of protein ($N \times 6.25$). Table 1 presents the data obtained with the two protein supplements with different levels of selenium as seleniferous corn. Without selenium in the diets growth was better with casein than with linseed oil meal. The same was true at 7 p.p.m. of selenium, but the difference was minor and survival and pro-

[2] Nutritional Biochemicals Corporation, Cleveland, Ohio.

tection against liver damage were better on the linseed oil meal diet. At 10 and 13 p.p.m. of selenium, growth was more severely retarded on the casein diet than on the linseed oil meal, and the survival and liver damage data indicated a much greater degree of protection against selenium poisoning by the linseed oil meal. Complete protection, however, was not

TABLE 1

The toxicity of various levels of selenium to rats fed casein and linseed oil meal
(8 rats per group)

PROTEIN SUPPLEMENT	SELENIUM [1] IN DIET	PROTEIN CONTENT OF DIETS	RESULTS OF 42-DAY EXPERIMENT			
			Average gain in weight of survivors [2]	Survival	Liver damage [3]	
	p.p.m.	%	*gm*	%		
8% casein [4]	0	15.4	256	100	None	(5.0)
	7	15.7	170	88	Moderate	(3.8)
	10	15.8	121	38	Severe	(2.4)
	13	16.0	. . .	0	Severe	(2.1)
21% linseed oil meal [5]	0	14.2	187	100	None	(5.1)
	7	14.5	157	100	Slight	(4.7)
	10	14.7	132	100	Slight	(4.6)
	13	14.8	80	63	Moderate	(3.5)

[1] Added as seleniferous corn.

[2] Initial weights averaged 66 to 69 gm among groups.

[3] The figures in parentheses are ratios of grams of liver weight per 100 gm of body weight at time of death or sacrifice.

[4] Vitamin-free (Nutritional Biochemicals Corporation), 85.4% protein.

[5] "Old process" expeller type (Spencer-Kellogg), 32.2% protein.

obtained on the linseed oil meal diets at any of the three levels of selenium. The data also indicated 10 p.p.m. of selenium to be satisfactory for use in further work.

Studies were then made with different preparations of linseed oil meal. Hexane-extracted expeller meal and petroleum ether-extracted raw ground flax proved to be as active as untreated expeller meal, thus indicating that the fat fraction was inactive.

Protective effect of linseed oil meal plus casein against naturally occurring and inorganic selenium

Experiments with combinations of casein and linseed oil meal (table 2) show a striking difference in the toxicity of the seleniferous diets containing casein alone and those containing linseed oil meal alone or with various casein levels. The addition of 6 or 12% casein in the presence of linseed oil meal complemented the protective effect of the meal somewhat as evidenced by increased growth and liver size. While this experiment did not include a group receiving casein alone at a level equivalent in protein to that of the linseed oil meal plus 12% of casein, data from an accessory study indicated that 21% of casein does not afford material protection.

The results in table 2 demonstrated that sodium selenite is similar in behavior to naturally occurring selenium, except that it appears somewhat more toxic in the present work. Therefore, inorganic selenium was used in all future work instead of seleniferous corn, thus simplifying the preparation of diets. Because of the satisfactory response obtained with linseed oil meal in the presence of casein, 12% of casein was included routinely in subsequent diets. Such a practice eliminated the possibility of protein deficiency with diets containing linseed oil meal fractions.

Feeding trials with ethanolic extracts of linseed oil meal

Attempts to extract the protective principle from linseed oil meal with acetone failed to remove any activity. Water extraction was unsatisfactory because of the mucilages. Continuous extraction with 95% ethanol in a Soxhlet greatly reduced the activity of the residue although none was observed in the extract. Since the prolonged heating of the solvent during extraction might have destroyed the protective principle, another experiment using cold solutions of absolute and of 50% ethanol was undertaken. Feeding tests with the cold extracts indicated only slight protection with the absolute ethanol preparation, but significant protection with the 50%

TABLE 2

Study of the selenium protective effect of linseed oil meal with diets containing added casein and selenium as toxic corn or sodium selenite

(6 rats per group)

DIET VARIANTS [1]			PROTEIN CONTENT OF DIETS	RESULTS OF 42-DAY EXPERIMENT		
Selenium form	Casein in diet	Linseed oil meal [2] in diet		Average gain in weight of survivors [3]	Survival	Average liver weight per 100 gm body weight
	%	%	%	*gm*	%	*gm*
No selenium						
1	12	0	18.7	262	100	5.9
Seleniferous corn (10 p.p.m.)						
2	12	0	19.5	88	33	2.7
3	0	21	16.2	163	100	5.5
4	6	21	20.8	185	83	6.0
5	12	21	25.3	197	100	5.9
Sodium selenite (10 p.p.m.)						
6	12	0	18.7	24	17	3.1
7	0	21	15.3	112	83	5.5
8	6	21	19.9	139	83	5.3
9	12	21	24.5	151	100	5.9

[1] Variants added at expense of corn; casein contained 85.4% protein, linseed oil meal 36.8%, control corn 9.2% and seleniferous corn 11.2%.

[2] Commercial hexane-extracted, unheated product.

[3] Initial weights averaged between 78 to 79 gm among groups.

ethanol extract. A reduced activity of linseed oil meal was also noted following extraction with 50% ethanol. Since recombination of the extracts with their residues produced full activity, it was evident that no material part of the

TABLE 3

Protection against selenium poisoning by hot ethanolic extracts of linseed oil meal [1]

(4 rats per group)

	LINSEED OIL MEAL OR FRACTION ADDED TO BASAL DIET [2]		RESULTS OF 28-DAY EXPERIMENT	
	Description	Level added	Average gain in weight of survivors [3]	Survival
		%	*gm*	%
No selenium				
1	(Control basal)	0	188	100
10 p.p.m. selenium as Na_2SeO_3				
2	(Control basal)	0	61	25
3	Linseed oil meal	20.0	153	100
4	50% EtOH extract,[4]			
	a. 20% linseed oil meal equivalent	3.4	140	100
	b. 40% linseed oil meal equivalent	6.8	167	100
5	50% EtOH residue,[4] 24% linseed oil meal equivalent	20.0	83	50

[1] Commercial hexane-extracted, unheated product.

[2] Basal diet contained 12% casein (18.7% total protein) and addition of linseed oil meal or extracts was at the expense of corn.

[3] Initial weights averaged between 75 to 77 gm among groups.

[4] The linseed oil meal was extracted by heating at 70°C. for two hours with 7 volumes of 50% aqueous ethanol. The extract was filtered off hot by suction and the residue was promptly washed by using about three volumes of fresh solvent at similar temperature. The combined extracts and washings were dried in flat pyrex pans in a 75°C. forced draft oven. The residue was air dried.

protective principle had been destroyed in the extraction procedure.

Although cold 50% ethanol extraction appeared to remove much of the protective principle, the procedure used to obtain it was lengthy and laborious. In the search for a simpler procedure, the meal was extracted with 50% ethanol at 70°C.

by soaking and then washing and the extract was fed at two different levels (20 and 40% linseed oil meal equivalent). The hot ethanol removed 17% of the dry matter from the linseed oil meal, and left essentially all of the mucilage in the residue. The data in table 3 indicate that practically all of the protective factor was removed by the hot 50% ethanol since the extract, equivalent to 20% linseed oil meal, was essentially as active as linseed oil meal itself. Further, the higher extract level (40% linseed oil meal equivalent) gave protection supe-

TABLE 4

Failure of lead acetate to precipitate the active principle

(4 rats per group)

DIET DESCRIPTION	AVERAGE GAIN IN WEIGHT AT 28 DAYS [1]
	gm
No selenium (control basal)	178
10 p.p.m. Se as Na_2SeO_3	
Seleniferous basal	72
20% linseed oil meal	154
7.5% lead non-precipitable fraction [2] (80% linseed oil meal equivalent)	151
1.5% lead precipitable fraction [2] (80% linseed oil meal equivalent)	93

[1] Average initial weights 78 gm for each group.

[2] Fractions prepared by treating a water extract of dried hot 50% ethanol extract with excess lead acetate. Hydrogen sulfide was used to remove lead from precipitate and from filtrate. Upon drying at 70°C. the non-precipitable and precipitable fractions contained 13 and 9 p.p.m. lead, respectively.

rior to that of 20% of linseed oil meal, although the growth response was still not equal that of the non-seleniferous controls. The poor response obtained with the residue fraction further indicated a nearly complete removal of the protective principle.

A subsequent experiment has shown that the ash of the active ethanolic extract is not responsible for its protective effect. Further, it was found that water removed the protective principle from the dried ethanolic extract and then an excess of lead acetate did not remove the activity from water solution (table 4).

DISCUSSION

In these studies linseed oil meal has been found markedly superior to casein in protecting against selenium poisoning. In view of this and of the results with 50% ethanol extracts, it appears that some fraction of the meal other than protein was responsible for the protective effect. The failure of lead to precipitate the extracted active principle from solution supports this view. Whereas the effect of protein on selenium poisoning has received considerable attention, these findings point to a need for the consideration of other food constituents in the search for practical control measures, as well as for a reconsideration of protein effects.

At present there is no explanation of the mechanism by which linseed oil meal prevents selenium poisoning. Halverson and Hendrick ('54) have demonstrated that the anti-vitamin B_6 principle (Kratzer and Williams, '48) is not involved. Linseed oil meal does not reduce deposition of selenium in the liver (Olson and Halverson, '54), and extraction of the active principle with 50% ethanol leaves the mucilage with the inactive residue. Therefore, prevention of selenium absorption by the mucilage, a mechanism suggested for the protective effect of beet pectin (Rosenfeld and Beath, '47), does not seem likely. Identification of the active principle of linseed oil meal and the determination of its mode of action may be helpful in the understanding of the metabolic effects of selenium.

The effectiveness of linseed oil meal in preventing selenium poisoning in farm animals has not been adequately determined. However, in studying the effect of protein content of the diet with sheep fed seleniferous extracts of *Atriplex canescens*, Rosenfeld and Beath ('46) used medium and high protein diets containing 16 and 25% of linseed oil meal and found both protective when compared to a low protein diet without linseed oil meal. Although other protein supplements were included in their diets, it is possible that the protection they observed was largely due to the linseed oil meal. Unpublished studies at this laboratory indicate that this protein supplement

is effective against selenium poisoning in hogs and chickens. Since it appears to supply a protective principle in addition to protein, linseed oil meal should probably be considered the supplement of choice in seleniferous areas.

SUMMARY

In rat studies the protective effect of linseed oil meal against selenium poisoning has been demonstrated under several conditions. The inclusion of 6 or 12% casein in the diets did not alter or mask the protective effect of the meal and meals prepared by different methods were all found active. The protective principle was effective against inorganic (selenite) selenium as well as against the form occurring in toxic corn.

The protective principle has been extracted from linseed oil meal with hot 50% aqueous alcohol. It is water-soluble, and an excess of lead did not precipitate it from solution. The ash of active extract was not protective.

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THE NUTRITIVE VALUE OF PROTEINS OF MUSCLE FROM HOGS FED DIETS SUPPLEMENTED WITH AUREOMYCIN OR TERRAMYCIN HYDROCHLORIDE [1]

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Certain antibiotic substances exert a vitamin-sparing and growth-promoting action when added to the diets of children, cattle, poultry, rats and swine, under some but not all experimental conditions. Their roles in nutrition have been reviewed recently by Braude, Kon and Porter ('53) and by Mickelsen ('53). Relatively little consideration has been given, however, to ascertaining whether or not the nutritive value of the proteins synthesized under the influence of antibiotics is the same as that of animals fed unsupplemented rations. The effect of supplements of aureomycin [3] and terramycin [4] hydrochloride upon feed consumption, growth and nitrogen balance of hogs, and upon histology, cooking qualities, biochemical properties and nutritive value of the pork obtained from them

[1] Contribution no. 181, Department of Home Economics, and no. 203, Department of Animal Husbandry, Kansas State Agricultural Experiment Station, Manhattan.

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[3] The trademark of American Cyanamid Co. for the antibiotic chlortetracycline is aureomycin.

[4] The trademark of Chas. Pfizer Co. for the antibiotic oxytetracycline is terramycin.

therefore was investigated. Data relative to the nutritional worth of the pork are presented herein.

PROCEDURE

Littermate weanling pigs were fed individually until each weighed approximately 225 pounds. Ten hogs were fed the basal ration only; 10 others received 10 mg of aureomycin hydrochloride per pound of basal ration; and 10 received 10 mg of terramycin hydrochloride per pound of ration. The basal ration contained corn, soybean meal, tankage, alfalfa meal, a mineral mixture and vitamin D. Initially it provided 18% of protein, during the growing period 15%, and later 12%. Food was made available to the hogs during the 24 hours prior to slaughter to obviate possible effects of withdrawal of food upon the concentration of antibiotic substances in the tissues.

Diets containing pork derived from hogs that were fed the experimental rations were offered ad libitum to weanling male rats of the Sprague-Dawley strain for 4 weeks. Nitrogen balances were measured during the last 7 days of the test, and increments in hepatic nitrogen during the 4-week period were estimated. Details of the procedure have been described elsewhere (Clark, Hooper and McCord, '55). Since half of the hogs reached slaughter weight in late summer, and half in early spring, 6 diets were prepared. The rats were divided into two lots, 15 being assigned to a given diet in Lot 1, and 10 to each diet in Lot 2. Pork from both right and left sides of all hogs was included in the sample, the posterior end of the loin beginning after rib 13 being used. The loin ends were wrapped, frozen, and stored at 0°F. Pork was ground, dehydrated at room temperature, and defatted with ether under the conditions employed for beef (Clark et al., '55). Analytical values for the 6 samples of pork so treated were similar, and averaged 13.7% nitrogen, 3.98% ash, 0.26% ether extract, and 9.20% water. Pork provided 1.6% nitrogen in all diets, which also contained 20% Crisco, 2% roughage,[5]

[5] Ruffex.

5% Richardson and Hogan salt mixture ('46), 5% vitamin mixture,[6] and dextrin to total 100%.

RESULTS AND DISCUSSION

In table 1 are summarized data pertaining to growth and nitrogen balances of rats that consumed pork from hogs that were fed the basal ration alone or with 10 mg of aureomycin

TABLE 1

Average daily food intakes and daily gains in body weight and in hepatic nitrogen during a 28-day interval, and nitrogen metabolism in 7 days of growing rats ingesting pork from hogs fed a basal ration with supplementary aureomycin or terramycin hydrochloride

MEASUREMENT	ANTIBIOTIC FED TO HOGS					
	Lot 1			Lot 2		
	None	Aureomycin	Terramycin	None	Aureomycin	Terramycin
Nitrogen in diet, %	1.60	1.64	1.61	1.64	1.62	1.64
Initial wt., *gm*	54.0	53.0	53.0	56.0	56.0	56.0
Food intake, *gm*	10.9	11.1	11.0	12.2	12.5	12.3
Nitrogen intake, *mg*	174.0	183.0	176.0	202.0	204.0	202.0
Gain, *gm*	3.8	3.9	3.8	4.7	4.9	4.9
Gain/gram N eaten, *gm*	21.9	21.3	21.8	23.2	24.0	24.0
Gain in liver N, *mg*	3.6	4.2	4.2	5.0	5.2	6.5
In 7-day balance test:						
Av. body wt., *gm*	148	151	149	172	175	173
Surface area, cm^2	252	254	252	275	278	276
N eaten, *mg/100* cm^2	551	583	573	629	652	630
N in urine, *mg/100* cm^2	136	162	148	164	173	172
N in feces, *mg/100* cm^2	72	74	71	84	84	93
N balance, *mg/100* cm^2	343	347	354	380	395	365

or terramycin hydrochloride added per pound of ration. Animals in the second lot were initially larger than those in Lot 1 and consistently ingested 20 to 30 mg more nitrogen daily. Temperature and other conditions were the same, however, for both lots of rats.

[6] This supplied in milligrams per kilogram of diet: ascorbic acid 100, biotin 0.1, choline chloride 1000, folacin 1, inositol 1000, niacin 50, para-aminobenzoic acid 1000, calcium pantothenate 20, pyridoxine-HCl 4, riboflavin 6, thiamine-HCl 4, vitamin B_{12} 0.03, and alpha tocopherol acetate 100. Also, 1,666 and 333 I.U. of vitamins A and D, respectively, were given orally per week per rat.

Neither daily gains in body weight nor gains per gram of ingested nitrogen were altered significantly by including aureomycin or terramycin in the rations of the hogs, nor were mean gains in each of the 4 weeks influenced. These data substantiated the findings of Huang and McCay ('53), who reported, while this investigation was in progress, that terramycin residues in pork, if any, did not produce growth effects in rats.

Nitrogen balances were calculated for a 7-day interval in terms of 100 cm^2 of surface area by Lee's formula ('29), and subjected to analysis of covariance. Data relative to each diet in Lots 1 and 2 then were pooled. The mean daily intake of nitrogen by the 25 rats receiving no supplement was 582 mg, and the adjusted nitrogen balance 372 mg. For those receiving aureomycin, the intake was 611 mg and the adjusted balance 351; and for those receiving terramycin, the intake was 596 and the adjusted balance 357 mg. The adjusted nitrogen balance of rats that ingested pork from the basal ration was significantly higher at the 5% level than were those representing hogs that were fed either of the antibiotic substances. Whether or not this implies superiority of the basal ration is not clear. Although mean gains and body weights were the same in all groups during the balance period, it was observed that certain rats fed pork from hogs receiving antibiotics gained more rapidly during the balance period than during the preceding week, whereas all of those receiving the basal ration gained at the same rate during both weeks. Possibly irregularity in the growth response was reflected by changes in nitrogen balance but not in mean body weight.

The failure of aureomycin and terramycin hydrochloride incorporated in hog rations to stimulate growth and nitrogen retention of rats ingesting the pork was consistent with data obtained by analysis of certain tissues from one lot of hogs. Samples of spleen, liver, and psoas major tissue, analyzed by Charles Pfizer Co., Terre Haute, Ind., did not indicate any positive values for terramycin; nor were significant amounts of aureomycin found in these tissues by Lederle

Laboratories, American Cyanamid Co., Pearl River, New York.

The responses of rats that ingested rations containing pork were compared with those of rats that consumed the same quantity of nitrogen from beef (Clark, Hooper and McCord, '55). The amino acid composition of pork closely resembles that of beef (Lyman and Kuiken, '49; Schweigert, Bennett, McBride and Guthneck, '52). Since the sulfur-containing amino acids may be present in inadequate amounts when the concentration of beef or pork in the ration is low, the content of methionine and cystine in the samples of beef and pork tested in these investigations was determined microbiologically by the Wisconsin Alumni Research Foundation. On the basis of ash-fat-moisture-free material, pork contained 23.1 mg and beef 23.5 mg of methionine per gram, whereas pork was slightly lower in cystine (6.9 vs 8.8 mg) than beef. Poling, Schultz and Robinson ('44) and Hoagland, Ellis, Hankins and Snider ('47) reported that these two sources of protein were equivalent for promotion of rat growth. Similarly, in the present investigation, gains in weight of rats fed the same quantity of nitrogen from beef and pork were almost identical, and nitrogen balances were similar.

SUMMARY

The nutritive value of proteins of pork was studied when hogs were fed (1) a basal ration adequate for growth; (2) the basal ration supplemented with aureomycin hydrochloride; or (3) the basal ration supplemented with terramycin hydrochloride. Growth and nitrogen balance of weanling rats that were offered 1.6% of nitrogen served as criteria. Inclusion of 10 mg of aureomycin or terramycin per pound of ration fed to hogs did not stimulate the growth of rats, but nitrogen balance, expressed in terms of surface area, was significantly higher ($P < 0.05$) when pork representing the basal ration was fed than when meat from hogs receiving either of the antibiotics was offered. The nutritive value of pork proteins was similar to that of beef for the growing rat.

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THE ABSORPTION OF CYANOCOBALAMIN [1] (VITAMIN B_{12}) FROM THE GASTRO-INTESTINAL TRACT OF DOGS [2,3]

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FOUR FIGURES

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The absorption of cyanocobalamin (vitamin B_{12}) from the intestinal tract of man and other animals has been studied by a variety of indirect methods. In man, absorption of the vitamin has been based on the hematopoietic response of patients with pernicious anemia following oral administration of the vitamin (Ungley, '51–'52), and the urine and fecal excretion of radioactive Co^{60} following ingestion of Co^{60}-labeled cyanocobalamin (Heinle et al., '52; Schilling, '53). In animals the presence of Co^{60} in various body tissues and fluids following oral administration of radioactive Co^{60}-labeled cyanocobalamin has been used as a measure of absorption (Barbee and Johnson, '51; Scheid et al., '51; Rosenblum et al.,

[1] Cyanocobalamin is used to designate crystalline vitamin B_{12} in this report. The term "vitamin B_{12} activity" denotes all substances representing such activity for *Lactobacillus leichmanii*. The major portion of this activity is presumed to be cyanocobalamin.

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[3] A preliminary report of this investigation has appeared in Fed. Proc., *12:* 428, 1953.

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'52). Yamamoto and collaborators ('51) attempted to measure plasma vitamin B_{12} following oral administration and absorption of the vitamin in dogs. They found demonstrable increases of the vitamin in the plasma of dogs given 900 μg of vitamin B_{12} per kilogram of body weight but not in dogs given 150 μg. The method of vitamin B_{12} analysis used by these investigators was apparently not suitable to measurements of relatively small changes in vitamin B_{12} activity. However, the development of chick- and rat-growth assay procedures by feeding graded amounts of the vitamin is ample evidence that sufficient vitamin is absorbed to maintain and support growth (Richardson et al., '51; Lewis et al., '49).

Recently, sensitive microbiological procedures for the direct estimation of vitamin B_{12} activity in blood plasma were developed using *Euglena gracilis* var *bacillaris* (Ross, '52; Mollin and Ross, '52) and *Lactobacillus leichmanii* (Rosenthal and Sarett, '52) as the test organisms. By means of these procedures it has been possible to demonstrate measurable quantities of vitamin B_{12} activity in blood plasma or serum of man and other animals (Rosenthal and Brown, '54; Unglaub et al., '54). It has also been shown that the oral administration of 3000 μg of cyanocobalamin results in increased serum vitamin B_{12} activity in normal subjects and in patients with pernicious anemia (Unglaub et al., '54).

