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WALTER CHARLES RUSSELL

(1892 – 1954)

WALTER CHARLES RUSSELL

WALTER CHARLES RUSSELL

(October 1, 1892 – March 10, 1954)

Walter Charles Russell's associates may remember him best as a quietly persuasive man who, between meticulous puffs at his pipe, ranged in his scientific thinking all the way from the ultra-conservative to the realm of science fiction. His comments, often punctuated with dry humor, were concise and straight to the point.

Walter C. Russell was born in Bellaire, Ohio, on October 1, 1892. His father was a railroad man and young Russell developed an interest in railroading which stayed with him all his life. A reminder of this was his father's solid gold railroad watch which he inherited and carried. Visitors, particularly graduate students, were usually reminded early in the interview that time was precious when the watch was pulled out with the remark, "I have to watch my time."

Young Russell, or Barney as he came to be known to his close associates, learned early in life that one must work and work hard to get ahead. Bellaire was a steel town and he spent several summers at a full-time job in the mills. This habit of hard work was a legacy which remained with him the rest of his life. After high school, he attended Ohio Wesleyan University where he received the B.S. degree in 1914. He taught chemistry and physics in Chillicothe, Ohio, High School for a year and then returned to Ohio Wesleyan as an instructor in chemistry from 1915 to 1917.

When the United States entered World War I he enlisted as a private in the Medical Department of the U. S. Army. He rose rapidly from the ranks and 4 months later, in November 1917, was commissioned a First Lieutenant in the Sanitary Corps. In February 1919, he was promoted to Captain. Lieutenant-Captain Russell spent 15 months in France in water

supply and troop sanitation service. Here one of the many "firsts" of his career involved the use on uniforms of live steam to kill off the famous World War I "cootie" or body louse. Another first was his initial publication, an article on the Chlorination of Water Supplies. During the war he was in great demand for parties to go back to Paris on leave, for his fellow officers knew he would stay sober and would shepherd the rest back to their units on time. At the war's end and before returning to the United States, Russell spent three months as a student at the University of Paris and the Pasteur Institute under the auspices of the Army Educational Service.

In 1919 Russell started his graduate work, becoming a teaching fellow at Harvard University under Otto Folin, but financial difficulties forced him to stop at the end of a year. He spent the years 1920 to 1923 at Syracuse University as an Instructor in Chemistry. One of the important happenings of this era was that he met Mildred Stephens, whom he married in 1923. They have one daughter, Ruth. From Syracuse he went to the University of Chicago where he held the Swift Fellowship under Stieglitz. He completed his work for the doctorate in 1925, with a research problem in the general field of cellulose chemistry. Three publications resulted from this work.

In 1925 he accepted the position as head of the Department of Agricultural Biochemistry at the College of Agriculture, Rutgers University, with the title of assistant professor. Promotions came rapidly to associate professor in 1929 and full professor in 1931.

The department was completely new in 1925, designed to utilize the newly appropriated Federal Purnell funds which were available for research in home economics and nutrition. There was no staff, no laboratory, no office, no equipment, nothing. He worked in a borrowed laboratory, with borrowed equipment, while the top floor of the Dairy Building, really the attic, was being outfitted for his needs. These quarters tended to be hot in the summer and cold in the winter and with driving rainstorms the water literally came in through the side of the

brick wall until it was given a water-proofing treatment. Yet it was Dr. Russell's and he had the ability to endow everything he touched with a little extra dignity and worth.

For several years afterward, he shared a secretary with one department and a utility man with another. When he first arrived on campus he told Dean Lipman he would like to do some teaching some day as well as research. It was barely a week later when graduate students came around wanting to sign up for his course. Many graduate students who got their first taste of biochemistry in his courses have since made significant contributions in this field.

Dr. Russell was strictly a self-made nutritionist and the fact that his early training was largely that of a chemist encouraged him to look at nutrition from the molecular level. In those early days that viewpoint was not as common as it is now. He chose first to work in the field of the antirachitic vitamin which was known better then as an effect due to sunshine rather than as an organic entity. Since he was in an agricultural college, he worked with chickens. He measured the amount of ultraviolet in New Brunswick sunshine, the passage of these wavelengths through glass and glass substitutes, their effect on bone development in chicks, the duration of such effects, the effect of sunlight and vitamin D on blood calcium and egg production, different sources of calcium for bone formation, and other related topics. Working to some extent with Hess of Columbia who was a pioneer in the field, Russell, too, became one of the authorities. Under a fellowship from the du Pont Company he carried out much of the preliminary work on the relative values of irradiated ergosterol and the cod liver oil type of vitamin D, which later led du Pont to the discovery of means for producing irradiated 7-dehydrocholesterol, just at a time when World War II cut off the supply of fish liver oil.

Dr. Russell also served for a number of years as associate referee on the assaying of vitamin D milks for the Association of Official Agricultural Chemists. Aside from a few publications on methodology, no startling results were obtained in

this field. Perhaps the most interesting discovery was made with the help of his associate, Dr. Adolph Zimmerli, who found that the isolation of the vitamin from vitamin D milk in a very concentrated form resulted in a loss of potency. For a long time the method was blamed, and there are fond memories of the time Russell and Zimmerli set up a whole 12-foot deskful of apparatus designed to saponify, extract, concentrate, resaponify, etc. a quart of milk under an atmosphere of nitrogen. It was one of the early attempts at automation, and it worked, except that it required an attendant with quick reflexes. Net results were that something in milk was adding to the efficacy of vitamin D rather than there being any loss on concentration. Work was also done on possible chemical methods for vitamin determination but the method devised, a modification of the antimony trichloride reagent, was subject to too much interference from various sterols and did not prove to be practical.

Dr. Russell started his rat colony in 1926 with a dozen animals from Wistar Institute. The colony was under the care of the wife of one of the farm foremen. She was not a trained scientist in any sense of the word but she was alert, quick to learn and loved animals. As a colony diet they used Sherman's diet B, one-third whole milk powder, two-thirds ground whole wheat and a little salt. According to Sherman this diet was adequate, but Mrs. Howard found quite the opposite results, especially as regards the raising of any young. Casting about for a possible supplement, it was found that raw beef gave marked improvement and that meat scraps were equally good. This diet of 30 parts whole milk, 60 parts wheat, 10 parts (sometimes 12) meat scraps, and 1.2 parts sodium chloride, has given excellent colony growth and rearing of young for over 30 years. Had Russell only thought of the phrase "animal protein factor" when he published in 1932, his paper might have become a classic.

Another early project was measuring the effect of the curing process on the carotene content of hays. Then, as now, the Walker-Gordon Laboratories in nearby Plainsboro was one

of the leaders in promoting progressive practices in dairying and one of these was a long tunnel-like home-made hay drier, augmented later by a commercial rotary drier. An early paper in 1934, when animal assays were being used rather than the chemical method, has been widely quoted, and a more comprehensive paper in 1938 added to the picture. Later when various types of silage began to replace hay, these too were assayed for carotene, before and after processing and using various ensiling additives.

Vitamin A was investigated rather thoroughly under Russell's leadership, with other reports showing the requirements of growing birds, of laying hens, and of turkey poults. The percentage of intake transferred by the cow from the feed into the milk and by the hen into the egg was measured, the bird showing much the greater efficiency. He observed the effect of the fat content of the diet on the absorption of carotene and vitamin A by the bird and, going over into another species, studied the picture of vitamin A deficiency in the dog. For a time he was active in the field of dog nutrition, serving on a committee of the American Veterinary Medical Association to test the nutritive value of dog foods.

The work on fat-soluble vitamins led to a consideration of the role of fat *per se*. Thus he showed that birds could exist quite normally on diets extremely low in fat, synthesizing most of the body and egg fat. However, when the diets were made high in fat, poorer performance resulted. Had Russell and his associates realized the imbalance that was created in the ratio of energy to other nutrients, we might have had high energy poultry rations much sooner.

An ambitious project he directed was in the use of the pig as an experimental animal which would be a near approach to the human. Or as Russell used to put it, "the human vs. the domestic pig." Pyridoxine and pantothenic acid deficiencies were studied and the experimental animals were examined by dermatologists, brain specialists, heart specialists, and many other medical men. The pigs were patients, though at times they seemed to have little patience with their physi-

cians. The fact that very few positive findings were reported only shows that these deficiencies had few obvious manifestations.

There were other studies, such as the balance between amino acid intake and deposition in the chick, purified amino acid diets for rats, protein quality of legumes, and the folic acid requirement of turkey poults. Dr. Russell published with his associates some 70 research papers and a half dozen reviews but his work covered a wide range of subjects. Had he not been so occupied with other responsibilities, the total would have been greater. It seemed almost as though he became bored with a subject when too much was known about it. As someone once said, "I enjoy Russell's papers. There's always something original about them."

In athletic parlance they often refer to a "take-charge guy." Russell was that type. Although there was nothing obtrusive about it, in most of the groups he was in, he usually was the one who ended up suggesting what the others should do. In his own department he was automatically consulted on all decisions of any importance. It wasn't that he required it in so many words, it just was the thing to do. And although he might suggest work to others, you knew that he was always doing plenty himself. As one colleague observed, "Russell must enjoy being on these committees or he wouldn't do such a good job on them."

As a consequence of his capacity for detail and organization, Dr. Russell was chosen in 1935 as the first Executive Secretary of the Graduate Faculty, a title which was changed to Dean in 1952 when the Graduate Faculty became the Graduate School of Rutgers University. "After I get things organized, probably in the next two or three months, it should only take me an hour a day, two or three times a week" he said. That time never came. Previously there had been no one with responsibility to whom problems could be taken. The faculty got along as best it could, each faculty member tending to handle his own problems, and a faculty committee settling the few big ones. Now the pent-up troubles came pouring in. Even

when they did not, Dr. Russell could foresee coming needs and take steps to meet them. He had two offices, one on each side of New Brunswick, and two secretaries as well as the staff of his department, and he kept them all busy.

A typical day might be something like this: stop in the Graduate Office, check the mail, leave transcribed dictation that he had recorded at home, possibly dictate more letters, a few phone calls and then go to the Agricultural College, arriving about 9:30 A.M. Check the mail there, lecture for an hour, sign letters dictated earlier, check with the staff on research projects and return to the Graduate Office about 11:30. Sign letters and go to the University Cafeteria for lunch in the Faculty Room. All kinds of weighty questions were settled there. Back to the Graduate Office at 1:30 for more mail, perhaps a committee meeting, a couple of students and a faculty member with special problems, work on a report for the President, consideration of material for a graduate bulletin and then possibly back to the Agricultural Biochemistry Department for a brief time.

These activities could not always keep a man busy so he managed to accumulate a few other duties. These included the following: Secretary, 1937, and Chairman, 1938, Division of Biological Chemistry, American Chemical Society; Chairman, Gibson Island Vitamin Conference 1941 and again 1943; Secretary, 1939 to 1943 and Chairman, 1952, Section on Graduate Work, Land-Grant College Association; Chairman, 1952, Council on Instruction, Land-Grant College Association; Consultant on graduate work, Board for Southern Regional Education, 1949–1950; member of United States Pharmacopeia Vitamin Advisory Board, 1949–1950; member of Division of Chemistry and Chemical Technology and later the Division of Biology and Agriculture, National Research Council, from 1942; member of Food and Nutrition Board, National Research Council; charter member of the American Institute of Nutrition and a member of its Council, 1950–1953; Editorial Board Journal of Nutrition, 1945–1949.

In between his many duties he was an enthusiastic hiker and active in the organization and program of the University Outing Club, serving as its president and on various committees. Another hobby seemed to be people, for he attended many meetings and made an attempt to meet all the people he could.

He was a member of the honarary societies Phi Beta Kappa, Phi Kappa Phi, Sigma Xi and Phi Lambda Upsilon and the social fraternities Alpha Chi Sigma and Delta Tau Delta. In 1947 he received the honorary degree of Doctor of Science from his Alma Mater, Ohio Wesleyan, an honor which meant a great deal to him. He was a member of the Presbyterian Church, serving as a deacon and elder.

Dr. Russell was prolific in ideas for research which he jotted down, with the date, on sheets from a scratch pad and filed. Many involved detailed analyses to correlate with a known deficiency or with some observed activity. Most would have required two or three years' work. They were big ideas. Although Dr. Russell was the picture of conservatism most of the time, on rare occasions he could go to the other extreme. One instance was the time when he wanted to see if there was any truth in the theory that growing root tips gave off some kind of beneficial emanation and he raised a cage of rats over trays of sprouting grains. It didn't work.

Around the laboratory Dr. Russell took his position very seriously. The graduate students were always Mister and the faculty Doctor. Yet at lunch and in social gatherings he was usually the life of the party. But one could always sense that innate reserve which made it easy to respect him but hard to become too intimate.

The laboratory was a place for work, not for play or idle chatter and Dr. Russell assumed that everyone else believed the same. At the same time he realized that not everyone could be perfect all the time and he apparently did not want to catch people in minor transgressions. Therefore, he invariably trod heavily on the steps of the last flight of stairs and jingled his keys loudly. It worked out well for everyone.

Perhaps an occasional individual thought he was getting by with something but Dr. Russell's powers of observation and deduction were keener than the student might suspect.

It was a shock to all of us when, in November of 1953, we learned that, over a weekend, he had suddenly had a major operation for a malignant growth in the sinus area under one eye. For several years he had had recurrent trouble with the growth of nasal polyps and had undergone occasional minor surgery. Whether this was the cause or not, no one knows, but the diagnosis of malignancy was made from such an operation and the decision made for the immediate extended operation. For a time it seemed that the operation would be a success and Dr. Russell was back on the job about a month later pulling together the loose ends which had accumulated during his absence. But such was not to be and 4 months after the original operation he succumbed. In his memory, the Walter C. Russell Memorial Fund was established by his many friends both at Rutgers and throughout the United States, the interest on the fund to be used to sponsor research or other appropriate activities. The first such occasion was a memorial lecture given April 21, 1958 by Dr. D. P. Cuthbertson, Director of the Rowett Research Institute of Aberdeen, Scotland.

Dr. Russell's passing can not be easily forgotten, for he put his mark on so many things. He established a strong program of teaching and research in his own department, he built a strong administrative foundation for the Graduate School, and his influence reached out far beyond Rutgers University. His death was a great personal loss to his many friends, a loss to the University as a whole, and a loss to the world of science.

M. WIGHT TAYLOR
JAMES B. ALLISON

HYDROXYANTHRANILIC ACID AS A SOURCE OF NIACIN IN THE DIETS OF THE CHICK, GUINEA PIG AND HAMSTER[1]

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(Received for publication July 14, 1958)

Numerous reports indicate that animal species differ in the dietary requirement for niacin. These observed differences in requirement probably result from the fact that tryptophan can substitute for this vitamin to varying degrees in different species (Krehl et al., '45a, b). The general pattern of the biosynthesis of nicotinic acid from tryptophan by *Neurospora crassa* has been well established. Present evidence also supports this pathway in animals.

3-Hydroxyanthranilic acid was identified as an intermediate in this conversion in studies with *Neurospora* (Mitchell et al., '48a; Bonner, '48). Its niacin replacing activity in the rat was approximately equal to that of tryptophan or about 2% of that of nicotinic acid (Mitchell et al., '48b; Henderson, '49). In this species 3-hydroxyanthranilic acid also produces a distinct increase in urinary excretion of niacin, N^1-methylnicotinamide (Albert et al., '48), and quinolinic acid (Henderson, '49).

While tryptophan has been tested as a substitute for niacin in a number of other species, 3-hydroxyanthranilic acid seems to have been examined only in the rat.

[1] Submitted in partial fulfillment of the requirement for the M.S. degree by R. H. Decker. Supported in part by a grant in aid from the National Science Foundation G–1810.

[2] Present address: Department of Biochemistry, Oklahoma State University, Stillwater.

In vitro studies, chiefly with rat tissues, have not led to complete duplication of the observations made with the intact rat. The oxidation of 3-hydroxyanthranilate by slices and cell-free preparations has failed to produce niacin. The products identified are an unstable intermediate (Bokman et al., '51), quinolinic (Henderson et al., '49), and picolinic acids (Mehler, '56). Suhadolnik et al. ('57) reported that liver filtrates from 10 mammalian species varied considerably in the rate at which they oxidized 3-hydroxyanthranilic acid. The rat liver contained less of the 3-hydroxyanthranilic acid oxidase than most of the species studied. The deviation of the results of *in vitro* studies with liver from the pattern observed in the whole animal suggests that each species must be studied *in vivo* as well.

The experiments reported here were done to determine whether 3-hydroxyanthranilate can substitute for niacin in the growth of three species of animals, the chick, the guinea pig and the hamster.

EXPERIMENTAL

The levels at which 3-hydroxyanthranilic acid was incorporated into the diets were established from results in the literature and from preliminary feeding studies as described below in the discussion.

Chicks. One-day-old male chicks from New Hampshire males X Columbian females were housed in electrically heated batteries with raised wire floors. Ten chicks were assigned to each of three experimental groups. Feed and water were supplied ad libitum, and the birds were weighed at weekly intervals during the course of the experimental period. Niacin and 3-hydroxyanthranilic acid were added to the niacin-free basal diet as a premix with sucrose in accordance with the experimental design shown in table 1.

Guinea pigs. Weanling, albino guinea pigs of mixed sexes were placed in individual cages with raised floors and given food and water ad libitum. Cages were cleaned every two days, and a commerical fox chow [3] was fed to the animals until their weights reached 250 gm. Eighteen animals were divided

[3] Purina.

TABLE 1

Composition of basal diets

CONSTITUENT	CHICKS	GUINEA PIGS	HAMSTERS
	%	%	%
Cerelose	62.36		38.50
Sucrose		10.00	
Casein [1]	18.00		9.00
Gelatin	10.00	10.00	
Corn oil	4.00		6.20
Salts IV [2]	5.34	4.00	4.80
Zein			40.00
Alfalfa meal		5.00	
Soybean oil meal		17.00	
Ground yellow corn		54.00	
L-Histidine			0.50
L-Lysine			1.00
DL-Methionine	0.30		
Choline·Cl	0.20	0.30	0.30

	EXPERIMENTAL DESIGN		
	CHICKS	GUINEA PIGS	HAMSTERS
Group 1	basal	basal	basal
Group 2	basal + 80 mg % 3-OHAA [3]	basal + 300 mg % 3-OHAA	basal + 75 mg % 3-OHAA
Group 3	basal + 2.5 mg % NA [4]	basal + 3 mg % NA	basal + 1.5 mg% NA

[1] Borden's Labco.
[2] Phillips and Hart ('35).
[3] 3-Hydroxyanthranilic acid.
[4] Nicotinic acid.

into three groups of 6 each and fed the diet described in table 1 ad libitum with additions to the diets made according to the experimental design. In addition to this diet 0.25 ml of a solution containing 100 mg per ml of ascorbic acid in 70% sucrose solution was administered to each guinea pig every second day. Animals were weighed every 5 days during the 14-day experimental period.

Hamsters. Twelve weanling golden hamsters were given the synthetic diet (table 1) supplemented with 2 mg of nicotinic acid per 100 gm of diet for three days. The hamsters were then weighed, placed in individual cages with raised wire floors and assigned to one of the three experimental groups. Food and water were supplied ad libitum, and the animals were weighed every 5th day during the experimental period.

In addition to the components shown in table 1, vitamins were supplied in the diets at the following levels (milligrams per kilogram of diet): thiamine·HCl, 15.0; pyridoxine·HCl, 10.0; *d*-calcium pantothenate, 50.0; inositol, 100; folic acid, 1.0; biotin, 1.0; *p*-aminobenzoic acid, 100; vitamin B_{12}, 0.1; 2-methyl-1,4-napthoquinone, 2.5; and α-tocopherol acetate, 2.0. Vitamins A and D_3 were supplied to the guinea pigs and hamsters by the oral administration of three to 4 drops of halibut liver oil to each animal once a week. The chick diet was supplemented with 10,000 I.U. of vitamin A acetate and 600 I.C.U. of the vitamin D_3 per kg.

The experiments were discontinued after the symptoms of deficiencies were noted and a definite growth curve could be established. Standard errors of the mean were calculated and recorded in table 2.

RESULTS AND DISCUSSION

Since many species of animals appear to utilize tryptophan as a source of niacin, diets were prepared to make this the limiting amino acid. Koeppe and Henderson ('55) observed that low levels of tryptophan in the diet supported growth in the absence of niacin least effectively when there was an excess

of all of the remaining essential amino acids. Therefore, all amino acids except tryptophan were provided at levels in excess of the minimum requirement by the addition of proteins low in tryptophan (corn proteins and gelatin). Substantial stimulation of growth by the addition of niacin was observed for each species with the diets used.

Data presented by Briggs et al. ('42, '45) indicated that the efficiency of conversion of tryptophan to niacin in the chick is about 4% or that this species could grow about as well on a niacin-free diet supplemented with 100 mg% of tryptophan as on the same diet supplemented with 2.5 mg% of niacin under the conditions of their experiments. The level of 3-hydroxyanthranilic acid selected for these experiments was 80 mg%. This level should result in a good rate of growth if one assumes that this compound is used as well as its precursor, tryptophan, for niacin synthesis. The results in table 2 indicate that while 3-hydroxyanthranilic acid is used to replace niacin, it is not as effective at 80 mg% as niacin is at 2.5 mg%. It thus appears that 3-hydroxyanthranilate is less than 4% as effective as niacin in supporting growth under the conditions of these experiments.

Reports in the literature showed that the guinea pig grows as well on a niacin-free diet supplemented with 300 mg% of tryptophan as with 3 mg% of niacin (Cannon et al., '46). Preliminary studies in which 100, 200, and 300 mg% of 3-hydroxyanthranilic acid were fed showed that 300 mg% supported better growth than either of the two lower levels of intake. The results in table 2 indicate that 300 mg% of this compound also gave better growth than 3 mg% of niacin. These results together with those of Cannon et al. suggest that 3-hydroxyanthranilic acid has niacin-replacing activity approximately equal to that of tryptophan for this species.

The results with hamsters were very striking. The synthetic diet, an adaptation of the diet used by Krehl et al. ('45b) for rats was adequate when supplemented with 1.5 mg of niacin per 100 gm. In preliminary experiments three levels of 3-hydroxyanthranilic acid were tested with this species. Since

75 mg% gave better growth than the two lower levels, this amount was added to the diet in the experiments reported here. The results in table 2 show that 75 mg% of hydroxyanthranilic acid will support growth comparable to that obtained with 1.5 mg of niacin. This species was affected markedly by a niacin deficiency. During the first 12 days of the growth studies, the weights of the animals in all three groups were about the same, but by the 14th day a loss of

TABLE 2

Growth response to 3-hydroxyanthranilic acid

SUPPLEMENTS PER 100 GM DIET	NUMBER OF ANIMALS	AVERAGE WEIGHT INCREASE (13 DAYS)	INCREASE OVER BASAL
		gm	%
	Chicks		
None	10	52.1 ± 6.6 [1]	—
80 mg 3-OHAA	10	80.0 ± 3.9	54.5
2.5 mg N.A.	10	104.0 ± 3.8	100.0
	Guinea pigs		
None	6	11.0 ± 4.2 [1]	—
300 mg 3-OHAA	6	28.0 ± 6.7	150.0
3 mg N.A.	5	24.0 ± 7.0	118.0
	Hamsters		
None	4 [3]	3.2 ± 1.82 [1,2]	—
75 mg 3-OHAA	4	13.5 ± 0.87	321.0
1.5 mg N.A.	4	13.8 ± 0.75	331.0

[1] Standard error of the mean.
[2] Average weight increase for 18 days.
[3] Two died on 18th day.

weight was observed in all of the negative control animals. Two of this group died on the 18th day, and the other two had developed diarrhea and unkempt fur. The animals receiving the two supplements continued to grow at the same rate observed during the first 12 days.

The results presented do not permit a quantitative evaluation of the conversion of 3-hydroxyanthranilate to niacin or its nutritional equivalent in the three species studied. However, these results together with the evidence previously presented clearly indicate that this compound has roughly the

same niacin-replacing activity for these species as tryptophan. This finding is consistent with the view that tryptophan is converted to niacin via 3-hydroxyanthranilate in these species as in the case of the rat.

SUMMARY

1. 3-Hydroxyanthranilic acid has been shown able to replace niacin for the growth of guinea pigs, chicks and hamsters. Moderate rates of growth were obtained with 300 mg%, 80 mg%, and 75 mg% for the three species, respectively.

2. The evidence presented supports the view that 3-hydroxyanthranilic acid is an intermediate in the conversion of tryptophan to niacin in these species.

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THE EFFECT OF DIETARY FAT ON THE DEVELOPMENT OF VITAMIN B_6 DEFICIENCY IN THE RAT [1,2]

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The evidence for a relationship between vitamin B_6 and lipid metabolism, distinct from the relationship with essential fatty acid metabolism (Witten and Holman, '52) is conflicting. McHenry and Gavin ('41) suggested that vitamin B_6 is required for the synthesis of carcass fat from protein. Sherman et al. ('50) reported that dietary fat spared vitamin B_6. Carter and Phizackerley ('51) concluded that vitamin B_6 deficiency decreased carcass lipid deposition from low-fat, but not from high-fat diets. In contrast, Beare, Beaton and McHenry ('53) found that vitamin B_6 deficiency reduced the percentage of carcass fat in rats fed either low-fat or high-fat diets.

In these reports, dietary fat was increased at the expense of carbohydrate without maintenance of a constant ratio of protein to food energy. Since food intake varies inversely with food energy concentration, protein intake would decrease as the percentage of dietary fat increased. The smaller nitrogen intake could then decrease the need for vitamin B_6 in amino acid metabolism to produce less rapid depletion of the vitamin. Thus, it is difficult to evaluate the effect of different levels of dietary fat on body composition and on the course of

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vitamin B_6 depletion unless a study is made of diets differing in fat concentration but equal in respect to the ratio of protein to food energy.

In the work reported here, we have studied body composition and the development of vitamin B_6 deficiency in rats fed diets in which the ratio of protein to food energy remained constant as the level of fat increased. We have also been unable to confirm the report of Beaton et al. ('54) that the ability of the rat to deposit body fat is decreased after only one week of vitamin B_6 deprivation.