Since the body tissues may take up and store orally administered vitamin (Barbee and Johnson, '51; Scheid et al., '51; Rosenblum et al., '52), it was felt that measurements of the serum or plasma vitamin B_{12} activity in peripheral blood after oral dosage might represent only that portion of the absorbed vitamin remaining in the blood stream after passage through the liver. If the rate of absorption of vitamin B_{12} from the gastrointestinal tract was sufficiently large, serum vitamin B_{12} activity would be greater in portal than in peripheral blood. In the present study, measurements of vitamin B_{12} activity in portal and peripheral blood of dogs

following the administration of the vitamin were undertaken and the results are presented in this report.[6]

MATERIALS AND METHODS

Twenty-four adult mongrel dogs of both sexes, ranging in weight from 6 to 17.5 kg were used in this study. They were maintained in the kennels on a mixed commercial cereal-base diet supplemented with fresh meat. The dogs were fasted for 16 to 24 hours prior to initiating the experimental procedures. They were anaesthetized with intravenous injections of nembutal (30 mg per kilogram of body weight). Supplementary doses of nembutal were given during the experimental period to maintain light anaesthesia and 5 to 10 ml of saline were injected subcutaneously every 30 minutes to prevent dehydration.

After opening the abdomen by a lateral or ventral incision, the portal vein was cannulated by insertion of a polyethylene tube into the portal vein through a suitable mesenteric vein. The cannula, anchored in place with ligatures, was brought to the surface through the incision and was maintained free of clots by filling the tube with heparinized saline after each withdrawal of blood. At termination of the experiments, the dogs were sacrificed and the correct position of the cannula confirmed.

Solutions of crystalline cyanocobalamin or saline in 5- to 10-ml volumes were placed in the stomach or duodenum by means of a rubber tube passed through the esophagus. When solutions were placed in the stomach, a ligature just prior to the pyloric sphincter prevented the administered solution from entering the duodenum. In some experiments, the solutions were placed in 4-inch segments of duodenum between ligatures placed at the oral and aboral positions. In other

[6] After this work was begun, Ungley ('51–'52), in his review of the clinical aspects of vitamin B_{12}, mentioned that the portal blood of dogs contained greater amounts of vitamin B_{12} than did the peripheral circulation following intestinal administration. To our knowledge, however, a complete report of these experiments has not as yet been published.

experiments, solutions were placed in the unligated duodenum. Heparinized blood samples were obtained from the jugular and from the portal vein prior to and at intervals following the administration of cyanocobalamin or saline.

In experiments where urine was required, 4-hour collections were obtained by bladder catheterization (with the dogs under anaesthesia) prior to and following the administration of the vitamin. The collected urine was adjusted to pH 6.8 ± 0.1 and frozen until analyzed.

The heparinized blood plasma was analyzed for vitamin B_{12} activity by the procedure of Rosenthal and Sarett ('52) using *Lactobacillus leichmanii* 4797 as the test organism. Urine vitamin B_{12} activity was determined by the procedure of Thompson et al. ('50) as modified by Register and Sarett ('51). Urine creatinine analyses by the alkaline picrate procedure were performed to check completeness of the urine formation and collection.

RESULTS AND DISCUSSION

Vitamin B_{12} activity of jugular and portal blood plasma of fasted dogs

The vitamin B_{12} activity of jugular blood plasma from 12 dogs in the post-absorptive state ranged from 0.12 to 0.50 mμg per milliliter of plasma with an average of 0.24 mμg per milliliter. The vitamin B_{12} activity of portal blood plasma obtained from these dogs was essentially the same and ranged from 0.14 to 0.40 mμg per milliliter of plasma and averaged 0.25 mμg per milliliter. Standard deviations for jugular and portal plasma were 0.10 and 0.08 respectively.

Vitamin B_{12} activity of jugular blood plasma and urine following duodenal administration of cyanocobalamin

The absorption of cyanocobalamin from the duodenum under a variety of experimental procedures was studied by measuring the vitamin B_{12} activity of jugular blood plasma.

The data demonstrate that the administration of saline in the *ligated* or *unligated* duodenum has no effect on either the plasma vitamin B_{12} activity (fig. 1) or on the excretion of the vitamin in the urine (table 1) during the experimental period.

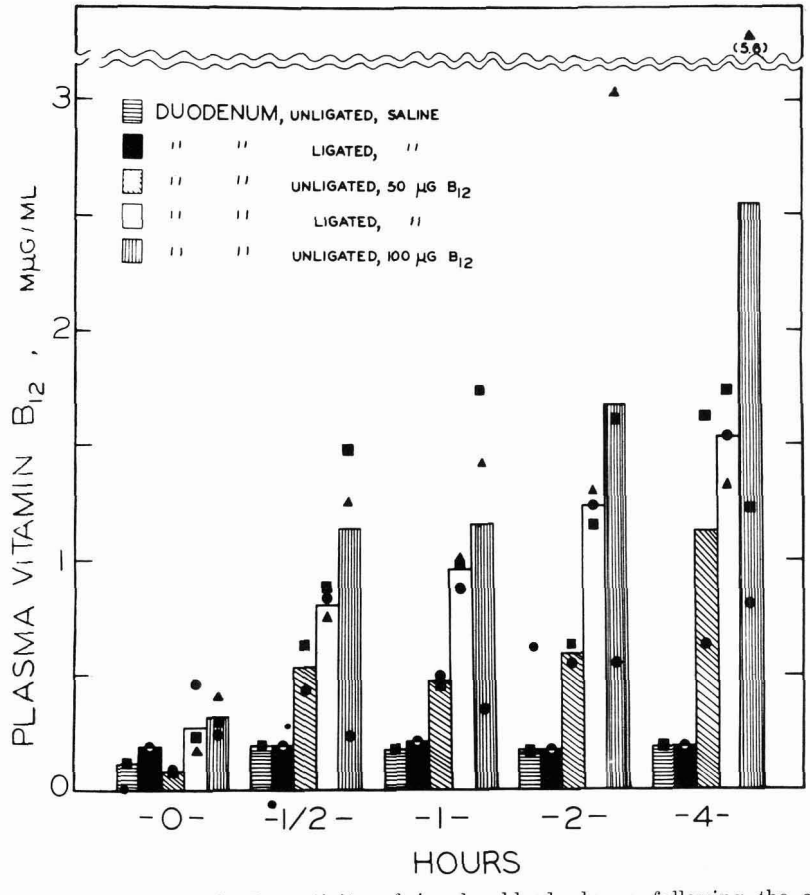

Fig. 1 The vitamin B_{12} activity of jugular blood plasma following the administration of cyanocobalamin or saline to dogs. The symbols represent values obtained on 10 animals.

However, the administration of 50 µg of cyanocobalamin per kilogram of body weight to three dogs with *ligated* duodenums and to two dogs with *unligated* duodenums results in increased plasma vitamin B_{12} activity. Similar increases in plasma vitamin avtivity were obtained in two dogs with *un-*

ligated duodenums given 100 μg of cyanocobalamin per kilogram of body weight. The plasma level of a third dog increased from the control value of 0.38 to 5.62 mμg/ml, a value far in excess of those of the other dogs. The urine vitamin activity was generally variable, but a definite increase of urine vitamin activity above that of the control period was apparent in 6 of 8 dogs given cyanocobalamin. Although the excretion of the vitamin in urine cannot be used as a measure

TABLE 1

Urinary vitamin B_{12} excretion following duodenal or intravenous administration of cyanocobalamin to 13 dogs. The values represent individual determination

ROUTE OF ADMINISTRATION	DOSAGE	URINE VITAMIN B_{12} ACTIVITY		PERCENTAGE OF DOSE EXCRETED
		Control	After B_{12}	
		mμg/4 hrs.		
Duodenum unligated	Saline	23	23	0.00
Duodenum unligated	50 μg/kg	32	498	0.12
Duodenum unligated	50 μg/kg	11	40	0.01
Duodenum unligated	100 μg/kg	15	43	0.01
Duodenum unligated	100 μg/kg	44	132	0.01
Duodenum unligated	100 μg/kg	32	6000	0.67
Duodenum ligated	Saline	21	18	0.00
Duodenum ligated	50 μg/kg	32	192	0.04
Duodenum ligated	50 μg/kg	22	2040	0.41
Duodenum ligated	50 μg/kg	22	2900	0.48
Intravenous	1.5 μg/kg	28	3270	25.6
Intravenous	1.5 μg/kg	10	1750	15.6
Intravenous	1.5 μg/kg	13	4020	23.3

of vitamin absorption from the intestinal tract, calculation indicates that less than 0.67% of the administered dose appeared in the urine during the experimental period.

The rate of absorption of the vitamin during this experiment, as measured by plasma vitamin B_{12} activity, is shown in figure 1. An increase in plasma vitamin B_{12} activity is apparent within one-half hour after dosage with the vitamin and the level continues to increase during the 4-hour experimental period. It may be noted that the average rate of absorption following the administration of 50 μg of cyanocobalamin per kilogram of body weight placed in the *ligated* duodenum is

slightly greater than the rate of absorption of a similar dose placed in the *unligated* duodenum. The data obtained in this experiment show that vitamin B_{12} placed in the intestinal tract of dogs is rapidly absorbed in measurable amounts.

The urine vitamin B_{12} activity is also greater for the *ligated* dogs as compared with that of *unligated* dogs given the same amount of vitamin (table 1). The elimination of part of the absorbed vitamin in the urine may be interpreted to mean that the rate of absorption is greater than the rate of binding of free vitamin B_{12} to blood and tissue proteins (Smith, '54) and the unbound free vitamin is probably excreted by the kidney.

Vitamin B_{12} activity of portal and jugular plasma during vitamin absorption from the intestinal tract

The vitamin B_{12} activity of the portal and jugular blood plasma of dogs following the administration of cyanocobalamin or saline under a variety of experimental conditions is shown in figure 2. In two dogs with cyanocobalamin placed in the stomach (50 μg per kilogram of body weight), the activity of the portal and jugular plasma of one dog increased slightly during the first hour, but returned to control values for the succeeding 7 hours of the experiment (fig. 2C). It may be noted that the difference in vitamin B_{12} activity between the portal and peripheral plasma for the half hour and one hour samples are within possible experimental error and are of doubtful significance. The vitamin B_{12} activity of the portal and peripheral plasma for the second dog remained constant. It is apparent, therefore, that vitamin B_{12} is not absorbed from the stomach in amounts measurable by our technique.

In two dogs given duodenal administration of saline, one dog with the duodenum *ligated* and one dog with the duodenum *unligated*, the vitamin B_{12} activity of the portal and peripheral blood plasma remained essentially constant dur-

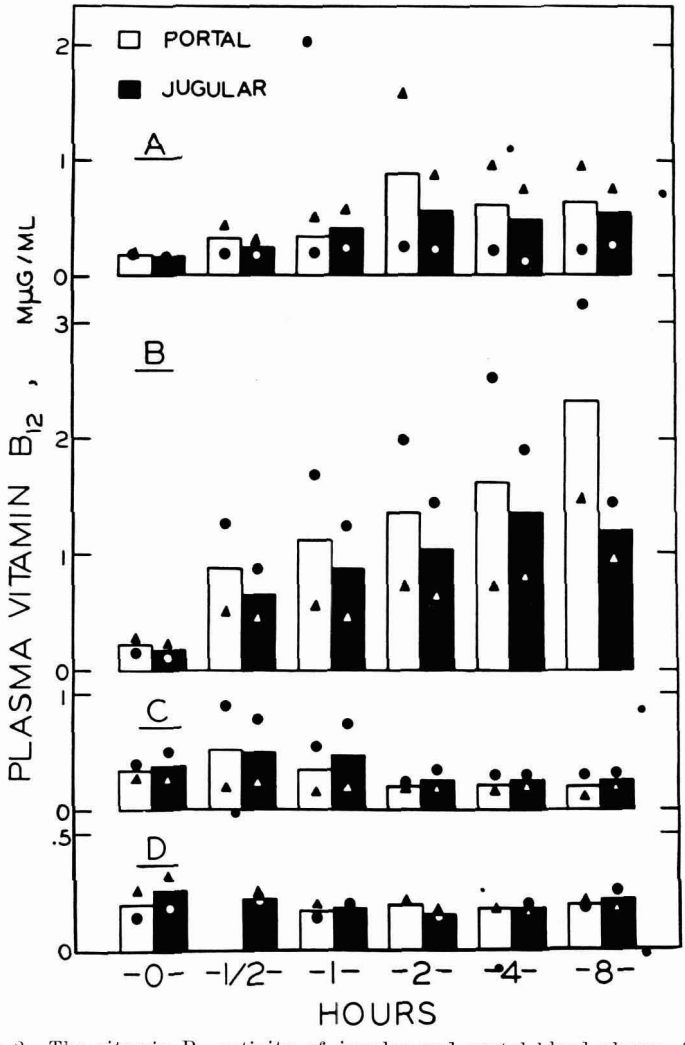

Fig. 2 The vitamin B_{12} activity of jugular and portal blood plasma following the administration of cyanocobalamin or saline to dogs. The symbols represent values obtained on individual animals.

A. Cyanocobalamin, 10 μg per kilogram of body weight placed in ligated duodenum of two dogs.

B. Cyanocobalamin, 50 μg per kilogram of body weight placed in unligated duodenum of two dogs.

C. Cyanocobalamin, 50 μg per kilogram of body weight placed in stomach ligated at the duodenal cap of two dogs.

D. Saline placed in unligated duodenum of one dog (○), and in ligated duodenum of another dog (△).

ing the 8-hour experimental period and averaged 0.18 and 0.21 mμg per milliliter of plasma respectively (fig. 2 D).

The administration of cyanocobalamin (50 μg per kilogram of body weight) in the *unligated* duodenum of two dogs resulted in a somewhat variable response (fig. 2 B). In one dog, the vitamin B_{12} activity of the portal plasma increased rapidly and continuously during the experiment and reached a maximum of 3.3 mμg/ml at the end of 8 hours. The vitamin B_{12} activity of the peripheral plasma increased in a similar fashion but at all times remained below the value attained in the portal blood. At the end of the experiment, the activity of the portal blood was still increasing while the peripheral blood was decreasing after reaching a maximum of 1.9 mμg/ml at the end of 4 hours. The response of the second dog was of a lower magnitude than that of the first dog. In this dog also, the peripheral plasma contained less vitamin B_{12} activity than portal plasma.

Of two dogs given 10 μg of cyanocobalamin per kilogram of body weight placed in the *ligated* duodenum, one dog failed to demonstrate changes in serum vitamin B_{12} activity while in the second dog, the plasma vitamin B_{12} activity did not increase until after the first hour and thereafter, increased to only a slight extent (fig. 2 A). However, in the latter dog, the portal plasma contained more vitamin B_{12} activity than the corresponding jugular plasma. In this dog, the degree of vitamin absorption (as indicated by the appearance of the vitamin in the plasma) from a limited portion of the duodenum was comparable to that of the smallest degree of absorption found in a dog given 50 μg of cyanocobalamin per kilogram of body weight in the *unligated* duodenum (fig. 2 B).

The administration of cyanocobalamin in a dose of 50 μg of cyanocobalamin per kilogram of body weight to three dogs with *ligated* duodenums is shown in figure 3. In all three dogs a rapid increase in the vitamin B_{12} activity of portal and jugular blood plasma was apparent during the experiment. The average vitamin B_{12} activity of the portal circulation was greater than the corresponding activity in the peripheral

circulation. It may be noted that the plasma vitamin B_{12} activity increased more rapidly and attained higher levels of activity than it did in the plasma of dogs given a similar dose of cyanocobalamin placed in the *unligated* duodenum (fig. 2 B).

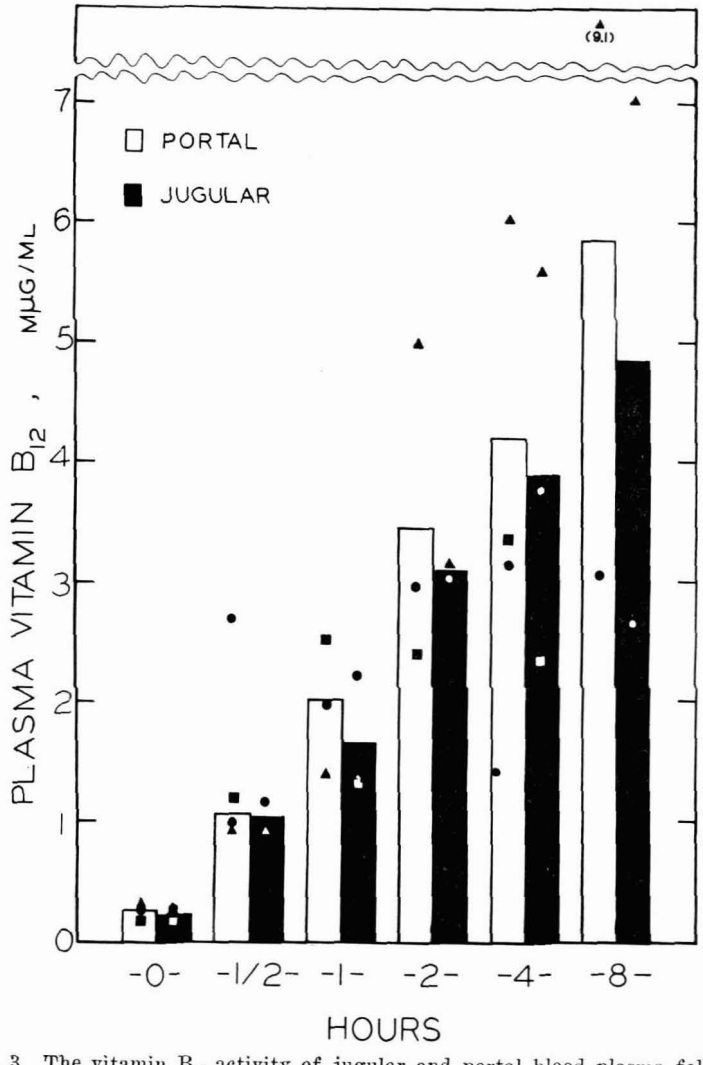

Fig. 3 The vitamin B_{12} activity of jugular and portal blood plasma following the administration of 50 μg cyanocobalamin per kilogram of body weight to three dogs with ligated duodenums.

The higher level of vitamin activity present in portal plasma during the absorption of vitamin from the intestinal tract indicates the removal of absorbed vitamin from the peripheral circulation by the liver and tissues of the body. This is consistent with previous studies in which Co^{60} was found to be deposited in the organs of rats following oral administration of Co^{60}-labeled cyanocobalamin (Rosenblum et al., '52). However, since the vitamin B_{12} activity of peripheral and portal blood plasma follows a similar pattern, the vitamin B_{12} activity of peripheral blood plasma may be used as an index of absorption of the vitamin from the gastrointestinal tract.

Vitamin B_{12} activity of blood plasma and urine following intravenous administration of cyanocobalamin

The elevation of urine vitamin B_{12} activity following duodenal administration of the vitamin prompted us to study the plasma and urine vitamin B_{12} activity after the intravenous administration of cyanocobalamin in doses that would result in plasma vitamin activity approximating that obtained by the intestinal route. Three dogs were injected with 1.5 μg of cyanocobalamin per kilogram of body weight and samples of blood and urine were obtained during a 4-hour experimental period. The rapid decrease in plasma vitamin activity (fig. 4) is due to elimination of free vitamin B_{12} by the kidney before binding to blood proteins and incorporation into tissues can take place, as has been indicated previously (Smith, '54). When such relatively small amounts of cyanocobalamin are injected, the major portion of the dose is removed from the blood during the first hour, and the rate of elimination or removal of vitamin from the blood decreases slowly thereafter. During the experimental 4-hour period, 15.6 to 25.6% of the administered dose was excreted in the urine (table 1).

As has been shown previously by Conley et al. ('51), Sokoloff et al. ('52) and Chow et al. ('50) in human subjects, and Yamamoto et al. ('51) in rats and dogs, large doses of cyano-

cobalamin injected into the body are rapidly eliminated in the urine. Concomitantly, plasma vitamin B_{12} activity decreases rapidly but small amounts of the vitamin above control levels may persist for at least 24 hours (Unblaub et al., '54). Our data obtained in dogs, substantiate these results. In the present experiments, small quantities of the vitamin were also eliminated in the urine in most of the dogs when

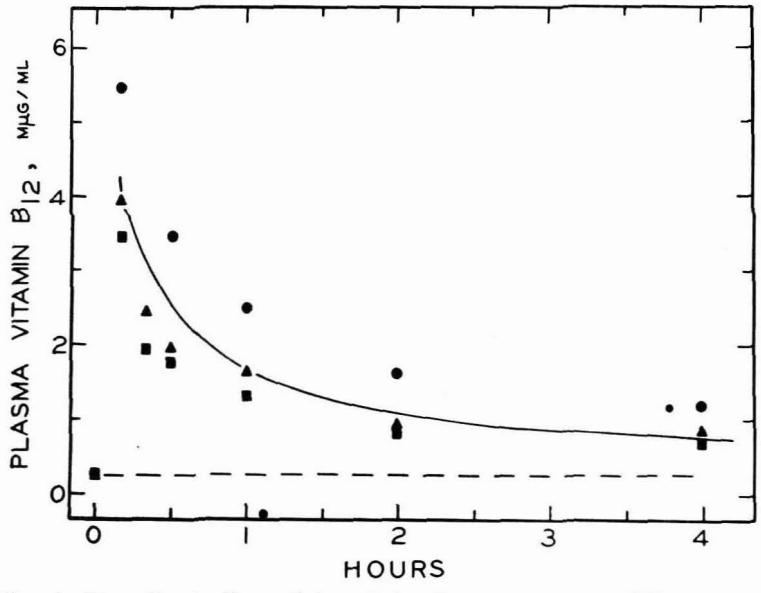

Fig. 4 The vitamin B_{12} activity of jugular blood plasma following the intravenous injection of 1.5 μg of cyanocobalamin per kilogram of body weight to three dogs. The solid line represents average data. The broken line represents the fasting control level of plasma vitamin B_{12} activity.

plasma vitamin B_{12} activity was elevated during absorption of the vitamin from the intestinal tract. Although it is not possible to correlate the quantity of vitamin excreted in the urine with the plasma vitamin activity due to the large variability and small number of observations presented in this report, these data indicate that some of the absorbed vitamin is excreted in the urine. The oral administration of cyanocobalamin to human subjects also results in a highly variable

but measurable elevated urinary excretion of the vitamin (Unglaub et al., '54). The apparent differences between these data and those previously reported in the literature which indicate that the vitamin is not excreted in the urine following oral administration may be due to differences in sensitivity of the various procedures used for the measurement of vitamin B_{12} activity.

The experimental data reported here constitute direct evidence that vitamin B_{12} administered to dogs is absorbed, in part, from the intestinal tract via the circulatory system in amounts which approach the concept of physiological quantities. These experiments demonstrate that vitamin B_{12} placed in the duodenum of dogs results in measurable increases in the vitamin activity of plasma, and an increase in the excretion of the vitamin in the urine. Although the absorption of cyanocobalamin varies widely among these dogs, it is apparent that the administration of 50 μg per kilogram of body weight to dogs with ligated or unligated duodenums results in increased vitamin activity in the blood stream. When the vitamin is localized in a portion of ligated duodenum, doses as low as 10 μg per kilogram of body weight may also increase plasma vitamin activity. The data obtained in dogs shows a greater rate of absorption of the vitamin when maintained in a limited portion of the intestine and may represent a mass action effect on the absorptive system. Glass et al., ('54) have recently proposed a mechanism for the absorption of vitamin B_{12} which involves a regulatory vitamin binding absorptive system similar to that of apoferritin in iron absorption, and that the B_{12} transferase system may be overcome with l rge doses of vitamin B_{12} by a mass action effect.

The procedures used to obtain these data may be criticized on the grounds that the operative ligating procedure could cause traumatic changes which would alter the intestinal absorption of the vitamin. Although trauma may effect such absorption late in the experimental period, the rapid and regular increase in plasma vitamin B_{12} activity during the first two hours indicate that traumatic changes were at a

minimum and probably played only a minor role during the early phases of the experiment. Furthermore, in those experiments where the vitamin was placed in the unligated duodenum, measurable increases in plasma activity were obtained under conditions where trauma was at a minimum.

SUMMARY

In the post-absorptive state, plasma vitamin B_{12} activity of dogs is the same in portal and peripheral blood plasma, but portal plasma contains greater amounts of activity than does peripheral plasma during active absorption of the vitamin from the intestinal tract.

Crystalline vitamin B_{12} placed in ligated segments of the duodenum or in the unligated duodenum of dogs results in the rapid appearance of vitamin B_{12} activity in the plasma of portal and peripheral blood. During absorption, small but definite amounts of vitamin activity also appear in the urine. In dogs subjected to operation but administered saline in place of vitamin B_{12}, plasma and urine activity remained constant. The plasma vitamin B_{12} activity of dogs with stomachs ligated at the duodenal cap remained essentially constant following the administration of vitamin.

The major portion of vitamin B_{12} administered by intravenous injection, in an amount approximately that found in plasma during oral absorption, results in a rapid elimination of the vitamin in the urine. These data are discussed in relation to the problem of vitamin B_{12} absorption in man and animals.

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THE DIGESTION OF PROTEINS IN VIVO[1]

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Many of the details of protein digestion *in vivo* are inferred from the results of isolated enzyme experiments *in vitro*. Few quantitative determinations have been made of free amino acids in contents of the alimentary tract removed during digestion, and most of these antedate the development of microbiological methods. Abderhalden et al. ('07) established by chemical means the presence of several amino acids in the combined chymes of large groups of animals. These pioneer researches established the fact that certain amino acids are present in the lumen of the intestine after feeding a protein meal. The evidence does not prove, however, that the amino acids found were derived solely from the ingested protein. Certain evidence on this point is described in the present paper. Quantitative estimations of 15 amino acids were made on gastric and intestinal contents after feeding egg albumin, zein, and non-protein test meals.

METHODS

Mongrel dogs, weighing 6.8 to 18.3 kg, were fasted at least 24 hours before being fed the test meals. Coagulated egg albumin (15.6% N) was prepared from "crystalline" egg albumin according to Chibnall et al. ('43); zein flakes (15.5% N) were prepared by dissolving powdered zein in 70% ethanol and reprecipitating by the addition of water. "Pro-

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tein'' test meals (30 to 50 gm) usually consisted of 80% protein, with 10% sucrose and 10% lard added for palatability. Non-protein test meals contained 45 or 40 gm of lard and 5 or 10 gm of sucrose. Water was always available. The animals were sacrificed approximately one and one-half hours after feeding. The abdomen was opened and the gastrointestinal tract chilled with ice during the time that double ligatures were placed at cardia, pylorus, ligament of Treitz, and at the upper quarter, midpoint and terminus of the remainder of the small intestine. For purposes of this paper these ligatures delimit stomach, duodenum, jejunum, upper, and lower ileum, respectively. The contents of each segment were removed promptly and completely without contamination with blood.

All samples of chyme were weighed and the semisolid ones diluted with water to form a slurry. They were then boiled for two minutes and evaporated to syrupy consistency on a steam bath. While still hot the samples were treated with 20 to 30 volumes of 70% ethanol. The flocculent precipitate which formed was allowed to settle in the cold room (5 to 8°C.) for approximately 18 hours and then separated from the supernatant liquid by centrifugation. The precipitate was washed once with 70% ethanol, the alcoholic extract and washings were combined, made to volume with 70% ethanol, and stored at 5 to 8°C. Since zein is soluble in 70% ethanol, only one of the two zein chymes was treated according to the scheme described above; the other was treated with water, instead of 70% ethanol, shaken with toluene and preserved at — 5°C. In either case it seems likely that the free amino acids were extracted.

Total nitrogen in each alcoholic extract was determined by means of a semi-micro Kjeldahl method. Alpha amino nitrogen content of each alcoholic extract and its acid hydrolyzate was determined manometrically (Peters and Van Slyke, '46). A prerequisite in this determination is the complete removal of alcohol, which was accomplished by evaporation in

vacuo at room temperature. The residue was dissolved in water for the determination of amino nitrogen.

Fifteen "free" amino acids were determined microbiologically, in the 70% ethanol-soluble fraction, essentially by the methods of Sauberlich and Baumann ('46). Leucine, valine, isoleucine, tryptophan and glutamic acid were determined with the aid of *Lactobacillus arabinosus* 17–5 (ATCC 8014). *Leuconostoc mesenteroides* P-60 (ATCC 8042), was used for the other amino acids, excepting threonine which was assayed with *Streptococcus fecalis* R (ATCC 8043). The total volume in each assay tube was 4.0 ml. After an incubation period of 64 to 72 hours the acid produced was titrated with 0.05 N NaOH. All standard curves, except those for serine and threonine were established with crystalline L-amino acids. In using DL-serine and DL-threonine the necessary assumption was made that the test organism is unable to utilize the D-forms.[2] From 90 to 103% of valine and 96 to 103% of lysine were recovered when added to the alcoholic extracts over the whole range of concentrations and dilutions encountered in this work. Duplicate determinations agreed within 10% of the mean value.

In two animals, several months prior to these experiments, pancreatic juice was eliminated from the small intestine by ligation of ducts and removal of the head of the pancreas.

RESULTS

The nitrogen in different portions of the alimentary tract, as affected by various test meals, is shown in table 1. As indicated, large portions of the protein test meals remained in the stomach. In the small intestine the total nitrogen recovered is greater after feeding protein test meals but even with a non-protein test meal considerable nitrogen is found in the contents. Zein is soluble in aqueous ethanol and hence the column marked A (alcohol) may include some undigested zein. The corresponding water-soluble fraction is included

[2] All amino acids were purchased from Nutritional Biochemicals Company, Cleveland, Ohio.

TABLE 1

Nitrogen in gastrointestinal contents

TEST MEAL	SEGMENT OF GI TRACT	NO. OF DOGS	NITROGEN			
			Total (a)	Soluble in 70% ethanol (b)	(b)/(a) × 100 (c)[1]	
			gm	*gm*		
Egg albumin (7.4; 4.7; 3.3 gm N)[2]	Stomach	3	3.087 ± 1.199[3]	0.267 ± 0.115	9	
	Duodenum	3	0.184 ± 0.017	0.080 ± 0.024	44	
	Jejunum	3	0.391 ± 0.025	0.189 ± 0.004	48	
	Upper ileum	3	0.396 ± 0.022	0.223 ± 0.053	56	
	Lower ileum	2	0.336 ± 0.040	0.186 ± 0.062	55	
Non-protein	Stomach	4	0.071 ± 0.027	0.041 ± 0.015	58	
	Duodenum	4	0.068 ± 0.014	0.020 ± 0.004	29	
	Jejunum	4	0.090 ± 0.030	0.033 ± 0.009	37	
	Upper ileum	4	0.109 ± 0.036	0.040 ± 0.009	37	
	Lower ileum	4	0.186 ± 0.063	0.068 ± 0.034	37	
				A[3] W	A	W
Zein (2.9; 3.3 gm N)	Stomach	2	1.234 ± 0.032	.296 .021	24	2
	Duodenum	2	0.184 ± 0.113	.191 .029	100 +	16
	Jejunum	2	0.408 ± 0.127	.297 .080	73	20
	Upper ileum	2	0.459 ± 0.030	.240 .071	52	16
	Lower ileum	2	0.369 ± 0.319	.027 .068	7	19
Effect of absence of pancreatic juice						
Egg albumin (4.7; 4.7 gm N)	Stomach	2	3.274 ± 0.030	0.224 ± 0.020	7	
	Duodenum	2	0.665 ± 0.015	0.009 ± 0.000	1	
	Jejunum	2	0.146 ± 0.051	0.014 ± 0.003	10	
	Upper ileum	2	0.236 ± 0.114	0.027 ± 0.018	11	
	Lower ileum	2	0.187 ± 0.096	0.030 ± 0.012	16	

[1] Soluble in 70% ethanol as percentage of total nitrogen.

[2] Grams nitrogen in test meals.

[3] Mean ± standard error of the mean.

[4] A refers to material soluble in 70% ethanol.
W refers to material soluble in water.

TABLE 2

Microbiologically available amino acids present in gastrointestinal contents

TEST MEAL	PART OF GI TRACT	LYSINE	LEUCINE	METHIONINE	THREONINE	HISTIDINE	VALINE	TRYPTOPHAN	PHENYL-ALANINE
		mg	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
Egg albumin (3) [3]	St.[1]	3.3 ± 1.5[2]	71.3 ± 33.3	9.0 ± 3.5	24.0 ± 10.8	1.5 ± 0.8	41.3 ± 19.8	4.3 ± 1.9	12.0 ± 3.5
	Du.	15.3 ± 1.1	28.7 ± 7.2	5.7 ± 1.7	16.7 ± 4.1	8.3 ± 2.3	19.3 ± 5.4	4.0 ± 0.7	15.0 ± 2.6
	Je.	41.7 ± 4.7	68.0 ± 16.0	11.7 ± 2.2	42.7 ± 13.4	15.3 ± 2.6	46.7 ± 16.7	11.0 ± 2.1	33.0 ± 6.4
	UI.	58.0 ± 9.1	98.0 ± 13.9	14.7 ± 2.6	47.3 ± 12.0	20.0 ± 4.1	61.3 ± 19.9	13.7 ± 2.9	43.3 ± 9.1
	LI.	48.0 ± 20.0	68.0 ± 11.0	16.5 ± 3.5	42.5 ± 20.5	18.0 ± 5.0	57.0 ± 23.0	15.5 ± 4.5	42.0 ± 13.0
Non-protein (4)	St.	4.5 ± 3.5	7.8 ± 5.5	1.4 ± 0.8	3.4 ± 1.6	1.6 ± 1.1	5.4 ± 3.4	2.2 ± 1.9	2.9 ± 2.4
	Du.	3.5 ± 1.5	4.3 ± 1.6	1.3 ± 0.3	3.0 ± 0.7	1.1 ± 0.3	2.3 ± 0.6	0.8 ± 0.1	2.3 ± 0.9
	Je.	7.5 ± 2.7	7.0 ± 2.0	1.4 ± 0.6	6.8 ± 3.2	2.8 ± 0.7	6.5 ± 2.4	1.8 ± 0.5	5.3 ± 1.9
	UI.	9.3 ± 3.7	13.0 ± 4.6	2.0 ± 0.7	6.8 ± 3.2	3.5 ± 0.9	6.8 ± 1.9	2.0 ± 0.4	6.3 ± 1.7
	LI.	26.5 ± 9.0	33.5 ± 13.2	6.5 ± 2.5	25.3 ± 9.8	9.0 ± 3.0	23.8 ± 9.5	5.8 ± 2.2	17.0 ± 6.1
Zein (2)	St.	2.5 ± 1.5	55.5 ± 50.5	1.2 ± 0.9	14.5 ± 13.5	1.0 ± 0	9.5 ± 7.5	0.6 ± 0.4	4.5 ± 3.5
	Du.	15.5 ± 10.5	27.5 ± 19.5	6.5 ± 4.5	14.5 ± 9.5	5.5 ± 3.5	12.0 ± 8.0	2.5 ± 1.5	9.5 ± 6.5
	Je.	38.5 ± 17.5	71.0 ± 37.0	13.0 ± 6.0	38.0 ± 22.0	15.5 ± 8.5	31.5 ± 13.5	6.5 ± 3.5	25.5 ± 12.5
	UI.	25.5 ± 8.5	45.0 ± 16.0	9.5 ± 3.5	23.5 ± 4.5	9.5 ± 3.5	17.5 ± 4.5	4.5 ± 1.5	17.0 ± 6.0
	LI.	9.0 ± 6.0	17.0 ± 14.0	2.5 ± 1.5	7.0 ± 6.0	3.0 ± 1.5	7.5 ± 5.5	1.2 ± 0.8	5.5 ± 3.5

[1] St = Stomach; Du = Duodenum; Je = Jejunum; UI = Upper ileum; LI = Lower ileum.

[2] Mean ± standard error of the mean.

[3] Numbers in parentheses indicate number of experiments done with each type of test meal.

under W. In the absence of pancreatic juice the 70% ethanol-soluble fraction is reduced by more than 80%.

Quantitative determinations were made of 15 amino acids; the results in table 2 include only those for 8 of the essential ones but they are typical of the whole group. All the amino acids sought were found in chymes from all parts of the gastrointestinal tract investigated, including chymes recovered after feeding non-protein test meals. As expected, the stomach contained the lowest concentration of amino acids. Their relative abundance in the stomach does not correspond well with that found in the duodenum and hence the mixture found in the stomach is probably not due primarily to regurgitation of intestinal contents. The egg albumin and non-protein test meals yielded chymes in the small intestine which contained somewhat larger amounts of free amino acids in the lower than in the upper portions. This was not found after feeding zein. Of great interest is the fact that, regardless of the test meal, whether it contained a complete protein, an incomplete protein, or no protein, all of the 15 amino acids sought were found. For example, histidine and lysine, which are absent, or nearly so, from zein, were present in all segments of the gastrointestinal tract whether egg albumin or zein was fed.

The pH of most samples was determined with a glass electrode. All specimens from the stomach, duodenum and jejunum were acid, and only 5 of 21 samples from the ileum were neutral or slightly alkaline. Of 11 samples of stomach contents, 8 ranged from pH 3.1 to pH 3.7 and three from pH 5.9 to pH 6.5. Two of the latter group were obtained with non-protein meals and the other with a meal which contained only 22% of protein. Twenty-two samples of duodenal and jejunal chymes ranged from pH 5.7 to pH 6.7.

DISCUSSION

The total nitrogen recovered after feeding a non-protein meal increases gradually from the duodenum to the lower ileum which may indicate an accumulation of the digestive

enzymes and other proteins contributed by the gastric, pancreatic, and intestinal juices. Auto-digestion of the enzymes and other proteins secreted into the small bowel could account for the presence of a complete assortment of essential and other amino acids after ingestion of a non-protein meal (table 2).

The intestinal chyme derived from a particular test meal contains a relatively constant percentage of nitrogenous substances soluble in 70% ethanol (table 1, column C). These substances include amino acids as well as the simple peptides. It is pertinent to point out here that this hydrolytic activity seems inconsistent with the classical concept of the optimum pH for the various digestive enzymes, according to which the pH observed here is too high for pepsin and too low for either the pancreatic or the intestinal enzymes. The qualitative uniformity of the microbiologically available amino acid mixture in the chyme, regardless of the test meal, is shown by the data in table 3, in which the molar ratios, on a comparable basis, are computed for test meal proteins as well as gastrointestinal contents. Owing to space limitations, results from duodenum, and upper and lower ileum were deleted. The 15 amino acids listed are the ones which were determined quantitatively by microbiological methods. The molar ratios are all based on the molar concentration of threonine as unity. Threonine was selected for this purpose because in the three types of chyme it occupies an intermediate position (table 2), and it is present in more than minimum amounts in both egg albumin and zein. The ratios for the test meal proteins were derived from analytical data reported by Block and Bolling ('51).

Lysine is particularly interesting because it is absent from zein but it is found in the zein-derived chyme in approximately the same ratio as in the other two chymes. Tryptophan is 10 times as concentrated in egg albumin as in zein but, except in the stomach, this difference cannot be detected in the chymes derived from feeding these different proteins. Zein contains relatively much more leucine and glutamic acid than egg

albumin but these differences are scarcely detectable beyond the stomach. The most astonishing results were obtained with the non-protein test meal. If this column (NP in table 3) were not labeled, it would be difficult, except for stomach contents, to determine that these results were obtained from chymes derived from non-protein test meals.

TABLE 3

Moles of amino acid per mole of threonine

AMINO ACID	IN INGESTED PROTEIN		IN GASTROINTESTINAL CONTENTS AFTER FEEDING THREE TYPES OF TEST MEALS [1]					
			Stomach			Jejunum		
	EA	Z	EA	Z	NP	EA	Z	NP
Lysine	1.25	0.00	0.11	0.13	1.31	0.80	0.83	0.90
Leucine	2.03	7.17	2.71	3.37	2.06	1.45	1.69	0.93
Methionine	1.04	0.61	0.30	0.07	0.31	0.22	0.28	0.16
Threonine	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Histidine	0.44	0.44	0.05	0.05	0.35	0.27	0.31	0.32
Valine	1.55	1.02	1.76	0.66	1.59	1.11	0.84	0.97
Tryptophan	0.21	0.02	0.11	0.02	0.38	0.15	0.10	0.16
Phenylalanine	1.28	1.54	0.36	0.22	0.62	0.56	0.49	0.56
Isoleucine	1.62	2.21	1.55	0.85	0.55	1.08	1.03	0.73
Arginine	0.99	0.41	0.15	0.16	0.72	0.94	0.61	1.12
Tyrosine	0.66	1.14	0.12	0.13	0.77	0.40	0.37	0.46
Serine	2.29	2.91	1.08	0.47	0.97	1.54	1.30	1.30
Glutamic acid	3.08	7.17	1.65	2.25	1.69	1.17	1.47	1.58
Aspartic acid	1.92	1.67	0.07	0.85	0.55	0.40	0.60	0.28
Proline	2.00	3.59	0.06	0.21	0.62	0.56	0.71	0.67

[1] Containing EA = Egg albumin, Z = Zein, or NP = No protein.

The simultaneous presence of the essential amino acids in a mixture is a prerequisite for the most efficient utilization of the nitrogen in such a mixture (Geiger, '47). The data presented here demonstrate that the amino acid mixture available for absorption in the small intestine tends to retain a relatively constant composition regardless of the composition of the ingested food. This circumstance provides a mixture for absorption into the blood stream that contains all of the essential amino acids and one which can, therefore, be used to advantage by the liver in the synthesis of new pro-

tein. The biological value of zein is not zero and this may be due to supplementation by the complete amino acid mixture provided by the intestine itself. Such advantage may be gained only at the expense of essential amino acids already present, presumably in proteins of the digestive juices, and must, therefore, be short lived. It is impossible in the long run to obviate the necessity for ingesting a full quota of the essential amino acids required for nitrogen equilibrium.

The relatively constant qualitative composition of the amino acid mixture found in the gastrointestinal tract during digestion suggests an unusual extension of the principle of homeostasis. It will be most interesting to determine whether an amino acid mixture, suggested by the present experiments, is superior nutritionally to other mixtures when fed to experimental animals. The essential amino acid requirements for the maintenance of nitrogen equilibrium in the adult rat have been reported over a period of years from this laboratory. In view of the results given in the present paper it may not be coincidence that the requirements of isoleucine (Anderson and Nasset, '48), valine (Nasset and Anderson, '50), phenylalanine (Nasset and Siliciano, '52), tryptophan (Nasset and Ely, '53) and histidine (Nasset and Gatewood, '54), expressed as moles of amino acid per mole of threonine required, yield ratios which are within 20% of those given for the same amino acids in table 3, taking egg albumin chyme in the jejunum for the comparison (almost any other sample will do as well). The ratios obtained for leucine (Anderson and Nasset, '50), methionine (Nasset and Anderson, '51), lysine (Nasset and Ely, '52), however, are one-half, 7 times, and one-fifth as great respectively as the comparable values in table 3.

It is well to emphasize here that the amino acid determinations were made on portions of the gastrointestinal contents which were soluble in 70% ethanol. This solvent extracts free amino acids as well as certain peptides and some unhydrolyzed zein. Free amino acids were readily demonstrated by means of paper partition chromatography in all parts of the

small intestine. It was not always possible to demonstrate all of the amino acids in the free state in gastric contents. Without the removal of water the concentration of free amino acids in the stomach was often too low for detection by the chromatographic method. The microbiological assay for free amino acids can doubtless be influenced by other compounds but at least the chromatographic method indicates the presence of each of the amino acids in the free state.

How do chymes derived from totally different test meals yield so soon a solution of free amino acids which apparently is relatively constant in composition? There is no evidence that free amino acids are normally absorbed from the stomach. Hence in this organ, aside from duodenal regurgitation and possible secretion of amino acids, the free amino acid content is determined by the hydrolytic cleavage of the various amino acids from both test meal and endogenous (e.g. pepsin and mucus) proteins. In the small intestine the process of absorption brings in another complicating factor. If there were large differences in rates of absorption of the various amino acids, some of them should tend to disappear toward the ileum and others should tend to accumulate. If this were true, the ratios given in table 3 should not remain so constant.

The average total recovery of nitrogen from the gastrointestinal tract after feeding non-protein meals was 0.524 gm, the equivalent of over 3 gm of protein. How much of this was present before feeding cannot be determined but the protein turnover of the gastrointestinal mucosa must be appreciable. Dreisbach and Nasset ('54) recovered more nitrogen from the gastrointestinal tract of rats 5 hours after feeding than was contained in the test meal. After such a large synthesis and secretion of enzyme protein the mucosa probably makes significant demands on the body's amino acid pool. This is confirmed in part by the work of Borsook et al. ('50) who reported that O^{14}-labeled glycine, L-histidine, L-leucine and L-lysine after intravenous administration are predominantly concentrated in the visceral proteins within 30 minutes.

SUMMARY

Dogs were fed protein-containing and non-protein test meals and sacrificed approximately one and one-half hours later. The contents of the gastrointestinal tract were analyzed for total nitrogen, 70% ethanol-soluble nitrogen, and 15 "free" amino acids.

In the small intestine all 15 amino acids were present in approximately constant proportions whether protein or non-protein test meals were fed. Lysine, for example, which is absent from zein, one of the test-meal proteins, was present in chyme derived from zein in approximately the same proportion as in chyme derived from egg albumin.

The mixture present during digestion appears to contain amino acids derived not only from the hydrolysis of the food proteins but also from autodigestion of the hydrolytic enzymes themselves and other endogenous proteins.

It is concluded, therefore, that the qualitative amino acid composition of intestinal contents is not greatly altered by changing from a non-protein to a protein-containing test meal.