MATERIALS AND METHODS

Long-Evans rats bred in our own colony were used in all experiments. Vitamin supplements were fed separately three times per week. The water-soluble vitamins were supplied in 20% ethanol, and vitamins A, D, and E were supplied in cottonseed oil. With this procedure, all animals received the same daily vitamin intake: thiamine hydrochloride, 43 μg; riboflavin, 43 μg; calcium pantothenate, 171 μg; niacin, 171 μg; folic acid, 8.5 μg; biotin, 8.5 μg; menadione, 50 μg; vitamin B_{12}, 200 mμg; choline chloride, 10 mg; vitamin A, 32 I.U.; vitamin D, 3 I.U.; α-tocopherol, 0.16 mg. Vitamin B_6 deficiency was created through the omission of pyridoxine from the diet. No antimetabolites were used. The pyridoxine-supplemented rats received 50 μg of pyridoxine hydrochloride/day. Body water was determined by drying the carcasses (minus liver) to constant weight in a forced-draft oven at 100°C. Fat was determined from the loss of weight of the dried, ground carcass after extraction with ethyl ether in a Soxhlet apparatus. After ether extraction, the carcasses were autoclaved for 6 hours in 20% sulfuric acid and the hydrolyzates filtered and diluted to a known volume. Nitrogen was determined on aliquots of the hydrolyzates by the semi-micro Kjeldahl procedure. The factor 6.25 was used to estimate protein from nitrogen. Liver cholesterol was determined by a modified Sperry-Webb ('50) procedure, and liver total crude fatty acids were determined by the method of Bloor ('28).

RESULTS

Experiment 1

The first experiment was undertaken to confirm the report (Beaton et al., '54) that the ability of the rat to deposit body fat was decreased after only one week of pyridoxine deprivation. Weanling male rats were fed the experimental diet,[3] supplemented with 50 μg of pyridoxine hydrochloride/day, until they reached a weight of 70 gm (less than one week). They were then placed in experimental groups for one, two, or three weeks, and the pyridoxine supplement continued or discontinued. One group was sacrificed at 70 gm body weight to determine the initial body composition. The procedure differs from the procedure of Beaton et al. ('54) in that cottonseed oil was substituted for corn oil, and the vitamin supplements were fed separately. Younger rats were also used. We originally hoped to duplicate their conditions, but found this not possible.

The results are given in table 1. For the first two weeks, there was no significant difference in weight gain between the pyridoxine-deprived and the pyridoxine-supplemented pair-fed controls. After three weeks, the difference in weight gain was greater, but still not significant. Also no significant differences appeared in body composition. Thus, under our conditions, pyridoxine-deprivation did not affect growth before the third week of deprivation, and even then, the composition of the gains made by the deficient and the supplemented animals did not differ significantly.

Experiment 2

This study was undertaken to determine whether the reported sparing action of fat on the development of vitamin B_6 deficiency reflects the decreased protein intake which occurs when the dietary fat level is increased without adjustment of

[3] Diet, grams per 100 grams: vitamin-free casein, 20; cottonseed oil, 20; U.S.P. salts 14, 4; sucrose, 56.

the protein/food energy ratio. The diets used supplied either 2 or 40% of cottonseed oil, with and without adjustment of the protein/food energy ratio.[4] To hasten the development of the deficiency, the dams of the rats were fed a pyridoxine-free diet one week before the young were weaned. At weaning, the young male rats were placed on the experimental diets,

TABLE 1

The effect of the duration of pyridoxine deprivation on weight gain and body composition of the rat

WEEKS ON DIET	TREATMENT	NO. OF RATS	GAIN	BODY WATER	BODY FAT
			gm	%	%
1	— Pyridoxine	9	13.7 ± 1.6 [1]	68.5 ± 0.6	7.6 ± 0.8
	+ Pyridoxine	9	16.1 ± 1.7	67.9 ± 0.9	8.9 ± 1.1
2	— Pyridoxine	6	21.2 ± 2.8	68.2 ± 0.4	6.9 ± 0.6
	+ Pyridoxine	6	21.6 ± 2.1	67.2 ± 0.4	8.7 ± 0.5
3	— Pyridoxine	7	38.4 ± 3.3	66.6 ± 0.4	8.5 ± 1.8
	+ Pyridoxine	7	46.4 ± 3.4	66.2 ± 0.6	8.8 ± 0.8

[1] Mean and standard error of the mean.

TABLE 2

The effect of the dietary levels of protein and fat on vitamin B_6 depletion

DIET	— PYRIDOXINE	THREE-WEEK GAIN [1] + PYRIDOXINE, PAIR-FED	+ PYRIDOXINE, AD LIB.
	gm	*gm*	*gm*
2% fat, 20% casein	11.5 ± 1.5 (14) [2]	27.3 ± 3.5 (7)	80.6 ± 4.0 (9)
40% fat, 29% casein	14.1 ± 2.0 (13)	41.7 ± 4.4 (7)	69.3 ± 4.9 (9)
40% fat, 20% casein	8.7 ± 1.3 (14)	27.0 ± 3.2 (7)	58.0 ± 4.8 (9)

[1] Mean and standard error of the mean.
[2] Number of rats.

which were fed for three weeks. Each deficient group was compared with a pair-fed and an ad libitum-fed control group.

Of the pyridoxine-deprived groups, the smallest weight gain was made by the group fed the 40 %fat-20% casein diet (table

[4] Diets, grams per 100 grams: (a) cottonseed oil, 2; vitamin-free casein, 20; U.S.P. salts 14, 4; sucrose, 74; (b) cottonseed oil, 40; vitamin-free casein, 29.3; U.S.P. salts 14, 5.8; sucrose, 24.9; (c) cottonseed oil, 40; vitamin-free casein, 20; U.S.P. salts 14, 4; sucrose, 36.

2). The difference in gain between this group and the group fed the 40% fat-29% casein diet was significant ($P < 0.02$). The difference in gain between the 2% fat-20% casein and the 40% fat-29% casein groups was not significant although animals in the latter group were still gaining slowly after three weeks. In the other deficient groups, nearly all the animals had plateaued in weight. The growth data for the ad libitum controls show that, with adequate pyridoxine, the 2% fat-20% casein and the 40% fat-29% casein diets, which provided 20% of metabolizable energy as protein, permitted greater gains than the 40% fat-20% casein diet which provided 13% of metabolizable energy as protein.

Experiment 3

The somewhat longer time required for the pyridoxine-deprived rats fed the 40% fat-29% casein diet to plateau in body weight raised the question of whether a similar effect would be observed at levels of fat lower than 40%. Experiment 3 studied the progress of vitamin B_6 depletion at intermediate levels of cottonseed oil. The levels of cottonseed oil tested were 5, 10, 20 and 40%, and the protein/food energy ratio of each diet was adjusted to the ratio of the 2% cottonseed oil-20% casein diet of experiment 2.

Of the deficient animals, equal gains were made by the 5, 20 and 40% fat groups (table 3). The group fed 10% fat gained significantly less. Thus, under our conditions, the course of vitamin B_6 depletion, as measured by weight gain, was not affected by the level of dietary fat. Comparison of table 3 with table 2 also shows that the rats fed the 40% fat-29% casein diet in experiment 3 gained significantly more than the comparable group in experiment 2 ($P = 0.02$) although experimental conditions were the same. The difference can be ascribed only to variations between litters or to coprophagy.

Carcass analyses (table 3) show that the percentage of body fat in the deficient groups rose significantly as the level of dietary fat increased from 10 to 20% or from 20 to 40%, but

TABLE 3

The effect of the level of dietary fat on vitamin B_6 depletion and body composition of the rat

COTTONSEED OIL	PYRIDOXINE-DEPRIVED			PYRIDOXINE-SUPPLEMENTED, PAIR-FED		
	THREE-WEEK GAIN	BODY WATER	BODY FAT	THREE-WEEK GAIN	BODY WATER	BODY FAT
%	*gm*	%	%	*gm*	%	%
5	21.5 ± 3.1 [1](8)[2]	71.9 ± 0.3	1.6 ± 0.3	35.6 ± 4.8 (8)	71.4 ± 0.7	1.8 ± 0.4
10	13.9 ± 2.2 (9)	71.2 ± 0.2	1.7 ± 0.2	47.8 ± 6.1 (9)	70.7 ± 0.7	4.1 ± 1.2
20	24.9 ± 2.7 (9)	71.2 ± 0.1	2.9 ± 0.2	40.8 ± 4.6 (9)	69.9 ± 0.8	4.5 ± 0.6
40	21.1 ± 1.6 (9)	70.6 ± 0.2	3.8 ± 0.3	39.2 ± 4.7 (9)	70.1 ± 0.6	4.8 ± 0.7
40 [3]	—	68.4 ± 0.4 (9)	6.8 ± 0.5	—	67.0 ± 1.0	9.5 ± 1.2

[1] Mean and standard error of the mean.

[2] Number of rats.

[3] Diet contained 40% fat and 20% casein (exp. 2).

not from 5 to 10%. Comparison of each deficient group with its pair-fed control shows that vitamin B_6 deficiency significantly decreased body fat if the diets contained either 10 or 20% of fat, but not if the diet contained either 5 or 40% of fat. Comparison of the deficient groups fed the 40% fat-29% casein diet and the 40% fat-20% casein diet of experiment 2 shows that the latter diet produced a significantly higher percentage of body fat.

Because of the relationship of vitamin B_6 and essential fatty acids (Witten and Holman, '52) and the increase in essential fatty acid requirement caused by high levels of saturated fatty acids (Deuel et al., '55), we repeated this experiment with different levels of coconut oil, a highly saturated fat. With the 10 or 20% level of coconut oil, the development of vitamin B_6 deficiency was similar to that observed with the same level of cottonseed oil. However, if the diet contained 40% of coconut oil or a combination of 38% of coconut oil and 2% of cottonseed oil, the condition of the pyridoxine-deprived rats became very poor. Satisfactory gains were observed with the pair-fed controls. This observation suggests an increased metabolism of vitamin B_6 when essential fatty acids are limited or when their utilization is increased, as with diets rich in saturated fatty acids, although the unusual composition of coconut oil itself is an uncontrolled factor.

Experiment 4

We attempted to reveal a relation between vitamin B_6 and lipid metabolism by studying the effect of vitamin B_6 deficiency on liver fatty acids and cholesterol in the presence or absence of dietary cholesterol. The diet [5] used facilitates maximum deposition of liver fat and cholesterol in rats fed 1% of cholesterol. Both pair-fed and ad libitum control groups were used.

[5] Diet, grams per 100 grams: vitamin-free casein, 18.0; cottonseed oil, 13.5; U.S.P. salts 14, 4.0; choline chloride, 0.07; sucrose, 64.4. -Cholesterol (1%) was substituted for an equal weight of sucrose.

The results are presented in table 4. In the absence of added cholesterol, vitamin B_6 deficiency prevented the significant fall in liver fatty acids which food restriction caused in the pair-fed controls, but had no effect on the percentage of total liver cholesterol. In the cholesterol-fed groups, vitamin B_6 deprivation lowered liver fatty acids to the same extent as did food restriction, but produced an even greater decrease in total liver cholesterol.

TABLE 4

The effect of vitamin B_6 deficiency on liver fatty acids and cholesterol in the presence or absence of dietary cholesterol

DIET	TREATMENT	NO. OF RATS	FATTY ACIDS	TOTAL CHOLESTEROL
			% wet wt.	*% wet wt.*
Basal	— Pyridoxine	13	8.1 ± 0.8 [1]	0.20 ± 0.01
	+ Pyridoxine pair-fed	9	5.2 ± 0.4	0.15 ± 0.02
	+ Pyridoxine ad libitum	9	9.8 ± 0.9	0.24 ± 0.05
Basal plus cholesterol	— Pyridoxine	13	19.9 ± 0.9	1.28 ± 0.10
	+ Pyridoxine pair-fed	8	20.6 ± 1.4	1.69 ± 0.15
	+ Pyridoxine ad libitum	10	28.5 ± 1.4	2.32 ± 0.19

[1] Mean and standard error of the mean.

DISCUSSION

Under our conditions, increasing the percentage of dietary fat increased the percentage of body fat in both vitamin B_6-deficient and pair-fed control rats. The increase occurred whether the protein/food energy ratio remained constant or decreased as the percentage of fat increased. On the other hand, the particular level of dietary fat determined whether a difference in the percentage of body fat appeared between the deficient rats and their pair-fed controls.

Carter and Phizackerley ('51) reported that vitamin B_6 deficiency did not affect the percentage of body fat in rats fed a 20% casein-20% margarine-5% cod liver oil diet, but significantly decreased body fat in rats fed a 20% casein-5% cod liver oil diet. Their animals were analyzed after typical

dermatitis appeared, but the time required for dermatitis to develop was not stated. Beare et al. ('53) could not confirm these results in their study of deoxypyridoxine-treated rats fed 20% casein-20% corn oil or 20% casein-5% corn oil diet for 27 to 29 days. Comparison of these reports is difficult because of differences in procedure. For the same reason, our results are not directly comparable.

From all these observations, it appears that no generalization can be made on the effect of vitamin B_6 deficiency on the percentage of body fat in the rat.

The decreased liver storage of dietary cholesterol in the vitamin B_6-deficient rat may indicate decreased absorption of dietary cholesterol although Carter and Phizackerley ('51) found that dietary fat absorption was not affected. The effect of vitamin B_6 deficiency on cholesterol absorption deserves attention because of the altered composition of bile acids in the deficient rat (Bergeret and Chatagner, '56).

SUMMARY

The effect of the level of dietary fat on the development of vitamin B_6 deficiency and on the body composition of vitamin B_6-deficient and pair-fed control rats was studied with diets in which the ratio of protein/food energy remained constant as the level of fat increased. In both deficient and control animals, the percentage of body fat increased as the percentage of dietary fat increased. Whether vitamin B_6 deficiency reduced body fat depended upon the level of dietary fat: there was no significant difference between the deficient and control groups at 5 or 40% of cottonseed oil, but a significant decrease occurred in the deficient animals fed a 10 or 20% of cottonseed oil diet. Vitamin B_6 deficiency also decreased the storage of liver cholesterol in cholesterol-fed rats.

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THE EFFECT OF HIGH ENVIRONMENTAL TEMPERATURE ON BASAL METABOLISM AND SERUM ASCORBIC ACID CONCENTRATION OF WOMEN [1]

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INTRODUCTION

Most investigators have found that in man acclimatized to hot countries energy exchange at the basal level tends to be lowered. A basal level 15 to 25% below that of similar individuals living in the temperate zone has been reported. Thompson et al. ('48) studied the basal metabolism of a large group of young women native to southern Arizona where the summers cover a relatively long period of time from May into October with high environmental temperature and low relative humidity. The basal metabolism was found to be much lower than that of similar subjects living in the temperate zone. In comparing the basal metabolism of 18-year-old subjects with that reported for the same age from 5 midwestern states they found that it diminished with increasing mean annual temperature. In studying young women confined to a room calorimeter, Du Bois et al. ('52) demonstrated a minimal basal metabolism in the region of about 79 to 87°F. Energy

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exchange at the basal level is believed by some to fluctuate with seasons of the year. Gessler ('25) reported that seasonal changes tend to parallel average daily temperature. He demonstrated a close inverse relation to mean monthly temperature throughout the year.

The effect of heat upon ascorbic acid concentration of animal tissues and biological fluids has been demonstrated in animal experimentation. In heat-treated albino rats Squibb et al. ('54) demonstrated in serum, and Gofine ('56) in urinary excretion, a diminished concentration of ascorbic acid. Parvis ('41) and Martini and Torda ('37) demonstrated a diminished concentration in the organs of heat-treated guinea pigs.

In studying the ascorbic acid requirement of young women on a constant intake of the vitamin Belser et al. ('39) could not duplicate in September the urinary excretion levels found the previous month. Results cited for one subject served to illustrate. When the daily intake was 100 mg of ascorbic acid the daily value obtained from August 5 to 11 (mean temperature 77°F) was 182 mg of ascorbic acid. From September 11 to 17 (mean temperature 55°F) a higher value of 231 mg was obtained. In a study of two human subjects kept in a high thermal environment for several hours Miura et al. ('51) demonstrated a diminished urinary excretion of the vitamin. The level was increased with return to normal temperature after a lag in time. Dodds et al. ('50) found no seasonal effect in young women in Tennessee with a mean temperature of 55°F in winter and 78°F in summer. Their calculations were made with mean values of experimental intakes of 25, 50, 75 and 100 mg/day for all subjects and no one subject was studied both seasons.

No study has been reported in the literature involving seasonal reduction in serum ascorbic acid concentration with controlled ascorbic acid intake in the same individual native to the region with adjustment to unit of body weight. Such an investigation was made comparing the ascorbic acid concentration in blood serum during the winter and summer seasons. Following a period of saturation the subjects received low but

constant intakes of ascorbic acid by means of the dietary and supplements of synthetic ascorbic acid referred to unit of body weight. Energy metabolism at the basal level was compared during the two seasons.

EXPERIMENTAL

Seven women students and faculty members, 19 to 62 years of age, participated in the study. All were natives of southern Arizona or had lived there many years. All were physically healthy and none was considered to be under or overweight. Average weight was 61.2 kg with a range in average of individual weights of 53.8 to 64.9 kg. The experimental periods, including saturation periods, varied, depending upon the subject, from 7 to 14 weeks in winter and 8 to 10 in summer. Six subjects were studied both seasons, one during winter only. Venulet ('53, '54) presented evidence in experimental animals and Musmanno ('51) and Goyanna ('55) in humans that cigarette smoking tends to lower the concentration of ascorbic acid in blood serum. Therefore, only non-smokers were accepted as subjects.

Daily minimum and maximum temperatures and relative humidity, as recorded at the Agricultural Experiment Station [3] on campus, were available for reference during all experimental periods.

Basal metabolism. Basal metabolism was determined on all subjects during each experimental period. Each determination was obtained on the basis of two 8-to-10-minute tests on each of at least two mornings. The subjects returned on another morning for additional tests until oxygen consumption from either of the tests on a given day agreed within 5% of that on another day. All tests within this range were averaged to represent oxygen consumption in cm^3/minute. Percentage deviations referred to age were calculated from the Harris-Benedict standard on the basis of weight and height and from the Mayo Foundation on the basis of surface area.

[3] Acknowledgment is made to Professor H. V. Smith, Agricultural Experiment Station, University of Arizona, who kindly made these data available.

Dietary ascorbic acid. Food consumption was weighed daily and nutrients calculated [4] to insure adequacy, except for ascorbic acid, and to prevent changes in body weight. The diet was analyzed for ascorbic acid and dehydroascorbic acid by Bessey's ('38) modification of the method of Mindlin and Butler ('38). In preparing food samples for analysis, daily aliquots were diluted to volume with 3% metaphosphoric acid, centrifuged, and aliquots buffered to a pH 3.5 to 3.6. The decrease in concentration of 2,6-dichlorophenol indophenol induced by addition of the buffered extract was measured for ascorbic acid by means of the Evelyn photoelectric colorimeter [5] at 520 mμ. To determine total ascorbic acid the buffered extract was treated with H_2S and washed free of H_2S with a stream of wet nitrogen.

Ascorbic acid supplement. The subjects received 0.4 mg of synthetic ascorbic acid per kilogram of body weight in capsule form before breakfast in addition to that present in the experimental diet. This amount had been demonstrated in early work in winter with cases 1 and 2 (table 4) to result in a serum level of 0.40 to 0.60 mg total ascorbic acid/100 ml serum. Before beginning the depletion period all subjects, except 1 and 2 who were saturated with the equivalent in orange juice, received 400 mg synthetic vitamin daily before breakfast for 5 to 7 days to insure a state of blood saturation.

Serum ascorbic acid. Fasting blood was collected from finger-tip puncture three times weekly. The serum was analyzed for total ascorbic acid by the micromethod of Lowry et al. ('45). Proteins were precipitated from the serum by trichloracetic acid. The remaining fluid was incubated after oxidation of the ascorbic acid to dehydroascorbic acid by copper sulfate in the presence of thiourea and 2,4-dinitrophenylhydrazine. After addition of concentrated sulfuric acid and formation of

[4] The USDA Agriculture Handbook No. 8 by Watt and Merrill, 1950, was used to calculate nutrients.

[5] Evelyn Photoelectric Colorimeter, Rubicon Company, Ridge Avenue at 35th Street, Philadelphia 32, Pa.

the red pigment, the samples were read in the Beckman spectrophotometer [6] at 520 mμ.

RESULTS

Environmental temperature and relative humidity. Monthly mean temperatures with maximum and minimum and relative humidity means for the two seasons are shown in table 1.

TABLE 1

Temperature (°F) and relative humidity [1] in Tucson, Arizona, 1955–58

	TEMPERATURE			HUMIDITY
	Mean	Maximum (Mean)	Minimum (Mean)	
	°F.	°F.	°F.	%
Jan.	55	68	41	39
Feb.	56	69	42	37
Mar.	60	77	43	26
Apr.	66	81	51	22
Av.	59	74	44	31
July	87	100	74	29
Aug.	85	98	72	37
Sept.	84	99	69	31
Av.	85	99	72	32

[1] Noon reading.

Basal metabolism. In winter deviations of basal metabolism (table 2) from the Harris-Benedict and Mayo Foundation standards (Carpenter, '48) were —0.85 to —13.82% and —3.21 to —15.95%, respectively; in summer —1.54 to —19.63% and —3.25 to —20.03%, respectively. Cases 1, 2, 3 and 4 diminished in summer between 7 and 11% below that in winter; cases 6 and 7 showed little change.

Ascorbic acid intake. The average daily intake of total ascorbic acid from food was 12.6 mg (ascorbic acid, 7.1 mg). Variations from day to day were found to be greater than seasonal. Average daily supplements for individual cases in winter were from 21.3 to 26.3 mg ascorbic acid and in summer

[6] Beckman Spectrophotometer, Model DU, Beckman Instruments, Inc., Fullerton, California. Equipped with micro attachment, Pyrocell Manufacturing Company, 207 East 84th Street, New York, N. Y.

TABLE 2

Physical data with basal metabolism and deviations from standards

CASE NO.	AGE	DATE	WEIGHT	HEIGHT	SURFACE AREA	BASAL CALORIES		DEVIATION	
								Harris-Benedict	Mayo Foundation
	Yrs.		*kg*	*cm*	m^2	*per 24 hrs.*	*per m^2/hr.*	%	%
1	18.9	Feb. '56	64.73	164.4	1.71	1460	35.56	— 2.01	— 3.21
	19.5	Aug. '56	65.10	164.5	1.72	1294	31.43	— 13.21	— 13.70
2	19.0	Feb. '56	56.18	164.4	1.61	1249	32.38	— 11.29	— 11.87
	19.5	Aug. '56	51.50	164.5	1.55	1108	29.76	— 18.59	—18.99
3	19.5	Feb. '57	60.77	161.6	1.64	1270	32.19	— 12.11	— 12.38
	19.1	Aug. '56	59.09	162.1	1.63	1147	29.38	— 19.63	— 20.03
4	62.3	Feb. '57	65.27	167.3	1.74	1287	30.89	— 0.85	— 5.27
	62.8	Aug. '56	66.37	166.8	1.74	1204	28.77	— 7.74	— 11.78
5	42.5	Jan. ,57	61.90	154.9	1.61	1237	32.06	— 7.34	— 10.20
	—[1]	—	—	—	—	—	—	—	—
6	19.2	Feb. '57	64.90	168.2	1.74	1290	30.88	— 13.82	— 15.95
	19.7	Aug. '57	64.73	168.2	1.74	1282	30.73	— 14.14	— 16.36
7	30.4	Jan. '58	57.45	164.1	1.62	1330	34.22	— 4.15	— 5.32
	29.9	July '57	56.90	164.2	1.62	1342	34.54	— 1.54	— 3.25

[1] Subject 5 was not available for the summer period.

from 20.5 to 25.9 mg. Body weights changed but little, therefore supplements for individual cases were found to vary between differences of only 0.5 and 1.2 mg in winter and 0.5 and 1.4 mg in summer. Total daily intakes averaged from 33.9 to 38.9 mg in winter and 33.1 to 38.5 mg in summer.

Ascorbic acid in serum. Saturation levels were somewhat lower in summer than in winter for cases 3, 4, 6, and 7 as shown in table 3. These differences may imply a seasonal effect. However, the curves of depletion obtained over the relatively long periods of time which were involved were found to be independent of these differences in saturation level.

TABLE 3

Saturation levels of total ascorbic acid in milligrams per 100 ml of serum during the summer and winter periods

CASE NO.	SUMMER	WINTER
	mg/100 ml	*mg/100 ml*
1	1.34	1.26
2	1.27	1.05
3	1.27	1.88
4	1.98	2.16
5	—	1.80
6	1.54	1.58
7	1.49	1.76

The level which was reached after 4 weeks for almost all cases was below 1.00 mg in winter and 0.90 mg in summer and within 6 weeks below 0.80 mg in winter and 0.60 mg in summer regardless of the saturation level. The major part of the loss occurred in the early stages of the study periods during the first three or 4 weeks which included 6 to 12 observation dates. This early loss affected the regression coefficient more than the smaller changes during the latter part of the period.

Although there was variation in ages of the subjects, and the initial rates of saturation were different, on the average there was a more rapid rate of depletion and less deviation from the curve of depletion in summer than in winter. This was confirmed by the fact that averages of the regression co-

efficients [7] (table 4) were significantly higher in summer than in winter. However, there appears to be a variability characteristic of the individual subject which is consistent over both seasons, no interaction appearing between genetic ability to retain ascorbic acid and the environment.

It would appear that basic information on rate of depletion of serum ascorbic acid can be obtained during relatively short periods so that when a serum level of 0.40 to 0.60 mg is reached no further observation will be necessary. In agreement with these results Lutz et al. ('54) found, in a study of serum ascorbic acid losses in two men and two women ingesting a total of 40 mg ascorbic acid daily (diet + synthetic ascorbic acid), that concentrations stabilized at 0.40 to 0.60 mg/100 ml serum in 6 weeks. Their subjects had been saturated previously with 800 mg ascorbic acid daily for 4 days.

It is suggested by the present investigators that for future study subjects be maintained for periods not longer than 6 weeks under controlled depletion, then resaturated and the depletion procedure repeated. This would provide more information within each season with a limited number of subjects and would confirm repeatability of individual results. For such a procedure to be feasible, the winter and summer periods should be of sufficient duration to permit adequate saturation periods.

Subjects 1, 2 and 6 were exposed to summer heat many hours during the day in classes held in buildings only a few

[7] In analyzing the data, the initial fit was made using the function $\log y = a + bx$ where y = mg of vitamin/100 ml serum and x = days from the beginning of the experiment. This was based upon results obtained earlier on cases 1 and 2 in which several types of functions were tried and the above function found to be the most suitable. It was observed, however, that for subsequent cases the length of the observation period was much shorter than in the earlier periods and the shape of the curve was less affected by the relatively constant values observed after the subjects reached their minimal level on the prescribed vitamin intake. Under such a situation, the function $y = ax^b$ was found to be superior to the previous function in that it gave higher correlation of the vitamin with time. This new equation was then used to fit the data for all cases. The equation $\ln y = \ln a + b \ln x$ was used in obtaining the actual least squares solutions, when ln refers to the natural logarithm as computed on the IBM 650 Calculator.

TABLE 4

Weekly values of total ascorbic acid in milligrams per 100 ml of serum during the winter and summer periods

WINTER

Case no.	1	2	3	4	5	6	7	Av.
Week								
1	1.23	0.84	1.65	1.92	1.59	1.43	1.47	
2	— [4]	1.06	1.38	1.17	1.26	0.95	1.22	
3	1.21	0.90	1.19	1.16	1.06	1.13	0.86	
4	— [4]	0.96	0.90	0.74	0.85	0.93	0.58	
5	1.01	0.85	0.95	0.74	0.79	1.03	0.62	
6	0.92	0.70	0.78	0.71	0.71	0.81		
7	0.97	0.77	0.75	0.68	0.69	0.82		
8	0.86	0.58	0.78	0.58	0.66	0.72		
9	0.95	0.59	0.79	0.61	0.78	0.80		
10	0.97	0.70	0.67	0.59	0.70	0.62		
11	0.77	0.64						
12	0.83	0.47						
13	0.70	0.47						
14	0.67							
15	0.63							
16	0.61							
b [2]	— 0.255	— 0.261	— 0.396	— 0.515	— 0.386	— 0.275	— 0.614	— 0.386
r [3]	0.85	0.91	0.98	0.94	0.96	0.90	0.95	0.93

SUMMER

Case no.	1	2	3	4	5 [1]	6	7	Av.
Week								
1	1.12	1.11	1.29	1.48	—	1.27	1.22	
2	0.80	0.82	0.87	0.96	—	1.00	0.92	
3	0.68	0.52	0.86	0.89	—	0.90	0.73	
4	0.53	0.48	0.86	0.75	—	0.75	0.52	
5	0.53	0.39	0.74	0.62	—	0.60	0.58	
6	0.48	0.40	0.55	0.48	—	0.60	0.42	
7	0.40	0.43	0.62	0.50	—	0.62	0.40	
8	0.49	0.41	0.51	0.52	—			
9								
10								
11								
12								
13								
14								
15								
16								
b [2]	— 0.399	— 0.453	— 0.402	— 0.544	—	— 0.415	— 0.586	— 0.467
r [3]	0.92	0.90	0.94	0.98	—	0.95	0.95	0.94

[1] Subject was not available for the summer period.

[2] Regression coefficient.

[3] Correlation coefficient.

[4] Spectrophotometer was out of order.

of which were air conditioned and in organized activities such as swimming, tennis and dancing. Subject 3 worked part time cataloguing in an air conditioned library. Subjects 4 and 7 worked full time in the air conditioned nutrition laboratory but were exposed to heat at other times. It would appear that varying degrees of a stress condition due to differences in physical activity and exposure to direct rays of the sun were important factors in the determination and evaluation of these results. Hamel ('37) reported increased requirements in ascorbic acid with increased physical exercise based upon loading experiments and determination of saturation deficits. On this campus out-of-door organized activities continue throughout both the academic year and summer session. Subjects 1, 2 and 6 were engaged in such activities during both periods but physical activity may have been increased, accompanied by increased fatigue in summer. This may have increased somewhat the requirement of ascorbic acid, or its destruction, thereby contributing to the increased rate of reduction in the serum.

Recent work (Kirch et al., '43; Johnson, '43; Henschel et al., '44; Sargent et al., '44; Shields et al., '45) does not confirm the earlier view (Bernstein, '37; Foulger, '42) that loss of ascorbic acid in profuse sweating may be considerable and suggests that losses are negligible. Lugg and Ellis ('54) reported that it appears to be unnecessary to supplement ascorbic acid intakes of European men working in a warm environment to offset dermal losses provided they already receive normal amounts of the vitamin in their daily diet. Ara et al. ('54), working in India, reported that ascorbic acid in sweat is present almost totally as dehydroascorbic acid. They found from 0.038 to 0.530 mg/hour in winter and 0.208 to 0.840 mg/hour in summer and concluded that such loss might be significant at low dietary intakes of the vitamin. In evaluating the possible role of dermal losses in the present study, it is to be emphasized that total intakes were low with serum approaching a relatively low level during a period of several weeks. This final level approximates the border line of ade-

quacy and the possibility exists that such losses may have significance under the conditions of this study.

Subjects 1, 2, 3 and 6 who were in their 19th year had lowest rates of reduction whereas the older subjects 4 and 7 had the highest for both seasons. It is suggested that increasing age may have been a factor in effecting this increase.

SUMMARY

The rate of depletion of total ascorbic acid in blood serum of women living in southern Arizona has been demonstrated to be significantly higher in summer than in winter. However, there appears to be a variability characteristic of the individual which is consistent over both seasons, no interaction appearing between genetic ability to retain ascorbic acid and the environment. Six subjects studied over periods of several weeks in both seasons received a constant but minimum amount of ascorbic acid. This was provided in the dietary with daily supplements of 0.4 mg synthetic ascorbic acid/kilogram body weight after a preliminary period of blood saturation.

Basal metabolism diminished to a significantly lower level in summer in the majority of the subjects.

Under the condition induced by climatic stress with a diminished rate of energy exchange as in this study, it is apparent that ascorbic acid metabolism was altered in some manner due to increased requirement or destruction. Differences among the subjects may have been due to age or degree of activity and exposure to the direct sun with increased fatigue. Dermal losses may have been of significance due to the border-line of adequacy in serum ascorbic acid maintained during most of the experimental period.

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MANGANESE DEFICIENCY IN THE GUINEA PIG [1,2]

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The value of studying the function of nutrients through the use of more than one species is well appreciated. In certain instances differences in the maturity of animals at birth is an important consideration. In other cases, investigation of a new species has revealed unexpected metabolic individualism. The present paper deals with the production of manganese deficiency in guinea pigs, studies which were conducted simultaneously with rat experiments concerned with the nature of the ataxia associated with manganese deficiency (Hurley, Everson and Geiger, '58).

Ataxia as it is manifested in manganese deficiency has not been observed in the case of the rat except when the maternal diet is restricted in the trace mineral. A high proportion of the surviving offspring of such females exhibit ataxia. Therefore, manganese studies with the guinea pig appeared to entail two accomplishments; first, establishing satisfactory reproduction in this species when a partially synthetic diet is continued over a major part of the life span, and second, reduction of the manganese content of such a diet to the extent that a manganese deficiency is apparent.

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[2] Presented in part at the 22nd annual meeting of the American Institute of Nutrition, April, 1958. (Federation Proc., *17:* 480, 1958).

EXPERIMENTAL

Young guinea pigs weighing approximately 200 gm were obtained from commercial sources. Female animals were fed three types of rations. A pelleted commercial feed recommended for guinea pigs was selected as a stock diet.[3] Animals receiving this ration were supplemented with greens and with ascorbic acid, α-tocopherol, and cod-liver oil by mouth. Ascorbic acid was given three times weekly in amounts to supply 15 mg per day. α-Tocopherol in corn oil was fed two times each week providing 1.5 mg per day for young animals and 3.7 mg daily for adult animals. Cod-liver oil was fed orally once per week providing 900 U.S.P. units of vitamin A and 9 U.S.P. units of vitamin D per day. Male animals were maintained on this stock diet except for short periods when they were put into mating cages. A partially synthetic diet which was pelleted was prepared in the laboratory and was regulated in manganese content by omission of the manganese sulfate of the salt mixture in the case of deficient groups. The ingredients of the synthetic diet were as follows: casein [4] (vitamin-free) 30, cornstarch 20, glucose [5] 10.6, sucrose 10, roughage [6] 10, agar [7] 5, salts [8] 6, cottonseed oil 5, potassium acetate 2.5, magnesium oxide 0.5, inositol 0.2, and choline chloride 0.2, and vitamin.[9] The ingredients were combined and mixed with 800 ml of distilled water per kilogram of dry diet. As the preparation began to solidify, it was pressed into a hopper of an electrically driven meat grinder to produce pellets. The

[3] Rockland Guinea Pig Diet; A. E. Staley Mfg. Company, Decatur, Illinois.

[4] Vitamin-free test casein; General Biochemicals, Inc., Chagrin Falls, Ohio.

[5] Cerelose.

[6] Solka Floc; Mefford Chemical Co., 5353 Jillson Street, Los Angeles, California.

[7] Agar was found to vary widely in manganese content. Sources used were chosen for low manganese concentration.

[8] Salts, in grams $CaCO_3$, 300 gm; K_2HPO_4, 325; NaCl, 168; $FeSO_4 \cdot 7HOH$, 25; $MgSO_4 \cdot 7HOH$, 28; KI, 0.8; $ZnCO_3$, 0.25; $CuSO_4 \cdot 5HOH$, 0.3. Salt mix used for the manganese-supplemented diet contained in addition 2.3 gm of $MnSO_4$.

[9] Vitamins were added in amounts to provide for each kilogram of diet the following in milligrams: thiamine · HCl, 16; riboflavin, 16; pyridoxine · HCl, 16; Ca Pantothenate, 40; nicotinic acid, 200; biotin, 1; folic acid, 10; 2-methyl-napthoquinone, 5; *p*-aminobenzoic acid, 100; α-tocopherol, 100; also vitamin B_{12}, 50 μg; vitamin A 6000, I.U.; and vitamin D 600, I. U.

preparation was then spread thinly on trays and dried by means of fans at room temperature. The dried pelleted ration was stored at sub-zero temperatures until needed. Animals receiving both manganese-low and manganese-adequate diets were given amounts of ascorbic acid and α-tocopherol equal to those fed the stock diet. The experimental diets were begun when the animals weighed approximately 200 gm and were continued in some instances throughout three pregnancies. Distilled water was used throughout the study. Concentrations of manganese found to be present in the three test diets were as follows: (a) for the manganese-low synthetic diet, under 2 p.p.m.; (b) for the manganese-adequate synthetic diet, 40 p.p.m.; (c) for the commerical feed, 137 p.p.m.

RESULTS

Growth records indicated that the synthetic diet including manganese supported good growth, equal to that of animals raised on the stock ration. In the case of the manganese-deficient diet, growth was somewhat suppressed. At 12 and 14 weeks of age, females receiving manganese weighed between 500 and 600 gm, which agrees closely with weights of healthy female guinea pigs described by Reid ('58). The absence of manganese during growth resulted in a 50-gm suppression of weight at 12 weeks.

Reproduction on the synthetic diet including ample amounts of manganese was as successful as that observed for stock females. The number of young born per litter, birth weights of the young, and the incidence of still-births indicated no disadvantage from the feeding of the synthetic diet.

It will be noted from table 1 that the omission of manganese from the synthetic diet had a marked influence on reproduction. A high proportion of the females lost their litters prematurely. In all such cases the young were delivered dead. The size of the litters was slightly smaller than was true when manganese was present in adequate amounts, and the number of young dead at birth was increased in the absence of manganese. The most striking difference between the effects of the two syn-

thetic diets was the complete absence of ataxia among young born to females receiving manganese, while 100% of the offspring born to manganese-restricted females showed ataxia. Birth weights of the young were not altered by the omission of manganese from the maternal diet.

The symptoms observed among manganese-deficient guinea pigs at birth varied to some extent. Some animals were totally helpless, being unable to walk or to remain upright when placed on their feet. In such cases some of the young were kept alive for several days by feeding them a fortified milk diet by dropper. In other young, which were not handicapped to this extent, the ataxia was characterized by a pronounced head

TABLE 1

Reproductive performance of guinea pigs

ITEM OF INTEREST	STOCK DIET	PELLETED SYNTHETIC DIET	
		+ Mn	− Mn
Number of pregnancies tested	19	22	29
Number of young born	63	57 [1]	43 [2]
a. Average per litter	3.3	2.9	2.5
b. Number of young dead at birth	13	8	18
c. Average birth weight, gm	99.2	109.7	99.4
d. Number of young with ataxia	0	0	25 (100%)

[1] Two litters were aborted.
[2] Twelve litters were aborted.

retraction and a poor sense of balance. Both types of cases often exhibited an apparently uncontrollable twitching of the limbs. While numerous defective young were sacrificed at 4 and 21 days of age to explore certain tissues, a small number of manganese-deficient young were continued on the manganese-low synthetic diet for three months. These animals continued to exhibit abnormal head movements, inability to turn over when placed on their side or back, and unsteadiness of gait. Uncontrollable head motion, continuing for several seconds at a time, and a tendency for the animals to tilt their heads have been the main differences in behavior observed at three months of age. Illustrations of the manganese-deficient guinea pigs at birth are shown in figures 1, 2, and 3.

Fig. 1 Example of a manganese-deficient guinea pig at birth unable to stand without assistance.

Fig. 2 Example of a newborn guinea pig deficient in manganese attempting to upright itself.

Fig. 3 Example of the head retraction typical of the manganese-deficient guinea pig.

TABLE 2

Enzyme activity of tissues in manganese deficiency

Part 1

Serum alkaline phosphatase values in guinea pigs

GROUP	AGE		
	Newborn	4 days	21 days
			Nitrophenol units [1]
Manganese (+)	8.26	5.76	6.54
	7.25	5.42	3.48
	7.08	5.37	3.25
	7.02	2.75	3.14
	6.70	2.49	3.11
	6.57		2.77
	6.37		2.77
	5.13		2.75
	4.27		2.69
	4.05		
Manganese (−)	6.23	2.54	3.90
	6.13	1.77	3.57
	5.20		3.35
			2.36

Part 2

Acetyl-cholinesterase activity in brains of guinea pigs

	MILLIEQUIVALENTS ACETYL CHOLINE HYDROLYZED PER MILLIGRAM FRESH TISSUE PER MINUTE × 10^6		
Manganese (+)	13.8	14.5	16.2
	13.6	14.3	14.4
	13.5	13.5	12.3
	12.7	12.4	12.3
	12.6		11.4
	12.6		11.3
	10.6		10.8
	10.5		
Manganese (−)	17.0	13.5	15.1
	14.5	13.3	11.5
	11.5		10.8
	11.3		

[1] Alkaline phosphatase activity is expressed as millimoles of *p*-nitrophenyl phosphate hydrolyzed per liter of serum per hour.

A limited number of analyses of the alkaline phosphatase activity of serum have been completed for both manganese-deficient and manganese-supplemented young at birth and at 4 and 21 days of age. These data are given in table 2. The method used was that of Bessey, Lowry and Brock ('46). Serum alkaline phosphatase values did not appear to have been influenced by the omission of manganese from the maternal diet. This conclusion is in agreement with the observations of Hurley, Everson and Geiger ('59) for rats.

The activity of acetyl-cholinesterase (Aprison, Nathan and Himwich, '54) of entire brain was also explored in a small number of guinea pigs at birth and at two additional ages. As will be found from table 2, the absence of manganese in the maternal diet did not alter the enzyme activity of the tissue tested. This finding is also in agreement with the previous observations for rats (Hurley, Everson and Geiger, '58). Further studies are in progress.

SUMMARY

A pelleted synthetic diet with and without manganese has been fed to young female guinea pigs and continued throughout the growth period and one, two or three gestation periods. The synthetic diet including manganese supported growth and reproduction equal to that observed for good stock diets. When manganese was omitted from the maternal diet, litter size was reduced, a high percentage of the young were born dead or delivered prematurely. One hundred per cent of the living young born to deficient females showed ataxic symptoms at birth. A small number of defective young have now been maintained on the manganese-deficient diet for three months and abnormal head movements and unsteadiness of gait have persisted.

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BIOLOGICAL AND CHEMICAL STUDIES ON COMMERCIAL FRYING OILS

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INTRODUCTION

During the past several years investigations have been made on the effect of heat and oxidation on the nutritive value of edible oils. These include studies on corn oil and cottonseed oil (Dyme, '39; Harrelson et al., '39; Dyme et al., '40), heated linseed oil, corn oil, peanut oil, soybean oil (Crampton and others, '51a, b); cottonseed oil (Kaunitz et al., '55). Other studies using laboratory heated and oxidized oil are those of Johnson et al., ('56), Witting et al., ('57) and Dangoumau and others ('57).

In the above studies the workers reported detrimental effects from the feeding of heated and oxidized fats ranging from marked growth depression to diarrhea and death.

Studies from other laboratories have been reported which are not in agreement with the foregoing; for example, Deuel et al. ('51) in experiments with margarine fat, Melnick ('57), Rice, Mone and Poling ('57) in experiments with commercially heated fats.

A large portion of the work reported in the literature was done with laboratory heated and aerated fat and included mainly unsaturated fats such as corn or cottonseed oil. The commercially heated fats used by some workers were obtained from relatively uncontrolled sources where actual heating times and temperatures were not accurately known.

In view of the conflicting results reported, it was deemed desirable to study the effect of hydrogenated fats heated in a controlled commercial frying operation as well as of unheated and laboratory heated fats upon the rate of gain and well-being of the growing rat.

METHODS AND RESULTS

Caloric studies

A caloric value study by means of the caloric restriction technique, as described by Rice et al. ('57b) and Oser and Oser ('57) was carried out on various samples of oil obtained from a commercial frying operation. Weanling male rats of the Sprague-Dawley strain were used. Five animals were assigned to each of the levels of the standard curve and to each of the samples of fat. A standard curve was established from the rat growth response obtained on 5 gm of basal diet (table 1) per day plus the following levels of cottonseed oil: 0.0, 0.5, 1.0 and 2.0 gm per day. The cottonseed oil was arbitrarily assigned a caloric value of 100%.

The test animals recieved 5 gm of basal diet plus 1.5 gm of sample per day. All animals were individually housed and water was given ad libitum. The experiments were continued for three-week periods during which time the animals were weighed twice weekly. The dose-response is shown in table 2. The caloric value of each sample was calculated from a standard curve constructed from these values.

In the first experiment only samples 1, 1a, 1b, 2, and 2a were available. In the second experiment these same samples plus 3 and 3a were tested. The caloric value of the samples as calculated from the standard curve is shown in table 3.

Difficulties encountered with the air-conditioning equipment for the animal rooms during the experiments may account for the differences in the caloric value obtained between the two experiments. Since a calorically-restricted diet was used, a small difference in average temperature may be magnified into a relatively large difference in caloric value. However, it

is evident from table 3 that although the caloric values differed in the two experiments, the order of the values remained relatively constant.

It seemed unusual that fats, which had been heated to a point where the color, flavor, and free fatty acid content indicated that some breakdown had occurred, had a higher caloric value than the original unheated fat. In searching for an explanation for these seemingly incongruous results it was noted that there was an apparent positive correlation

TABLE 1

Composition of the basal diet

CONSTITUENT	AMOUNT	VITAMIN MIX	
		Constituent	Amount
	gm/100 gm		*gm/100 gm*
Vitamin-free casein	20.0	Thiamine	0.500
Sucrose	73.6	Riboflavin	0.800
Cottonseed oil	2.0	Niacin	4.000
Mineral mix (Jones-Foster)	4.0	Pyridoxine	0.500
Cystine	0.2	Calcium pantothenate	4.000
Choline	0.1	Biotin	0.040
Vitamin mix	0.1	Folic acid	0.200
Vitamin A	2000 units	Menadione	0.500
Vitamin D	200 units	Cyanocobalamin	0.003
α-Tocopherol	10 mg	Inositol	10.000
		p-Aminobenzoic acid	10.000
		Cornstarch	to make 100.000

TABLE 2

Dose response to increasing levels of cottonseed oil (3-week data)

COTTONSEED OIL [1] PER DAY	WEIGHT GAIN	CRITICAL RATIO OF FOLLOWING INCREMENT [3]
gm	*gm*	
0.0	21.2 ± 1.6 [2]	
0.5	35.2 ± 3.1	14.0
1.0	48.8 ± 1.3	24.2
1.5	55.4 ± 2.8	9.6

[1] Wesson oil.

[2] Standard deviation.

[3] A critical ratio of 3.0 is regarded as statistically significant at the 99% confidence level.

TABLE 3

Caloric availability based on cottonseed oil equal to 100%

SAMPLE NO.	DESCRIPTION	EXPERIMENT 1	EXPERIMENT 2	PERCENT TOTAL NON-CONJUGATED DOUBLE BONDS
1	Hydrogenated cottonseed oil	50.0 (66.3) [1]	68.8 (65.2)	2.87
1a	Hydrogenated cottonseed oil used commercially 14 days [2]	68.8 (85.0)	80.0 (76.0)	5.95
1b	Same as 1a but used 24 days	75.3 (100.0)	95.5 (90.5)	8.58
2	Hydrogenated cottonseed oil used 10 days (different run than series 1)	71.1 (94.0)	90.0 (85.2)	6.62
2a	Same as 2 but used 14 days	69.2 (92.0)	85.5 (81.0)	6.90
3	Hydrogenated cottonseed oil (different brand than series 1)	——	77.0 (73.6)	4.41
3a	Same as 3 but used 18 days	——	105.5 (100.0)	10.21

[1] Values within parentheses are caloric availability based on per cent of highest value sample.

[2] About 10% hydrogenated cottonseed oil was added daily to all commercially heated oils during the operation to replenish the oil removed by the frying.

between the increase in caloric value and the total non-conjugated double bonds present in the fat as measured spectrophotometrically (A.O.C.S., '51). A linear regression line was calculated, using the method of least squares, relating total non-conjugated double bonds present in the various samples to the caloric content. From the equations obtained, the lines best fitting the experimental points were constructed (fig. 1), and correlation coefficients (R) were determined. From the variations observed, the regression of total non-conjugated double bonds Y on caloric content X is $Y = 0.21X - 7.64$ with a correlation of $R = 0.986$ for the first experiment and $Y = 0.20X - 10.7$ with a correlation of $R = 0.983$ for the second experiment.

In order to correct for the temperature variation in the two experiments, the highest caloric value (75.3 in experiment 1 and 105.5 in experiment 2) in each experiment was assigned a value of 100 and the percent of this value calculated for each of the other samples, as shown within parentheses in table 3. The data were then pooled and the regression equation and correlation coefficient for the combined data was calculated (fig. 2). These data yielded a regression of total non-conjugated double bonds Y on caloric content X of $Y = 0.17X - 7.93$ with a correlation of $R = 0.919$. These results again indicated a very high correlation between the total non-conjugated double bonds present in a fat sample and its available calories. Other factors besides increased unsaturation which could conceivably increase the available caloric content of the fat upon heating might be: (1) An altered molecular structure which makes the fat more easily absorbed by the animal or (2) the formation of an oxidative biological intermediate which is more readily utilized by the animal than the unaltered fat. An examination of the ultraviolet and infrared spectra of the unheated and heated fats gave little support to either of the above explanations.

To study further the relationship between total non-conjugated double bonds and available calories, fat samples in different stages of hydrogenation were tested. The caloric

value of unhydrogenated cottonseed oil (iodine value, 112.1), semi-hydrogenated cottonseed oil (iodine value, 86.4) and hydrogenated cottonseed oil (iodine value, 69.5) were determined using the caloric restriction technique previously described. The results obtained are shown in table 4.

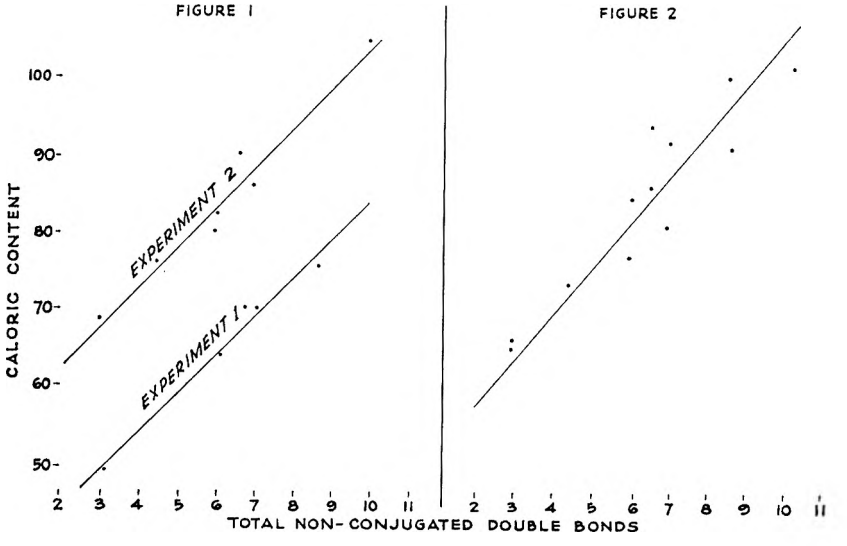

Fig. 1 A plot of the data obtained in experiments 1 and 2 showing the correlation of the caloric content with the total non-conjugated double bonds.

Fig. 2 A plot of the combined data of experiments 1 and 2 based on the highest caloric content showing the correlation of the caloric content with the total non-conjugated double bonds.

A correlation coefficient of $R = 0.988$ was obtained from these data, again indicating a high correlation between the total non-conjugated double bonds and the caloric value of the oil.

Chemical studies

It was surprising to find that hydrogenated cottonseed oil heated in a commercial deep-fat fryer became more unsaturated as heating was continued. These observations are in contrast to reports that heating under similar conditions

results in considerable polymerization and a consequent decrease in unsaturation (Bailey, '51).

A typical example of the increase in polyunsaturated fatty acids upon heating is shown in table 5. It should be noted that the increase in polyunsaturated fatty acids can be almost completely accounted for by the increase in the percentage of dienoic acids. However, since the trienoic and tetraenoic acids must be included in any growth-stimulating property of an oil, the relationship of growth to the total polyunsaturated acids (as indicated by the total non-conjugated double bonds) was selected as the criterion used in these studies. A critical examination of the proportions of component fatty acids of frying oil and chicken fat, as well as the oil replacement rate, indicated that the increased unsaturation observed could not be entirely attributed to simple dilution with chicken fat from the chicken parts being fried.

TABLE 4

Caloric value of cottonseed oil in various stages of hydrogenation

SAMPLE	IODINE VALUE	CALORIC VALUE	PERCENT TOTAL NON-CONJUGATED DOUBLE BONDS
Unhydrogenated	112.1	109.3	41.32
Semi-hydrogenated	86.4	92.0	21.83
Hydrogenated	69.5	66.6	3.91

TABLE 5

Mixed fatty acid composition of frying fats

SAMPLE	DAYS OF USE [1]	FATTY ACIDS					
		Dienoic	Trienoic	Tetraenoic	Oleic	Saturated	Conjugated diene
		%	%	%	%	%	%
A	0.0	0.64	0.00	0.00	72.9	21.9	0.14
B	1.0	1.45	0.31	0.18	69.6	23.9	0.18
C	4.4	2.78	0.00	0.28	69.0	23.3	0.26
D	9.1	4.98	0.19	0.19	65.2	24.7	0.37
E	13.9	5.49	0.23	0.22	64.0	25.3	0.40
F	18.6	5.95	0.21	0.24	62.6	26.1	0.47

[1] Each "day" is equal to an 8 hour frying period.

A possible explanation of further unsaturation could be a dehydrogenation of the oxygenated fatty acids formed at frying temperatures. Such a reaction has been postulated by Holman ('54) for oxygenated vegetable oils subjected to either bleaching or deodorization. Similar results were observed when a sample of frying oil used about three days was heated to 121°C for one hour with intermittent stirring, alone or in the presence of 20% (by weight) of either starch or a commercial filter aid (table 6). Thus, the desaturation appears to be promoted by heat alone or heat in the presence of two substances often found in contact with the frying oil in commercial frying operations. Another instance wherein heat and perhaps moisture apparently caused desaturation is found in the work of Melnick ('57), who reported increases up to 14 iodine units in hydrogenated vegetable oils used for potato chip frying.