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FATTY LIVER OF PORTAL TYPE: EFFECTS OF CHOLINE, METHIONINE, AND VITAMIN B_{12} [1,2]

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In previous reports (Shils et al., '54a, b) we have described a procedure for rapidly producing a portal type of fatty liver in rats. It consists of maintaining stock weanling rats on diets containing certain plant foodstuffs and devoid of animal protein. This experimental fatty liver resembles the portal type of fatty liver seen in human disease, kwashiorkor (Davies, '51; Sénécal et al., '53; World Health Organization, '53) and, in addition, differs morphologically from a central type of fatty liver seen in choline deficiency (Lillie et al., '42; Hartroft, '50; Shils et al., '54c). This latter type appears to be caused by a dietary deficiency of "methyl groups" and it has been thoroughly established that it responds not only to choline but also to methionine (reviewed by Best and Lucas, '43) and under certain circumstances to vitamin B_{12} (Best et al., '53; Hawk and Elvehjem, '53; Drill, '54). These compounds are believed at present to have little or no effect in kwashiorkor and related diseases (Trowell, '54; Waterlow, '48), although the clinical evidence is still scanty. It was, therefore, of interest to determine whether they would be effective in preventing or curing the portal type of fatty liver which we have produced in rats by feeding them diets containing corn, cassava, or rice as the only source of protein.

[1] Supported in part by a grant from the National Vitamin Foundation.

[2] Preliminary report of portions of this study were presented before the American Institute of Nutrition, Atlantic City, April 1954.

METHODS AND PROCEDURES

Black rats taken from mothers fed a diet of Rockland "D-free" pellets supplemented with fresh milk were placed on the experimental diets at weaning (21 days).

The three diets with protein contained corn meal[3] ground white rice,[4] or cassava flour,[5] at a level of 74 parts. To this proportion of each was added the following basal mixture: hydrogenated vegetable oil (Crisco) 15, salts (Hubbell, Mendel and Wakeman, '37) 3, 5% cod liver oil in corn oil 2, and vitaminized glucose 3. The latter contributed in milligrams: thiamine HCl 0.5, riboflavin 1, niacin 5, pyridoxine HCl 1, calcium pantothenate 2.5, folic acid 0.02, and biotin 0.01. A choline-deficient diet was also fed to some of the rats. This contained casein [6] 9, glucose 67, salts 1, plus the basal mixture given above. When choline chloride was incorporated into the diets it was always at a level of 0.25%; DL-methionine was added at the various levels given in the appropriate tables, and vitamin B_{12} was injected intraperitoneally once weekly (3 μg in 0.2 ml physiological saline).

Histological studies including Sudan IV staining for fat in the liver and determination of levels of hepatic lipid as total fatty acids and unsaponifiable matter were performed as previously described (Shils and Stewart, '54c). In addition, determinations of free and total cholesterol (Sperry and Webb, '50) and iodine number (Hanus reagent) (A.O.A.C., '50) were made on unhydrolysed lipid extracted from the diet, liver and perirenal (body) fat by a slightly modified method of Artom and Fishman ('43). For cholesterol determination an aliquot of the chloroform extract was evaporated at 37°C. then dissolved in a mixture of equal parts of acetone and absolute ethanol prior to analysis. Only littermate rats were used for

[3] Bolted, water ground, white cornmeal, "Indian Head" brand from Wilkins-Rogers Milling Co., Washington, D. C.

[4] Polished white uncoated rice ground finely in the laboratory.

[5] A flour supplied through the courtesy of Dr. R. J. Smit, Department of Nutrition, Pretoria, Union of South Africa.

[6] Plain, untreated, General Biochemicals, Inc.

intergroup comparison to obtain the mean difference for the "t" test.

RESULTS

As shown in table 1, neither vitamin B_{12} nor choline had any significant effect in preventing the accumulation of hepatic lipid when administered to rats on the corn meal diet. While preliminary data (Shils et al., '54a) had suggested some preventive action of choline, the additional experiments reported here did not sustain this view. Of considerable interest is the finding that, in male rats, 0.5% DL-methionine actually increased the amount of hepatic lipid, a finding which was statistically highly significant. The amino acid at this level, however, had no effect on the amount of lipid in the livers of females. There was no significant influence on growth by any of these three "lipotropic" substances in the amounts given in this experiment. Microscopic examination of liver sections stained with Sudan IV revealed that treatment with these substances did not alter the site of lipid deposition. The majority of the treated rats continued to have the fat localized in the portal areas. The increased amounts of fat noted chemically in the methionine-treated males is reflected in the microscopic findings of diffuse and heavy fat infiltration in many of the livers.

The unexpected finding that 0.5% methionine increased the hepatic fat in male rats suggested that other levels of this amino acid be tested. Accordingly, groups of male rats were placed on diets containing various amounts of methionine (table 2). At the 0.5% level there was observed again an increase in in the amounts of fat in the liver. However, higher percentages caused decreasing amounts of lipid accumulation, until, at the 2% level, all rats had normal levels of hepatic lipid. It is to be noted, however, that poorer growth was associated with the higher methionine levels and this may have been related to the lower lipid concentrations. While the growth inhibition at 1% methionine was equivocal, there is no doubt that 1.5 and 2% are toxic to rats on the corn meal diet.

TABLE 1

Influence of various lipotropes on concentration of hepatic lipid, its location in the lobule, and on the growth of rats on the corn meal diet (after 28 days)

	SUPPLEMENT											
	None		Vitamin B_{12}		None		0.25% choline		None		0.5% methionine	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
	NO. OF RATS											
	10	8	10	10	14	10	14	10	21	12	21	12
Liver lipid (%)[1]												
Average	9.5	8.0	9.0	8.4	10.7	11.4	8.7	10.7	9.4	10.9	14.6	11.9
± S.E.	0.92	1.11	1.36	1.24	1.45	1.95	1.70	1.91	0.80	1.64	0.99	1.75
Average wt. gain (gm)	9	12	9	9	9	15	12	18	10	10	9	8
Site of lipid:												
Portal			6	7			9	8	15	9	10	7
Central			0	0			0	0	0	0	0	0
Diffuse			1	1			2	1	4	2	11	3
None			2	0			3	1	2	1	0	2

[1] Statistical analysis of lipid data of paired littermates:

	SUPPLEMENT					
	None	Vitamin B_{12}	None	Choline	None	Methionine
	♂	♀	♂	♀	♂	♀
No. of littermate pairs	10	8	6	6	21	12
Mean lipid difference (%)	+ 1.66	0.0	+ 1.51	+ 0.05	— 5.02	— 1.08
Significance of mean diff., P =	0.28	1.0	0.16	> 0.56	< 0.001	0.56

The ability of 2% methionine to prevent accumulation of excess liver led us to examine its efficiency in reducing excess lipid once it had accumulated in the liver. In preparation for this experiment, 15 littermate rats were placed on the

TABLE 2

The influence of increasing levels of DL-*methionine in corn meal diet on liver lipid content and growth of male rats*[1]

	% DL-METHIONINE IN DIET				
	0	0.5	1.0	1.5	2.0
	NO. OF RATS				
	13	12	13	6	13
% Lipid, mean ± S.E.	9.5± 1.1	14.3± 1.3	6.8± 0.96	4.5±0.71	3.2±0.22
% Lipid, range	5.3 – 15.6	5.3 – 18.8	3.4 – 13.5	2.4 – 7.0	2.5 – 3.8
No. below 4.0%	0	0	2	3	12
Wt. gain, av., gm	10	9	7	3	0.6
Wt. gain, range, gm	3 – 20	5 – 14	1 – 9	−2 to +6	−2 to +4

[1] After 28 days on diets.

TABLE 3

Curative effect of 2% DL-*methionine added after 18 days on the corn meal diet*

DAYS ON DIET	NO. OF RATS	LIPID		GROWTH	
		Mean	Range	Mean	Range
		%	%	gm	gm
18	5	10.2	7.1–14.4	7	5–12
28	5	12.2	5.9–19.5	11	9–15
28 (methionine)[1]	5	3.4	2.5–3.9	−1	−4 to +1

[1] Methionine added only for the last 10 days.

corn meal diet. The fact that these rats had developed a fatty liver was shown by killing and analyzing a group of 5 on the 18th day (table 3). At this time, half of the rats which were continued on the diet were given a supplement of 2% methionine. Ten days later, those without this supplement had

elevated hepatic lipids, and had continued to gain weight, whereas the methionine was found to have cured the fatty liver. However, the amino acid also caused a marked decrease in growth or actual weight loss. Here, again, weight loss rather than a "true" lipotropic action may have been an important influence on the levels of hepatic lipid. Further experiments are needed to assess the relative importance of these factors.

Rice and cassava diets: The preventive influence of choline and methionine was also tested in conjunction with diets containing either polished rice or cassava flour. Table 4 summarizes the results of these experiments. Choline had no effect upon the level of hepatic lipids in the females on the rice diet. It may have decreased somewhat the fat in the animals on the cassava diet but more data are needed to assess the significance, if any, of this change. Methionine did not prevent accumulation of fat in animals on either of these two diets. Neither supplement influenced growth . The pattern of distribution of lipid in the lobules was not altered by treatment; the fat was initially and preponderantly localized in the portal areas. However, 0.5% methionine supplementation to the rice diet tended to cause so much fat deposition, particularly in the females, that the entire lobule frequently became diffusely involved, as had occurred in males on the corn meal diet supplemented with 0.5% methionine.

Chemical nature of liver lipids: The portal type of fatty liver, then, does not appear to respond significantly to the usual lipotropic agents, a striking contrast to their beneficial effect on the central type of fatty liver seen in choline deficiency. It was of interest to know whether there were, in addition, any chemical differences in the types of lipid deposited under the different dietary conditions. In another experiment, groups of rats were placed on each of the 4 diets shown in table 5. Histological examination of the livers stained with Sudan IV showed that the fat was deposited preponderantly as is shown in the table. Comparison was then made of the iodine number and cholesterol content of the lipids

TABLE 4

The influence of choline or DL-*methionine on liver lipids and growth of rats on rice and cassava diets*[1]

SUPPLEMENT	NO. AND SEX	RICE				NO. AND SEX	CASSAVA			
		Lipid		Wt. gain			Lipid		Wt. gain	
		Av.	Range	Av.	Range		Av.	Range	Av.	Range
		%	%	*gm*	*gm*		%	%	*gm*	*gm*
None	4 ♂	14.5	10.5–18.5	13	12–16	2 ♂ + 2 ♀	6.7	5.4–9.2	—7	—5 to —10
	10 ♀	14.8	6.0–20.2	12	10–15					
Choline Cl (0.25%)	..	...	...	..	...	2 ♂ + 2 ♀	5.3	3.9–7.5	—7	—5 to —8
	8 ♀	14.2	7.5–21.4	12	6–18					
Methionine (0.5%)	4 ♂	15.1	12.9–18.5	11	7–16	2 ♂ + 2 ♀	9.1	6.5–13.2	—6	—6 to —7
	6 ♀	19.7	17.6–23.6	9	2–13					

[1] Twenty-one days on diets.

TABLE 5

Comparison of average iodine numbers and cholesterol in total lipids extracted from rats on various diets[1]

DIET	CORN MEAL	CORN MEAL + CHOLINE	CHOLINE-DEFICIENT	CHOLINE-DEFICIENT + CHOLINE
Site of lipid in lobule	Portal	Portal	Central	Little or no fat
Total liver lipid (av., %)	15.7 (9)[2]	16.4 (16)	14.6 (8)	5.0 (4)
Liver wt. (av., gm)	2.60	2.62	3.67	2.25
Wt. gain (av., gm)	12	11	48	32
Iodine no. of:				
Liver lipid	91 (9)	89 (16)	86 (8)	73 (4)
Perirenal lipid	81 (3)	78 (8)	74 (4)	76 (3)
Dietary lipid	97	97	91	91
Total cholesterol in liver lipid (%)	1.86 (8)	1.85 (14)	1.96 (8)	3.67 (4)

[1] = After 28 days on diets.
[2] Number of animals in parentheses.

extracted from the livers and perirenal fat (as a sample of body fat) of animals on each diet. The lipids extracted from these two types of fatty livers had iodine numbers lying roughly about two-thirds of the way between those of the perirenal fat and the dietary fat. These results indicate that moderately unsaturated fat was deposited to about the same degree on both types of diets. This suggests that the excess hepatic lipid was derived, at least in part, from the dietary fat or newly synthesized moderately unsaturated fatty acids rather than from the depot fat alone. In contrast, the lipids from livers with a nearly normal fat content (last column, table 5) had an iodine number very close to that of perirenal fat.

Cholesterol comprised only a small percentage of the total liver lipid and the values were very similar in all the animals with fatty livers (table 5). The percentage of cholesterol in the lipids extracted from the livers of rats with normal levels of hepatic lipid was almost twice as high, suggesting that when excess fat is deposited, it contains a smaller proportion of cholesterol than is found in the lipid of the more normal liver. While the total cholesterol per gram of liver was increased in the fatty livers over that in normal livers, the increase was not proportional to the rise in total fat. It was also noted that with increasing amounts of liver fat the ratio of free to total cholesterol tended to decrease, so that instead of ratios of 0.45 to 0.55, there often occurred values of 0.20 to 0.35 when the lipid exceeded 20% of the wet weight.

DISCUSSION

The failure of choline, vitamin B_{12} and of methionine at 0.5% to cause a significant decrease in liver lipids in these experiments with certain plant foods, strongly suggests that a mechanism other than "methyl group" deficiency is responsible for this portal type of fatty liver. Others, also, have noticed types of fatty liver which did not respond to the usual lipotropic agents. For example, diets containing mixtures of purified amino acids deficient in lysine or threonine

caused fatty liver despite the presence of choline and vitamin B_{12} (Dick et al., '52, Singal et al., '53a,b). However, the fat appeared initially in the central portion of the lobule (Dick et al., '52). Singal et al. ('53a) found that on their amino acid-deficient diets the iodine number of hepatic fat decreased, whereas it increased in our experiments. Our findings with cholesterol are in general agreement with theirs. In addition, various investigators have produced a type of fatty liver by feeding diets containing 9% of casein but supplemented with choline and which did not respond to vitamin B_{12} (Litwack et al., '52; Singal et al., '53a; Harper et al., '53, '54a). The microscopic appearance of the fatty livers developed on the 9% casein diet with added choline has been described (Niño-Herrera, Harper and Elvehjem, '54). While they describe a "network-like distribution of fatty cells," it is not clear to us just where the fat is located in the lobules.[7]

The "antilipotropic" action of 0.5% methionine noted with males on the corn meal diet which we have described previously (Shils et al., '54a) has also been noted by Harper et al. ('54b) in young male rats subsisting on 9% casein diets containing choline and supplemented with 0.1 to 0.6% of DL-methionine. Methionine supplementation improved growth in their experiments, while it had no effect when added at the 0.5% level to our diets.

It is not clear from our experiments whether the preventive and curative effect of the high levels of methionine is a true lipotropic effect or is the result of an altered state of metabolism caused perhaps by an amino acid imbalance. Various workers have noted that methionine at 1.5 to 2% or higher levels is deleterious to growth, particularly on low-protein diets (Fishman and Artom, '44; Kade and Shepherd, '48). High levels of methionine appear to increase fat catabolism (Roth and Allison, '49, '50). Furthermore, weight loss *per se* may have been important in our experiment just

[7] In a personal communication to one of us (W.B.S.), Dr. A. E. Harper has indicated that more recent studies have revealed that the lipid in the livers of his rats on the 9% casein diet is apparent initially in the portal areas.

as it plays a role in reducing the fatty livers produced by choline deficiency (Best and Ridout, '38). Certainly, the ineffectiveness of choline and vitamin B_{12} would suggest an action for methionine, under our experimental conditions, other than that of a "methyl donor."

In studies with second generation rats which developed portal fatty liver on the corn meal diet supplemented with 3% casein, Shils and Stewart ('54c) found that choline was effective in preventing fatty liver, methionine (0.5%) partially effective and vitamin B_{12} very erratic in its effect. In addition to the differences in the history of the rats and the presence of casein, the experimental procedure differed from that reported here in that the animals were continued on the supplemented diet for 65 days rather than 28 days. These differences in results suggest the need for information on (a) the effect of small amounts of animal protein in response to methyl-donating lipotropic substances (b) the effect of length of supplementation and of age on the response and (c) the effect of previous dietary history of the animals on the response.

SUMMARY

Choline and vitamin B_{12} failed to prevent accumulation of excess hepatic lipid in the portal areas of rats fed diets containing either corn meal, cassava or rice as the only source of protein.

DL-methionine (0.5%) not only failed to prevent lipid accumulation in the livers, but in the instance of males on the corn meal diet had a tendency to increase the fat still further. Higher levels of methionine (1, 1.5 and 2%) added to the corn meal diet progressively reduced hepatic lipids, and at a 2% level, methionine caused normal hepatic lipid levels. At these higher levels, however, growth was correspondingly inhibited.

Determination of iodine number and cholesterol content revealed no significant differences between hepatic lipids deposited in the portal areas on corn diets or central areas on a choline-deficient diet with casein as the source of protein.

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BLOOD ERGOTHIONEINE AND DIETARY OATS [1]

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The biological origin and function of ergothioneine remain unknown. Evidence has accumulated that diet affects the level of this compound in the erythrocyte (Eagles and Vars, '28; Potter and Franke, '35; Hunter, '51; Spicer et al., '51; Baldridge and Lewis, '53) and other tissues (Melville et al., '54). It has been established that oats and cabbage constitute an ergothioneinogenic diet for the rabbit (Baldridge and Lewis, '53). The experiments described in this paper were planned to determine which of these two foodstuffs is responsible for this effect. Since it has been suggested (Latner and Mowbray, '49) and denied (Heath et al., '52) that ergothioneine might influence thyroid function, histologic studies were made of thyroid tissue from rats with various levels of blood ergothioneine.

EXPERIMENTAL

Male white rabbits and rats were fed selected diets, and blood ergothioneine determinations were performed following several weeks of dietary control. Ergothioneine concentrations were determined by a modification of the Hunter diazo procedure previously described (Baldridge and Lewis, '53). Blood was obtained periodically from ear veins from rabbits and at time of sacrifice from rats by cardiac puncture following

[1] A report of this work was presented at the meeting of the Federation of American Societies for Experimental Biology, Atlantic City, April, 1954 (Baldridge, '54).

nembutal anesthesia. Heparin was added as the anticoagulant. Packed red blood cells from 2 ml of blood were washed twice with 0.9% sodium chloride solution and made to 10 ml with dilute acetic acid prior to deproteinization (Hunter, '49). The results are expressed as milligrams of ergothioneine per 100 ml of whole blood. Aliquots of up to 1 ml of the protein-free filtrates were applied to paper sheets for ascending chromatography. The solvents and reagents were the same as those used previously (Baldridge and Lewis, '53).

In preliminary experiments one rabbit was fed fresh cabbage added to a purified (20% casein) diet (Wooley and Sebrell, '45), a second oats with the casein diet, and a third both of the natural foodstuffs plus the purified diet. The last rabbit ate little except the cabbage portion of the diet. Blood ergothioneine determinations were made before and after 10 weeks of these dietary regimes. The ergothioneine level in the blood of the rabbit fed cabbage with the casein diet decreased from 11.1 mg% to 5.0 mg% during this period. The level in the rabbit fed oats plus the casein diet increased from 4.6 mg% to 9.4 mg% in the same time. Little change was noted (7.0 mg% initially versus 8.4 mg% after 10 weeks) in the blood level of the rabbit fed cabbage plus oats and the casein diet. Ergothioneine concentrations were estimated from volumes of blood filtrates which were just sufficient to yield detectable spots on paper chromatograms. The chromatographic findings agreed with the results obtained by colorimetric analysis.

Seven groups of male white rats (weight about 200 gm) were also fed various dietary combinations including oats, fresh and dehydrated [2] cabbage, purified (casein) diet and rat chow. There were 4 rats each in groups M-1 through M-5. The animals in group M-1 were each fed 10 gm per day of a purified diet which consisted of casein 20; sucrose 10; cellulose 5[3]; cornstarch 56; salts (Hubbell et al., '37) 2; corn oil

[2] Obtained through Dr. Howard B. Lewis from the U. S. Army Medical Nutrition Laboratory, Chicago, Ill.

[3] Ruffex.

4; cod liver oil 2; vitamin mixture 1. The vitamin mixture contained thiamine hydrochloride 37 mg; riboflavin 75 mg; pyridoxine hydrochloride 37 mg; calcium pantothenate 300 mg; p-aminobenzoic acid 300 mg; niacin 375 mg; corn starch 99 gm. The rats in group M-2 were each fed 1 gm and those in group M-3, 2 gm of dehydrated cabbage plus 9 and 8 gm respectively of the purified (casein) diet per day. The rats of group M-4 were each fed 9 gm of the purified diet plus 15 gm of fresh cabbage daily. Those in group M-5 were given 15 gm of ground oats plus 20 gm of fresh cabbage per day. Two rats (group

TABLE 1

Diet and ergothioneine of rat blood

GROUP	NO. OF RATS	DIET [1]	ERGOTHIONEINE		CHROMATOGRAPHIC EVIDENCE
			Range	*Mean*	
			mg %	*mg %*	
M-1	4	Purified	0.2 — 0.4	0.3	—
M-2,3,4	12	Cabbage + casein	0.2 — 0.9	0.4 ± 0.2 [2]	—
M-5	4	Oats + cabbage	8.0 — 12.5	11.2 ± 1.6	+
M-6	2	Oats only	8.2 — 13.8		+
M-7	7	Chow	1.2 — 1.7	1.3 ± 0.2	—

[1] See text for complete description of diets fed.

[2] *Average deviation from the mean.*

M-6) were fed 20 gm of ground oats only, and a final group (M-7) of 6 rats was fed 15 gm of Rockland rat chow daily per rat. Animals from various groups were sacrificed between the 4th and 13th week of dietary control. The blood ergothioneine values are shown on table 1. From the data obtained, it is apparent that there is no relationship between ingestion of cabbage and blood ergothioneine levels. Elevated ergothioneine concentrations were observed only in the blood of rats fed oats as part or all of the diet.