Toxicity studies

Concurrently with the caloric value study, experiments were carried out to determine any toxic effects of commercially heated oils. A comparison was made between the growth response of rats receiving a hydrogenated cottonseed oil and rats receiving the same oil after it had been used in a commercial frying operation. These oils were fed in the presence of 2% cottonseed oil as a source of essential fatty acids. Three different levels of samples were used: 13, 18, and 4.7%. The oils used were samples 3 and 3a which were previously described. A group containing cottonseed oil at the 4.7% level was included. The basal diet was that shown in table 1 with fat replacing sucrose to make 100%. The experiment was continued for 7 weeks; the results are shown in table 7. There were no apparent symptoms of toxicity, such as diarrhea, rough hair coat, etc. It appears, therefore, that the feeding of these oils in the presence of an adequate diet causes no gross symptoms of toxicity. Furthermore, the growth response of rats which had received the heated oil was greater than that of the rats which had received the cottonseed oil

TABLE 6

The effect of heat and additives on the fatty acid composition of a commercially used frying fat

SAMPLE	TREATMENT	FATTY ACIDS					
		Dienoic	Trienoic	Tetraenoic	Oleic	Saturated	Conjugated diene
		%	%	%	%	%	%
S	None [1]	2.78	0.00	0.28	69.0	23.3	0.27
ST	1 hr. 121°C, starch	3.31	0.14	0.08	73.64	18.14	0.29
FA	1 hr. 121°C, filter aid	3.14	0.22	0.05	67.63	24.10	0.46
HC	1 hr. 121°C	3.43	0.11	0.10	66.52	25.18	0.26

[1] Hydrogenated cottonseed oil used in frying for three days.

control. These results are in agreement with those found using the caloric restriction technique. The animals were sacrificed and the liver weights determined. The results are also shown in table 7. There was no significant difference between the liver weights of the heated and unheated fats at 13, 18 or 4.7% of the diets.

TABLE 7

Comparison of weight gain and liver weight of rats receiving used or unused hydrogenated cottonseed oil at various dietary levels

SAMPLE [1]	PERCENT OF DIET	AVERAGE 7 WEEK GAIN	AVERAGE LIVER WEIGHT
		gm	*gm/100 gm body wt.*
3a	13	308.4 ± 17.6 [2]	4.92 ± 0.46
3	13	289.4 ± 13.0	4.83 ± 0.38
3a	18	301.2 ± 20.1	5.45 ± 0.77
3	18	295.9 ± 10.0	5.52 ± 0.32
3a	4.7	296.6 ± 18.4	4.92 ± 0.33
3	4.7	289.3 ± 13.7	5.22 ± 0.78
Unhydrogenated cottonseed oil	4.7	295.6 ± 18.4	4.83 ± 0.35

[1] For sample description see table 3.
[2] Standard deviation.

Laboratory heated and oxidized fats

Since there did not appear to be any adverse effects of feeding commercially heated hydrogenated cottonseed oil in the presence of a source of essential fatty acids (cottonseed oil), it was decided to determine if toxicity symptoms would appear in its absence. In this comparison the cottonseed oil was replaced by sucrose. Furthermore, it was thought advisable to heat and aerate a hydrogenated cottonseed oil in the laboratory to see if toxic symptoms would be produced when this material was fed to rats. In this case the level of cottonseed oil was raised to 4% to be certain of an adequate source of essential fatty acids in the event that laboratory heated and oxidized oil contained antagonists for these nutrients. The B vitamin mix was increased from 0.1 to 0.5 gm per 100 gm of diet in both cases to approximate the vitamin level

of the diet of Johnson et al. ('56). Ten weanling male rats of the Sprague-Dawley strain were fed each diet.

The experiment was continued for 5 weeks. The results are shown in table 8. These results indicate that the addition of cottonseed oil to a diet containing 18% of an unheated or commercially heated hydrogenated cottonseed oil does not significantly increase the caloric value of the oil as measured

TABLE 8

Comparison of weight gain and liver weight of rats receiving used or unused cottonseed oil with laboratory heated and oxidized cottonseed oil at a level of 18% of the diet

SAMPLE NO.	DESCRIPTION	AVERAGE 5 WEEK WEIGHT GAIN	AVERAGE LIVER WEIGHT
		gm	*gm/100 gm body wt.*
4	Hydrogenated cottonseed oil	193.4 ± 17.1 [1]	6.41 ± 0.89
5	Hydrogenated cottonseed oil used 9 days	208.5 ± 12.8	6.38 ± 0.55
4-OH	Laboratory heated and oxidized hydrogenated cottonseed oil	167.6 ± 8.1	7.05 ± 0.39
4a	Same as 4 plus 4% cottonseed oil	205.5 ± 15.7	6.26 ± 0.37
5a	Same as 5 plus 4% cottonseed oil	205.4 ± 17.7	6.37 ± 0.56
4-OHa	Same as 4-OH plus 4% cottonseed oil	189.6 ± 11.1	7.46 ± 0.64

[1] Standard deviation.

by rat growth. Furthermore, the addition of cottonseed oil to a laboratory heated and oxidized sample of hydrogenated cottonseed oil significantly increases ($P < 0.01$) the caloric value of the oil as measured by rat growth. It was also observed that the feeding of 18% of hydrogenated cottonseed oil that has been commercially heated or that has been heated and oxidized in the laboratory causes no apparent symptoms, i.e., diarrhea, rough hair coat, in the growing albino rat whether or not a source of essential fatty acids is included

in the diet. There was no significant difference between the liver weights of the various groups.

It should be noted that although laboratory heated and oxidized oil has a lowered caloric value, commercially heated oil has not. Again, a somewhat higher caloric value was ob-

TABLE 9

Comparison of various fats in the presence and absence of 2% cottonseed oil

SAMPLE	DESCRIPTION	AVERAGE 4 WEEKS WEIGHT GAIN
		gm
6	20% unheated hydrogenated cottonseed oil	163.6 ± 21.5[1]
7	18% sample 6, plus 2% cottonseed oil	170.8 ± 15.0
8	20% hydrogenated cottonseed oil commercially heated for 9 days	172.9 ± 17.5
9	18% sample 8, plus 2% cottonseed oil	177.7 ± 16.4
10	20% sample 6, laboratory heated and oxidized as previously described	144.6 ± 16.4
11	18% sample 10, plus 2% cottonseed oil	160.3 ± 14.9
12	20% corn oil	140.5 ± 15.6
13	18% sample 12, plus 2% cottonseed oil	181.0 ± 8.6
14	20% sample 12, laboratory heated and oxidized	128.0 ± 22.3
15	18% sample 14, plus 2% cottonseed oil	174.8 ± 10.9
16	20% hydrogenated cottonseed oil, unheated (old batch)	127.9 ± 12.5
17	18% sample 16, plus 2% cottonseed oil	158.6 ± 18.8
18	20% sample 16, laboratory heated and oxidized	104.5 ± 9.5
19	18% sample 18, plus 2% cottonseed oil	163.8 ± 17.2

[1] Standard deviation.

tained by this oil than the unheated control oil. In order to extend this study to include other oil samples, 10 weanling male rats were fed the basal diet previously described.

The cottonseed oil was used at a level of 2% since previous experiments indicated that this level was sufficiently high. The experiment was continued for 4 weeks; the results are shown in table 9. There were no apparent symptoms of toxicity in any of the animals in any group. These results confirm the findings previously described and extend them to include corn oil as well as hydrogenated cottonseed oil.

It will be noted that the unheated corn oil and the unheated hydrogenated cottonseed oil (old batch) each gave a rather low growth response. These samples had been in the laboratory for an extended period of time and changes may have taken place which might account for this low response. Since no gross symptoms of toxicity from commercially or laboratory heated and aerated fat, as described by Johnson et al. ('56) were obtained, it was decided to compare the basal diet used in our laboratories in the presence of heated and unheated corn oil at a level of 20% with that used by the above authors. The composition of the diets are shown in table 10.

TABLE 10

Comparison of diet used in this study with that used by Johnson and associates ('56)

JOHNSON ET AL. ('56)		CAMPBELL	
Sucrose	44.0%	Sucrose	54.8%
Casein	31.9%	Casein	20.0%
Mineral mix	5.0%	Mineral mix	4.0%
Vitamin mix	0.2%	Vitamin mix	0.5%
Oil	20.0%	Oil	20.0%
Vitamin A	20,000 units/kg diet	Cystine	0.2%
Vitamin D	2,000 units/kg diet	Choline	0.5%
Vitamin E	100 mg/kg diet	Vitamin A	20,000 units/kg diet
		Vitamin D	2,000 units/kg diet
		Vitamin E	100 mg/kg diet

VITAMIN MIX		VITAMIN MIX	
Vitamin	Amount	Vitamin	Amount
	gm		*gm*
Choline	93.50	Thiamine	0.500
Thiamine	1.24	Riboflavin	0.800
Riboflavin	1.24	Niacin	4.000
Calcium pantothenate	2.48	Pyridoxine	0.500
Folic acid	0.30	Calcium pantothenate	4.000
Pyridoxine	1.24	Biotin	0.040
		Folic acid	0.200
		Menadione	0.500
		Cyanocobalamin	0.003
		Inositol	10.000
		PABA	10.000
		Cornstarch to make	100.000

The experiment was continued for 8 weeks, during which time food consumption was measured daily and the animals weighed weekly. The results are presented in table 11.

The toxicity symptoms consisted of diarrhea, rough hair coat, and in many instances loss of hair. Since the toxicity symptoms did not appear in rats which were fed laboratory heated and oxidized corn oil in the presence of the diet used in our laboratories, it would appear that the diet used by Johnson was possibly inadequate. This possibility is further strengthened by the weight gain and food consumption data.

These show that rats which had received laboratory heated and oxidized corn oil in the presence of the diet used in our laboratories grew significantly better ($P < 0.01$) and had a significantly higher food efficiency ratio ($P < 0.01$) than those which received unheated corn oil in the presence of the diet used by Johnson. When the latter diet is examined it will be noted that a rather high casein diet (31.9%) was used. Furthermore, this diet is lacking in several nutrients, i.e., cystine, niacin, vitamin B_{12}, inositol, *p*-aminobenzoic acid, vitamin K, and biotin. Although it might be argued that the rat does

TABLE 11

Results of comparing different diets in the presence of 20% heated and unheated corn oil (8-week data)

DIET	NO. OF ANIMALS	WEIGHT GAIN	FOOD EFFICIENCY [1]	INCIDENCE OF SYMPTOMS [2]
		gm		
Johnson + unheated corn oil	10	171.8 ± 30.5 [3]	0.294 ± 0.01	0
Johnson + laboratory heated and oxidized corn oil	10	164.0 ± 35.4	0.285 ± 0.02	10
Campbell + unheated corn oil	10	253.8 ± 28.3	0.357 ± 0.007	0
Campbell + laboratory heated and oxidized corn oil	10	252.8 ± 25.6	0.344 ± 0.006	0

[1] Grams gained per gram of feed consumed.

[2] Symptoms consist of diarrhea, loss of hair, rough hair coat, etc.

[3] Standard deviation.

not necessarily require some of these nutrients under certain conditions, it would appear from a comparison of the results obtained with the diet used in our laboratories with those reported by Johnson, that the diet used by Johnson does not appear adequate. Since a high-protein-high-fat diet was used by Johnson, the inclusion of all known nutrients in the diet might have yielded different results. A more recent paper by the same group, Witting et al. ('57), reported data which showed there was no significant difference between the weight gain of rats receiving fresh or used hydrogenated shortening. The diet used in these experiments contained cystine, glycine, inositol, niacin, *p*-aminobenzoic acid, and biotin, all of which were absent in the previous work.

DISCUSSION

From the data presented, it can be seen that hydrogenated cottonseed oil which has been heated for several days in a commercial frying operation has no adverse effect on the growth or well-being of growing albino rats when fed at levels as high as 20% of the diet. Furthermore, there is a strong indication that commercial heating of hydrogenated cottonseed oil actually increases the caloric value of the fat, apparently due to the increase of polyunsaturated fatty acids caused by the heating. This increase in caloric value of commercially heated hydrogenated fats has been noted in the literature, although attention has not usually been called to it. For example, Rice et al. ('57a) presented data comparing various heated and unheated fats. When the heated hydrogenated fats are compared to the unheated hydrogenated sample (to compare this work directly with ours) rather than with the prime steam lard control, an increase in caloric value is apparent in most of the hydrogenated samples (table 12).

In the paper of Deuel et al. ('51) the weight gains of rats, both male and female, as well as the efficiency of the diet were increased when commercially heated margarine type fat was compared to the unheated control. Melnick ('57) reported

iodine value changes of fresh oil, heated oil and shortening blends employed in the commercial manufacture of potato chips. There were variations in these changes from a decrease of 8.5 to an increase of 14.15. These differences, according to the author, were due to errors in sampling the blends. However, in examining these data it will be noted that out of 16 different samples, 8 showed increases in iodine value. Furthermore, the average change was that of an increase. A further indication that heated fats may, under certain conditions, give a better rate of gain than unheated fats, is found in the work of Selva ('56) who fed rats diets containing 15% of fat (raw or heated lard, olive oil, and butter), heated lard gave the best growth. Only with lard did heating enhance growth over the raw fat.

TABLE 12

Comparison of the caloric availability of various fats and oils with corn oil and hydrogenated cottonseed oil

FAT OR OIL	CONTROL CALORIES [1]	HYDROGENATED VEGETABLE OIL [2]
	%	%
Corn oil	100	
Hydrogenated soybean oil	108	
Cottonseed oil	102	
Hydrogenated vegetable oil	92	100
Lab. heated hydrogenated vegetable oil	65	
Lab. oxidized hydrogenated vegetable oil	64	
Samples heated 1–5 days commercially		
A. Corn oil	90	98
B. Winterized cottonseed oil	111	121
C. Hydrogenated cottonseed oil	97	106
D. Hydrogenated vegetable oils	100	109
E. Hydrogenated lard and hydrogenated vegetable oils	95	103
F. Hydrogenated lard	90	98
G. Lard	100	109
H. Lard + hydrogenated vegetable oil	97	106
I. Animal fat + hydrogenated vegetable oil	95	103
J. Hydrogenated vegetable oils	108	118

[1] From Rice et al. Presented at the Annual Meeting of the Federation of American Societies for Experimental Biology, Chicago, Ill., April 15–19, 1957, and personal communication.

[2] Our calculations based on hydrogenated cottonseed oil equal to 100%.

It is apparent that further investigation of published data as well as continuing research will be necessary to completely elucidate this problem.

SUMMARY

1. Hydrogenated cottonseed oil heated in a commercial deep-fat fryer under actual production conditions for as long as 24 days had no deleterious effects on rats when fed at levels as high as 20% of the diet.

2. Hydrogenated cottonseed oil heated in a commercial deep-fat fryer under actual production conditions for as long as 24 days had a higher available caloric value than the unheated control oil as measured by the caloric restriction technique as well as by the rat growth method.

3. The increase in available caloric value of the commercially heated hydrogenated cottonseed oil may be associated with the finding that heating under these conditions apparently causes unsaturation of the fat. A highly significant positive correlation was found to exist between the nutritive value of the fat, as measured by the caloric restriction technique, and the total non-conjugated double bonds present in the fat.

4. Fats which have been heated and oxidized under laboratory conditions yield a lower growth rate in rats than the unheated oils but exhibit no apparent symptoms of toxicity (i.e., diarrhea, rough hair coat, etc.) when tested using nutritionally adequate diets.

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THE EFFECT OF THE CARBOHYDRATE AND FAT CONTENT OF THE DIET UPON THE RIBOFLAVIN REQUIREMENT OF THE CAT[1]

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Little is known of the nutritional requirements of cats. Not only have the quantitative aspects of cat nutrition been neglected but also descriptions of various nutritional deficiency diseases in cats are lacking. In this paper the results of studies of the riboflavin requirements of cats fed diets varying in carbohydrate and fat content will be reported.

EXPERIMENTAL

In this work three- to 6-months-old kittens of mixed breed and sex were used. All cats before being admitted to the laboratory were dusted with an insecticide, dewormed and vaccinated against feline distemper. The animals were housed individually in wire mesh cages and maintained on food and water ad libitum. The two diets used in these studies (table 1) differed considerably in their fat and carbohydrate content. In the low-carbohydrate, high-fat diet, 46% of the calories came from fat and in the high-carbohydrate, low-fat diet, 11%. In both diets, 25% of the calories were provided by casein.

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Two separate experiments were performed. In the first, 9 kittens were fed the low-carbohydrate, high-fat diet without riboflavin for two months by which time all were suffering from acute riboflavin deficiency. Four of the cats were sacrificed for histologic study. The others were given 5 mg of riboflavin subcutaneously and then fed diets with graded amounts of riboflavin. Two cats received 0.5 mg, two cats 1.0 mg, and one cat 1.5 mg of riboflavin per kilo of diet. All of these cats died, presumably of riboflavin deficiency within

TABLE 1

Composition of cat diets

CONSTITUENT	LOW CARBOHYDRATE	HIGH CARBOHYDRATE
	%	%
Casein	32.1	25.5
Sucrose	37.6	65.2
Corn oil	12.5	2.0
Hydrogenated fat	12.5	2.0
Cod liver oil	1.0	1.0
Salts IV [1]	4.0	4.0
Choline	0.3	0.3
Vitamins [2]		

[1] Hegsted et al. ('41).

[2] Vitamins: 4 mg thiamine, 40 mg niacin, 20 mg Ca pantothenate, 4 mg pyridoxine, 1 mg folic acid, 0.2 mg biotin, 1 mg menadione per kilo of ration. Riboflavin was added as required.

4 months of being fed the supplemented diets. These animals were autopsied generally within one to two hours of death.

In the second experiment, groups of cats were fed the two experimental diets with varying amounts of riboflavin for periods up to 34 months. After the cats had been on experiment for three weeks, the food intake of two cats on each of 5 dietary regimes was measured and their urine and feces were collected and analyzed for riboflavin microbiologically (Snell and Strong, '39).

In all, 25 cats were autopsied. In the 9 animals in experiment 1, complete gross and microscopic studies were carried out, including examination of brain and spinal cord in selected

animals. Tissues were fixed in 10% neutral formalin and paraffin sections were stained with Hematoxylin and Eosin and in the case of nervous tissues, with Luxol Fast Blue. Frozen sections were stained with Sudan IV and Hematoxylin. Autopsies were performed on all 16 animals in experiment II although microscopic studies were carried out on selected tissues only.

RESULTS

The symptoms of acute deficiency in cats fed diets without any added riboflavin consisted of anorexia accompanied by weight loss terminating in death. In the first experiment 7 of the 9 cats showed some loss of hair about the eyes and ears after two months of the unsupplemented diet. Of the 5 cats showing hair loss, and then receiving riboflavin supplements, only one demonstrated regrowth of hair, the supplement in this case being 1 mg per kilo of diet. A second cat, changed to a diet containing 0.5 mg of riboflavin per kilo, developed cataracts and increased hair loss extending to its chest and feet.

The results of the second experiment are summarized in table 2. In acute riboflavin deficiency those animals fed the low-carbohydrate diet became moribund sooner than those fed the high-carbohydrate diet. Chronic riboflavin deficiency differed from acute deficiency not only in the more protracted course but also in the occurrence of cataracts. The 4 animals which developed cataracts were, like the one instance in the first experiment, receiving the low-carbohydrate diet. In one animal receiving 1 mg of riboflavin per kilo, cataracts appeared after 3½ months. In the other three animals, supplemented with 2 mg of riboflavin per kilo, cataracts were noted after 6 to 8 months. In cat 124, two 20 mg riboflavin supplements given parenterally and transfer to a low-carbohydrate diet containing 3 mg of riboflavin per kilo did not affect the appearance of the cataracts. The cat died 4 months later after a total of 13½ experimental months. In none of the cats in either experiment were there lesions of the cornea, changes about the mouth, anemia, paralysis, or the riboflavin collapse syndrome.

TABLE 2

The effect of riboflavin-deficient diets on cats

RIBOFLAVIN /KILO DIET	CAT NO.	EXPERIMENTAL AGE		FATTY LIVER	CATARACTS
		At death	At maximum weight		
mg		*months*	*months*		
		Low carbohydrate diet			
0	120	1½	¾	+	—
0	121	1¾	¾	+	—
1	8	4½	3¼	—	+
1	15	3¼	2¼	+	—
2	1	10¼	5½	+	+
2	3	11¾	9	—	+
2	122	6½	5¾	—	—
2	123	13	3¼	+	—
2	124	13½	3½	—	+
2.5	125	10¼	6	+	—
2.5	127	5½	3	—	—
		High carbohydrate diet			
0	111	3	2	+	—
0	112	2½	2½	+	—
0	113	2¼	0	+	—
2	115	6	2¼	+	—
2	116	12¾	6	+	—

TABLE 3

Effect of dietary carbohydrate on riboflavin excretion[1]

DIET	WEEKS ON DIET	DIETARY RIBOFLAVIN /2 CATS /WEEK	FECAL RIBOFLAVIN /2 CATS /WEEK	URINARY RIBOFLAVIN /2 CATS /WEEK
		μg	*μg*	*μg*
Low carbohydrate				
no added riboflavin	4	0	428 ± 107	24 ± 6
+ 2 mg riboflavin/kilo	12	873 ± 20	1687 ± 301	44 ± 10
+ 2.5 mg riboflavin/kilo[2]	6	1074 ± 47	3524 ± 1179	322 ± 30
High carbohydrate				
no added riboflavin	7	0	744 ± 155	53 ± 10
+ 2 mg riboflavin/kilo[2]	12	1256 ± 41	3171 ± 517	1329 ± 157

[1] All values include standard error of the mean.

[2] Both diets provide approximately 0.5 mg of riboflavin per 1000 Cal.

Two cats have been kept in excellent health for more than 34 months on the high-carbohydrate diet plus 3 mg of riboflavin per kilo and cats have been routinely maintained in this laboratory on the low-carbohydrate diet plus 4 mg of riboflavin per kilo for periods up to two years.

Urinary and fecal riboflavin values shown in table 3 demonstrate a marked effect of both the high- and low-carbohydrate diets in promoting riboflavin synthesis in cats since considerably more riboflavin was recovered in the feces and urine than was fed. The urinary excretion data indicate that more of the synthesized riboflavin was available to cats receiving the high-carbohydrate than those receiving the low-carbohydrate diets.

PATHOLOGY

Bilateral cataracts have been seen in 5 cats as sharply circumscribed, often lobulated opacities. Localization of the cataracts was quite variable involving the nucleus, the cortex, or the posterior subcapsular region. The Y-suture lines, which are quite prominent in the adult cat lens, usually appeared to be accentuated. Cataractous lenses were distinctly softened and could readily be cut. In one instance, all of the lens substance appeared diffusely involved with shrinkage in the anterior-posterior diameter and irregularities of the surfaces. The capsules in two cases were loosened and there were small amounts of subcapsular fluid, rich in large birefringent rhombohedral crystals. Following alkaline hydrolysis of these lenses, a positive Liebermann-Burchard reaction was obtained, maximum optical density occurring after 25 to 30 minutes, comparable to that obtained with pure cholesterol.

Grossly and microscopically, there was a total loss of fibrillar structure in the cataractous foci. The lens substance appeared as an anuclear, homogenous, highly eosinophilic coagulum. There were granular areas, small and large ovoid fluid spaces and occasional foci of Morgagnian globules. The lens epithelium appeared atrophic. Foci of subepithelial vacuoles suggested lipid though frozen sections were negative. No

birefringent crystals could be seen within the lens substance. There were no significant corneal changes.

Fat was generally, though not invariably, present in the parenchymal cells of the livers of deficient cats in moderate to large amounts. In minimally involved livers, the fat was present as small droplets in the mid-zonal portions of the liver lobule. With increasing amounts of fat, the periportal liver cells became involved. There was sparing of the liver cells centrally except in those cases of massive involvement. In the latter, large fat globules were seen and rarely the formation of cysts of apparent multicellular origin. An occasional instance of cholangitis with pericholangitis was seen without evidence of parasites.

Alopecia, which was seen only in the first experiment, was not accompanied by other gross skin changes. It was quite focal being limited to the area about the ears and periocular involvement was never prominent. The skin from areas of alopecia showed a distinct atrophy of the epidermis and all dermal appendages. Involvement of hair follicles and sebacious glands was more prominent than that of sweat glands. There were moderate numbers of dystrophic hair shafts, which did not appear to be emerging in the usual fashion, but were twisted and associated with an increase in keratohyalin in the follicle neck. These changes were more pronounced in the supplemented animals at 6 months than in acutely deficient animals. In addition, among the former animals there were several instances of edema of the dermis as well as a peculiar hyperplasia of the sebacious glands combined with follicular atrophy. Inflammation was rare and apparently incidental.

All of the 6 male cats, whose testes were studied histologically, showed distinct testicular hypoplasia. This was true of completely deficient cats at the end of two months as well as of cats supplemented with 2 mg of riboflavin per kilo for periods up to 10 months. There was generally complete aspermia and decreased numbers of spermatids and spermatocytes. Occasionally, multinucleated spermatogonia were seen as well as

an apparent increase in Sertoli cells. The other endocrine glands were unremarkable.

No alterations in the myelin structures of the brain or spinal cord were seen nor were there any apparent cellular changes. There were several instances of acute ulceration of the prepyloric gastric mucosa with inflammation and occasionally hemorrhage. This is apparently an incidental lesion that has been noted not uncomomnly in our laboratory cats maintained on a variety of dietary regimens. There was no evidence of atrophy of the gastro-intestinal mucosa, nor were there changes in the papillary structures of the tongue. There was no increase in fat in the convoluted tubules of the kidney, which is normally abundantly present in control cats.

DISCUSSION

Although it has been reported that diets containing large quantities of dextrin or corn starch decrease the requirements of rats for riboflavin (Mannering et al., '41), the effect of dietary fat and carbohydrate on riboflavin requiremets has not been extensively studied. Potter et al. ('42) in dogs and Mannering et al. ('44) in rats did not observe a sparing influence of carbohydrate on the dietary requirements for riboflavin when sucrose was fed. Urinary and fecal excretion in the present study indicate a marked effect of sucrose in promoting riboflavin synthesis in cats. The development of riboflavin deficiency in cats, although they were excreting large amounts of riboflavin in their feces, indicates that much of the synthesized riboflavin was not available. Some of it was probably utilizable as indicated by the higher excretions of urinary riboflavin and increased survival in acute deficiency of cats receiving the high carbohydrate diets. However, the data do not allow a clear cut distinction between the possibility of a higher metabolic need for riboflavin on high-fat diets versus an explanation based solely upon intestinal synthesis.