Since ergothioneine was not detected previously (Baldridge and Lewis. '53) in the blood of rats fed oats as 50% of an other-

wise purified diet, the effect of various dietary levels of oats was investigated. Male white rats (weight about 100 gm) were fed 4 diets. The purified diet was fed to the animals in group T-1, (in addition 1 μg of vitamin B_{12} was administered intraperitoneally three times weekly to three of the rats in this group). The diet fed group T-2 was the same as that for group T-1 except that ground oats replaced the cellulose, one-half of the casein and part of the corn starch to make a total of 50% of the diet. The diet for group T-3 consisted of ground oats 75; casein 10; sucrose 8; salts (Hubbell et al., '37) 2; corn oil 2; cod liver oil 2; vitamin mixture 1. Group T-4 was fed only ground oats. There were 6 rats in

TABLE 2

Effect of dietary oats on ergothioneine of rat blood

GROUP	NO. OF RATS	OATS IN DIET	ERGOTHIONEINE		CHROMATOGRAPHIC EVIDENCE
			Range	Mean	
		%	*mg %*	*mg %*	
T-1	6	0	0.1 — 0.3	0.2	—
T-2	6	50	1.0 — 2.6	1.6 ± 0.6 [1]	±
T-3	6	75	2.0 — 8.4	4.4 ± 1.7	+
T-4	9	100	3.3 — 13.3	8.2 ± 2.2	+

[1] Average deviation from the mean.

each group except T-4 in which there were 9. Food was limited to 10 gm per day per rat. Some of the animals on the 100% oats diet failed to eat all of the food offered. Animals from each group were sacrificed at intervals from the 4th to 10th week of dietary control. The blood ergothioneine values obtained are shown on table 2. It is observed that the average blood ergothioneine concentrations are related to the amount of oats in the diet. Again the determination of the minimum volumes of blood filtrates required to yield detectable spots on paper chromatograms provided parallel evidence for the indicated levels of blood ergothioneine. In one of the rats fed a diet of 100% oats for only 4 weeks, the blood ergothioneine concentration was over 9 mg%; however, perhaps due to individual variations among the animals studied, no trend was

noted toward increased blood concentrations with increased time of ingestion of oats.

Thyroids from these animals were removed at autopsy. The tissues were weighed and examined for relative amounts of colloid and the status of the acini and epithelial cells.[4] No marked differences were noted in thyroid weight or in microscopic appearance between the thyroids from rats with elevated blood ergothioneine levels and those from animals with little or no blood ergothioneine.

DISCUSSION

It appears that oats contain a factor which when ingested gives rise to ergothioneine in the erythrocytes. Ergothioneine has not been detected in oats or any other foodstuff to date. Melville et al. ('53) reported that histidine labelled with carbon-14 in carbon atom number two of the imidazole ring is not incorporated into ergothioneine when the labelled compound is administered to rats. Similarly, the same workers found no transfer of methyl groups from labelled methionine to ergothioneine methyl. Heath et al. ('53) stated that sulfur-35 from labelled methionine was incorporated in small amounts into ergothioneine isolated from seminal fluid of the boar. Ergothioneine was not isolated from the erythrocytes of the animals studied; however, the bromine-oxidizable sulfur fraction (presumably due to ergothioneine sulfur) was radioactive.

In the present work ergothioneine was incorporated in fairly large amounts into the red blood cells of rats fed a diet of oats only. From analyses of whole oat protein (Heathcote, '50) it can be calculated that the diet fed probably contained inadequate amounts of both histidine and methionine[5] for optimal growth of the young white rat (Rose, '37). This

[4] The author is grateful to Dr. A. R. Peale, Department of Pathology, Temple University School of Medicine, for preparation and examination of the thyroid sections.

[5] The oats fed probably supplied adequate methyl groups for the rat. Samples of rolled oats analyzed by Dr. R. W. Carrol contained about 100 mg of choline per 100 gm (personal communication).

observation might be interpreted as additional evidence that histidine and methionine are probably not precursors of ergothioneine in the animal body.

Ling and Chow ('53) noted slight increases in the ergothioneine concentration of the erythrocytes of some anemia patients following administration of vitamin B_{12}. In the present work, the blood ergothioneine concentrations observed in rats injected with vitamin B_{12} were no different (i.e., less than 1 mg%) from those of other animals fed the same diet.

SUMMARY

1. Ergothioneine appears in the erythrocytes of the rabbit and the white rat following ingestion of oats. The blood level of the rat is related to the percentage of oats in the diet. The compound was not detected in the blood of rats fed purified (casein) diets or such diets supplemented with cabbage.

2. Administration of vitamin B_{12} has no effect on blood ergothioneine levels in the white rat.

3. Elevated blood levels of ergothioneine are not related to thyroid function as revealed by histologic examination and relative weight of rat thyroid tissue.

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ADDENDUM

Since this paper was submitted for publication, Melville et al. have reported the isolation of ergothioneine from oats (Fed. Proc., *14:* 254, 1955).

THE RELATIONSHIP BETWEEN PYRIDOXINE INGESTION AND TRANSAMINASE ACTIVITY

I. BLOOD HEMOLYSATES [1,2]

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SIX FIGURES

(Received for publication November 19, 1954)

Despite some apparently conflicting data (Cammarata and Cohen, '51, and Shwartzman and Hift, '51), most workers are in agreement with the concept that pyridoxal phosphate acts as the coenzyme for the transaminase enzymes. The published work on this relationship has been concerned chiefly with analyses on tissues from small animals maintained on normal or sub-normal intakes of pyridoxine (Schlenk and Snell, '45; Lichstein, Gunsalus and Umbreit, '45; Ames, Sarma and Elvehjem, '47; Shwartzman and Hift, '51 and others).

This report is concerned with a study of the blood levels of transaminase and vitamin B_6 and the alterations in those levels in both monkey and man on varied intakes of pyridoxine.

EXPERIMENTAL

By employing the method of Tonhazy, White and Umbreit ('50), which is sufficiently sensitive to permit analysis on small samples, measurable quantities of the aspartic-glutamic en-

[1] This investigation was supported by research grants from the National Vitamin Foundation and American Cancer Society.

[2] Presented before the Federation of American Societies for Experimental Biology, April 16, 1954.

zyme have been found in hemolyzed blood. Little or no activity was found in plasma or in fresh unlysed blood by this method.

While this manuscript was in preparation, however, an article by LaDue, Wróblewski and Karmen ('54) on the aspartic-glutamic transaminase reported the presence of small but measurable quantities of this enzyme in serum as well as in whole blood hemolysates. The authors employed a spectrophotometric method based upon the oxidation of DPNH to DPN by malic dehydrogenase, a method which appears to be more sensitive than that of Tonhazy et al.

The latter method was found to serve adequately the purposes of this study which were concerned primarily with comparative values on differing regimens. It was not satisfactory, however, for the determination of absolute levels of the enzyme in blood for several reasons.

One difficulty encountered in the determination was the poor proportionality found between the pyruvate formed and the concentration of the enzyme (the volume of the blood sample). Although greater values are to be had with samples of 0.05 ml of blood, the results obtained with 0.10 ml samples were used in calculating the values reported throughout this study because this amount gave more consistent results in duplicate determinations than did the smaller sample.

A further deterrent to a determination of the absolute level of the enzyme in blood, by this method, is the presence in blood of substances which have an inhibiting effect upon color formation in the presence of the substrates. Pyruvate, in amounts varying from 40 to 75 μg, added to inactivated blood, was recovered to the extent of only about 75% when compared with standards containing no blood.

By the use of an internal-standard method and the addition of inactivated blood to the reagent blank, values were obtained which exceeded those found with the regular method by 50% or more.

The data given in this paper, however, are the lower values obtained by use of the regular method. This consists of incubating, at 37°C., samples of 0.05 and 0.10 ml of blood,

hemolyzed by repeated freezing and thawing, with aspartate and alpha-ketoglutarate as substrates for a period of exactly 10 minutes. The substrates were added in the amounts and concentrations employed by Tonhazy et al. The color developed was read in a Klett colorimeter against a reagent blank containing no blood. The values calculated as units of transaminase (TAA) are micrograms of pyruvate formed by 0.1 ml of blood during 10 minutes, calculated for 1.0 ml of blood.

Control tubes indicated the presence in many blood samples of small amounts of preformed pyruvate and alpha-ketoglutarate, in agreement with the reports of other workers (Cavallini, et al., '49 a.b; Hawary and Thompson, '53). The quantities are too small to have significance in the tests since Hawary found the total keto-acid content of fasting human blood to be only 7.3 ± 2.1 μg/ml.

The method usually gives good reproducibility in determinations carried out at the same time. Repeat determinations at a later date show greater variation. The values obtained in a second test carried out on 84 different samples of stored, frozen blood, at intervals ranging from one to 30 days after the initial analysis, varied from these latter values by not more than 23%. The range was from -20 to $+23$%. In 96% of these samples variations between the first and the second analyses did not exceed 20%, and in 77% it did not exceed 10%.

That this variation is not due to a gradual loss of the enzymatic activity is evidenced by the fact that in more than half of the second tests the values were higher than those of the first tests. Further proof of the stability of the transaminase in frozen blood is to be found in the data, presented in table 1, of determinations carried out at intervals for 26 days upon the same sample of blood stored in the frozen state. These data show no loss of activity over that period of time. The variation among these tests was of the same order of magnitude as that found in the preceding group of analyses. An average value of 305 showed a standard deviation

of $\pm$ 19.4 units. Variations of two or of three standard deviations would correspond to differences of 13 or of 19%, respectively.

TABLE 1

The stability of the transaminase activity (TAA) in a sample of blood stored in the frozen state

DATE	*TAA*	DATE	*TAA*	DATE	*TAA*
9/24	289	10/6	289	10/15	322
9/30	325	10/6	286	10/19	289
10/5	290	10/9	341	Average	305 [1]
10/6	313	10/12	307		

[1] $\sigma = 19.4$ $\sigma m = 6.1$

From these two sets of data it would appear that any change in transaminase levels in blood exceeding 15 or 20% may be considered significant.

The vitamin B_6 determinations were carried out by a modification of the microbiological method of Atkins et al. ('43), as described in an earlier publication (Greenberg and Rinehart, '49). The method measures not only the pyridoxine but all forms of the vitamin, i.e., the total vitamin B_6.

RESULTS

Monkeys

The monkeys employed in these experiments were maintained on a synthetic diet, the composition of which has been given in a previous report together with a description of the care and handling of the animals (Greenberg and Rinehart, '49). The pyridoxine hydrochloride was administered daily by mouth. All blood samples were drawn in the morning before the pyridoxine was given.

Figures 1, 2 and 3 summarize the results obtained, respectively, on (a) 5 young immature monkeys weighing 2.0 to 2.5 kg, (b) two young adult monkeys weighing approximately 4.0 kg, and (c) one older monkey weighing about 12.0 kg. The transaminase and vitamin B_6 blood levels were determined at intervals during periods in which the pyridoxine intake per day varied between zero and 4.0 mg.

It may be seen from each of the three graphs that an increase in the pyridoxine intake was followed by a significant rise in the transaminase level as well as in the vitamin B_6 level of the blood.

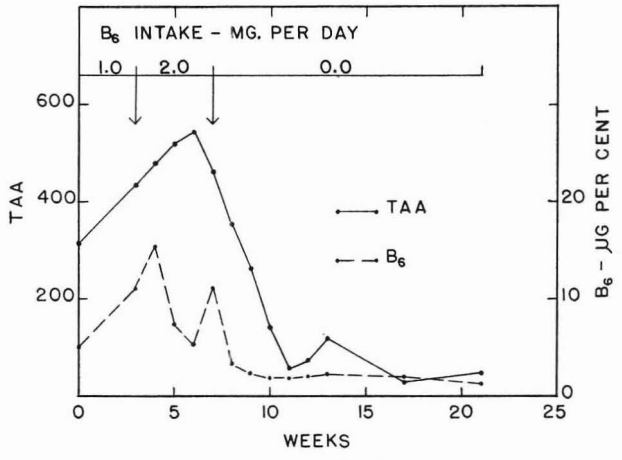

Fig. 1 The effect of varied pyridoxine intakes upon the transaminase and the vitamin B_6 levels in the blood of young immature monkeys. Monkeys No. 87–90; 92. Initial weight: 2.0 to 2.5 kg.

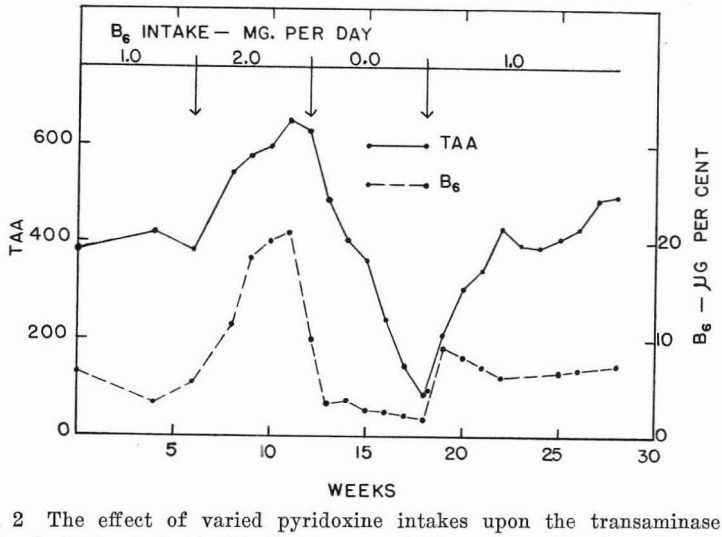

Fig. 2 The effect of varied pyridoxine intakes upon the transaminase and the vitamin B_6 levels in the blood of young adult monkeys. Monkeys No. 57 and 58. Initial weight: 4.0 kg.

In the immature monkeys (fig. 1) the 1.0 mg dosage of pyridoxine increased the transaminase level obtained soon after the animals were received at the laboratory from 319 to 432 units. The increase in the daily dosage to 2.0 mg caused a further rise to 568 units after three weeks. In the two young adult monkeys (fig. 2) the level of approximately 400 units on the 1.0 mg intake of pyridoxine increased to 600 and over when the dosage was doubled. In both groups of animals, the changes in blood levels of vitamin B_6 more or less paralleled those in the transaminase.

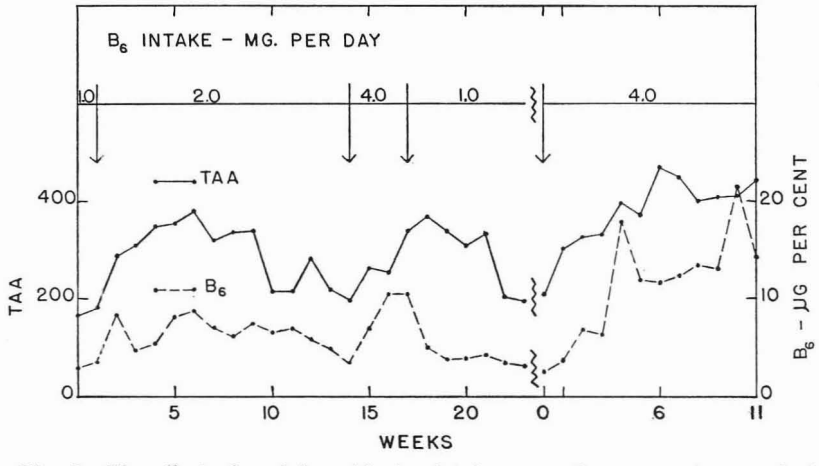

Fig. 3 The effect of varied pyridoxine intakes upon the transaminase and the vitamin B_6 levels in the blood of an older monkey. Monkey No. 34. Initial weight: 12.0 kg.

The withdrawal of all pyridoxine was followed in all the monkeys of these two groups by a rapid decrease in both the transaminase and the vitamin B_6. The enzyme concentration soon reached a level of 100 units or less and, in many instances, no activity was found. The decrease in vitamin B_6 was even more precipitous than that noted for the transaminase, but the level never decreased below 1.0 to 2.0 µg% in these studies.

In the one case (fig. 3) in which 2.0 mg of pyridoxine was administered for a longer period of time, the higher levels

were not maintained. After 13 weeks, both the transaminase and the vitamin B_6 had returned to the original values in spite of continued administration of the 2.0 mg dose. For this monkey, which weighed approximately 12 kg, the 2.0 mg intake represented a much smaller dose, on the basis of body weight, than the smaller monkeys had received. Increasing the intake to 4.0 mg for a period of three weeks produced increases in the vitamin B_6 of the blood which were slightly greater than those obtained by the administration of 2.0 mg for a corresponding period. The increase in transaminase, however, was not greater with the higher intake. In neither period did it reach 400 units. After an interval of many months on an intake of 1.0 mg of pyridoxine daily, during which time the transaminase remained around 200 units and the vitamin B_6 around 3.0 μg%, the intake was again increased to 4.0 mg. The maximum concentration in transaminase (463 units) was not reached until the 6th week, but it was then maintained at or above 400 units during the following 7 weeks.[3] These data make it appear probable that a 4.0 mg intake would sustain a permanent concentration of 400 units of transaminase.

Although the blood vitamin B_6 showed some irregularity, it reached greater concentrations during this 11-week period than it had during the earlier three-week period on a 4.0 mg intake of pyridoxine.

From data obtained on the monkeys discussed above and also on one additional older monkey (No. 18), and on other young immature monkeys receiving suboptimum amounts of pyridoxine, either 50, 100 or 150 μg per day, it appears that there is, in general, a relationship between the maximum concentration of enzyme in the blood and the intake of pyridoxine based on body weight. This is illustrated in figure 4 in which the maximum transaminase value obtained has been plotted against the logarithm of the corresponding daily dosage of pyridoxine expressed as micrograms per kilogram of body weight.

[3] Data were obtained for two additional weeks not represented in the chart.

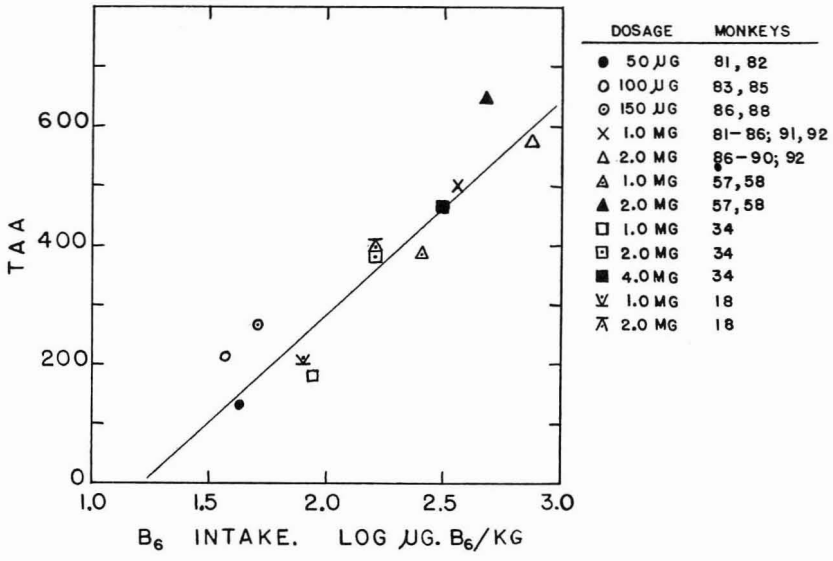

Fig. 4 Relationship bewteen the logarithm of the pyridoxine intake per kilogram of body weight and the maximum transaminase activity found at each level of intake. Data include determinations upon 16 monkeys.

Humans

The levels of blood transaminase and vitamin B_6 have been followed in three groups of humans comprising a total of 18 subjects. The human beings studied included both men and women; they were all normal, healthy individuals, either laboratory workers or medical students. Extra vitamin B_6 in the form of pyridoxine was administered either three times per day as a B-complex tablet [4] containing 5 mg of pyridoxine hydrochloride or once per day in the form of a 10 mg tablet of pyridoxine hydrochloride.

In table 2 are presented the data obtained upon 4 laboratory workers in whom the daily administration of 15 mg of pyridox-

[4] Composition of B-complex tablets:

Thiamine mononitrate	1.0 mg	Folic acid	1.0 mg
Riboflavin	1.0	Ascorbic acid	50.0
Niacinamide	20.0	Pyridoxine HCl	5.0
Calcium pantothenate	23.0	Vitamin B_{12}	0.01

ine for a period of 4 weeks resulted in an average increase in the transaminase concentration of the blood from 385 to 542 units, an increase of 41% (range 33 to 52) above the initial control level.

One week after the supplements were discontinued, the transaminase had returned to the control level in the two subjects (J. E. and T. G.) showing the smaller increase, but in the two subjects (M. D. and M. G.) with increases of 43 and 52%, the levels were still 14 and 20%, respectively, above their corresponding control levels.

TABLE 2

Transaminase and vitamin B_6 levels of blood initially, after 4 weeks of oral administration of pyridoxine (5 mg 3 ×/day) and one week after supplements were discontinued

SUBJECT	TAA UNITS			VITAMIN B_6 μG %		
	Control	B_6 for 4 weeks	1 week later	Control	B_6 for 4 weeks	1 week later
M.D.	361	518	412	1.6	8.4	3.8
M.G.	406	616	487	1.6	8.5	3.9
J.E.	369	491	373	1.7	5.5	3.1
T.G.	404	543	378	1.7	11.5	4.9
Averages	385	542	412	1.65	8.5	3.9
% Increase		41	7			

The changes in the vitamin B_6 of the blood paralleled the changes in the transaminase in three of the subjects. In the 4th subjects (T. G.) the increase in B_6 was greater, and the return to normal level was apparently slower.

In a second experiment, the same dosage of pyridoxine was administered to 8 medical students for $9\frac{1}{2}$ weeks, with blood samples analyzed after two, 4, 6 and $9\frac{1}{2}$ weeks. As may be seen in figure 5, there was a progressive increase in the transaminase values, from an average of 433 to a final value of 571 units (32% increase). The vitamin B_6 rose from 1.9 to 6.8 μg% after two weeks and showed no further increase. At $9\frac{1}{2}$ weeks the value had decreased to 5.1 μg. One week after the pyridoxine supplement had been discontinued, the

transaminase had decreased only to 528 units and the vitamin B_6 to 3.3 μg%.

Blood transaminase and vitamin B_6 were followed in another group of 6 subjects who received supplemental vitamin B_6 in the form of a 10 mg tablet of pyridoxine once daily for a period of 6 weeks or longer. After the extradietary pyridoxine was discontinued, analyses were carried out at intervals until the transaminase had returned to the initial level. The average values obtained in this series of tests are presented in figure

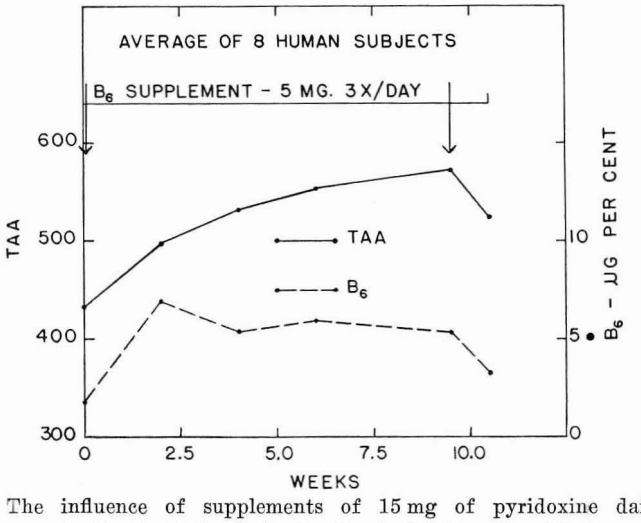

Fig. 5 The influence of supplements of 15 mg of pyridoxine daily upon transaminase and vitamin B_6 levels in the blood of 8 human subjects.