Although urinary riboflavin was raised when the low-carbohydrate diet with 2.5 mg of riboflavin per kilo was fed and was very much increased when the high-carbohydrate diet with

2 mg of riboflavin per kilo was fed, the deaths of the cats fed these diets after periods of 6 to 13 months even though no specific signs of riboflavin deficiency were observed, indicate that these quantities of riboflavin were subminimal. The urinary riboflavin excretion after long periods upon these diets is not known. However, 3 mg of riboflavin per kilo of high-carbohydrate diet and 4 mg of riboflavin per kilo of low-carbohydrate diet provide sufficient riboflavin for growth and health in cats over long periods.

Riboflavin deficiency has been associated with cataracts in the rat (Day et al., '31; O'Brien, '32; Day et al., '37), mouse (Langston et al., '33; Lippincott and Morris, '41), and pig (Patek et al., '41; Wintrobe et al., '44). The actual incidence of cataract production in the rat is highly variable ranging from none (György, '35) to 100% (Day and Langston, '34). The explanations for this lack of agreement range from that of Day and Darby ('38) that small amounts of riboflavin may prevent occurrence of the lesion to that of Baum et al. ('42) that minute amounts of the vitamin are essential for cataract formation. The lesions are generally cortical in location (Langston et al., '33; Wintrobe et al., '44), though in the rat their variability has been emphasized (O'Brien, '32; Buschke, '43).

The 5 cataracts seen in the deficient cats varied as to localization, in several instances the nucleus being involved. Histologically, the lesions resembled those described in the rat, although there was atrophy of the lens epithelium rather than the usual hyperplasia. This atrophic change has been seen in mice (Langston et al., '33; Lippincott and Morris, '41). It is presumed that the crystalline material in the subcapsular fluid of the cataractous lenses was unesterified cholesterol, which has been noted in the rat (O'Brien, '32). It was noteworthy that several of the animals developed cataracts while maintaining adequate weight responses and without other evidence of malnutrition. Corneal vascularization and related changes that have been described in other species (Bessey and

Wolbach, '39; Lippincott and Morris, '41; Potter et al., '42; Patek et al., '41) were not seen in the deficient cat.

Fatty livers have been described in riboflavin-deficient dogs (Sebrell and Onstott, '38; Street and Cowgill, '39; Potter et al., '42), though one of these groups concluded after further work (Street et al., '41) that such changes could be explained on the basis of inanition. Wintrobe et al. ('44) have noted fatty livers in riboflavin-deficient pigs. Normal cats maintained on the 26% fat purified diet for control purposes in this laboratory show small quantities of liver fat seen as small globules in the periportal liver cells. Cats maintained on the alternate control diet, containing 5% fat, show only minute traces of liver cell fat. The quantity of liver fat in the riboflavin-deficient cats was quite variable but was in a number of instances far in excess of what we have seen in instances of inanition, whether secondary to infectious disease or accompanying other deficiency states.

Unequivocal testicular atrophy associated with riboflavin deficiency has been described only in the rat (Shaw and Phillips, '41). The histologic picture of the testes in riboflavin-deficient Cebus monkeys (Mann et al., '52) appears to represent sexual immaturity. In riboflavin-deficient cats, a prominent testicular hypoplasia of nonspecific character was seen, somewhat less marked than that in vitamin A-deficient cats (Gershoff et al., '57), but distinctly more pronounced than the changes we have found in inanition.

Partial paralyses due to myelin degeneration of the central nervous system and peripheral nerves so prominent in other species (Zimmerman and Burack, '34; Phillips and Engel, '38; Shaw and Phillips, '41; Lippincott and Morris, '41; Wintrobe et al., '44; Mann et al., '52), and anemia reported in some of these species (Spector et al., '43; Wintrobe et al., '44; Waisman, '44; Greenberg and Rinehart, '56), were not seen in riboflavin deficient cats.

Skin manifestations of riboflavin deficiency have been described in a number of species, rats (Sullivan and Nicholls, '41; Wolbach and Bessey, '42), mice (Lippincott and Morris,

'41), hamsters (Routh and Houchin, '42), dogs (Street et al., '41), pigs (Patek et al., '41), monkeys (Waisman, '44; Mann et al., '52), and man (Sebrell and Butler, '39; Hou, '41). The lesions are usually described as a dry, scaling, seborrheic dermatosis associated with hair loss. These changes are seen about the face and over the trunk and limbs. In contrast, the riboflavin-deficient cat showed no gross dermatosis and only alopecia, generally quite limited in extent. The histologic appearance of the lesions in the cat bear out the essentially non-inflammatory atrophy described in other species. The lesions in the rat described by Wolbach and Bessey ('42), are very similar to those in the cat though these authors, unlike Sullivan and Nicholls ('41), found atrophy limited to the skin appendages. The sporadic sebacious gland hyperplasia found in the cats was not associated with any apparent seborrhea in life. This hyperplasia would appear quite distinct from the peculiar changes described by Sullivan and Nicholls ('41) preceding the stage of atrophy in rats. The ulcerative tendency seen in the mouse (Lippincott and Morris, '41) was not observed.

The absence of alopecia in the second experiment would appear anomalous, in that 7 of the 9 animals in acute deficiency in the first experiment showed hair loss. However, in the second experiment there were only two acutely deficient animals fed the high-fat, low-carbohydrate diet which were strictly comparable to those in the first. These two animals died or were moribund in less than two months, the time at which alopecia was noted in the first experiment.

Bro-Rasmussen ('58) has summarized and evaluated much of the data available on the riboflavin requirements of different species and concluded that the minimal requirement of different species is similar if expressed upon a calorie basis. He arrives at a minimum requirement during early life of 0.7 to 0.8 mg per 1000 Cal. This is assumed to be an optimal intake at all ages. The minimum need of adult man is concluded to be approximately 0.25 mg per 1000 Cal. and 0.5 to 0.6 mg per 1000 Cal. is assumed to be an adequate intake. This

author also reviews some of the evidence upon intestinal synthesis and concludes that, "These results seem to leave no doubt that the intestinal flora may be of importance also for man's riboflavin supply." His analysis of the data is somewhat contradictory since he concludes that the requirement per 1000 Cal. was essentially the same in various studies on adults in which the composition of the basal diets were as variable as in the studies of (Horwitt et al., '49; '50) and the study of Burgess ('46) on prisoners of war.

There are insufficient data to make a quantitative estimate of the importance of the kind and amount of dietary carbohydrate and fat on human riboflavin requirements, and further study appears indicated. In addition to the papers referred to by Bro-Rasmussen, the studies of Widdowson and McCance ('54) in German orphanages provide substantial evidence that the intestinal synthesis and the urinary excretion of riboflavin depend upon the ratio of fat to carbohydrate calories in the diet. Iinuma ('55) has concluded that there is less riboflavin deficiency in Japan than might be expected upon the basis of the dietary intake and demonstrated that vegetable diets high in crude fiber promote intestinal synthesis. This effect of crude fiber is also inferred from the studies of Yasuda ('51) upon rats.

In view of the established effect of dietary carbohydrate and fat content on the riboflavin need of experimental animals and qualitatively similar data on human beings, it seems likely that riboflavin standards arrived at under particular dietary conditions may not be applicable in parts of the world with different dietary conditions. Since riboflavin deficiency is common in many parts of the world and dietary sources of riboflavin are limited, a thorough investigation of this problem may be of considerable practical importance.

SUMMARY

Riboflavin deficiency has been produced in cats fed isonitrogenous purified diets varying in carbohydrate and fat content and containing different quantities of riboflavin. In a number

of instances cataracts developed in cats receiving amounts of riboflavin slightly below their minimal requirement. Fatty livers and testicular hypoplasia were present as well as skin changes which were less dramatic than in other species. Anemia and central nervous system changes were not observed. High-carbohydrate, low-fat diets appear to exert a sparing influence on the cats' riboflavin requirement. Urinary and fecal riboflavin determinations indicate that this effect is largely due to increased intestinal synthesis and utilization of riboflavin.

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THE EFFECT OF PROTEIN AND ENERGY ON THE POTASSIUM REQUIREMENT OF THE CHICK

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Ben Dor ('41) demonstrated the necessity for including potassium in the diet of young chicks. His results indicated that at least 0.17% potassium was needed to achieve maximum growth rate, while lesser amounts were necessary to prevent excessive mortality. Gillis ('48, '50) concluded that the potassium requirement of chicks was 0.20 to 0.24% of the diet. The higher quantity of potassium was found to be needed when the phosphorus content of the diet was borderline or suboptimum. Gillis also showed that potassium is required for proper bone formation. Burns and associates ('53) reported that the potassium requirement of the chick was between 0.23 and 0.40% of the diet. They concluded that the requirement was in part dependent upon the growth rate of the chicks. Sodium and potassium were found to be toxic if one of these minerals was fed greatly in excess of the other. The toxicity was overcome by raising the level of the other element.

Recent results obtained at this laboratory suggested an increased need by the chick for potassium when a high-protein, high-energy purified diet is fed. The present paper is concerned with the results obtained during a reinvestigation of the potassium requirement of young chicks fed a purified diet of this character. Particular attention has been focused on possible non-mineral dietary factors which might have an effect on the potassium requirement of the chick.

EXPERIMENTAL

The composition of the basal diet used in the investigation is given in table 1. The soybean protein in the basal diet was purified by repeated washings in tap water at pH 4.6 with a final washing in demineralized water followed by pressing to remove as much water as possible and subsequent drying in a forced draft electric oven at 65°C.

TABLE 1

Composition of the basal diet

INGREDIENT	AMOUNT
	%
Glucose	45.10
Corn oil	10.00
Purified soybean protein [1]	33.36
Cellulose	3.23
DL-Methionine	0.84
Glycine	0.36
Mineral mixture [2]	5.84
Vitamin mixture [3]	1.25
Butylated hydroxytoluene (BHT)	0.02
Metabolizable energy, Cal./gm	3.68
Protein content, %	30.36

[1] Drackett Assay Protein C-1, purified by repeated washings.

[2] Supplies the following per 100 gm of diet: $CaHPO_4$, 2.31 gm; $CaCO_3$, 1.61 gm; KH_2PO_4, 0.93 gm; NaCl, 0.65 gm; $MgSO_4$, 0.27 gm; $FeSO_4 \cdot 7H_2O$, 36 mg; $MnSO_4 \cdot H_2O$, 36 mg; KI, 0.28 mg; $CuSO_4 \cdot 5H_2O$, 1.8 mg; $ZnCl_2$, 12.5 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.34 mg.

[3] Supplies the following per 100 gm of diet: choline chloride, 161.6 mg; inositol, 26.9 mg; niacin, 5.4 mg; calcium pantothenate, 4.3 mg; pyridoxine HCl, 0.48 mg; folic acid, 0.43 mg; menadione sodium bisulfite, 1.03 mg; biotin, 22 mcg; vitamin B_{12}, 5.4 mcg; alpha-tocopheryl acetate, 7.1 IU; vitamin A, 540 IU, and vitamin D_3, 108 ICU; thiamine HCl, 1.1 mg; riboflavin, 1.1 mg.

The diet was developed by Dam and associates ('57) for use in studies of unidentified chick growth factors, from a diet used previously for this purpose by Morrison ('56) and shown by him to be adequate in sodium and potassium. In developing the new diet the energy content was increased from 3.30 to 3.68 Cal. of metabolizable energy per gram and the protein content from 23 to 30%. The quantities of vitamins and min-

erals included in the diet of Dam and associates were increased over those in the Morrison diet in proportion to the increase in energy content. This raised the level of sodium from 0.24 to 0.26% of the diet and the potassium from 0.25 to 0.27%.

The diets and ingredients were analyzed for potassium content using the flame spectrophotometric method of the Association of Official Agricultural Chemists ('55). The results of the analyses showed that the washing procedure used to purify the soybean protein in the diet reduced the potassium content of this material from 0.26 to 0%. They also indicated that the only measurable source of potassium in the diet was supplied by the monobasic potassium phosphate in the mineral mixture. This was evidenced by the close agreement between calculated (0.27%) and analytical values for the potassium content. In the experiments, the results of which are presented in tables 4, 5 and 6, the sodium chloride and potassium phosphate in the mineral mixture were replaced with sodium acetate and dicalcium phosphate. Potassium was added in the chloride and bicarbonate forms in such a manner that the chloride content of the diet was maintained constant at 0.38%.

The nitrogen content of the soybean protein was determined using the macro-Kjeldahl procedure. Protein content was calculated on the basis of $N \times 6.25$. Calculations for the metabolizable energy content of the diets were made using the values of Hill and associates ('58).

Duplicate lots of 13 to 15 one-day-old Vantress X White Plymouth Rock male chicks were used in all experiments. The chicks were placed in electrically heated battery brooders with raised wire-screen floors and were supplied the experimental diets and demineralized water ad libitum. Individual weights were taken at weekly intervals during the 4-week experimental period. Records of feed consumption were made at the time the chicks were weighed.

RESULTS AND DISCUSSION

Since preliminary results indicated that the basal diet was borderline in mineral content, two experiments were conducted in which the effect of increasing the sodium and potassium content of the diet was studied. The results of these experiments are given in table 2. Increasing the sodium from 0.26 to 0.33% and potassium from 0.27 to 0.50% resulted in consistent in-

TABLE 2

Effect of increasing the sodium and potassium content of the basal diet

TREATMENT	AV. WT.		INCREASE OVER BASAL	
	2 wk.	4 wk.	2 wk.	4 wk.
	gm	*gm*	%	%
	Experiment 1			
0.27% potassium 0.26% sodium	188	546 (29)[1]	—	—
0.50% potassium 0.33% sodium	208	579 (30)[1]	11.0	6.0
	Experiment 2			
0.27% potassium 0.26% sodium	174	526 (25)[2]	—	—
0.50% potassium 0.33% sodium	206	586 (26)[2]	18.0	11.0

[1] Survivors of duplicate lots of 15 chicks per lot.
[2] Survivors of duplicate lots of 13 chicks per lot.

creases (6 to 11%) in growth rate. The magnitude of this response was greater (11 to 18%) when the chicks were two weeks of age. A third experiment was conducted to ascertain which mineral was responsible for the growth increases. The results of this experiment are given in table 3. Although the responses (4%) to the mineral additions were not as great as those noted in the previous experiment, the mineral responsible for this growth increase appeared to be potassium. In the next experiment, graded levels of potassium were fed for the purpose of determining the approximate potassium requirement of chicks fed the basal diet. The results which are

given in table 4 indicated that the potassium requirement for optimum growth was approximately 0.30%, while 0.20% was required to prevent excessive mortality. The chicks which received 0.10% potassium exhibited deficiency symptoms similar to those described by Gillis ('48).

TABLE 3

Effect of increasing the sodium and potassium content of the basal diet

TREATMENT	AV. WT.		INCREASE OVER BASAL	
	2 wk.	4 wk.	2 wk.	4 wk.
	gm	*gm*	%	%
0.27% potassium 0.25% sodium	178	517 (27)[1]	—	—
0.52% potassium 0.25% sodium	194	537 (112)[2]	9.0	4.0
0.52% potassium 0.40% sodium	191	538 (112)[2]	7.0	4.0

[1] Survivors of duplicate lots of 15 chicks per lot.

[2] Survivors of duplicate lots of 15 chicks per lot fed 4 different sources of potassium.

The results of the first 4 experiments indicated that the potassium requirement of chicks receiving the type of basal diet fed in this investigation is somewhat more than 0.27%. Therefore it seemed desirable to determine what effect dietary factors such as protein and energy content might have on the potassium requirement of young chicks.

TABLE 4

Effect on growth rate and mortality of adding graded amounts of potassium to the basal diet

ADDED POTASSIUM	AV. WT.		MORTALITY [1]
	2 wk.	4 wk.	
%	*gm*	*gm*	%
0.10	64	148	78.6
0.20	123	396	14.3
0.30	181	528	7.1
0.40	186	528	3.6
0.50	196	546	3.6
0.60	182	512	0

[1] Duplicate lots of 14 chicks per lot at start of experiment.

Several investigators have suggested an interaction between protein and potassium. Wooley and Mickelson ('54) obtained greater growth in rabbits by increasing the quantity of a mixture of sodium, potassium and calcium when either the protein or the fat content was increased. In further work sodium was found to be as effective as the combination of all three minerals. The authors suggested that an animal's requirement for minerals may be more intimately associated with the composition of the diet than previously thought. Cannon and associates ('52) and Frost and Sandy ('53) reported that potassium was necessary for maintenance of nitrogen balance in rats. Potassium also influences the utilization of injected amino acids by surgical patients according to the results of Frost and co-workers ('53). Eckel and co-workers ('54) and Iacobellis and asociates ('56) have shown that potassium is involved in the maintenance of certain free amino acids in cellular tissue.

The results of an experiment designed to study the effect of protein on the potassium requirement are given in table 5. In this experiment diets containing 25, 30 and 37% protein were fed. The diets were maintained isocaloric at approximately 3.68 Cal. of metabolizable energy per gram by varying the fat content $\pm$ 0.5%. When the level of potassium was inadequate, increasing the protein content from 25 to 37% of the diet markedly depressed growth rate, decreased the grams gain per gram of potassium, and increased mortality. However, protein level had no effect on the chicks which received sufficient potassium to achieve optimum growth rate.

An estimate of the potassium requirement was obtained through the use of the method described by Almquist ('53). In this method, the growth response is plotted against the logarithm of the quantity of potassium in the suboptimal region. The intersection of the linear curve obtained with a line representing optimum response is taken as the requirement. The results indicated that the potassium requirement increases as the protein level of the diet is increased. These increases in

TABLE 5

Effect of protein level on the potassium requirement of the chick

ADDED POTASSIUM	PROTEIN, 25.8%			PROTEIN, 30.4%			PROTEIN, 37.9%		
	Av. wt. 4 wk.	Gm gain/ gm K	Mortality [1]	Av. wt. 4 wk.	Gm gain/ gm K	Mortality [1]	Av. wt. 4 wk.	Gm gain/ gm K	Mortality [1]
%	*gm*		%	*gm*		%	*gm*		%
0.15	252	331	42.8	218	282	53.6	118	183	60.7
0.20	489	350	7.1	440	323	25.0	335	308	21.4
0.25	585	294	0	537	289	3.6	485	286	10.7
0.40	595	189	0	593	184	3.6	612	189	0
0.50	615	152	0	605	153	0	589	152	0
Estimated requirement, %									
2 week		0.31			0.33			0.38	
4 week		0.27			0.29			0.32	
Protein increase, %		100			118			147	
K requirement increase, %		100			108			120	

[1] Duplicate lots of 14 chicks per lot at start of experiment.

potassium requirement were not, however, proportional to the increase in protein content.

The data from this experiment were subjected to analysis of variance by the methods outlined by Snedecor ('55). The retarding effect on growth of increased protein in the presence of potassium deficiency, and the improved growth obtained with increasing amounts of potassium, were found to be highly significant ($P < 0.01$). In addition, a highly significant interaction was observed between quantity of protein and quantity of potassium. These results may indicate a function of potassium in protein metabolism, especially catabolism, since the two higher levels of protein were in excess of the chick's need.

The results of an experiment designed to study the effect of energy on potassium requirement are given in table 6. In this experiment, diets containing from 3.35 to 4.25 Cal. of metabolizable energy per gram were fed. The relationship of energy to protein and other ingredients was kept constant throughout the experiment. Potassium levels varying from 0.15 to 0.45% were included in the 3% corn oil diet, while the graded levels in the 10 and 20% corn oil diets were increased according to increases in the energy content of the diet. This resulted in a constant ratio of potassium to energy for each potassium level.

In this experiment some difficulty was encountered in estimating the potassium requirement of chicks fed the 3% fat diet. This difficulty involved the establishment of a value for maximum response. On the basis of the "Law of Diminishing Increment," the growth response obtained with either the 0.36 or the 0.45% level of potassium appeared to be out of line. This is made evident by plotting the average 4-week weights against the quantity of potassium supplied in the experimental diets. Therefore, the requirement is given as a range in which the growth response of the chicks fed 0.36% and those fed 0.45% potassium were used as the values for maximum response. The estimated requirement appeared to be, therefore, between 0.23 and 0.25% of the diet. However, on the basis of growth rates obtained in other experiments using this diet,

TABLE 6

Effect of increasing the energy content of the diet on the potassium requirement of the chick

K: ME [1]	CORN OIL, 3%; ME, 3.35 CAL./GM			CORN OIL, 10%; ME, 3.72 CAL./ GM			CORN OIL, 20%; ME, 4.25 CAL./GM		
	Added K	Av. wt. 4 wk.	Gm gain/ gm K	Added K	Av. wt. 4 wk.	Gm gain/ gm K	Added K	Av. wt. 4 wk.	Gm gain/ gm K
	%	*gm*		%	*gm*		%	*gm*	
2.22	0.15	260 (25)[2]	325	0.17	282 (20)	310	0.19	309 (20)	337
1.77	0.19	436 (26)	310	0.21	458 (25)	317	0.24	410 (25)	311
1.49	0.23	501 (28)	280	0.25	530 (25)	281	0.28	518 (28)	288
0.93	0.36	526 (27)	180	0.40	590 (27)	186	0.45	597 (28)	181
0.74	0.45	572 (28)	141	0.50	610 (28)	147	0.57	600 (28)	149
Estimated requirement, %									
2 week		0.26–0.29			0.30			0.37	
4 week		0.23–0.25			0.27			0.32	
Energy increase, %		90			100			114	
K requirement increase, %		85–93			100			119	

[1] Ratio of milligrams of potassium to metabolizable energy per 100 gm diet.

[2] Survivors of duplicate lots of 14 chicks per lot.

and by applying Duncan's multiple range test (Federer, '55) to the differences between the means, the growth response of the chicks fed 0.45% potassium appeared to be maximum, and therefore the estimation of requirement of 0.25% appeared to be the more reliable under the conditions of the experiment.

The results of this experiment indicated that the potassium requirement is related to the energy content of the diet rather than the fat content *per se*. The percentage increase in the potassium requirement was found to be in close agreement with the increase in the energy content of the diets. Also, in the diets in which potassium was the limiting factor, the grams of gain per gram of potassium was markedly uniform at each level of potassium regardless of the fat content of the diet. If the effect of fat was mediated through some mechanism other than calories, these values would have not agreed but would have varied with the change in dietary fat content. In further confirmation of this conclusion, the results of an analysis of variance showed that highly significant growth increases ($P < 0.01$) were obtained with all diets by increasing the potassium content but no interaction was revealed between level of fat and level of potassium. The improvement in growth obtained by increasing the fat content from 3 to 10% was also highly significant.

The chick's requirement for potassium was observed to decrease with increasing age. For the purpose of brevity the two-week results were not presented for the experiments the results of which are presented in tables 5 and 6. However, the estimated requirements determined from the two-week data are presented. Examination of these data showed that chicks require on the average 0.04% more potassium during the first two weeks of life than is indicated by the findings covering the entire 4-week period. The comparison of two- and 4-week results in tables 2 and 3 lends further support to this conclusion.

Some variation in the potassium requirement of chicks fed the basal diet containing 10% corn oil was observed in this investigation. This is revealed by a comparison of the results

presented in tables 2, 3, 5 and 6. These results indicate that the minimum potassium requirement of chicks of the same breed and source varies from experiment to experiment by approximately 7.5%. This is believed to be caused by uncontrollable conditions inherent in research work with the chick.

SUMMARY

The results of the experimental work presented in this report revealed that the protein and energy content of the diet affects the potassium requirement of the chick. A definite interrelationship between potassium requirement and protein level was demonstrated. Increasing the protein content of the diet increased the potassium requirement of the chick for optimum growth and survival. The increase in the potassium requirement, however, was not in proportion to the increase in protein content. On the other hand the effect of fat on the potassium requirement of the chick appeared to be mediated through changes in the caloric content of the diet. The changes in the requirement were found to parallel closely the changes in energy content of the diet. The chick's requirement for potassium was also observed to decrease with increasing age.

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THE NUTRITIONAL EFFECT OF POLYMERS ISOLATED FROM THERMALLY OXIDIZED CORN OIL [1,2]

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INTRODUCTION

In a recent report from the National Research Council (Gortner, '58) it was stated that "Chemical alterations produced in fats during heating should be defined and studies should be made of the heated oils produced in food processing and under home cooking conditions." The chemical alterations which occur in an unsaturated oil at commerical food frying temperatures of approximately 200°C have already been reported elsewhere (Perkins et al., '58; Johnson and Kummerow, '57). The length of time a specific triglyceride molecule of the oil is exposed to this temperature is dependent on the turnover rate of the oil. As this rate is governed by the amount of oil absorbed on the "cooked" food item, the turnover rate is variable. In order to eliminate variables in the composition of oil samples and to note only the effects of temperature on the

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nutritional value of an edible oil heated for a specific period of time, corn oil was heated continuously at 200°C for 48 hours in the present study. The heated oil was subjected to urea fractionation and molecular distillation and various fractions fed to weanling rats for 21 days.

EXPERIMENTAL

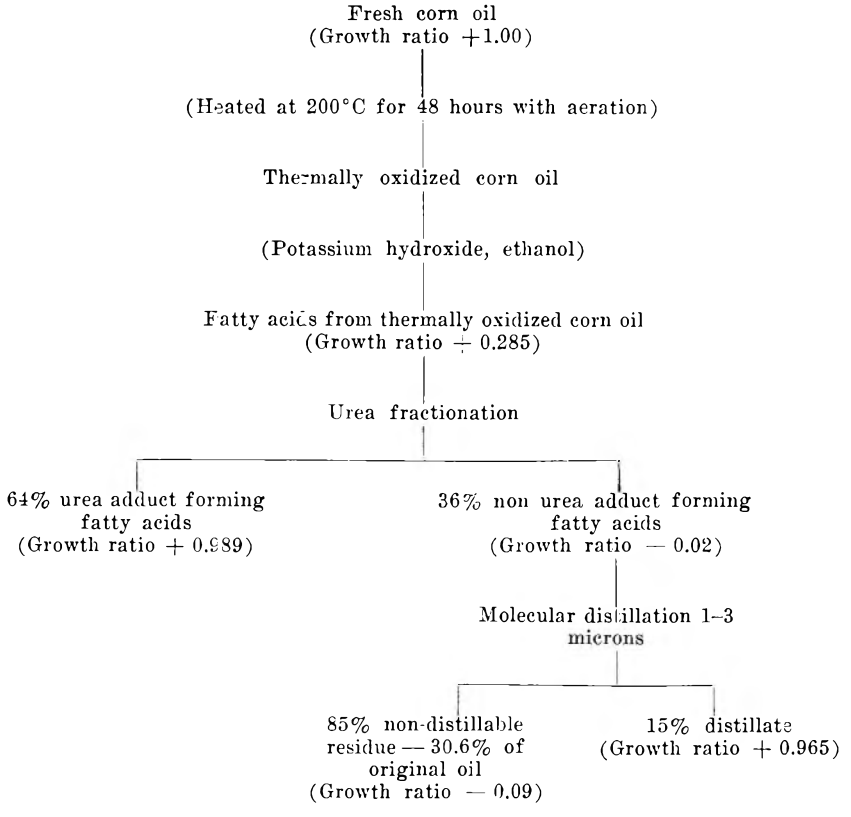
A basal diet which consisted of 50% glucose,[4] 31% casein, 5% Wesson ('32) salt mix and 14% fat was used in all the feeding experiments. The test fat or fatty acid fraction represented 12% and fresh cottonseed oil 2% of this 14% of fat. Two grams of a water-soluble vitamin mix were added to each kilogram of food. This mixture was composed of choline chloride 93.5 mg, thiamine hydrochloride 1.24 mg, riboflavin 1.24 mg, pyridoxine hydrochloride 1.24 mg, calcium pantothenate 2.48 mg, folic acid 0.30 mg, and 1.9 gm of glucose. The fat-soluble vitamins were given by dropper once each week.[5] Groups of 7 animals each were kept in single cages, weighed daily and all animals arbitrarily restricted to the same amount of food intake as those fed the non-urea-adduct-forming acids.