6. Only the first 6 weeks of the supplementation period are charted since two of the subjects were not continued beyond that time. The average levels at the beginning of the second control regimen were, however, the final values obtained during the supplementation period irrespective of time. The transaminase increased from 423 to 615 in 6 weeks, an average rise of 44%. Two weeks after the supplements were discontinued, the transaminase was 36% above the initial level, and after 6 weeks it was still plus 14%. The vitamin B_6 increased from about 4 μg% to about 10 μg% and returned to the original level two weeks after the extra pyridoxine was discontinued.

In several different samples of blood hemolysates from vitamin B_6-deficient monkeys, we have been unable to increase the activity of the enzyme system by the addition of pyridoxal phosphate [5] or pyridoxamine phosphate [5] in quantities of 0.01 to 0.50 μg (table 3). This, however, does not necessarily mean that the enzyme system is not catalyzed by one or both of these coenzymes. Others (Schlenk and Snell, '45; Ames,

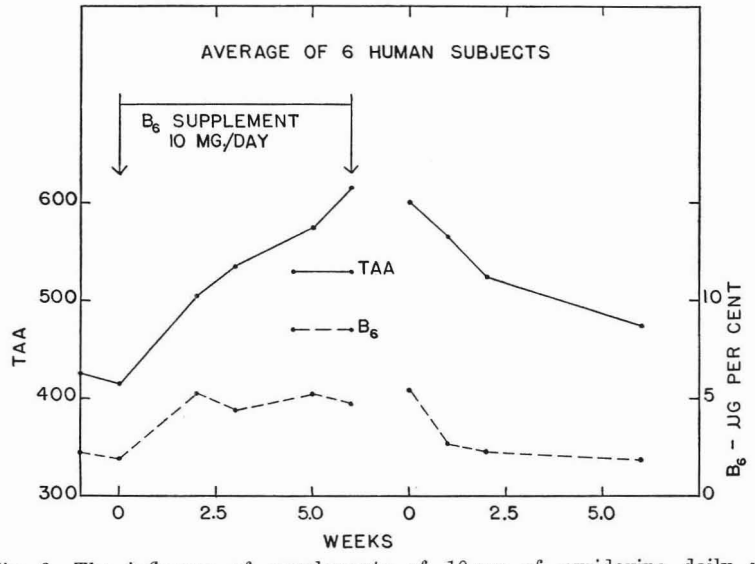

Fig. 6 The influence of supplements of 10 mg of pyridoxine daily upon transaminase and vitamin B_6 levels in the blood of 6 human subjects.

Sarma and Elvehjem, '47; Shwartzman and Hift, '51) have been unable to increase significantly the aspartic-glutamic acid transaminase of tissues of vitamin B_6-deficient animals by the addition of these coenzymes. Our observations on the variation of the activity of hemolysates from both monkey and man as influenced by the intake of pyridoxine certainly support the idea that the enzyme system probably represents a vitamin B_6-catalyzed reaction. It is possible that the failure to

[5] We are grateful to Dr. W. W. Umbreit of Merck Institute of Therapeutic Research for the pyridoxal phosphate and to Dr. Alton Meister of the National Cancer Institute, National Institutes of Health, for the pyridoxamine phosphate.

observe stimulation of activity by the addition of the phosphorylated pyridoxal and pyridoxamine may be related to the presence of an active phosphatase in blood which brings about their cleavage at a rapid rate.

TABLE 3

The effect of the addition of pyridoxal or pyridoxamine phosphates upon the transaminase of blood of vitamin B_6-deficient monkeys

MONKEY NO.	ADDITIONS	QUANTITY	TRANSAMINASE UNITS	
			Control	With addition
		μg		
72	Pyridoxal phosphate	0.01	46	46
74	Pyridoxal phosphate	0.01	27	32
76	Pyridoxal phosphate	0.10	33	33
72	Pyridoxal phosphate + ATP — 1.0 mg	0.20	50	61
72	Pyridoxamine phosphate	0.01	26	37
73	Pyridoxamine phosphate	0.10	0	0
76	Pyridoxamine phosphate	0.50	0	0

SUMMARY

The method of Tonhazy et al. for the estimation of aspartic-glutamic transaminase has been adapted to the measurement of the enzyme in whole blood.

A study was made of the transaminase and vitamin B_6 levels of the blood of monkeys receiving graded doses of pyridoxine hydrochloride and also of humans receiving extradietary supplements of pyridoxine hydrochloride.

In both monkeys and man, increasing the intake of pyridoxine resulted in significant increases in the blood level of the transaminase and of the vitamin B_6. Reductions in the pyridoxine intake were followed by a lowering of the blood concentration of both factors. The changes in the level of transaminase were often more gradual, however, than were those of vitamin B_6. The time required to reduce the levels to a minimum after withdrawal of all pyridoxine or to the initial levels after the supplemental dosage was discontinued seemed to be dependent to some extent upon the duration of the period of the increased intake.

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MATERNAL DIET AND OTHER FACTORS AFFECTING THE LIPID CONTENT OF LIVERS OF VERY YOUNG RATS [1]

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In previous studies from this laboratory (Schultze, '49; Halvorson and Schultze, '50; Schultze et al., '52), it was reported that young rats born to mothers fed certain vitamin B_{12}-deficient rations incurred a high incidence of death within 48 hours after birth. An abnormally high concentration of urea in the blood preceded the death of the young. Hence, the syndrome was referred to as "acute uremia of the newborn" (Schultze, '49). When vitamin B_{12} was added to the ration of the mothers or injected into the young immediately after birth, the increase in blood urea was much smaller and the young survived. During these studies, it was observed that the livers of uremic young rats frequently had a yellowish color suggestive of fatty infiltration. This paper records the results of a study of the concentration of liver lipids during the early life of rats as affected by maternal diet and some other factors.

We are not aware of prior studies on the lipid concentration of the liver of newborn rats. Guggenheim and Jürgens ('44) and Beauvallet ('53) observed that some of the young born to mothers fed a fat-free ration developed fatty livers. A lipotropic effect of vitamin B_{12} has been observed in several

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laboratories (Strength et al., '51; Bennett et al., '51; Morgan and Lewis, '53; Shils and Stewart, '54; Drill, '54) but not under all conditions (Hawk and Elvehjem, '53).

EXPERIMENTAL

Rations and animals. The young rats used for this study were born to mothers that had been fed since weaning one of the following three types of rations:

(a) Ration CS-1, previously described (Halvorson and Schultze, '50), consisting of a mixture of ground yellow corn, soybean oil meal, alfalfa leaf meal and salts. It is deficient in vitamin B_{12} and produces usually a high incidence of acute uremia of the newborn. As shown in table 2, each kilogram of this ration was supplemented in specific instances with 40 μg of vitamin B_{12}, 3.35 gm of DL-methionine or 1 gm of choline chloride.

(b) Ration OC_1, previously described (Schultze, '54), which consists of a mixture of 84% of ground, rolled oats, 6.5% of casein, 0.33% of DL-methionine, 3% salts, 2% wheat germ oil, 1% corn oil and a complete mixture of fat-soluble and water-soluble vitamins including 40 μg of vitamin B_{12} and 1 gm of choline chloride per kilogram.

(c) Stock ration, which consists of a mixture of 61.49% of yellow corn, 10% of dried skimmilk, 9% each of alfalfa leaf meal, soybean oil meal, fish meal, 1.5% of a salt mixture, and 0.01% of irradiated yeast.

With few exceptions, the rats were of the black strain of our Line 3. Towards the end of pregnancy, each animal was housed separately and her young, when required, were removed soon after birth before they had obtained milk.

Analytical. The livers were removed from the decapitated rats; adhering blood was removed by blotting and the tissue was weighed, dried at 70°C. for 48 hours, and weighed again. The liver thus dried had a residual moisture content of 3% and this was taken into account in the calculations. Dried livers from individual rats were transferred to a 25 × 200 mm test tube, crushed to a powder and refluxed for one hour with

10 ml of a mixture of 95% ethanol-acetone (2:1 v/v). The extract was decanted into a beaker and the extraction with ethanol-acetone repeated three times. The combined extracts were evaporated to dryness, the lipid residue extracted exhaustively with petroleum ether, and the latter extract was filtered and washed through glass wool into a tared vial. The petroleum ether was evaporated to dryness and the residue weighed. Control analyses demonstrated: (1) that further extraction of the liver residue with 10 ml portions of carbon

TABLE 1

Factors affecting liver lipids

GROUP [1]	MATERNAL DIET	EXPERIMENTAL CONDITIONS	NO. OF INDIVIDUALS	AGE OF YOUNG	LIVER LIPIDS, % OF DRY WEIGHT
				hours	
1	Stock ration	Young not nursed	6	0	10.1 [2]
2	Stock ration	Young nursed	7	36	18.4 [2]
3	CS-1	Young not nursed	16	0	15.7 ± 0.8 [3]
4	CS-1	Young nursed	18	36	42.4 ± 2.7
5	CS-1	Young not nursed	10	0	17.5 ± 1.3
6	CS-1	Young not nursed	13	24	17.8 ± 1.9
7	CS-1	Young nursed	16	24	40.2 ± 1.0
8	OC_1	Young not nursed	13	0	15.4 ± 0.8
9	OC_1	Young not nursed	27	24	18.4 ± 1.2

[1] Animals of groups 1 and 2; 3 and 4; 5, 6 and 7; 8 and 9 respectively were littermates.

[2] Analyses performed on pooled livers.

[3] Standard error of the mean.

tetrachloride, methanol, benzene and ethanol-acetone (2:1 v/v) in turn failed to remove any petroleum ether-soluble compounds; (2) that amounts of dry liver ranging from 25 to 150 mg yielded the same percentage of lipids; (3) that lipids added to pre-extracted ground liver could be quantitatively recovered. The results were calculated on a dry weight basis.

RESULTS AND DISCUSSION

Factors affecting liver lipids. As shown in table 1, the lipid content of the liver of very young rats, under the conditions of this experiment, is affected by at least three conditions: (1)

the maternal diet before parturition; (2) the age of the young; (3) the availability of milk to the young. Thus, in livers removed immediately after the young were born to mothers fed the stock ration (group 1), the lipid concentration was lower than in those removed from young born to mothers fed the CS-1 or the OC_1 rations (groups 3, 5 and 8). A subsequent increase in liver lipids occurred only when the young were nursed (compare group 5 with 6 and 7), but the magnitude of this increase was affected by the maternal diet and through it, presumably by the composition of the milk (compare group 2 and 4). While in older animals of various species including the rat (see Harrison, '53 for discussion and bibliography) starvation induces a rapid increase in liver lipids, with newborn rats only a small (group 9) or no (group 6) increase was observed in these experiments.

To determine the effect of lipotropic compounds added to a maternal ration of poor nutritional quality, weanling female rats from the stock colony were raised to a weight of about 150 gm with ration CS-1 and then bred. At that time they were divided into 4 groups and their rations were supplemented during the last 10 days of pregnancy as shown in table 2. Assuming that published analyses for choline (McElroy et al., '48) and methionine (Block and Bolling, '43; Sheldon et al., '48) in the yellow corn, soybean oil meal and alfalfa apply approximately to the specimens of these feeds used in this work, the choline and methionine content of the supplemented rations were about 100% and 67% respectively higher than in the unsupplemented rations. Included in table 2 are the results obtained with young born to mothers that had been fed the rolled oats-casein ration since weaning. After birth of the young, littermates were used randomly at different time intervals for lipid analysis.

According to the data summarized in table 2, there was, after birth, in all groups a rapid increase in the percentage of liver lipids. In some cases this reached within 15 hours double the concentration at birth. The maximum concentration was reached some time during the second and third day

of life and thereafter it decreased gradually. Of the lipotropic compounds added as supplements to the maternal ration, only vitamin B_{12} was effective in decreasing the magnitude of the rise in liver lipids (group 2). The lower concentration of liver lipids attained by rats in group 5 is presumably also a reflection of the vitamin B_{12} content of the maternal ration.

TABLE 2

Effect of age and maternal ration on liver lipids of young rats

AGE OF RATS	GROUP 1	2	3	4	5
	MATERNAL RATION AND ADDITIONS				
	CS-1	CS-1 + 40 μg B_{12}/kg	CS-1 + 0.335% DL-Methionine	CS-1 + 0.1% Choline chloride	OC_1 [1]
	LIVER LIPIDS, % OF DRY WEIGHT				
0 hours	15.7 ± 0.8 [2]	12.3 ± 1.0 [2]	14.4 ± 0.7 [2]	13.2 ± 0.9 [2]	13.0 ± 0.7 [2]
6 to 15 hours	25.4 ± 1.9	22.1 ± 1.0	27.2 ± 1.8	29.2 ± 1.4	19.6 ± 1.8
16 to 25 hours	39.8 ± 1.7	28.3 ± 1.6	37.2 ± 1.4	37.7 ± 2.0	32.4 ± 1.2
26 to 40 hours	41.4 ± 1.1	30.2 ± 1.8	40.6 ± 1.3	39.5 ± 1.5	28.3 ± 1.9
41 to 72 hours	41.9 ± 2.1	32.4 ± 1.5	34.3 ± 1.7	33.2 ± 2.0	30.3 ± 1.4
73 to 96 hours	.....	26.5 ± 1.8	37.1 ± 2.0	36.4	22.8 ± 1.9
97 to 144 hours	.....	27.2 ± 2.1	29.0 ± 2.2	35.2	23.0 ± 1.8
6 to 15 days	.....	20.8 ± 1.2	28.6 ± 1.3	38.8	21.5 ± 1.2
21 days	.....	23.1 ± 1.5	24.2 ± 2.3	...	23.5 ± 1.1

[1] Ration OC_1 contained 40 μg vitamin B_{12}, 3.3 gm DL-methionine and 1.0 gm choline chloride added per kilogram of ration.

[2] The number of specimens from which the means and standard errors were calculated ranged from 9 to 21 in group 1; from 10 to 14 in group 2; from 10 to 19 in group 3; from 10 to 17 through 40 hours and 6, 3, 3, 2 respectively for the later intervals in group 4; from 10 to 23 for group 5. High mortality of the young left no survivors in group 1 after 72 hours and only the numbers indicated in group 4 after 40 hours.

In another experiment, 4 groups of rats were fed the rations shown for groups 1 to 4 in table 2 from weaning. The livers of their young, 36 hours after birth, had a mean concentration of liver lipids of 42.4% (18 rats), 27.3% (20 rats), 34.6% (19 rats) and 35.0% (29 rats), respectively. In this instance, when the supplements to the CS-1 ration were fed throughout the life of the mother, instead of only during the last phase of

pregnancy, choline, methionine and, particularly vitamin B_{12}, had a definite effect in preventing a maximum rise in liver lipids. The probability, P, that the difference between the unsupplemented group and those fed choline or methionine was due to chance was 0.04, for the group fed vitamin B_{12}, the P value was 0.008. The effects of including methionine and choline at higher levels in the maternal ration were not investigated.

Since in all instances where the young had access to milk, there was a postnatal rise in the concentration of liver lipids this appears to be a "normal" occurrence. Certainly, there is no evidence that the rise of lipids in the liver to a concentration of about 30% is harmful because the early growth and survival of the young from mothers fed the rolled oats-casein ration has, in many instances, been very satisfactory (Schultze, '54). There is also no evidence that the high concentration of liver lipids in the young from mothers fed vitamin B_{12}-deficient CS-1 ration is responsible for the high incidence of acute uremia of the newborn encountered among such animals.

Prenatal and postnatal effects. The data in table 3 show that the effect of vitamin B_{12} administered to the mothers in reducing postnatal increases in the concentration of liver lipids was exerted mainly before birth of the young. These results were obtained by the following technique: at birth, the young of the mothers fed the vitamin B_{12}-deficient ration CS-1 were allotted to three groups; one group was killed immediately for analysis of the livers; the young of the second group were permitted to nurse their own mothers, and those of the third group were placed with mothers fed the vitamin B_{12}-supplemented ration OC_1 and had cast litters at about the same time. The young from these mothers were, before they had suckled, likewise allotted to three groups, one of which was placed with lactating mothers fed the CS-1 ration, those in one group were permitted to nurse their own mothers and the third group was killed at birth. After 24 hours the surviving young were killed for analysis. The young born to

mothers fed the OC_1 ration had apparently stored enough vitamin B_{12} in utero or were otherwise able to prevent a maximum rise in the liver lipid concentration in 24 hours in spite of consuming milk from mothers whose own young developed a much greater fatty infiltration of the liver. The young born to mothers fed the vitamin B_{12}-deficient CS-1 ration, however, had essentially the same concentration of liver lipids whether fed by their own mothers or by foster mothers that consumed the vitamin B_{12}-supplemented ration.

TABLE 3

Liver lipids of young rats nursed by foster mothers for 24 hours

TREATMENT	NUMBER OF INDIVIDUALS	MEAN LIVER LIPIDS, % OF DRY WEIGHT
		Rats born to mothers fed ration CS-1
Killed at birth	11	16.2 ± 0.8 [1]
Nursed by own mothers	25	39.2 ± 1.8
Nursed by foster mothers fed ration OC_1	23	37.0 ± 1.9
		Rats born to mothers fed ration OC_1
Killed at birth	11	13.0 ± 0.7
Nursed by own mothers	30	31.2 ± 1.1
Nursed by foster mothers fed ration CS-1	31	29.4 ± 1.4

[1] Standard error of the mean.

Granting that consumption of milk is a prerequisite to the establishment of fatty livers in the very young rat, our data show that prenatal effects can determine the extent to which this takes place. When 0.06 μg of vitamin B_{12} (in a mannitol solution) was injected subcutaneously shortly after birth into rats born to mothers fed ration CS-1, these animals had a significantly smaller liver lipid concentration after 24 hours (mean of $32.7 \pm 1.2\%$ for 24 rats) than their littermates which were injected with the same volume of a mannitol solution free of vitamin B_{12} ($36.2 \pm 1.5\%$ for 24 rats). This observation suggests that young which nursed mothers fed

the OC_1 ration did not obtain an effective quantity of vitamin B_{12} through the milk. However, none of these young rats developed symptoms of acute uremia of the newborn whereas their littermates did so while suckling their own mothers fed the CS-1 ration.

Changes in other components of the liver. The increase in liver lipids in the newborn rat referred to above could be the result of a corresponding decrease in non-lipid components of the dry liver. In a separate experiment with rats born to

TABLE 4

Absolute contents of water, lipid, "crude protein" and other components in the liver of newborn rats [1]

AGE IN HOURS	WEIGHT OF LIVER	WEIGHT OF WATER	WEIGHT OF LIPID	WEIGHT OF PROTEIN	WEIGHT OF OTHER COMPONENTS [2]	DRY LIVER
	mg	*mg*	*mg*	*mg*	*mg*	*mg*
0	258 ± 15.2 [3]	202	8	41	6	55
6 to 15	219 ± 12.1	166	16	32	5	53
16 to 25	204 ± 19.4	150	19	30	6	55
26 to 40	226 ± 25.2	166	20	33	7	60
41 to 72	269 ± 20.9	197	24	39	9	72
73 to 96	299 ± 18.5	222	23	43	11	77
97 to 144	351 ± 29.2	248	32	58	12	102

[1] Born to mothers fed ration CS-1 with or without supplements shown in table 2.

[2] Calculated as : Dry weight — (Lipid + N × 6.25).

[3] Mean ± standard error of mean. The values in each of the first 5 lines are means of 40 determinations, those in the last two lines of 20 determinations.

mothers fed the CS-1 ration it was established that during the first 40 hours after birth there was a decrease in moisture content from 76 to 73% and a decrease from 74 to 55% in "crude protein" concentration (N × 6.25), if the latter is expressed in terms of dry weight. From these and other available data collected during this study an estimate was made, as shown in table 4, of the absolute amounts of moisture, lipids, "crude protein" and "other components" in the livers of young rats. The figures for the last term were obtained by subtracting the sum of the weights of the lipids plus "crude protein" from the dry weight of the liver. It includes ash, carbohydrates, as well as other components not analyzed for, and

the errors inherent in the methods used. Although the data in table 4 are admittedly approximations they indicate that during the first day of life of young rats born to mothers fed the CS-1 ration (including the various supplements shown in table 2), there was a decrease in the fresh weight of the liver due to a loss of moisture and protein. This decrease was about compensated by an increase in the amount of liver lipids. The "other components" and the dry weight of the liver remained about the same during this period. Afterwards, as would be expected from the growth of the young, including the liver, there was an increase in the absolute quantity of all components. As a result of the early shrinkage of the liver and the increase in the amount of liver lipids, the fatty infiltration of the liver of the very young rats became accentuated.

SUMMARY

1. Within 6 hours after birth of rats, there was an increase in the absolute amount and in the concentration of lipids in the liver. This increase continued until in about 40 hours it reached a level of about 40% of the dry weight, compared to about 15% at birth.

2. The increase in liver lipids was contingent upon consumption of milk by the young. The inclusion of vitamin B_{12} in the maternal ration and to a lesser extent of choline and of methionine decreased the extent of the rise in liver lipids significantly but did not prevent it.

3. During the first 24 hours after birth, there was a decrease in the fresh weight of the liver due to a loss of moisture and of crude protein, but a simultaneous increase in the absolute amount of liver lipids.

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THE RELATIONSHIP OF CARBOHYDRATE METABOLISM TO PROTEIN METABOLISM

IV. THE EFFECT OF SUBSTITUTING FAT FOR DIETARY CARBOHYDRATE

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ONE FIGURE

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In the early literature on protein metabolism there are a number of papers showing that nitrogen balance is adversely affected when fat is substituted isocalorically for the carbohydrate of the diet (see review by Munro, '51). More recently, it has been observed that the carbohydrate need not be removed from the diet. Mere separation in the time of eating the protein and carbohydrate is sufficient to cause a deterioration in nitrogen balance (Cuthbertson and Munro, '39; Cuthbertson, McCutcheon and Munro, '40; Munro, '49; Geiger, Bancroft and Hagerty, '50) and the question arises whether the disturbance in protein metabolism produced by substituting fat for dietary carbohydrate is due solely to lack of carbohydrate in the protein-containing meals. It was convenient to use rats in order to study this question but a survey of the literature (Munro, '51) revealed that, although there was convincing evidence in the case of man and the dog that substitution of fat for dietary carbohydrate causes a deterioration in nitrogen balance, the evidence in the case of the rat was contradictory. It was therefore necessary to determine the conditions under which substitution of fat for carbohydrate affects the nitrogen balance of the rat before

proceeding to compare the effect of substitution with the effect of eating carbohydrate separately from the dietary protein.