The thermally oxidized oil was prepared by heating fresh corn oil continuously for 48 hours at 200°C with agitation in the presence of air. The oil was then saponified with 4% potassium hydroxide in 95% ethanol, acidified with dilute hydrochloric acid, the lipids extracted with Skellysolve F and subjected to urea fractionation (Johnson et al., '57). The crystalline urea adducts of the straight chain fatty acids were removed by filtration and the adducts decomposed in warm water according to the scheme on opposite page.

[4] Cerelose.

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

Fractionation of thermally oxidized corn oil



The fatty acids were extracted from the aqueous phase with Skelly-solve F and freed from solvent. The non-urea-adduct-forming fraction was freed of urea and solvent and subjected to molecular distillation under 1 to 3 μ pressure at 150°C in a small falling film type still.

RESULTS

Weanling rats which had been fed the non-distillable residue from the non-urea-adduct-forming fatty acids of thermally oxidized corn oil all died within 7 days (fig. 1). These animals had lost approximately 7 gm, while those on the fatty acids of fresh corn oil had gained an average of 16 gm in weight on

the same amount of food intake during this 7-day period. Dilution of the non-urea-adduct-forming fatty acids with an equal volume of the fatty acids from fresh corn oil assured survival of the animals for the 21-day test period, but counteracted

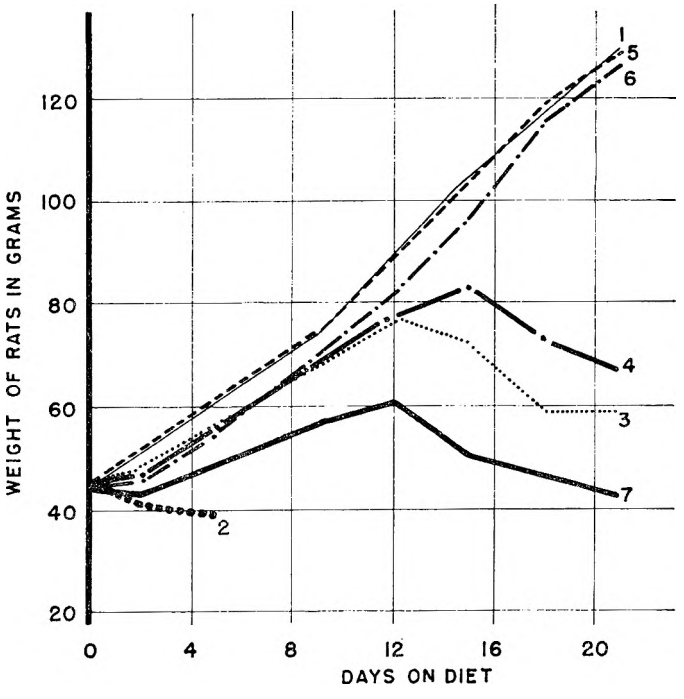


Fig. 1 Change in weight of rats fed various fractions of thermally oxidized corn oil. (1) Fatty acids from fresh corn oil; (2) Non-distillable residue from non-adducting acids from thermally oxidized oil; (3) 6% non-distillable residue, 6% fatty acid from fresh corn oil; (4) Thermally oxidized fatty acids; (5) Urea-adduct-forming acids from thermally oxidized oil; (6) Molecular distillate of non adducting acids from thermally oxidized oil; (7) Non-urea-adduct-forming acids from thermally oxidized oil.

only partially the growth depressing effect of the non-urea-adduct-forming fatty acids. The animals which had received this mixture had gained only 14 gm, while those on the fatty acids of fresh corn oil had gained approximately 85 gm in weight during the 21-day test period.

The major portion of the fatty acids in the thermally oxidized corn oil did not seem to be damaged by the severe heat treatment. Although the fatty acids from thermally oxidized oil depressed growth significantly, the rats fed the urea-adduct-forming fatty acids, which represented 64% of the oil, gained as much weight as those on the fatty acids of fresh corn oil. Furthermore, the molecular distillate from the non-urea-adduct-forming fatty acids, which represented 15% of this fraction, also did not depress growth significantly. It is evident, therefore, that only a minor portion of the fatty acids in the triglycerides of corn oil is susceptible to heat damage.

The proportion of non-urea-adduct-forming material and the molecular weight seem to represent a better index of nutritional value (table 1) than the iodine value (Melnick, '57; Melnick et al., '58). The urea-adduct-forming fatty acid had an iodine value of 62 as compared to 126 for the fatty acids of fresh corn oil, yet both gave similar weight gains. Characterization of the non-distillable residue from the non-urea-adduct-forming material indicated that this fraction contained polymers with molecular weights ranging from 692 to 1600. These polymers contained oxygen in the form of hydroxyl as well as carboxyl groups and double bonds which resisted hydrogenation (Perkins and Kummerow, '59).

It has been reported previously that heat and oxygen damaged the nutritional value of an edible oil at 95° (Kaunitz et al., '55) as well as at 275° (Crampton et al., '51). Differences in the temperature of heating and the presence or absence of oxygen can alter the rate and the type of polymerization which may occur but it is evident that heat damage is not limited to any specific temperature between 95 and 275°. The length of time that an oil is exposed to heat and oxygen and the percentage of heated oil in the diet also influence the nutritional value of the oil (Johnson et al., '56).

An oil heated for 6 hours at frying temperature may not be damaged enough to cause significant growth depression (Deuel et al., '51). However, the enlarged livers in rats fed oil which had been heated for 48 hours (Johnson et al., '57) seems to

TABLE 1

Comparison of constants and biological response to various fractions of the fatty acids from fresh and thermally oxidized corn oil [1]

DIET SUPPLEMENT	CONSTANTS OF TEST FATTY ACIDS			LINOLEIC ACID CONTENT	CHANGE IN WEIGHT IN 21 DAYS	PERCENTAGE OF LIVER/BODY WEIGHT
	Non adduct	Iodine value	Molecular weight (Rast)			
	%					
Fatty acids from fresh corn oil	5	126	294	55.4	+ 84.9 ± 6.9 [2]	4.8 ± 0.7
Urea-adduct-forming acids from T.O. oil	0	62	300	3.5	+ 84.0 ± 0.3	4.3 ± 0.3
Non-urea-adduct-forming acids from T.O. oil	74	83	512	9.5	— 2.0 ± 0.0	8.2 ± 1.4
Molecular distillate of non-adducting acids from T.O. oil	0	113	320	33.6	+ 82.1 ± 0.3	5.5 ± 0.2
Non-distillable residue from non-adducting acids from T.O. oil	100	70	692	6.0	— 7.6 ± 0.3 [3]	8.7 ± 1.2
Thermally oxidized fatty acids	36	71	454	10.0	+ 21.9 ± 0.9	7.1 ± 0.2
6% non-distillable residue + 6% fatty acids from fresh corn oil	50	98	460	31.7	+ 14.2 ± 1.0	8.1 ± 0.9

[1] Corn oil heated for 48 hours at 200°C with 150 ml of aeration per min./kg oil.

[2] Standard error of the mean.

[3] All died in 7 days.

imply that such oils do elicit a biological response. Whether thermally oxidized oil will manifest biological activity may depend on the amount of dietary protein (Witting et al., '56), the presence of sufficient amounts of pyridoxine or other vitamins and possibly other factors.

The present data cannot be projected to predict whether a specific unsaturated oil will be damaged sufficiently during commercial frying operations to be harmful to human consumers, as fresh oil is continually introduced into the frying vat, and the "used" oil adsorbed on the fried product and withdrawn. However, small amounts of thermally oxidized oils, similar in character to the one used in the present study, are sometimes added to salad oils as crystallization inhibitors, in order to prevent the crystallization of higher melting triglycerides from the oils. The present study indicates that the use of such oils for this purpose may not be desirable from a nutritional point of view.

SUMMARY

Weanling rats were fed for 21 days a diet composed of 50% glucose, 31% casein, 5% Wesson salt, 2% fresh cottonseed oil, 12% of the test fat or fatty acid fraction, and all of the known required water- and fat-soluble vitamins. Those fed the non-distillable residue from the non-urea-adduct-forming fatty acids of corn oil which had been heated at 200°C for 48 hours and represented approximately 30% of the original oil all died within 7 days. Dilution of the non-urea-adduct-forming fatty acids with an equal volume of the fatty acids from fresh corn oil assured survival of the animals for the 21-day test period, but counteracted only partially the growth depressing effect of the non-urea-adduct-forming fatty acids. The major portion of the fatty acids in the thermally oxidized corn oil did not seem to be damaged by the severe heat treatment. Although the fatty acids from thermally oxidized oil depressed growth significantly, the rats fed the urea-adduct-forming fatty acids, which represented 64% of the oil, gained as much weight as those on the fatty acids of fresh corn oil.

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METABOLIC STUDIES ON THE SODIUM FLUORIDE-FED RAT [1]

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The actively metabolizing cells of the animal body are very sensitive to fluoride (F^-). A two- to three-fold increase in the protoplasmic F^- concentration may become toxic. The exact locus of the physiological action of F^- has not been fixed in the fluoridated animal, aside from its effect upon the skeleton and teeth.

Certain evidence has directed attention to the effects of F^- in lipid metabolism. Kastle and Lovenhart ('00) reported that F^- inhibited lipase. Johnson and Lardy ('50) demonstrated that fatty acid oxidase activity was inhibited by 0.01 M F^-. The closely related acetate activating system is inhibited by 0.0001 to 0.0005 M F^- *in vitro,* which is within the concentrations present in soft tissues in fluorosis (Aisenberg et al., '55). Miller and Phillips ('55) found that high levels of dietary fat enhanced the toxicity of F^-. Growth rates were reduced in rats fed 0.10% of NaF when the dietary fat was increased from 5% (normal) to 15%. This effect was independent of the chain length of the constituent fatty acids.

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An attempt was made to determine the *in vivo* effects of F^- upon fatty acid oxidase reactions, the enhancement of fluoride toxicity by high-dietary fat and the acetylation capacity of tissues in fluorosis.

TABLE 1

The percentage composition of experimental diets

INGREDIENT	DIET NUMBER						
	1F	2F	3F	4	4F	5	5F
Sucrose	68.4	60.5	38.0	65.0	64.9	42.5	42.4
Casein	24.0	24.0	24.0	24.0	24.0	24.0	24.0
Salts IV [1]	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Vitamin mix [2]	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Celluflour	—	4.4	16.9	—	—	12.5	12.5
Cottonseed oil	1.5	5.0	15.0	5.0	5.0	15.0	15.0
Sodium fluoride	0.1	0.1	0.1	—	0.1	—	0.1

	6	6F	7	7F	8	8F
Sucrose	65.0	64.9	42.5	52.4	42.3	42.2
Casein	24.0	24.0	24.0	24.0	24.0	24.0
Salts IV [1]	4.0	4.0	4.0	4.0	4.0	4.0
Vitamin mix [2]	2.0	2.0	2.0	2.0	2.0	2.0
Celluflour	—	—	12.5	12.5	12.5	12.5
Cottonseed oil	5.0	5.0	15.0	15.0	15.0	15.0
Cystine	—	—	—	—	0.2	0.2
Sodium fluoride	—	0.15	—	0.15	—	0.15

[1] Hegsted et al., ('41).

[2] For composition of vitamin mix see text.

EXPERIMENTAL

Weanling rats (Holtzman strain) weighing between 40 and 45 gm were used. They were housed in cages with screen floors or in metabolism cages. Fecal and urine samples were collected quantitatively when desired.

Semipurified diets were used into which the F^- was incorporated as NaF. All diets containing increased fat levels were made isocaloric with the normal fat diets by the substitution of roughage [3] for an equivalent of sucrose. When necessary, paired feeding was resorted to as a means of controlling the

[3] Solka Floc, The Brown Company, Berlin, New Hampshire.

dietary effects of F^-. The various diets used are presented in table 1. The amount of vitamin mix used per kilogram of diet contained: choline chloride, 1 gm; inositol, 100 mg; calcium pantothenate, 20 mg; niacin, 10 mg; thiamine hydrochloride, 5 mg; riboflavin, 5 mg; pyridoxine hydrochloride, 3 mg; folic acid 200 μg; biotin, 100 μg; vitamin B_{12}, 10 μg. The fat-soluble vitamins were supplied to all animals by the weekly administration of fortified Haliver Oil.

At the close of each experiment the animals were sacrificed and the tissues needed were quickly excised, chilled and homogenized in isotonic sucrose; mitochondria were isolated by a modification of the method of Schneider ('48). The mitochondria were resuspended in isotonic sucrose so that 0.5 ml of suspension contained mitochondria from 0.25 to 0.50 gm of organ. The fatty acid oxidase activity was determined with the Warburg apparatus. The results were corrected for endogenous respiration and were expressed as microliters of oxygen uptake/hour/milligram of mitochondrial nitrogen, or/mitochondria from 1 gm of fresh tissues. Assay flasks contained 0.2 ml of 5% KOH in the center well and 0.5 ml of mitochondrial suspension in a final reaction volume of 3.0 ml in the main well. The final concentrations of other components present (liver) were as follows: $MgCl_2$, 0.005 M; adenosine triphosphate, pH 7.3, 0.002 M; KCl, 0.085 M; sodium caprylate, pH 7.3, 0.00066 M; KH_2PO_4-Na_2HPO_4 buffer, pH 7.3, 0.0166 M. In one experiment a KH_2PO_4-HPO_4 buffer of the same pH and molarity was used. In assays of kidney the final reagent concentrations were: $MgCl_2$, 0.005 M; adenosine triphosphate, pH 7.3, 0.002 M; KCl, 0.082 M; KH_2PO_4-K_2HPO_4 buffer, pH 7.3, 0.01 M; potassium fumarate, pH 7.3, 0.002 M; cytochrome c, 1.33×10^{-5} M; sodium caprylate, pH 7.3, 0.00133 M. Here the oxygen uptake was calculated from three successive 10-minute respiration periods.

Nitrogen was determined by the method of Hiller et al. ('48) or by the AOAC ('55) method, depending on the size of the sample. The water content was established by drying samples

to constant weight; fat content was determined by ether extraction of the dry sample.

RESULTS

The effects of two levels of fat and two levels of NaF upon fatty acid oxidase activity in the female rat were studied after the diets were fed for 6 to 8 weeks. The growth rates were retarded by dietary F^- and were further depressed by high dietary fat in the fluorotic rat. These observations were made upon rats fed diets 4, 4F, 5, 5F, 6, 6F, 7, 7F, 8 and 8F.

The mitochondrial fatty acid oxidase activity of the livers of these fluorotic rats and of their controls were essentially the same; hence there was no evidence of a direct effect of F^- on this enzyme system. Moreover, the high-fat diet had no influence upon the fatty acid oxidase values despite its growth rate retardation. These results were interesting since it has been reported that F^- inhibition of fatty acid oxidation was negligible in a soluble system prepared from rat liver mitochondria (Drysdale and Lardy, '53) but was complete in a kidney cyclophorase system (Johnson and Lardy ('50). Since in liver the end product of the oxidation is mainly acetoacetate and since kidney and other organs further metabolize this compound to CO_2 and water, these reports were interpreted to mean that the locus of the inhibitory action of F^- in the fatty acid oxidase system occurred at a step beyond the formation of acetoacetate. This view of the problem is supported by the work of Cheldelin and Beinert ('52).

Inasmuch as the kidney, which was shown to carry the oxidation to completion, has a QO_2 sufficient to measure manometrically, an experiment was designed to test the effect of NaF feeding upon kidney fatty acid oxidase. Diets 8 and 8F (table 1) were used in this study. Prolonged feeding of high levels of F^- has been reported to cause structural changes in the kidney. Hence the time interval was varied in this study to determine if kidney changes were involved.

The data are summarized in table 2. A significant decrease in fatty acid oxidase activity was observable as early as the 4th to 5th day after the beginning of F^- ingestion. Gross inspection of the kidneys at the 4 to 5-day interval indicated that they were unaffected, but chemical analysis showed a highly significant increase in kidney water content. After two weeks of the regimen, the kidneys from the treated animals were yellowish in color, edematous, and presented a granular

TABLE 2

The effect of duration of NaF feeding on the kidney fatty acid oxidase activity of rats

DIET [1]	DAYS ON DIET	MITOCHONDRIAL OXYGEN UPTAKE IN μl O_2/HR./MG MITOCHONDRIAL NITROGEN		MG MITOCHONDRIAL NITROGEN/GM FRESH KIDNEY	
		$(\bar{x} \pm s)$ [2]	n [3]	$(\bar{x} \pm s)$ [2]	n [3]
8	4–5	340 ± 51.0	4	2.41 ± 0.771	4
8F	4–5	240 ± 49.7 [4]	4	1.74 ± 0.526	4
8	17	326 ± 49.6	3	3.82 ± 0.191	3
8F	17	76.6 ± 90.6 [5]	6	2.09 ± 0.444 [5]	6
8	31–47	315 ± 93.0	7	3.52 ± 1.01	7
8F	31–47	115 ± 105 [5]	4	1.94 ± 0.619 [5]	4

[1] See table 1 for composition of diets.
[2] Mean ± standard deviation.
[3] Number of rats per group.
[4] Significant at the 5% level by Student's t-test.
[5] Significant at the 1% level by Student's t-test.

appearance; chemical analysis revealed a striking decrease in fat and nitrogen concentrations. Moreover, the kidney weight : body weight ratio was double that of the control animals (table 3). At present these data do not define the proper sequence between the enzymatic, chemical and anatomical changes resulting from elevated F^- ingestion. The striking decrease in fatty acid oxidase activity could not be accounted for on the basis of the decreased mitochondrial nitrogen content observed in the fluorotic rat kidney.

Since the high-fat diet (15%) did not affect the liver fatty acid oxidase system, a study was initiated to determine its

mode of action in growth inhibition. Three groups of weanling female rats were fed diets 1F, 2F, and 3F (table 1) respectively. These rations were isocaloric and were pair-fed to match the food intake of the group having the lowest voluntary food consumption, i.e., to those animals receiving the 15% fat diet. The weight gains in the three groups were alike for the 9-week test period. Because these results failed to explain the deleterious effect of high-fat diets in fluorosis, two groups of 4 animals each were fed ad libitum for 4 weeks, receiving diets 8 and 8F respectively. Fecal samples were collected and pooled by lots, and were then analyzed for fat and nitrogen. Estimates of the motility of the small intestine were obtained at the end of the experiment by the method of Perdue and Phillips ('52).

The data showed that in fluorotic rats the fecal fat excretion was two times greater than in control animals; similarly the nitrogen excretion increased by about 40%. The individual results for several collection periods were 8.0, 9.4, and 12.6% fecal fat excreted by the NaF animals as against 4.4, 6.0 and 5.2% for the controls. Similarly, the milligrams of nitrogen excreted per gram of feces were 17.0, 18.2, 16.5, 15.0, 16.0, 14.0 for the fluoridated rats, and 13.4, 12.4, 10.5, 10.8, 10.3 for the controls. Neither prolonged fluorine ingestion nor a single dose of NaF administered at the time of assay altered the average motility rating of the small intestine.

Since fluorosis in the rat is accompanied by semistarvation food intakes, an experiment was designed to compare food utilization in the fluorotic rat with that of ad libitum and of pair-fed control rats. Results obtained during two periods of observation (7 days each) showed a much greater excretion of fat and dry matter by the fluorotic animals than by either control group (fig. 1). These data suggest a better utilization of food in the unfluoridated animal. During both experimental periods the fat excretion of the fluoride-fed rats was 3 to 4 times greater than that of the pair-fed controls. These results make it evident that fluorosis *per se* and not the starvation accompanying it was responsible of the altered excretion pat-

TABLE 3

Kidney composition of fluorotic and normal rats[1]

DIET[2]	TIME ON DIET	KIDNEY WT. ($\bar{x} \pm s$)[3]	KIDNEY WT. GM ÷ BODY WT. GM	H_2O ($\bar{x} \pm s$)[3]	FAT ($\bar{x} \pm s$)[3]	MG NITROGEN /GM KIDNEY ($\bar{x} \pm s$)[3]
		mg		%	%	
8	4 days	662 ± 80.8 (4)	1.01 (4)	78.0 ± 1.63 (4)	1.55 (2)	27.4 ± 1.35 (4)
8F	4 days	543 ± 41.7 (4)	1.29 (2)	81.0 ± 0.568 (4)[4]	1.40 ± 0.132 (4)	25.7 ± 1.49 (4)
8	2 weeks	1067 ± 198.2 (8)	0.904 (8)	77.9 ± 0.255 (8)	2.40 ± 0.149 (8)	26.8 ± 0.443 (8)
8F	2 weeks	1264 ± 220.5 (8)	2.14 (8)	84.1 ± 1.26 (8)[4]	1.64 ± 0.181 (8)[4]	19.4 ± 1.35 (8)[4]
8	5–6 weeks	1500 ± 92.16 (4)	0.878 (4)	78.5 ± 0.338 (4)	2.59 ± 0.223 (4)	27.2 ± 0.841 (4)
8F	5–6 weeks	1519 ± 142.8 (6)	1.73 (6)	84.6 ± 0.886 (6)[4]	1.69 ± 0.207 (6)	19.7 ± 1.03 (6)[4]

[1] All data are reported on a kidney fresh weight basis. Figures within parentheses represent the number of animals per group.

[2] See table 1 for composition of diets.

[3] Mean ± standard deviation.

[4] Significant at the 1% level by Student's t-test.

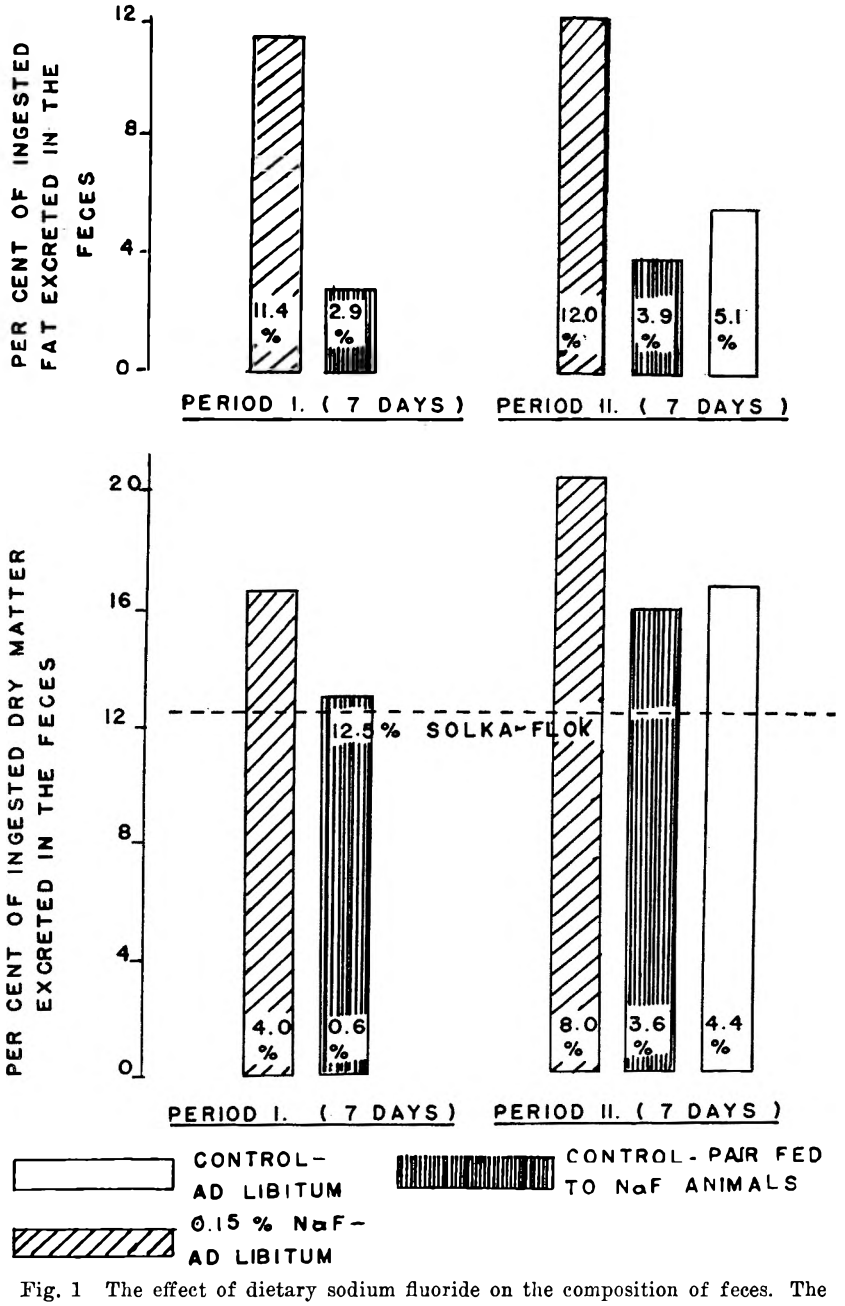

Fig. 1 The effect of dietary sodium fluoride on the composition of feces. The values given for the percentage of dry matter excreted in the feces have been corrected for the excretion of non-utilizable roughage (Solka Floc).

TABLE 4

The effect of dietary NaF on the per cent acetylation of urinary p-amino benzoic acid in PABA injected rats

EXPERIMENT	TYPE OF RAT USED	DOSE OF PABA [1]	DIET [2] AND TYPE OF FEEDING	NO. RATS	INJECTIONS BEFORE TREATMENT	INJECTIONS DURING TREATMENT
A	Female weanling	2.5 mg	8F ad libitum	4	3	3
			8 pair-fed to NaF-fed rat	4	3	3
			8 ad libitum	2	3	3
B	100 gm males	2.5 mg	8F ad libitum	3	4	6
			8 pair-fed to NaF-fed rat	3	4	6
			8 ad libitum	1	4	6
C	Female weanling	75 mg/100 gm rat	8F ad libitum	5	2	6
			8 pair-fed to NaF-fed rat	5	2	6
			8 ad libitum	2	2	5

[1] PABA injections were given subcutaneously at the start of each 24-hour urine collection period, as described in the text.

[2] See table 1 for composition of diets.

terns observed in fluoride-fed rats. Neither the cause nor the origin of the elevated fecal fat and dry matter observed in fluorotic rats are known.

Lipid metabolism is intimately associated with coenzyme A (CoA) and acetyl CoA. One of the functions of CoA in the body is in the transfer of acetyl groups. In these studies it seemed pertinent to test the effect of fluorosis on acetyl transfer. Since Riggs and Hegsted ('48) found that the fraction of injected *p*-aminobenzoic acid (PABA) excreted in the acetylated form dropped sharply in pantothenic acid deficiency, it seemed possible to determine the effect of dietary NaF on acetylation by measuring the capacity of the rat to acetylate.

Three groups of animals were fed diet 8 (table 1) for 7 to 12 days while normal excretion levels were established (Exp. A, B, and C, table 4). The PABA was injected at the beginning of each 24-hour urine collection period. At least three days were allowed to elapse between injections to ensure complete elimination of the drug between tests. The PABA was dissolved in saline and adjusted to pH 6 for injection.

In none of the three experiments was there any consistent difference noted in the per cent acetylation of PABA by fluoride-fed rats or by their pair-fed of their ad libitum-fed controls. There was no evidence that the ability of the intact rat to acetylate aromatic amines was inhibited by dietary F^-.

DISCUSSION

The experiments reported here represent a survey of some loci where fluoride toxicosis might inhibit fat metabolism, fat utilization, or dietary efficiency. They focus attention upon certain enzymes, dietary constituents and metabolic processes which might be adversely affected by the administration of a high-fat diet to fluorotic animals. No attempt was made to use less than growth-retarding levels of dietary F^- in these studies, since a frank fluoride toxicosis was desired. NaF fed to growing rats to supply 0.10% of the ration permits a steady small growth increment, whereas 0.15% of NaF restricts the growth

increment to nearly zero. These NaF levels assured that "fluorosis" was attained within a few weeks and that the effects on the tissue F^- would be most pronounced.

No inhibitory effect of fluorosis, either alone or in combination with high dietary fat, was observed on the liver fatty acid oxidase system or on liver fat and nitrogen content. The marked decline in the fatty acid oxidase activity of the kidney may initiate other changes which include a decrease in the fat and nitrogen content of the organ; conversely, an initial fall in kidney fat and nitrogen may produce secondary changes which profoundly affect the level or function of the fatty acid oxidase system in the kidney. It would appear that the kidney changes, i.e., the lowered concentrations of fatty acid oxidase, lipids and nitrogen, may be of sufficient importance and magnitude to account for many of the effects observed in the animals with "fluorosis." The decreased kidney fatty acid oxidase activity, whatever its cause, must seriously interfere with the animal's ability to metabolize fat.

SUMMARY

Fat metabolism in the rat was studied in a series of experiments designed to determine the effects of fluoride toxicosis upon fatty acid oxidase activity, on fat utilization and on the acetylation capacity of the intact rat.

1. Mitochondrial fatty acid oxidase activity was unaffected in the liver but was strikingly decreased in the kidney of the fluorotic rat. Concurrent changes in the kidney were a decrease in the fat and nitrogen content, and hypertrophy of the organ (wet wt.).

2. The decreased growth of ad libitum-fed fluorotic rats receiving a 15% fat diet, as compared with those receiving a 5% fat diet, was confirmed. However, when the caloric intake was held the same in rats given diets varying in fat content but identical in fluorine content, growth was the same in all groups. Fluorotic rats which were pair-fed isocaloric diets with their controls, were retarded in their rate of gain in weight.

3. Fluoride-fed rats excreted more fecal fat, nitrogen and dry matter than the control animals. The decreased retention of these nutrients could not be ascribed to an increase in intestinal motility or to starvation *per se*.

4. Dietary fluoride did not interfere with the acetylation of *p*-aminobenzoic acid by the intact rat. This suggests that the utilization of coenzyme A in this acetylation reaction, and presumably in those of fat metabolism, was not inhibited in the fluorotic rat.

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EFFECTS OF THE PREVENTION OF COPROPHAGY IN THE RAT

V. ESSENTIAL FATTY ACID DEFICIENCY [1]

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In general, all animal species are resistant to the development of the Burr and Burr ('29) syndrome of essential fatty acid deficiency. The weanling rat, which has been studied most extensively, usually requires three to 4 months on a completely fat-free diet to manifest the complete picture of the deficiency. Adult animals are either totally resistant or take much longer to become deficient (Holman, '54). Total exclusion of the essential fatty acids from the diet, even to the point of avoiding starch which contains traces of linoleic acid, must be employed, and low initial body stores are of great importance. One potential source of uncontrolled essential fatty acid ingestion is from feces. Sinclair ('30) noted that rats on a fat-free diet, having access to their feces, did not develop the dermal signs of the deficiency, although when maintained in raised wire screen-bottom cages, the deficiency signs developed as expected. Norcia and Lundberg ('54) reported the presence of small amounts of polyunsaturated fatty acids in the feces of rats on fat-free diets. Since it has been shown that rats on raised screen normally consume a

[1] This research has been supported in part by funds provided by the State University of New York and by the National Science Foundation.

large portion of their feces (Barnes et al., '57), it was of interest to evaluate the contribution of coprophagy to the development of essential fatty acid deficiency.

Interesting changes in plasma and liver cholesterol concentrations in essential fatty acid-deficient rats have been reported (Alfin-Slater et al., '54) (Mukherjee and Alfin-Slater, '58). An extension of these observations has been attempted in this study.

EXPERIMENTAL

Male weanling rats purchased from Holtzman (experiment 1) or Charles River Breeding Laboratories (experiment 2) were maintained in the laboratory for two days on a complete purified diet, then divided into groups to equalize weight distribution. All rats were individually caged on raised wire mesh and were fed the experimental diets ad libitum. Coprophagy was prevented by the use of a small plastic cup that fitted around the anus and was held in place by a felt collar attached to the tail (Barnes et al., '57). Blood samples were collected by heart puncture from lightly etherized rats. Serum cholesterol was determined by the method of Abell et al. ('52) and liver fat and cholesterol were measured in the extract of alkali digested liver as described in a previous report (Barnes et al., '59).

The fat-free diet contained per 100 gm: casein,[2] 25; cerelose, 69; salts (Hubbell, Mendel and Wakeman, '37), 4; choline dihydrogen citrate, 0.3; B vitamins in finely ground sucrose, 2; fat soluble vitamins in 95% alcohol, 1. The B vitamin mixture contained in 2 gm sucrose: thiamine·HCl, 0.4 mg; riboflavin, 0.8 mg; pyridoxine·HCl, 0.4 mg; Ca pantothenate, 4.0 mg; niacin, 4.0 mg; inositol, 20.0 mg; biotin, 0.02 mg; vitamin B_{12} (crystalline) 0.03 mg; menadione, 1.0 mg. The fat soluble vitamins in alcohol contained per 1.0 gm: vitamin A acetate 0.31 mg; vitamin D (calciferol), 0.0045 mg; α-tocopherol, 5.0 mg.

[2] Vitamin Test, General Biochemicals Corporation, Cleveland, Ohio

When the fat-free diet was supplemented with 1% of corn oil,[3] this oil was used in place of alcohol to dissolve the fat-soluble vitamins. When hydrogenated coconut oil[4] was used, it was added to the diet in place of an equal weight of cerelose.

Experiment 1. Five groups of male weanling rats were set up as follows: group 1, 10 rats received a low-fat diet containing 1% of corn oil (diet A); group 2, 10 rats received the same diet, but were maintained with fecal collection cups; group 3, 20 rats received a fat-free diet (diet B); group 4, 20 rats received the fat-free diet, but were maintained with fecal collection cups; group 5, 10 rats received a diet deficient in essential fatty acids that contained 15% hydrogenated coconut oil (diet C).

Growth responses are shown in figure 1. The prevention of coprophagy with the plastic fecal collection cups reduced the growth rate in both groups in which they were employed. Some reduction in growth rate caused by the collection cups has been noted in all studies regardless of diet (Barnes and Fiala, '58). However, it is evident that a greater than expected depression in growth was obtained when the rats were receiving an essential fatty acid-deficient diet. This observation supports the hypothesis that even during the ingestion of a fat-free diet, a significant quantity of essential fatty acids is obtained by the practice of coprophagy. The importance of the essential fatty acids in the feces in delaying the development of the deficiency syndrome was even more obviously illustrated in comparing the time of appearance of skin signs. These observations were made during the winter months when relative humidity was low. The conventional rats receiving the fat-free diet began to show scaliness of the feet about the 5th week, and 17 of the 20 had slightly scaly feet during the 6th week. The fat-free rats with fecal collection cups began to develop scaly feet by the third week and

[3] Mazola.

[4] Hydrol, made without added lecithin, Glidden Company, Cincinnati, Ohio.

in the 4th week, 7 out of 20 were scaly. It has long been known that saturated fat speeds the development of essential fatty acid deficiency (Evans and Lepkovsky, '32) and this effect was seen again in the group receiving 15% hydrogenated coconut oil. By the 4th week, 8 out of 10 rats had developed scaly feet.

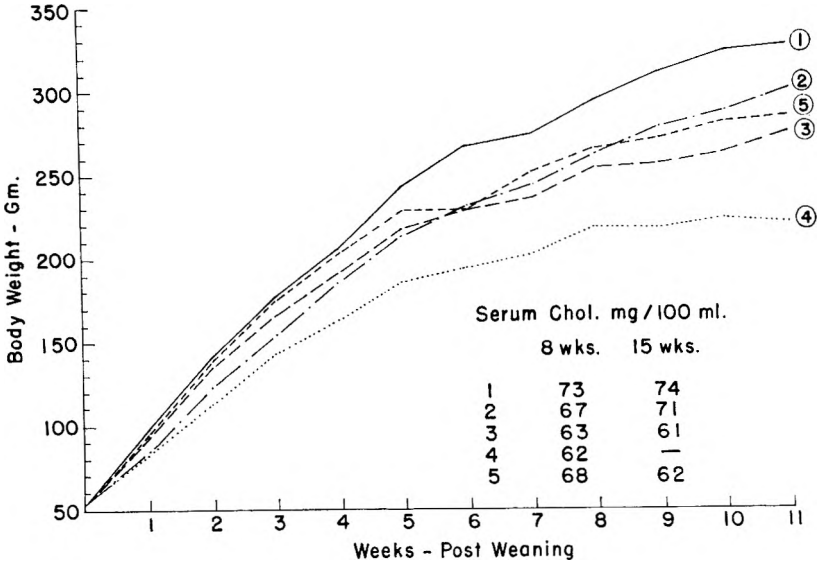

Fig. 1 Growth rate and serum cholesterol values from experiment 1. Group 1, 1% corn oil diet; group 2, 1% corn oil diet plus fecal collection cups; group 3, fat-free diet; group 4, fat-free diet plus fecal collection cups; group 5, 15% hydrogenated coconut oil diet.

A comparison of the speed of development of scaliness of the tails probably has little meaning, for the constrictive effect of the felt band that holds the fecal collection cups in place obviously affected circulation in the tail. In the conventional rats, 15 out of 18 showed scaly tails by week 9. In the group in which coprophagy was prevented only 4 out of 20 had scaly tails by week 9, although all eventually developed the characteristic severe scaliness.

Blood samples were taken during the 8th week from all the rats in groups 1, 2 and 5 and from 10 rats in groups 3 and 4. This was repeated except for group 4 at 15 weeks. Serum cholesterol values are shown in figure 1. Low values were noted for the rats deficient in essential fatty acids. Hydrogenated coconut oil, which in other studies has shown a hypercholesterolemic effect, did not seem to raise the serum cholesterol in the deficient rats as much as would be expected. Therefore, another experiment was set up to examine the effect of dietary fat in the essential fatty acid deficient rat.

Experiment 2. Forty male weanling rats were divided into 4 groups and given the dietary treatments described in table 1. Forty young adult male rats were divided into 4 groups and given the same dietary treatments. After 8 weeks the rats were bled and the livers were taken for lipid analyses. When rats were fed the diets starting at weaning, poor growth was observed in those fed the diets deficient in essential fatty acids. This study was conducted during the summer in rooms that were maintained at a constant temperature, but the humidity was relatively high and skin signs of the deficiency were not observed. The young adults obviously had adequate storage of essential fatty acids during the experimental period and the only differences in growth rate were related to the presence of the 15% fat in the form of hydrogenated coconut oil. It is of some interest that the hydrogenated fat in the presence of corn oil gave a slightly greater gain than the hydrogenated fat alone (diet B compared with diet D).

The serum cholesterol values of the essential fatty acid-deficient rats (weanling groups, diets A and B) were clearly lower than for those receiving the small supplement of corn oil. In the young adult groups, only the rats receiving the fat-free diet showed lowered serum cholesterol (diet A). The hydrogenated fat, therefore, caused higher serum cholesterols only when compared with a fat-free diet, and exerted only a minimal effect in the presence of essential fatty acid deficiency.

Liver fat and cholesterol values follow the general pattern described by Alfin-Slater et al. ('54). There was a slight

TABLE 1

Body weight, liver total fat and cholesterol and serum cholesterol of growing and young adult rats as affected by essential fatty acid deficiency in the presence and absence of saturated fat

TREATMENT	BODY WEIGHT		LIVER			SERUM CHOLESTEROL
	Start	8 wks.	Weight	Total fat	Cholesterol	
	gm	*gm*	*gm*	*mg/gm*	*mg/gm*	*mg/100 ml*
Weanling rats						
A EFA[1]-deficient	55	273	9.24 ± 0.40 [2]	32.14 ± 2.7 [2]	3.47 ± 0.30 [2]	51 ± 2.4 [2]
B EFA-deficient + 15% Hydrol [3]	55	291	9.92 ± 0.47	41.33 ± 1.1	3.08 ± 0.25	58 ± 5.6
C EFA-deficient 1% corn oil	55	320	10.05 ± 0.37	27.47 ± 2.1	2.77 ± 0.58	72 ± 3.2
D EFA-deficient 1% corn oil + 15% Hydrol	55	331	10.53 ± 0.60	39.79 ± 2.7	2.43 ± 0.14	73 ± 4.6
Young adult rats						
A EFA-deficient	247	397	13.38 ± 0.28	31.99 ± 2.4	2.40 ± 0.16	60 ± 2.1
B EFA-deficient + 15% Hydrol	247	431	14.42 ± 0.65	42.72 ± 4.9	2.28 ± 0.18	67 ± 2.9
C EFA-deficient 1% corn oil	246	396	12.34 ± 0.40	30.15 ± 2.9	1.82 ± 0.07	66 ± 5.8
D EFA-deficient 1% corn oil + 15% Hydrol	246	458	14.67 ± 0.32	37.70 ± 1.2	1.92 ± 0.06	69 ± 3.3

[1] Essential fatty acid.

[2] Standard error of the mean.

[3] Hydrogenated coconut oil, made without added lecithin, Glidden Company, Cincinnati, Ohio.

elevation of total liver fat in the weanling rats that were deficient in essential fatty acids and were receiving a fat-free diet. Hydrogenated coconut oil increased the liver fat equally in the deficient rats and in those receiving 1% of corn oil. In the young adults the fat-free group showed no elevation of liver fat, but, as was pointed out previously, these rats did not show any evidence of being essentially fatty acid deficient. The presence of hydrogenated coconut oil in the diet did elevate total liver fat to about the same extent as that found in the weanling rats.

The absolute values for liver cholesterol appear to be higher in the weanling rats than in the young adults. If comparisons are made only within one age group the rats receiving the essential fatty acid-free diets have slightly higher cholesterol values than those receiving 1% of corn oil. No consistent effect due to the presence of hydrogenated coconut oil in the diets is evident.

DISCUSSION

Although there is no direct evidence that linoleic acid is synthesized by intestinal bacteria, unsaturated acids have been found in feces. By preventing coprophagy it has now been shown that these fecal unsaturated fatty acids are at least in part essential. The fact that coprophagy prevention hastened the development of signs of essential fatty acid deficiency suggests that these fatty acids can be added to the list of nutrients that probably are not absorbed, to any major extent, from the cecum and large intestine.

The cholesterol results present an intriguing possibility that the presence of a certain level of essential fatty acids in the tissues is necessary for saturated fats to exert a hypercholesterolemic effect. Mukherjee and Alfin-Slater ('58) have shown that there is a decreased synthesis of cholesterol from acetate by livers of rats on essential fatty acid-deficient diets. This was true even in the presence of saturated fat in the form of hydrogenated coconut oil. The altered synthesis rate could be an explanation of the low serum cholesterols, but as these

authors point out synthesis may be inversely related to the level of cholesterol in the liver (Taylor and Gould, '50) and thus the effect of essential fatty acids may be on some other process such as cholesterol degradation which would cause an accumulation of liver cholesterol. In comparing the serum cholesterols in the two parts of experiment 2, a major variable was the extent of essential fatty acid deficiency. However, the age of the two groups was different and in other studies, it has been noted that young animals show greater changes in blood cholesterol due to dietary treatments than do older animals.[5] Unfortunately, the rat has proven to be a most unsatisfactory animal to use in studies of the dietary fat — blood cholesterol relationship. In a sense, the homeostatic mechanisms for the control of blood cholesterol are too effective in this species. Equally unfortunate is the fact that the rat, one of the animal species most susceptible to the development of essential fatty acid deficiency, was chosen for this study. Other species should be explored to unravel the potential relationship between essential fatty acid deficiency and the influence of dietary fat upon the level of blood cholesterol that has been suggested by this as well as other investigations.

The fact that liver cholesterol was increased in the young adults in which signs of deficiency were absent confirms the observation of Deuel et al. ('55). These authors showed that liver cholesterol increased in weanling rats within one week after being put on a fat-free diet. Obviously within this short time, depletion of tissue stores of essential fatty acids to the point of gross manifestation of deficiency had not yet been obtained. Therefore, one may interpret these findings as evidence that the increase in liver cholesterol is a rapid response to the lack of essential fatty acids in the diet and not necessarily dependent upon severe depletion of body stores that is necessary for the well established signs of deficiency to appear. The accumulation of liver fat in the deficient animals was minimal, but is a phenomenon that has been recognized for a number of years (Engel, '42). The results presented

[5] Unpublished observations.

here do not warrant a firm conclusion although it seems that total fat accumulation is a later manifestation of essential fatty acid deficiency than cholesterol accumulation in the liver. The increased liver fat of rats having hydrogenated coconut oil in their diets regardless of the existence of essential fatty acid deficiency must be recognized as an effect of dietary saturated fat that is now well documented (Channon and Wilkinson, '36) and is in addition to the essential fatty acid effect per se.

SUMMARY

Essential fatty acid deficiency in the rat is hastened by complete prevention of coprophagy. This observation does not imply that these fatty acids are synthesized in the large intestine, but does show that the unsaturated fatty acids of the feces are, in part at least, of an essential nature.

The serum cholesterol of the essential fatty acid-deficient rat is lower than is obtained by merely feeding a fat-free diet without the development of the deficiency syndrome. Furthermore, it appears that the level of serum cholesterol in the deficient rat may not respond to dietary changes such as the inclusion of saturated fat in the same manner that is observed in the non-deficient animal. Accumulation of liver cholesterol in the non-deficient as well as the deficient rat receiving a fat-free diet has been confirmed.

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COMPARATIVE PERFORMANCES OF BABY PIGS FED INFANT AND BABY PIG DIETS [1]

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There are many commercial liquid and powdered human infant diets on the market today, and they are widely used throughout the Western Hemisphere; however, very little research has been reported on the comparative evaluation of their nutritive value and general performance.

Roine et al. ('54) used rats, three, 9, and 22 weeks of age, to evaluate cow's milk and human milk. The human milk proved unsuitable for the rats, causing them to lose weight, whereas the feeding of cow's milk resulted in an average gain of 13.3 gm during the one- to two-week experimental period. Mortality was also higher among those rats fed human milk. The work of Scheunert and Sommers ('56) and others demonstrates that the weanling and adult rat has a low tolerance for lactose and galactose as measured by growth rate and urinary excretion patterns. This could account for the poor performance on human milk since it contains approximately 57% lactose on a dry matter basis as compared to about 9% in rat's milk. These reports indicate that the rat is not a satisfactory laboratory animal for testing human infant formulas.

The pig can utilize lactose very efficiently as demonstrated by the work of Hudman ('58) in which lactose was found to

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be the carbohydrate of choice for the pig one to three weeks of age. The research reported herein was conducted to study the performance of baby pigs fed commercially prepared human infant diets as compared to those fed baby pig diets which had previously resulted in satisfactory weight gains and feed conversion. The ultimate objective was to determine if the baby pig could be used as a test animal for evaluating human infant diets.

EXPERIMENTAL

Animals. Experiment 696. Sixty-four crossbred pigs averaging 11.1 days of age and 5.8 pounds of body weight were selected and randomly allotted by weight within replication to the 8 ration treatments. The restriction was placed on the allotment that no two litter mates would receive the same treatment. A randomized block design in two replications was employed.

The pigs (4 pigs per pen) were housed in an insulated, concrete-block building with radiant-heated concrete floors. The room temperature was maintained at 70°F. and heat lamps were used to provide additional restricted space heating to maintain a temperature of 85° F. at a level of 5 inches above the floor. The lamps were raised weekly so as to reduce the temperature 5° F. per week and they were removed completely at the end of the third week.

Feed and water were provided ad libitum. Consumption records were kept twice daily for those pigs receiving the liquid milk diets and weekly for those pigs receiving dry diets. Weight records were taken weekly.

Experiment 758. Seventy-two crossbred pigs averaging 7.1 days of age and 6.3 pounds of body weight were selected and randomly allotted by weight within replication to the 8 ration treatments. Four replications of 4 pigs per pen were offered a dry meal pig ration, whereas two pens of 4 pigs each were offered each of the liquid infant diets. Management of the pigs and environmental conditions were maintained similarly to those of experiment 696.

Rations. Experiment 696. Three commercially available milk formulas for infant feeding were selected for comparison with three previously tested baby pig diets. The infant formulas will be referred to as C, D and E (table 1). These formulas were reconstituted with water to provide isocaloric diets of 20 Cal. per fluid ounce and 2.7, 1.7 and 1.5% protein, respectively. They were fed in liquid form as was the synthetic milk for baby pigs. Formula C was also tested in its dry form (C_1) and in an aseptic liquid form (C_2). The three standard pig feeding regimens consisted of pre-starter diet, containing 40% dried skim milk, (modification of pre-starter "75," Speer et al., '54) fed throughout the 4-week experimental period, the pre-starter fed the first week and then a starter fed the remaining three weeks (table 2) and a synthetic milk (Catron et al., '53) fed throughout the 4-week experimental period (table 3). Since the common practice among pediatricians is to recommend supplementary vitamins and iron in addition to the regular formula, it was deemed desirable to fortify all diets with equivalent quantities of vitamins and trace minerals. All diets were supplemented with the same vitamins and trace elements, as was the pre-starter diet (table 2).

The liquid diets were prepared twice daily and stored under refrigeration until offered ad libitum in galvanized poultry founts which were thoroughly cleaned and fresh milk added twice daily. The dry diets were offered in standard baby pig self-feeders. Water was withheld from the pigs receiving liquid diets until they had started drinking the liquid milk.

Experiment 758. The same three infant formulas used in the previous experiment were again selected and in addition, 4 other infant formulas were tested. Two were milk protein base formulas (A — 3.4% protein, B — 2.7% protein) and the other two were soya protein base formulas (F — 3.2% protein, G — 3.1% protein). These formulas were reconstituted and fed in the manner described for the previous experiment. The previously described dry meal prestarter-starter regimen was used as the standard for comparative purposes.

TABLE 1

Label analysis of the liquid infant formulas when reconstituted to an isocaloric basis

ITEM	DIET						
	MILK PROTEIN					SOYBEAN PROTEIN	
	A	B	C	D	E	F	G
Calories per ounce	20	20	20	20	20	20	20
Solids, %	14.3	14.0	14.0	12.0	12.0	14.2	12.5
Protein, %	3.4	2.7	2.7	1.7	1.5	3.2	3.1
Fat, %	2.7	2.8	2.8	3.4	3.5	2.6	4.0
Calcium, %	0.12	0.10	0.10	0.06	0.05	0.10	0.13
Phosphorus, %	0.09	0.08	0.08	0.05	0.04	0.05	0.11
Carbohydrate, %	7.5	7.8	7.8	6.5	7.0	7.7	4.5
Source	Lactose Dextri-maltose	Lactose Dextri-maltose	Lactose Dextri-maltose	Lactose	Lactose	Dextri-maltose Sucrose	Dextri-maltose Sucrose Dextrose

TABLE 2

Composition of prestarters and starter rations

INGREDIENTS	PRESTARTER		STARTER	
	Exp. 696	Exp. 758	Exp. 696	Exp. 758
Ground yellow corn	—	11.65	11.05	40.00
Toasted corn flakes	8.55	—	—	—
Sucrose	5.00	10.00	15.00	15.00
Dextrose	15.00	5.00	—	—
Rolled oats	—	—	40.00	10.00
Solvent soybean oil meal	12.10	17.50	13.00	15.65
Dried skim milk (low-heat, spray-dried)	40.00	40.00	10.00	10.00
Dried sweet whey	2.50	2.50	2.50	2.50
Fish meal	2.50	—	2.50	2.50
Condensed fish solubles	—	2.50	—	—
Corn steep water	1.00	1.00	—	—
Dried brewers' yeast	1.00	1.00	—	—
Stabilized lard	5.00	5.00	—	—
Dried beet pulp	2.00	2.00	—	—
Lecithin (soybean)	1.00	—	—	—
Calcium carbonate	0.50	0.15	0.50	0.30
Dicalcium phosphate	1.20	0.60	—	1.40
Steamed bonemeal	—	—	2.75	—
Iodized salt	0.50	0.50	0.50	0.50
Vitamin-antibiotic premix [1]	2.00	0.45	2.00	2.00
Trace mineral mix [2]	0.15	0.15	0.20	0.15
Totals	100.00	100.00	100.00	100.00
[1] Vitamins and antibiotics added per pound of ration:				
Vitamin A, I.U.	10,000	5,000	3,000	3,000
Vitamin D_2, I.U.	1,000	500	1,000	500
Riboflavin, mg	0.3	2.5	0.3	2.3
Calcium pantothenate, mg	1.9	3.5	1.9	3.6
Niacin, mg	22.7	20	22.7	18.5
Choline chloride, mg	32.7	50	32.7	—
Vitamin, B_{12}, μg	20	20	20	20
Alpha tocopherol acetate, mg	10	—	10	—
Folic acid, mg	9	—	0.5	—
Ascorbic acid, mg	300	—	300	—
Thiamine, mg	2	—	2	—
Pyridoxine, mg	2	—	2	—
Para-amino-benzoic acid, mg	8	—	8	—
Menadione, mg	1	—	1	—
Chlortetracycline, mg	40	—	40	25
Oxytetracycline, mg	40	—	40	—
Bacitracin, mg	15	—	15	—
Penicillin, mg	5	50	5	25
Mycostatin, mg	—	—	10	—

[2] 0.15% Trace mineral mixture, contributed the following amounts (mg) per pound of complete diet: Mn, 38; Fe, 48; Cu, 3.3; Co, 1.14; Zn, 55.65; Mg, 19.8; Na, 348; Cl, 537.