EXPERIMENTAL

Animals and diets. In each experiment young adult male albino rats of about 200 gm body weight were distributed between the various treatments, using the randomized block technique to diminish the statistical effects of differences in body weight (Snedecor, '46). They were housed individually under thermostatic conditions in glass containers and fed the experimental diet twice daily. In order to prevent scattering, all food was moistened before feeding. The first meal, containing minerals, vitamins and roughage in the quantities previously described (Munro, '49), was given at 10 A.M. and the dishes were removed at 1 P.M. The second meal, given at 5 P.M., provided all the protein of the diet, namely 2.5 gm of casein made into a paste as described previously (Munro, '49). This meal was eagerly consumed. In addition to these fixed constituents of the diet, carbohydrate (glucose) and fat (olive oil) were added to one meal or the other according to the purpose of the experiment. During each experiment, changes were brought about either by substituting isocaloric amounts of olive oil for glucose or by transferring glucose from one meal to the other without altering the total amount given per day.

In the experiments involving isocaloric substitution of olive oil for glucose, physiological fuel values were taken to be 3.75 Cal. per gram for glucose and 9.0 Cal. per gram for olive oil. Subsequently it was found by direct determination in a bomb calorimeter that the heats of combustion of the glucose and olive oil used were 3.75 and 9.56 Cal. per gram respectively and, furthermore, that isocaloric exchange of olive oil for glucose in the diet of the rat caused no appreciable change in fecal energy loss. Consequently, the use of the factors 3.75 and 9.0 Cal. per gram for the physiological fuel values of glucose and olive oil resulted in a slightly higher energy intake during the period of fat administration. However, the

difference in energy intake does not exceed 1 Cal. in any of our experiments. Such a change in energy intake would alter the nitrogen balance of the rat by only 3 to 4 mg per day (Munro, '51), which is insignificant in comparison with the changes produced in the experiments reported here.

Collection and analysis of excreta. Wide-mouthed glass bottles were used to house the rats during the experiment. The bottom of each bottle was removed. It was then inverted. The rat was then placed in the bottle on top of a wire mesh. The urine and feces falling from the neck of the bottle were caught in a large glass funnel, the feces being separated by a fine wire mesh placed in such a way that they rolled to one side and were not contaminated by the urine. The urine was passed directly into bottles containing 20 ml of 6 N HCl and the glass and metal parts of the cages and feces separators were washed down daily with 250 ml of water into the urine bottles. A recovery experiment in which a urea solution was sprayed inside the cages on 4 successive days gave an average recovery in 8 experiments of 99.6%, with a range of 99.2 to 100.3% for individual cages. Since the urine passed during the experiments contained about 300 mg nitrogen per day, this would introduce an error of only from -2 to $+1$ mg in the recoveries from individual animals. The feces were marked every 4 days by giving ferric oxide in the food. Before sampling for analysis, they were homogenized with water in a blendor. The diet, feces and urine were analyzed for nitrogen by micro-Kjeldahl estimation, using mercury as the catalyst.

RESULTS

The effect of substituting fat for carbohydrate in the diet of the rat. Two groups of 7 rats each were fed initially on the same diet which provided 40 Cal. per rat per day (1310 Cal. per square meter of body surface area calculated according to the formula of Lee, '29). The morning meal contained 1 ml of olive oil in addition to vitamins, minerals and roughage; 5 gm of glucose were given together with the casein of the evening meal. In this experiment the starch

normally used in making up the vitamin-mineral-roughage mixture (Munro, '49) was omitted, so that the 5 gm of glucose fed in the evening meal provided all of the carbohydrate in the diet. This diet was given for one week before excreta were collected. During the first 4 days of collection, both groups continued to eat the same diet and excreted essentially the same amount of nitrogen (table 1). During the remaining 8 days of the experiment, one (control) group remained on this diet whereas the other (experimental) group

TABLE 1

Changes in nitrogen excretion produced by exchanging fat for carbohydrate in the diet. (During period I both experimental and control groups received the same diet; during periods II and III all the dietary carbohydrate of the experimental group was replaced by fat. Each figure is the mean result obtained with 7 rats.)

PERIOD	MEAN DAILY URINARY NITROGEN		MEAN DAILY FECAL NITROGEN		MEAN DAILY NITROGEN BALANCE	
	Experimental group	Control group	Experimental group	Control group	Experimental group	Control group
	mg	*mg*	*mg*	*mg*	*mg*	*mg*
I	296	295	23	22	+ 28	+ 29
II	323	290	25	21	— 1	+ 36
III	299	293	24	19	+ 23	+ 34

Analysis of variance demonstrates a significant difference ($P < 0.01$) between the nitrogen balances of the experimental and control groups during period II. This was due to a significant difference ($P < 0.01$) in urinary nitrogen output limited to period II. There was no significant effect of diet on fecal nitrogen output.

received 2.32 ml (2.09 gm) of olive oil given as an isocaloric substitute for the 5 gm of glucose fed in the evening meal.

Daily analyses of the urines revealed that the complete substitution of fat for the carbohydrate of the diet caused an immediate rise in urinary nitrogen output which persisted for 4 to 5 days and then returned to the level of output of the control group. Table 1 provides a summary of the urinary outputs over 4-day periods together with statistical analysis of the data. This picture is confirmed by statistical analysis of the nitrogen balances (table 1) which demonstrates a significant difference between the experimental and control

groups only during the first 4-day period after substitution of fat for carbohydrate. In consequence of this finding subsequent experiments were limited to the first 4 days after effecting a change in diet. There was no significant change in fecal nitrogen output (table 1).

Comparison of the effect of substituting fat for carbohydrate given along with or apart from the protein of the diet. In this experiment 4 groups, of 14 rats each, were given a diet providing 35 Cal. per rat per day (1290 Cal. per square meter body surface area). All groups received the vitamins,

TABLE 2

The effect on nitrogen balance of substituting fat isocalorically for carbohydrate in a protein-containing meal or in a protein-free meal

(Each figure is the mean result obtained with 14 rats)

GROUP	SUBSTITUTION OF FAT FOR CARBOHYDRATE	MEAN INITIAL BODY WEIGHT	NITROGEN BALANCE (4-day period)	DIFFERENCE IN NITROGEN BALANCE
		gm	*mg*	*mg*
1.	In protein-containing meal	179	— 158	— 123
2.	Control group	177	— 35	
3.	In protein-free meal	178	— 127	— 52
4.	Control group	180	— 75	

Analysis of variance shows that substitution of fat for carbohydrate adversely affected nitrogen balance, the effect being significantly greater when substitution was carried out in the meal containing protein ($P < 0.05$ for interaction). Differences in nitrogen balance exceeding 50 mg between groups are significant ($t < 0.05$).

minerals and roughage together with 1 ml of olive oil in the morning meal and casein in the evening meal. In addition, groups 1 and 2 were given 3 gm of glucose in the evening (protein-containing) meal and groups 3 and 4 received 3 gm of glucose in the morning (protein-free) meal. After the rats had eaten these diets for a week, 1.4 ml (1.26 gm) of olive oil was given as an isocaloric substitute for the glucose fed to groups 1 and 3, the other two groups continuing on the previous diets as controls.

Excreta collected over the next 4 days showed that all groups were in negative nitrogen balance (table 2). The

nitrogen balances of both groups 1 and 3 were adversely affected by isocaloric substitution of fat for carbohydrate, but the effect in the case of group 1 (substitution in the protein-containing meal) was significantly greater than in the case of group 3 (substitution in the protein-free meal). Although substitution in group 3 led to a greater nitrogen output than in the case of its control group, the change was statistically the borderline of significance ($P = 0.05$). From the much greater effect in the case of group 1 than in the case of group 3, it may be concluded that substitution of fat for carbohydrate causes a deterioration in nitrogen balance principally by affecting the utilization of dietary protein.

The effect of partial substitution of fat for carbohydrate. The object of this experiment was to determine whether nitrogen balance in the rat is affected by partial substitution of fat for carbohydrate in protein-containing meals, and also whether removal of carbohydrate is the sole cause of the change in nitrogen output or whether the introduction of fat into the protein-containing meal plays a part.

Four groups of rats were started on the same diet which consisted of vitamins, minerals, roughage and 1 ml of olive oil as the morning meal and casein with 3 gm of glucose as the evening meal. This provided 35 Cal. per rat per day (1100 Cal. per square meter of body surface area). After a preliminary week on this diet, each group received a different treatment and excreta were collected over a 4-day period. Group 1 (controls) continued to receive 3 gm of glucose in the evening meal. Group 2 were given only 1 gm of glucose, and an amount of olive oil isocaloric with 2 gm of glucose was added to this meal. Group 3 received the same diet as group 2, except that the olive oil was fed in the morning (protein-free) meal. In group 4 fat was substituted completely for the carbohydrate of the evening meal. Nitrogen balance determinations made during the first 4 days of feeding of these diets (table 3) show a significant deterioration in group 2 as compared with the control group 1; this indicates that protein utilization is affected by partial substitution of fat

for carbohydrate. The effect of complete substitution (group 4) was not significantly greater than partial substitution. Comparison of the nitrogen balances for groups 2 and 3 shows that it is immaterial whether the fat replacing the carbohydrate is given with the dietary protein or at another time of day. It may accordingly be concluded that the effect of substituting fat for carbohydrate is essentially due to removal of carbohydrate from the protein-containing meal and not to an adverse effect of feeding fat with protein.

TABLE 3

Comparison of changes in nitrogen balance following partial or complete substitution of fat for dietary carbohydrate

(Each figure is the mean result obtained with 6 rats)

GROUP OF RATS	MEAN INITIAL BODY WEIGHT	COMPOSITION OF EVENING MEAL			NITROGEN BALANCE (4-day period)
		Casein	Glucose	Olive oil	
	gm	*gm*	*gm*	*gm*	*mg*
1.	219	2.5	3	0	— 69
2.	219	2.5	1	0.83	—152
3.	217	2.5	1	0	—131
4.	217	2.5	0	1.26	—179

Analysis of variance shows that the different treatments had a significant effect on nitrogen balance ($P < 0.01$). This is due to a significant difference between group 1 and all the other groups, which do not differ significantly among themselves.

Nitrogen balance in relation to the amount of carbohydrate fed with protein. Since the most important factor in causing nitrogen balance to deteriorate when fat is substituted for carbohydrate appears to be the removal of carbohydrate from the protein-containing meal, it was of interest to determine the relationship between the amount of carbohydrate in the protein-containing meal and the nitrogen balance. For this purpose rats were fed in the morning meal with the vitamins, minerals and roughage together with 3 gm of glucose and 1 ml of olive oil; the casein was given alone in the evening meal. This diet provided 35 Cal. per rat (1170 Cal. per square meter of body surface area). After they had been on this diet for a week, different amounts of glucose were transferred

from the morning to the evening meal and the excreta collected during the succeeding 4 days.

The results (fig. 1) indicate that the presence of small amounts of carbohydrate in the protein meal was insufficient to produce maximum improvement in the nitrogen balance. On the contrary, the improvement in nitrogen balance was related linearly to the amount of glucose added ($P < 0.01$).

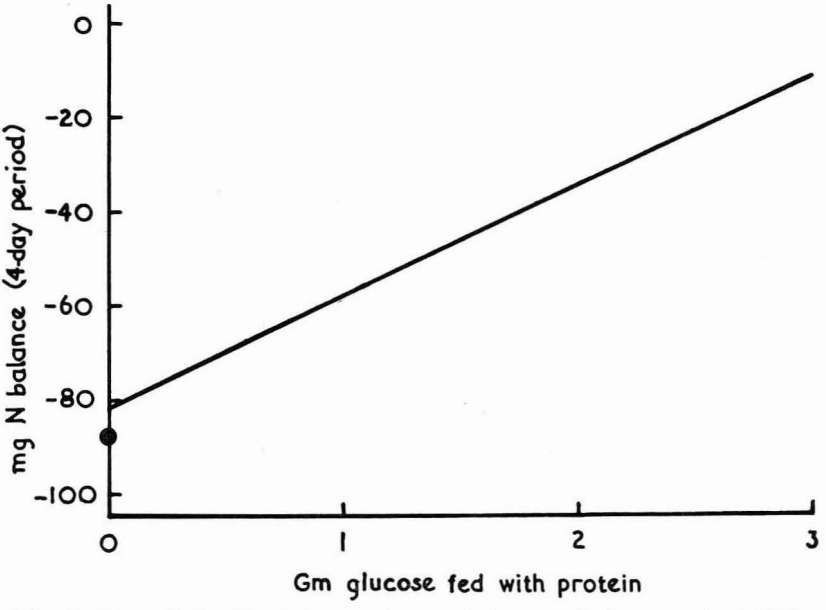

Fig. 1 The relationship between nitrogen balance and the amount of dietary carbohydrate fed with protein. All animals received the same total amount of food, but the quantity of glucose given along with the dietary protein was varied.

The relationship is expressed by the equation $Y = 23.2X - 81$, where X is the amount of glucose in grams given along with the dietary protein and Y is the nitrogen balance in milligrams per 4-day period. Analysis of variance revealed no significant deviation from linearity of response.

DISCUSSION

Our observations show a significant deterioration in nitrogen balance when carbohydrate is wholly or partly replaced

by fat in the diet of the rat. Data published by other authors fail to agree on this point. Some (Desgrez and Bierry, '20, '21; Lathe and Peters, '49) also obtained evidence of the superiority of carbohydrate. On the other hand no beneficial effect was observed by Maignon ('34) in his numerous partial and complete substitution experiments. In an experiment reported by Samuels, Gilmore and Reinecke ('48) the nitrogen output was not significantly greater on a protein-fat diet than on an isocaloric protein-carbohydrate diet. Similarly, in the extensive studies of Forbes and his associates (Forbes, Swift, Elliott and James, '46; Forbes, Swift, Thacker, Smith and French, '46) there was no evidence of an increased nitrogen output when fat was exchanged for part of the carbohydrate at a maintenance level of energy intake and in only one of the two experiments at a higher plane of energy intake.

In considering these experiments as a whole, it is significant that the investigators who observed a favourable effect of carbohydrate on nitrogen balance had collected the excreta immediately after changing the amount of carbohydrate in the diet, whereas all the negative findings were obtained in experiments in which the rats had been on the high-fat intake for a number of days before the study of nitrogen output was made. We have observed (table 1) that the increase in nitrogen output which follows substitution of fat for carbohydrate is quite transitory. It is thus not surprising that the result obtained by different investigators should be dependent on the length of time elapsing between substitution of fat for carbohydrate and the collection of excreta for nitrogen balance determinations.

The short duration of the disturbance in protein metabolism caused by substituting fat for carbohydrate is similar to the transitory increase in nitrogen output observed after carbohydrate has been transferred from a protein-containing meal to a protein-free meal (Munro, '49). Both types of experiment involve removal of carbohydrate fed along with the dietary protein, in one case by substituting fat for it, in the other case by feeding the carbohydrate separately from the

protein of the diet. In the case of exchange of fat for carbohydrate, the importance of the presence of protein in the same meal is shown by the finding that substitution has very little effect on nitrogen balance if carried out apart from the protein-containing meals of the diet (table 2). The increased nitrogen output following substitution is thus mainly dependent on an interaction between the protein of the diet and the other nutrients fed in the same meal. The presence and amount of fat in the protein-containing meal plays no part in the change in nitrogen balance, since it is immaterial whether fat eaten in place of carbohydrate is taken along with the dietary protein or separately from it (table 3). This finding is in agreement with a previous observation (Munro, '49) that nitrogen balance is unaffected by the transfer of fat from protein-containing to protein-free meals in the same diet. In consequence, the increased nitrogen output which follows substitution of fat for carbohydrate in a protein-containing meal can be attributed solely to withdrawal of carbohydrate from the meal. The magnitude of the effect of carbohydrate withdrawal is proportional to the amount of carbohydrate involved (fig. 1). The significance of this action of carbohydrate in protein-containing meals has been discussed elsewhere (Munro, '51).

On the question of whether nitrogen balance is in any way influenced by substituting fat for carbohydrate in protein-free meals, our experimental findings are equivocal (table 2). It is, however, well known that carbohydrate is superior to fat as a sparer of endogenous protein metabolism, since the feeding of an exclusively fat diet does not lower nitrogen output below the fasting level, whereas the feeding of carbohydrate does so (see review by Munro, '51). It would therefore seem probable that both "endogenous" and "exogenous" protein metabolism are affected by substituting fat for carbohydrate, but that the effect is much more pronounced in the latter case and is then dependent on the carbohydrate being fed with the dietary protein.

SUMMARY

1. When fat is exchanged isocalorically for carbohydrate in the diet of the rat, urinary nitrogen output increases for a few days and then returns to its former level. The transitory nature of the response may account for the failure of some investigators to observe any difference in nitrogen balance between groups of rats receiving equicaloric diets of differing fat and carbohydrate content.

2. Exchange of fat for carbohydrate in protein-containing meals leads to a much greater increase in nitrogen output than occurs after a similar exchange in meals devoid of protein. Thus the main consequence of replacing the carbohydrate of a mixed diet by fat is a deterioration in the utilization of dietary protein.

3. The change in nitrogen balance resulting from substitution of fat for carbohydrate is of similar magnitude, whether the fat is fed with the protein of the diet or apart from it. This indicates that the phenomenon is essentially due to removal of carbohydrate from the protein-containing meal and not to an adverse effect of feeding fat with protein.

4. There is a linear relationship between the amount of carbohydrate fed along with the protein of the diet and the nitrogen balance of the rat.

ACKNOWLEDGMENTS

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EFFECT OF PROLONGED ANTIBIOTIC ADMINISTRATION ON THE WEIGHT OF HEALTHY YOUNG MALES [1]

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ONE FIGURE

(Received for publication November 29, 1954)

That the administration of antibiotic prophylaxis during periods of epidemic streptococcal infections markedly reduces the number of infections, complications, and sequelae in the population has been clearly demonstrated (Gezon et al., '53; Naval Medical Research Unit No. 4, '53 — '54; Seal et al., '54; Wannamaker et al., '53). Little information is available, however, on other less specific effects of such widespread antibiotic administration to large groups of people. In the course of a study in this laboratory on the effects of antibiotic prophylaxis on the immune response in humans (Haight et al., in preparation), it was recognized that there was a unique opportunity to observe the influence of prolonged antibiotic administration on the weight behavior of healthy young males. Definite nutritional effects of the antibiotics have been noted for some time (Jukes and Williams, '53) and indeed this information has been applied practically in the feeding of livestock. To date there have been few studies of the nutritional effects in humans, and what little evidence is available is largely concerned with young children. The present report

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seems of interest, therefore, because of the results obtained in a controlled observation of several hundred young American males.

POPULATIONS, MATERIALS AND METHODS.

Initially the height and weight of the Navy recruits were recorded only as corollary observations in the overall study in progress. When it became apparent that certain results were of significance, a second study was instituted as a further control on the first experiment. The details of these two experiments follow. The first group consisted of 6 companies of Navy recruits, of 55 men each, who were selected immediately upon their arrival and formation on the station. These 330 recruits were interviewed for details relative to a history of infection by or immunization for diphtheria and typhoid fever. Each company was then divided at random into three treatment groups, so that each group contained approximately the same proportion of men with a presumptive positive or negative history for past exposure to these two antigens. At reveille each morning for 7 weeks every recruit received one yellow capsule which contained one of the following preparations: (1) 250 mg of chlortetracycline (Aureomycin), (2) 100,000 units of buffered procaine penicillin, or (3) a placebo (calcium carbonate). These were specially prepared to appear identical and were supplied by Dr. Stanton M. Hardy of Lederle Laboratories, Pearl River, New York. The various capsules were additionally coded by labeling the supply bottles for each company with the letters A, B, or C. For example, preparation A in a third of one of the companies might thus be the same as preparation B or C in another company. Although each man received the same preparation for the 7 weeks of administration, it was thus impossible for the observers to suspect the contents of any capsule by similarities in group behavior or untoward reactions in the same third of the various companies. Only when all the data had been tabulated was the code removed from the safe. Complete data were available on 310 men at the completion of this first experiment.

Following the initial interview, the nude weight in pounds and the height in inches to the nearest quarter pound or inch were recorded for all subjects. Capsule administration was instituted the following morning at reveille, and the test antigens were given one week later. These antigens were the purified O and Vi antigens of *S. typhosa* and combined alum-precipitated diphtheria and tetanus toxoid. Blood specimens were obtained at the time of the initial interview and also when subsequent measurements of the weight and height were made after 4 weeks and 7 weeks of "treatment." Care was exercised to make all measurements under identical conditions and at the same time of day. From the age, height and weight, the relative body weight (RBW) could be calculated, this being defined as the actual weight expressed as a percentage of the Medico-actuarial standard weight for the age and height (Association of Life Insurance Medical Directors and Actuarial Society of America, '12). Following completion of the study the individual and group changes in actual and relative body weights were tabulated and subjected to statistical analysis. Only a representative portion of these calculations will be presented here.

When it was noted that there were some group to group differences in weight behavior in this first study, and before the capsule code was known to the authors, a second experiment was instituted. The selection of subjects was the same as before, as were the interview and random assignment to one of three "treatment" groups. These men did not, however, receive any capsules or any test antigens. As before, the weight and height were recorded initially and again 4 and 7 weeks later, using the same precautions to maintain nearly identical conditions and timing of these measurements. Calculations were made for the relative body weights of these recruits, and the data were analyzed for statistical significance. Complete information was available on 242 subjects in this simulated experiment.

RESULTS

Some of the pertinent observations made during the period of antibiotic prophylaxis are recorded in table 1. Although minor group to group variations occurred, it will be noted that the average age, height, and weight on entry into training were essentially the same for all treatment groups. After 4 weeks of capsule administration there was an average gain

TABLE 1

Effect of daily antibiotic prophylaxis on weight of Navy recruits during a 7-week period

OBSERVATION	TREATMENT GROUP [1]			TOTAL GROUP
	Aureomycin	Penicillin	Placebo	
Number of men	102	105	103	310
Average age, years	18.5	18.6	18.4	18.5
Average height, inches	68.8	68.3	68.7	68.6
Average first weight, lbs.[2]	149.8	147.1	151.6	149.5
Average second weight, lbs.	153.4	150.2	153.6	152.4
Average third weight, lbs.	154.6	151.2	154.3	153.4
Average total gain, lbs.	4.8	4.1	2.7	3.9
Average first RBW, % [3]	103.6	103.2	105.3	104.0
Average second RBW, %	106.1	105.4	106.7	106.1
Average third RBW, %	107.0	106.1	107.3	106.8
Average total gain RBW, %	3.4	2.9	2.0	2.8
Poor appetite, number of men	3	5	5	13
Lower intestinal complaints, number	2	1	3	6

[1] See text for details of capsule administration.

[2] Weights were obtained initially and again 4 weeks and 7 weeks later.

[3] RBW = Relative body weight: Actual weight expressed as a percentage of 1912 Medico-actuarial standard weight for age and height.

in weight in all groups, but those subjects who had received either of the antibiotic-containing capsules had about equal but greater gains than those who received the placebos. The same trend was evident in the total change observed after 7 weeks (Aureomycin group, 4.8 pounds; penicillin group, 4.1 pounds; placebo group, 2.7 pounds). The calculations of relative body weight (RBW) are similarly recorded in table 1. It will be recalled that this computation is the actual weight ex-

pressed as a percentage of the 1912 Medico-actuarial standard weight for the age and height. Essentially the same degree of change is observed with the per cent gain in RBW as with the actual weight gain in pounds. Although all "treatment" groups showed an average gain in RBW during the 7 weeks of capsule administration, there was again a distinct difference between the results in the groups receiving either Aureomycin (3.4%) or penicillin (2.9%) and the group receiving the

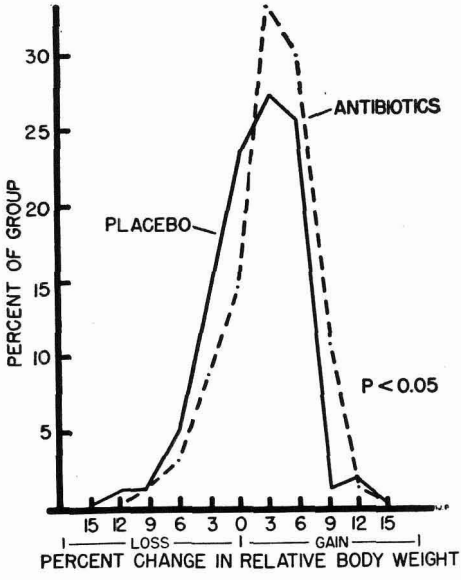

Fig. 1 Comparison of losses and gains in relative body weight in Navy recruits receiving antibiotics (Aureomycin or penicillin) or placebos for 7 weeks.

placebos (2.0%). The differences between chlortetracycline and penicillin in either actual or RBW weight changes are not statistically significant, but the difference between these two groups singly or combined and the placebo group appears quite significant by the Chi-square test ($p<0.05$).

It is not feasible to include in the present report all the observations and calculations that were made, for example, the distribution of the individual weight gains or losses of these 310 men. In figure 1, however, are plotted the percentage

of men in the two antibiotic groups combined and in the placebo group who gained or lost in relative body weight, and the percentage of this change. The antibiotic groups are combined here for purposes of easy visualization of the graph. Separate plotting of these data would not be particularly different. The distribution curve for the placebo group is consistently to the left, indicating more losses and fewer gains in RBW, and the peak is lower for the percentage of men in the placebo group who gained. On statistical analysis these differences appear to be significant and would be attributable to chance alone less than 5% of the time ($p<0.05$). The mean differences for all companies are implicit in table 1, but it should be pointed out that the group differences were consistent from company to company.

To determine if the observed differences were possibly due to other factors, each man was carefully interviewed at the conclusion of the study concerning his dietary habits before coming to this station, his current dietary habits, appetite, and any gastrointestinal complaints. Because voluntary dieting, for example, when coupled with the process of recruit basic training, might alter the calculations, this factor had to be excluded as a cause of the differences. Few subjects admitted to having tried purposely to lose weight, whereas most had either made no particular efforts or had tried to gain weight. Furthermore, as seen in table 1, very few of these men complained of poor appetites, and the number of lower gastrointestinal complaints was small, with no marked group to group differences. Because illness also is an important factor in weight behavior, this was carefully checked. The numbers of complaints, types of complaints, sick call visits, and hospitalizations were roughly the same in all three groups.

The second experiment was conducted along similar lines, and the major observations are shown in table 2. The two populations involved in these two studies were quite comparable, as judged by the same criteria on which the individual sub-groups were compared, namely, age, height, and weight

on entry into training. The three randomized groups in the simulated "antibiotic prophylaxis" programs were likewise composed of approximately equal elements. The initial gain in weight, as well as the total gain, whether actual or relative body weight, was somewhat less than in the first experiment. Even at a glance, however, it can be seen that there are no major differences between the three groups by either method of comparison. The notion was entertained that the dif-

TABLE 2

Observations on weight behavior of Navy recruits in a simulated antibiotic prophylaxis program

OBSERVATION	SIMULATED GROUP [1]			TOTAL GROUP
	I	II	III	
Number of men	84	77	81	242
Average age, years	18.6	18.8	18.7	18.7
Average height, inches	68.9	68.6	68.3	68.6
Average first weight, lbs.[2]	152.7	151.1	146.6	150.1
Average second weight, lbs.	153.6	150.7	146.7	150.3
Average third weight, lbs.	153.4	150.9	147.8	150.7
Average total gain, lbs.	0.7	— 0.2	1.2	0.6
Average first RBW, % [3]	105.0	104.8	102.6	104.1
Average second RBW, %	105.7	104.4	102.7	104.3
Average third RBW, %	105.6	104.7	103.5	104.6
Average total gain RBW, %	0.6	— 0.1	0.9	0.5

[1] No antibiotics were given, but recruits were randomized into three groups as in previous study (table 1).

[2] Weights were obtained initially and again 4 weeks and 7 weeks later.

[3] RBW = Relative body weight: Actual weight expressed as a percentage of 1912 Medico-actuarial standard weight for age and height.

ference between — 0.2 pounds in group II and + 1.2 pounds in group III might be significant and in the opposite direction of the results in the first experiment. On analysis this did not appear to be significant in groups of this size and distribution. The other minor group to group differences were similarly found to be insignificant (group I, 0.7 lb. or 0.6%; group II, —0.2 lb. or — 0.1%; group III, 1.2 lb. or 0.9%).

Although the detailed results of the corollary study on the effect of the period of antibiotic prophylaxis on the immune

response will be presented elsewhere, it is perhaps germane to this report to mention them here briefly. With each "treatment" group containing roughly the same number of men with some degree of antibody titer to these antigens and others with no antibody, it was possible to compare the results fairly well. It appears that whether one considers the primary immune response or the secondary immune responses, such as from a booster dose of diphtheria toxoid, there is no appreciable difference in the antibody titers obtained whether the subjects are receiving an antibiotic or a placebo. These data can probably be interpreted as representative of the whole general pattern of antibody response, since the antigens used were selected as prototypes of the three main antigenic types, namely, an acidic polymer, a lipopolysaccharide, and a protein antigen.

DISCUSSION

The unexpected finding of significant differences in weight gain in groups of Navy recruits during a period of 7 weeks of antibiotic prophylaxis is extremely interesting in view of the well documented specific nutritional effects of small doses of antibiotics in livestock and in certain instances in premature infants and undernourished children (Jukes and Williams, '53). Although the first experiment contained a means for estimating potential error, it seemed worth repeating. It was for this purpose that a second group of recruits was studied in a simulated program, to determine the amount of group to group variation by chance alone.

The failure of the entire population in the second experiment to gain as much as the placebo group in the first experiment is not surprising and should not invalidate these data. It has been pointed out previously that Navy recruits tend to gain more during the winter than during the spring and summer (Gibbons et al., '53). The first study was conducted during the winter and the second during the late spring and early summer. It would, therefore, appear that the additional controlled experiment has failed to provide evidence that a

natural group to group variation in weight gain would alone account for the observed differences. Moreover, such factors as dietary habits, appetite, illnesses, and living habits were investigated and could not be related to these observations.

If the findings are valid, then one is entitled to some speculation. It is interesting to wonder about the mechanism whereby small doses of either a broad-spectrum antibiotic (Aureomycin) or penicillin might affect the growth of healthy young American males who are presumably in a slow final phase of maturation. The theories which have been advanced for children and animals would not necessarily apply in this instance. Perhaps the observation of the marked metabolic effects of *large* doses of an antibiotic (Gabuzda et al., '52) may by deduction offer some support to the notion that very small doses critically upset certain delicate balances of homeostasis just as much, but with the reverse end results. No data are available, but further experimentation along these lines is certainly needed. It would be interesting, for example, to study a large, stable population as in the present study but over a prolonged period of administration rather than only 7 weeks. This approach would compensate for the cyclic changes in weight behavior that are attributed to the seasons.

The concept of relative body weight was introduced into the manipulation of these weights to reduce to a minimum the influence on the average changes that would be brought about, for example, by an obese recruit who purposely lost weight. The loss in pounds would be far greater than the percentage of his RBW. In fact, however, the changes were so definite that the computation of RBW did not alter the interpretations. When separate calculations were made for the individual changes in each group in both experiments, grouping those with an initial RBW of less than 95%, those between 95 and 115%, and those greater than 115%, the results were not materially different. The heavier men, of course, tended to gain less as an overall average, but there were no important group to group differences.

SUMMARY

Six companies of Navy recruits were distributed at random into one of three "treatment" groups, and all subjects received once daily for 7 weeks one of the following identical-appearing preparations: (1) Aureomycin, 250 mg; (2) penicillin, buffered, oral, procaine, 100,000 units; or (3) a placebo (calcium carbonate). Observations on the nude weight and height were made initially and again 4 and 7 weeks later. The individual and group weight changes were then calculated, both in pounds and in relative body weight (actual weight expressed as a per cent of the standard weight for age and height). Final data were available on 310 men.

Both the initial changes after 4 weeks and the total changes after 7 weeks revealed a distinct difference between the placebo group and the antibiotic groups. The Aureomycin and penicillin groups were not significantly different. This difference in weight gain between the placebo group (2.7 pounds) and the antibiotic groups (average 4.5 pounds) was reflected in roughly an equal degree of difference in relative body weight gain, and this would be expected due to chance alone less than 5% of the time ($p<0.05$).

A similar study was conducted but without administering any capsules to determine normal group to group variation in this population. No significant differences were encountered in this simulated experiment. These studies were exploratory and suggest further avenues of approach in evaluating the specific nutritional effects of antibiotics in humans.

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METABOLISM OF FOLIC ACID AND CITROVORUM FACTOR BY HUMAN SUBJECTS [1]

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ONE FIGURE

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INTRODUCTION

From the evidence of various *in vitro* and *in vivo* experiments, folic acid is generally regarded as a pro-vitamin and citrovorum factor its active form, possibly the predominant form in all living tissue.[4] Since the urinary excretion of citrovorum factor has been shown to be influenced by the ingestion both of pteroylglutamic acid and of ascorbic acid, the levels of intake of these two factors were varied for human subjects in the present experiments, in the expectation that patterns of excretion might suggest significant relationships between folic acid and citrovorum factor in metabolism.

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[4] Recent reviews include Baumann ('53); Bessey et al. ('53); Editorial Staff and Advisory Board, Nutrition Reviews ('53).

EXPERIMENTAL

Modifications of published turbidimetric microbiological methods (Luckey et al., '45; Sauberlich and Baumann, '49) were used to determine urinary folic acid activity (FA) measured against a pure pteroylglutamic acid (PGA) standard, and citrovorum factor activity (CF) against reticulogen (study I) and leucovorin (study II); the respective test organisms were *Streptococcus faecalis* and *Leuconostoc citrovorum*. In study I, commercially prepared acid-hydrolyzed casein was used in the culture medium for the folic acid assay, laboratory-prepared hydrolysate, for citrovorum factor; in study II, commercial media (Difco) were used for the two assays.

Aliquots of 24-hour urinary collections, adjusted to pH 6.8, were frozen until assayed in study I, while the samples from study II were assayed at once. The food composite (study II) was incubated with hog kidney extract, prepared and frozen within a few hours after removal of the organ from the animal.

Details of study I [5]

Five women and 10 men, graduate students, served as subjects. The diets, self-administered, were qualitatively restricted in regard both to the exclusion of rich sources of folic acid and to the use of 8 oz. of orange juice as the only important source of ascorbic acid throughout the various periods. Urinary excretions of total folic acid and citrovorum factor were determined at dietary levels of folic acid and on supplementation with 1 mg and 5 mg, respectively, of PGA.

Details of study II [6]

Four young women, graduate students, served as subjects for the 22 consecutive days of the study. The constant weighed diet comprised the following foods with portions listed in grams: apple juice, 100; canned peaches, 117; canned pears,

[5] See footnote 3, page 163.
[6] See footnote 2, page 163.

117; canned beets, 103; canned carrots, 117; processed cheese, 25; ground beef, 135; vitamin D-enriched milk, 732; butter, 35; enriched white bread, 70; cornmeal mush, 240; steamed rice, 135; rice flakes, 30; brown sugar, 28; and syrup, 40. Individual caloric needs were met by additions of butter and sucrose. Thiamine hydrochloride, 0.6 mg, and nicotinic acid, 2 mg, were included as part of the basal diet to bring its contents to the level of the recommended allowances of the Food and Nutrition Board of the National Research Council ('48) except for the test substances. The assayed content [7] of folic acid, 65 μg, fell well within the calculated range (Toepfer et al., '51; Tepley et al., '53) of the constituent foods. The ascorbic acid content of the diet was restricted to roughly 25 mg, a level which, though low, was nevertheless in excess of the 20 mg intake found by a committee of the British Medical Research Council (Peters, '48) to be adequate for curing scurvy and permitting some tissue storage. The sequence of periods and dosages is given in the legend of figure 1.

RESULTS

Studies I and II were in agreement with published reports [8] that citrovorum factor accounts for a minor part of the total urinary folic acid activity for human subjects on various diets. The ratio of the former factor to the latter averaged 1:4 for the 4 subjects on a quantitative weighed diet (study II) as well as for the 15 subjects on restricted self-administered diets (study I) and the 14 subjects of Register and Sarett ('51) on self-selected diets.[9] This agreement among

[7] This value obtained on a composite of foods from the same sources as those in study II, in a comparable experiment, replaces the preliminary food assay in Fed. Proc., *13:* 525 (1954). From a thesis offered by Martha McMillan in partial fulfillment of the requirements for the degree of Master of Science, University of Wisconsin, 1954. Present address: Nutrition Research Laboratory, University of Illinois, Urbana.

[8] See footnote 4, page 163.

[9] The average value given for citrovorum factor by Register and Sarett was multiplied by the presently accepted factor 0.5 to compensate for the racemization of the commercial synthetic standard, leucovorin.

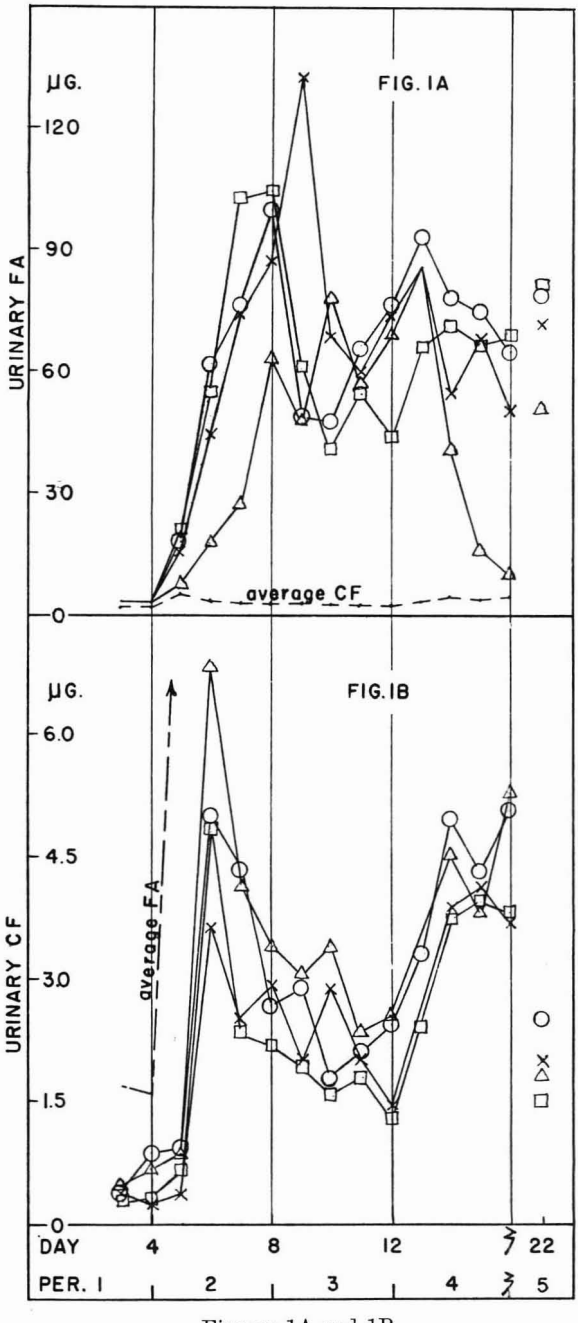
the three studies strengthens the suggestion of Register and Sarett ('51) that another folic acid metabolite is implied by the low ratio.

When graded doses of PGA were administered (study I) the excretion of citrovorum factor on the first day of dosage increased roughly in proportion to the increments in intake, in agreement with an earlier observation (Sauberlich, '49). The augmentation in the excretion of folic acid, however, progressed at a greater rate than this, the gap widening between the two urinary products at the higher intakes of PGA. Listed below is a series of three average values representing the following experimental periods in the same order: diet without supplement; dose of 1 mg PGA; and dose of 5 mg PGA. For the 5 subjects for whom these data were available, the total folic acid excretions averaged; 3.9 μg, 61 μg, and 1915 μg; citrovorum factor [10] excretions, 0.7 μg, 1.5 μg, and 5.0 μg; and CF : FA ratio, 1 : 5, 1 : 40, and 1 : 380, respectively.

When a 1 mg dose of PGA was administered (study II) the average folic acid excretion rose sharply from its base line of 1.7 μg on the weighed diet alone, to a maximum of 89 μg in 4 days (fig. 1a). After this, the excretion values for the individual subjects fluctuated rather widely throughout the succeeding 16 days of the PGA supplementation without clear relationship to the varying intakes of ascorbic acid; the sharp drop in the terminal period for one subject is unexplained. In a 9-day period on daily doses of 2 mg PGA, the maximum difference in urinary folic acid between any two consecutive days after the initial augmentation ranged

Fig. 1A and 1B Individual and average urinary excretions of FA and CF plotted on two different scales for subjects: RB, ○; DJ, △; KC, □; and MM, ×. On day 13, FA values for MM and DJ were plotted as an average, due to mixture of urines. Supplements of weighed diet: period 1, none; period 2, 1 mg PGA; periods 3 and 5, 1 mg PGA plus 80 mg ascorbic acid; period 4, 1 mg PGA plus 750 mg ascorbic acid. On days 17 through 20 (omitted) diet was not weighed but contained similar amounts of supplements as in period 3 and the two days of period 5 on weighed diet.

[10] These values were calculated from the conversion figures then available.



Figures 1A and 1B

from 200 to 370 μg for the 4 subjects of Murphy [11]; the daily average excretion for the group and period was 540 μg. The self-administered diets of her study were qualitatively controlled in regard to the avoidance of rich sources of folic acid and the maintenance of ascorbic acid from food close to a 100 mg level.

In contrast to folic acid, the values for urinary citrovorum factor (study II) showed a general uniformity from subject to subject, and formed a pattern of excretion for the different periods not reported previously, to the knowledge of the authors. Most notable was the fact that the maximum peak of excretion of citrovorum factor was reached in two days instead of the 4 required for folic acid so that, in the following two days, the level of citrovorum factor in the urine was falling off sharply at a time when the average folic acid excretion was doubling. These relationships were substantially confirmed in a somewhat similar study in this laboratory.[12]

In study II, the decline in the output of the citrovorum factor continued for 4 to 6 days, little influenced if at all, by the addition of 80 mg of ascorbic acid, but was sharply reversed when an unphysiologically large dose (750 mg) of ascorbic acid replaced this; each subject at least doubled her output of citrovorum factor in the 4 days on the higher dose of ascorbic acid. By the 6th day after the termination of this high dose and the resumption of the 80 mg dose, the excretion of citrovorum factor had returned to the level previously noted on the latter dose (periods 3 and 5, fig. 1B).

DISCUSSION

The relationships between folic acid, citrovorum factor and ascorbic acid are obviously complex. The reason for the transitory nature of the augmentation in urinary output of citrovorum factor when 1 mg of PGA supplemented the basal diet in period 2 (study II) is obscure. A tenable hypothesis

[11] Unpublished data from research carried out by Judith Murphy in 1952 in this laboratory.

[12] See footnote 5, page 164.

might be that something favoring the initial high rate of conversion of PGA to citrovorum factor was rather rapidly diminished by the accelerated reaction; this may have been the tissue supply of reducing substances. The decline in urinary citrovorum factor in periods 2 and 3 of study II may even have been an indication of a partial shift in the conversion of PGA away from citrovorum factor to some other metabolite. However, the known role of ascorbic acid in the conversion has some limitations; a greater average urinary excretion of citrovorum factor than that noted in study II on doses of 1 mg of PGA and 750 mg of ascorbic acid was elicited in study I on an ordinary intake of ascorbic acid (about 80 mg) when the dose of PGA was raised to 5 mg. Stimulation of the FA to CF conversion mechanism by large intakes of ascorbic acid appears to have been demonstrated when the level of PGA supplementation has been high, but similar intakes of this reducing agent have not been shown to be effective in increasing the excretion of citrovorum factor when the dosage of PGA has been 0.5 mg or less (Welch, '50).

The formation of a folic acid metabolite other than citrovorum factor has been postulated by Dietrich et al. ('52) to account for the effectiveness of injected folic acid for the methylation of nicotinamide in the rat in contrast to the lack of effect of injected leucovorin, under the conditions of their experiment. It is notable that Silverman and Keresztesy ('53) have reported the presence of a metabolite of folic acid in a liver autolysate, which they have shown is not measured by the assay for citrovorum factor; it appears to be an intermediate in the conversion of the bound form of folic acid to citrovorum factor. On the other hand, the folic acid-like substances (FS) reported to be present in human blood by Toennies et al. ('54) and Usdin ('54) are stated by them to be characteristic of individuals and not responsive to the intake of food. It has not been determined whether or not these substances are present in the urine.[13]

[13] Statement by Usdin ('54) in discussion of his paper.

SUMMARY

Citrovorum factor, on the average, accounted for only one-fourth of the total folic acid activity in the urines of 4 human subjects on a weighed diet containing approximately 25 mg of ascorbic acid and of 15 subjects on restricted self-administered diets containing approximately 100 mg of ascorbic acid. When folic acid had reached its maximum excretion 4 days after the initiation of a 1 mg daily dose of pteroylglutamic acid, citrovorum factor accounted for only one thirty-second of the folic acid activity of the urine.

Citrovorum factor showed a pronounced but transitory increase in the urine in response to the dose of pteroylglutamic acid; a sharp decline started before folic acid had reached its maximum level of excretion. An excessive daily dose of ascorbic acid (750 mg) superimposed on the dose of pteroylglutamic acid restored the excretion of citrovorum factor to its previous high level. Various hypotheses to explain these relationships are discussed. The production of a folic acid metabolite not measured by *Leuconostoc citrovorum* may possibly have been favored in metabolism at the expense of citrovorum factor during certain periods of the study.

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