TABLE 3

Composition of synthetic milk for baby pigs [1]

INGREDIENTS	AMOUNT
	%
Dried skim milk (low-heat, spray-dried)	64.5
Lard-lecithin premix [2]	33.3
Vitamin-antibiotic premix [3]	0.4
Vitamin B_{12} concentrate [4]	0.3
Trace mineral mix [3]	1.5
	100.0

[1] Reconstituted and fed as a 10% solids solution.

[2] 30% Fat (24% lard and 6% soybean lecithin) 70% dried skim milk (low-heat, spray-dried).

[3] Vitamins and trace minerals were added in the same amounts (dry basis) as in the prestarter diet, exp. 696.

[4] Lederle Profactor B (10 mg vitamin B_{12}/lb.).

RESULTS

Experiment 696. A summary of the gains and feed (dry matter) required per pound of gain is presented in figure 1.

As a whole, the performance in this experiment was quite satisfactory. Mild, yet transitory scouring (diarrhea) occurred in some of the pigs during the second to 4th week on experiment. The persistency or severity of scouring could not be associated with a specific ration treatment; however, the scouring was more prevalent among those pigs consuming liquid diets.

The most rapid gains were observed for those pigs fed the pig prestarter diet (24% protein) for the first week and then the starter diet (18% protein) for the following three weeks; however, gains on the other two pig diets and the liquid formulas, C and C_2, were quite satisfactory, with average 4-week gains ranging from 14.0 to 16.6 pounds. The pigs receiving formula C in dry form gained 11.1 pounds, and the gains for the pigs receiving formulas D and E were 8.0 and 9.5 respectively.

The feed conversion data indicated that formulas C and C_2 were the most efficient infant formulas. Formula D was more efficient than the dry pig rations. The synthetic milk

diet for pigs was the most efficient with a feed conversion of 1.01 pounds of dry matter required to produce a pound of gain. Formula C, fed in dry form, was the least efficient; however, the conversion figure of 1.66 for this diet was quite satisfactory.

Experiment 758. A summary of the gains and feed required per pound of gain is presented in figure 2.

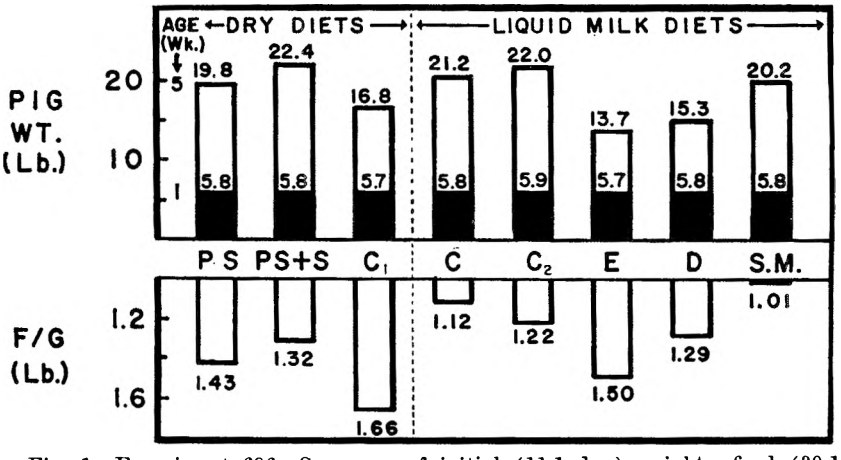

Fig. 1 Experiment 696. Summary of initial (11.1 day) weights, final (39.1 day) weights and feed (F) required per pound of gain (G). Coefficient of variability for gain, 11.7% and for F/G 6.4%.

The gains made by the pigs fed the prestarter-starter diet were considerably less than that observed for the previous experiment. The reported data for this ration treatment represent the average of 4 pens of 4 pigs per pen, whereas the other observations represent the average of two pens of 4 pigs per pen. The reason for the difference in performance between the two experiments is unknown; however, as can be seen from the figure, the average initial weight for these pigs was less than that for the other ration treatments. A covariance analysis of gains on initial weight indicated that gains were correlated with initial weight; however, adjustments of the gains to equal starting weights did not appreciably alter the relative performance on the various ration treatments.

Formulas A, B and C resulted in the most satisfactory performance. Again, formula D was not as efficient in producing gains as was C, and formulas E, F, and G were successively less effective in promoting gains and efficiency of feed conversion.

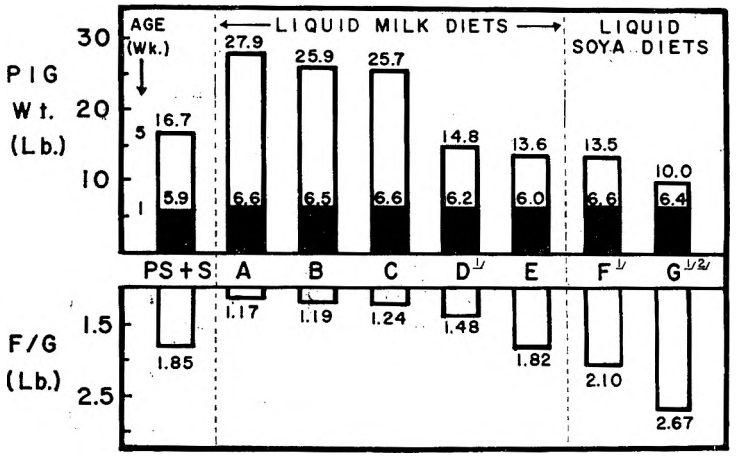

Fig. 2 Experiment 758. Summary of initial (7.1 day) weights, final (35.1 day) weights and feed (F) required per pound of gain (G). Gain coefficient of variability, 25%; regression coefficient of gain on initial weight, 2.704. [1] One pig removed from experiment because of extremely poor performance. [2] One pig died during experiment.

The physical properties of formulas F and G could be partially responsible for the poor performance of these diets. These formulas were much less soluble than the other formulas, resulting in a separation of the liquid and solid phases. Even though these formulas were thoroughly mixed before being placed in the fountains for ad libitum feeding, a uniform distribution of nutrients was not maintained in the solution as it automatically fed down from the reservior into the feeding trough of the poultry fount.

DISCUSSION

Baby pigs performed well on formula C in each of the experiments, and the performance on formulas A and B was very satisfactory in experiment 758. This satisfactory per-

formance was obtained without altering recommended procedure for feeding the formulas other than supplementation with vitamins and trace elements. Although the vitamin and trace element fortification was more complete than usually recommended by pediatricians, it is realized that the need for such a complete fortification of the formulas was not established.

Previous research (Lewis et al., '55) has demonstrated that the baby pig does not perform satisfactorily on soya protein diets; thus the observation for the soya type diets, F and G, are understandable. In addition to the pig's inability to satisfactorily utilize soya protein, performance was also affected by the physical properties of the soya formulas in that the solids were not completely and constantly maintained in a water suspension and as a result, the pigs did not have access to a uniform concentration of nutrients. The composition taken from the labels (table 1) did not list the proportion of each ingredient; thus a reason for the poor performance of formulas D and E can only be conjectured. The level of milk protein in these formulas was considerably lower than that of the other milk base diets. Also, the level of fat in these two formulas was much higher, thus the low level of protein, the high level of vegetable fat or the combination of the two could be responsible for the decreased rate of gain and increased feed required per pound of gain.

These data would need to be corroborated with carefully controlled clinical studies and general observations with the human infant before definite conclusions could be drawn concerning the baby pig's usefulness as a test animal in evaluating human infant diets; however, these experiments do demonstrate that the baby pig can biologically differentiate and evaluate human infant diets on the basis of growth rate and feed conversion.

SUMMARY

Two experiments involving 136 pigs were conducted to compare the relative value of commercially available human infant

milk formulas and previously tested baby pig diets as measured by rate of gain and feed conversion.

The one- to 5-week gains and feed conversion were quite satisfactory for the pigs receiving formulas containing milk as the source of protein when the protein levels were approximately 2.7 to 3.4% of the liquid diet (20 Cal. per fluid ounce). The feeding of formulas containing 1.7 and 1.6% protein and 3.4 and 3.5% fat, respectively, resulted in less rapid growth and required more dry matter per pound of gain as compared to the baby pig diets or the infant formulas containing 2.7% protein and 2.8% fat. The feeding of formulas containing soya protein at 3.2 and 3.1% resulted in lowered rate of gains and increased feed required per pound of gain, which is a reflection of the baby pig's inability to adequately utilize soya protein. The baby pig shows promise as a test animal in biologically evaluating human infant diets, however these data need to be corroborated with carefully controlled clinical studies and general observations with the human infant.

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METHIONINE, HOMOCYSTINE, CHOLINE, FOLIC ACID AND VITAMIN B_{12} IN THE NUTRITION OF THE MOUSE [1]

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Numerous studies have established the existence of relationships among methionine, choline, folic acid and vitamin B_{12} in the rat, chick and certain other species (Schaefer et al., '49, '50, '51; Bennett, '50; Stekol et al., '52; Jukes and Stokstad, '52). These relationships apparently involve the synthesis, transfer and utilization of methyl groups (Welch and Nichol, '52; Stekol et al., '53; Arnstein and Neuberger, '53; Smith, '54; Pfiffner and Bird, '56).

However, few studies have appeared on the interrelationship of these compounds in the nutrition of the mouse. Some evidence exists that mice require folic acid for growth and reproduction (Nielsen and Black, '44; Cerecedo and Mirone, '47). Anti-folacin compounds produce a growth-depression in mice that may be reversed with folic acid or leucovorin (Broquist et al., '52).

Several studies have shown that under certain conditions, such as with the use of thyroid-active compounds or a low-fat, high-protein diet, vitamin B_{12} stimulates the growth of the

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mouse (Bosshardt et al., '50; Meites, '53). Lee et al. ('53) reported that a condition of fetal resorption in the mouse could be prevented with supplements of either α-tocopherol or vitamin B_{12}. Mirone ('54) noted normal growth in mice fed a diet containing 23% of casein, but deficient in choline and vitamin B_{12} and apparently also in folic acid. However, reproductive difficulties and a high mortality rate were encountered after three or 4 months of feeding. Supplements of choline gave partial protection, while vitamin B_{12} had little, if any, effect. Jaffe ('52), however, found that vitamin B_{12} was necessary for normal reproduction in mice.

Reports indicate that the mouse is more resistant to a choline deficiency than the rat or chick, and therefore, probably has been little used in transmethylation studies (Barret et al., '38). In view of this, the present investigations were undertaken to study some of the possible interrelationships of methionine, choline, folic acid and vitamin B_{12} in the nutrition of the mouse.

EXPERIMENTAL

Commercial weanling male albino mice of the Rockland Swiss-Webster strain, averaging 10 gm in weight, were placed in individual wire-bottom cages in an air-conditioned room. Food and water were given ad libitum and the animals were weighed twice a week. A methionine- and methyl-free basal diet was used in nearly all experiments. The basal diet had the following percentage composition: oxidized casein [2] or methanol-extracted casein,[3] 12; corn oil, 4; cod liver oil, 1; salts,[4] 4; sucrose, 72.5; L-cystine, 0.3; and DL-tryptophan, 0.2. The tryptophan was omitted when regular casein was used. The following vitamins were added, in milligrams per kilogram of diet: inositol, 1,000; calcium pantothenate, 30; α-tocopherol, 50; α-tocopherol acetate, 50; niacin, 25; riboflavin, 6; pyridoxine, 6; thiamine, 6; 2-methyl-1,4-naphthoquinone, 5; and biotin,

[2] Hove et al. ('49).
[3] Schaefer and Knowles ('51).
[4] Salmon ('47).

0.5. Supplements of choline, homocystine, betaine, folic acid, vitamin B_{12}, methionine and other amino acids were added to the above basal diet as indicated in the text and tables. Microbiological analyses demonstrated the oxidized casein to be free of methionine. No attempt was made in these studies to enhance the vitamin deficiencies through the use of depletion, sulfa drugs, high fat levels, thyroid-active compounds or similar means.

In part of the animals, fat content and the transmethylase and choline oxidase activities of the liver were determined. The dried, pulverized liver samples were extracted with anhydrous ether in a Nolan extractor [5] and the fat expressed as total ether-extractable material. The liver transmethylase activity was determined by a procedure similar to that previously employed (Sauberlich, '53). The mice were decapitated and the livers removed and chilled with ice. Weighed portions of the liver were homogenized in a glass homogenizer with Ringer's phosphate buffer (1 part of liver — 5 parts of buffer) and 2.0-ml aliquots placed in beakers of a Dubnoff metabolic shaking apparatus. To each beaker was added 0.5 ml (4 mg/ml) of DL-homocysteine hydrochloride solution prepared with Ringer's phosphate buffer. In certain beakers, 0.1 ml of a neutralized 0.128 M (20 mg/ml) choline chloride or 0.128 M (19.7 mg/ml) betaine hydrochloride solution was added. Blanks were prepared by adding distilled water in place of the betaine or choline. The beakers were incubated for 90 minutes at 37°C and the reactions were then stopped with heat. The contents were analyzed microbiologically with the use of *Leuconostoc citrovorum*. The enzyme activity is expressed, corrected for blanks, as milligrams of methionine formed per gram of fresh liver during the 90-minute incubation period. The choline oxidase activities were determined by the method of Williams ('51b) simultaneously with the transmethylase activities.

[5] Nolan ('49).

RESULTS

The effect of dietary supplements on growth

The effect of various dietary supplements on the growth and survival of the weanling mice fed the methionine- and choline-free diet are presented in table 1. Animals fed the diet supplemented with choline, folic acid, and vitamin B_{12} (group 1) failed to grow and survive. However, further supplementation of the diet with methionine (group 18) permitted normal growth and survival (12 to 13 gm/mouse/4 wks.). This would indicate that the basal diet was devoid or nearly so of

TABLE 1

Effect of various dietary supplements on the growth of weanling mice fed a methionine- and methyl-free basal diet

GROUP NO.	DIET AND SUPPLEMENTS [1]	AV. WEIGHT GAINS/MOUSE/4 WEEKS [2]	
		Experiment I	Experiment II
		gm	*gm*
1	Basal + choline + B_{12} + FA	− 4.4 ± 0.2 (2/10)[3]	—
2	Basal + homocystine	0.0 ± 0.6 (8/10)	0.0 ± 1.0 (2/5)
3	Basal + homocystine + B_{12}	0.6 ± 0.8 (3/5)	1.9 ± 0.6 (5/5)
4	Basal + homocystine + FA	2.8 ± 0.7 (2/3)	2.0 ± 0.9 (2/5)
5	Basal + homocystine + FA + B_{12}	3.9 ± 1.8 (7/7)	4.3 ± 1.1 (14/14)
6	Basal + homocystine + choline	2.9 ± 0.8 (6/11)	3.0 ± 1.0 (13/14)
7	Basal + homocystine + choline + B_{12}	5.2 ± 1.6 (5/5)	5.5 ± 2.2 (3/5)
8	Basal + homocystine + choline + FA	14.7 ± 2.3 (3/3)	10.3 ± 0.8 (6/6)
9	Basal + homocystine + choline + FA + B_{12}	13.0 ± 0.5 (4/4)	13.2 ± 1.3 (10/10)
10	Basal + homocystine + betaine	6.8 ± 1.7 (6/6)	6.8 ± 1.3 (11/11)
11	Basal + homocystine + betaine + FA	—	10.0 ± 1.3 (4/4)
12	Basal + homocystine + betaine + FA + B_{12}	—	13.3 ± 0.1 (4/4)
13	Same as no. 5 + glycine + threonine	—	5.9 ± 1.1 (4/4)
14	Same as no. 13 + serine	—	6.7 ± 1.3 (10/10)
15	Basal + methionine	10.1 ± 2.2 (6/6)	9.9 ± 0.6 (9/9)
16	Basal + methionine + B_{12} + FA	10.8 ± 2.0 (2/2)	10.8 ± 0.9 (8/8)
17	Basal + methionine + choline	11.0 ± 3.3 (3/3)	10.1 ± 1.2 (6/6)
18	Basal + methionine + choline + B_{12} + FA	12.2 ± 0.9 (10/10)	13.0 ± 1.4 (9/9)
20	Casein [4] + choline + B_{12} + FA	14.1 ± 1.5 (3/3)	12.7 ± 1.0 (5/5)

[1] Where indicated, the supplements were added at the following levels per kilogram of diet: vitamin B_{12} (B_{12}), 100 μg; folic acid (FA), 10 mg; choline chloride, 4.0 gm; DL-homocystine, 5.0 gm; betaine hydrochloride, 4.0 gm; glycine, 4.0 gm; DL-threonine, 3.0 gm; DL-serine, 4.0 gm; DL-methionine, 5.0 gm.

[2] Average weight gain ± standard error of the mean; average initial weight was 10.0 gm.

[3] Two of the 10 mice started survived the experiment, etc.

[4] Methanol-extracted casein (not oxidized).

methionine, substantiating the microbiological analyses. The addition of 0.4% of DL-homocystine to the basal diet improved the survival of the mice, but it did not permit growth. Further supplementation of the diet singly with folic acid or with vitamin B_{12} neither improved growth appreciably nor prevented death of some rats (groups 2 to 4). However, when the basal diet contained the combined supplement or homocystine, vitamin B_{12} and folic acid, the mice survived the 4-week period and grew about 30% of normal (4.1 gm/mouse/4 wks.; group 5). The further addition of glycine, serine and threonine increased the growth to that of about 50% of normal (6.7 gm/mouse/4 wks.; groups 13 and 14).

When the basal diet was supplemented with choline in the presence of homocystine, deaths, following only slight growth, occurred in the experimental animals. Further supplementation with vitamin B_{12} improved growth only slightly, but additions of folic acid permitted normal or nearly normal growth (groups 6 to 8). Vitamin B_{12} in the presence of folic acid occasionally had a little further effect (group 9).

In comparison, supplements of betaine in the presence of homocystine permitted survival and about 50% of normal growth (group 10). A further supplementation with folic acid improved the growth equal to that obtained with a comparable supplement of choline and folic acid (group 8 vs. 11). A combined supplement of folic acid and vitamin B_{12}, in the presence of betaine and homocystine, produced normal growth.

The addition of methionine to the basal diet permitted nearly normal growth. Further supplements of vitamin B_{12} and folic acid or of choline had little, if any, effect. However, normal growth was obtained when the mice were fed a combined supplement of methionine, choline, vitamin B_{12}, and folic acid (groups 15 to 18). Additional experiments indicated that the presence of ethanolamine in the above diets exerted little or no effect on the response of the mice to the various supplements.

The effect of various dietary supplements on the liver choline oxidase activity of the mouse

The results of determinations for the liver choline oxidase activity are presented in table 2. Since extremely fatty livers were encountered in mice fed certain diets, it was desirable to express and compare the activities on the basis of wet tissue, dry residue, and fat-free dry residue.

The choline oxidase activity of mice fed the methyl-free basal diet supplemented with homocystine was very low. When the animals received in addition folic acid, the activity was increased to essentially normal values (expressed on the fat-free dry residue basis). Supplements to the basal diet of homocystine and vitamin B_{12} partially restored normal activity, while normal activity was obtained with a further supplement of folic acid (groups 1 to 4; fat-free dry residue basis).

Choline, in the presence of homocystine, also increased the choline oxidase activity. However, in the presence of choline,

TABLE 2

Effect of various supplements on the liver choline oxidase activity of mice fed a methionine- and methyl-free basal diet

GROUP NO.	DIET AND SUPPLEMENTS [1]	CHOLINE OXIDASE ACTIVITY OF LIVER AS μL O_2 UPTAKE/HOUR/GM: [2]		
		Fresh basis	Dry basis	Fat-free dry basis
1	Basal + homocystine	27 ± 7 (7)	50 ± 13	227
2	Basal + homocystine + B_{12}	49 ± 6 (6)	94 ± 11	346
3	Basal + homocystine + FA	64 ± 10 (2)	142 ± 22	436
4	Basal + homocystine + FA + B_{12}	67 ± 6 (4)	122 ± 11	456
5	Basal + homocystine + choline	94 ± 23 (3)	298 ± 73	370
6	Basal + homocystine + choline + B_{12}	79 ± 9 (3)	251 ± 28	318
7	Basal + homocystine + choline + FA	110 ± 12 (5)	351 ± 38	440
8	Basal + homocystine + choline + FA + B_{12}	131 ± 15 (6)	418 ± 48	479
9	Basal + homocystine + betaine	68 ± 14 (3)	159 ± 32	202
10	Basal + methionine + choline + B_{12} + FA	127 ± 8 (4)	405 ± 25	460
11	Casein [3] + choline + B_{12} + FA	126 ± 8 (3)	412 ± 26	462

[1] Levels of supplements employed same as indicated in table 1.

[2] Average ± standard error of the mean; values within parentheses indicate number of livers analyzed individually.

[3] Methanol-extracted casein (not oxidized).

further supplementation with vitamin B_{12} failed to increase the activity, whereas essentially normal values were again found in the livers of mice receiving supplements of folic acid (groups 5 to 8). When the basal diet was supplemented with homocystine and betaine, very low choline oxidase activities were observed, although the growth of these animals was greater than certain groups of animals with normal choline oxidase activities (group 9 vs. groups 3 and 4; see also table 1, group 10 vs. groups 3 to 5). Normal activities were observed in the livers of mice fed the basal diet supplemented with methionine, choline, folic acid and vitamin B_{12} or the methanol extracted casein basal diet (groups 11 and 12).

The effect of various supplements on liver fat and transmethylase activity in the mouse

The results of these studies are summarized in table 3. In general there was a correlation between transmethylase activity and the growth of the animals (compare with table 1). The transmethylase activity was reduced in mice fed the methyl-free basal diet supplemented with homocystine (wet-tissue basis; groups 1 and 18). However, the effect was considerably less when the activity was expressed on a fat-free dry residue basis. Further supplementation of the diet with both folic acid and vitamin B_{12} increased somewhat the transmethylase activity. The liver fat content was very high in animals receiving the methyl-free basal diet supplemented with homocystine (77.8%; dry basis). Additional supplements of folic acid and vitamin B_{12} had little, if any, effect on the fat content of the liver (groups 1 to 4). Similarly, supplements of glycine, serine and threonine, although permitting 50% of normal growth, had no apparent effect on lowering the liver fat content (73.0%; dry basis; group 5).

When the basal diet was supplemented with both homocystine and choline, the transmethylase activity was only about 60% of normal. When the diet was further supplemented with folic acid, normal values were observed. Supplements of vita-

TABLE 3

Effect of various dietary supplements upon the liver fat and transmethylase activity of mice fed a methionine- and methyl-free basal diet

(Weanling Rockland albino mice; 28 days on experiment)

GROUP NO.	DIET AND SUPPLEMENTS [1]	TRANSMETHYLASE ACTIVITY OF LIVER AS METHIONINE FORMED [2]		LIVER ANALYSIS		
				Av. water content	Av. fat content	
					Wet basis	Dry basis
		mg/gm	*mg/gm*	%	%	%
1	Basal + homocystine	0.97 ± 0.10 (4)[3]	81.5	46.2	41.9	77.8 (4)
2	Basal + homocystine + B_{12}	1.15 ± 0.24 (4)	81.0	47.5	38.3	72.9 (8)
3	Basal + homocystine + FA	0.95 ± 0.22 (2)	65.0	55.3	30.1	67.4 (3)
4	Basal + homocystine + FA + B_{12}	1.34 ± 0.27 (6)	91.2	45.3	40.0	73.1 (5)
5	Same as no. 4, + glycine, serine, threonine	—	—	43.7	41.0	73.0 (7)
6	Basal + homocystine + choline	1.52 ± 0.48 (4)	60.7	68.6	6.1	19.5 (4)
7	Basal + homocystine + choline + B_{12}	2.20 ± 0.43 (4)	88.3	68.5	6.6	20.9 (5)
8	Basal + homocystine + choline + FA	2.66 ± 0.41 (6)	106.0	68.7	6.2	19.9 (9)
9	Basal + homocystine + choline + FA + B_{12}	2.61 ± 0.21 (4)	95.6	68.7	4.0	12.9 (9)
10	Basal + homocystine + betaine	2.89 ± 0.96 (3)	85.2	56.9	9.2	21.3 (7)
11	Basal + homocystine + betaine + FA	—	—	65.0	4.8	13.6 (4)
12	Basal + homocystine + betaine + FA + B_{12}	—	—	61.3	6.2	16.1 (3)
13	Basal + methionine	—	—	47.4	30.1	57.3 (5)
14	Basal + methionine + FA + B_{12}	—	—	59.8	17.6	43.7 (5)
15	Basal + methionine + choline	—	—	65.2	4.1	11.9 (6)
16	Basal + methionine + choline + FA + B_{12}	2.75 ± 0.63 (5)	100.0	68.7	3.8	12.1 (6)
17	Casein [4] + choline + FA + B_{12}	2.67 ± 0.40 (3)	97.9	69.4	3.4	11.0 (3)
18	Stock diet	3.20 ± 0.21 (3)	106.0	65.1	4.7	13.6 (3)

[1] Levels of supplements employed same as indicated in table 1.

[2] Betaine was employed as the methyl donor in these determinations.

[3] Average ± standard error of the mean; values within parentheses indicate number of livers analyzed individually.

[4] Methanol-extracted casein (not oxidized).

min B_{12} also appeared to have some influence. The liver fat content was markedly lowered when 0.4% choline was fed, and further supplementation with folic acid and vitamin B_{12} permitted normal levels (20.9 and 12.9%, respectively; dry basis). The transmethylase activity of mice receiving betaine was lower than normal, but it was higher than that of animals receiving choline. Liver fat was also greatly reduced by the supplement of 0.4% betaine hydrochloride, and the effect appeared to be improved by the presence of folic acid in the diet (21.3 and 13.6%, respectively). When the basal diet was supplemented with methionine, choline, folic acid and vitamin B_{12}, normal liver fat values and transmethylase activities were observed (group 16). Supplements of methionine alone (0.5%), only partially reduced the liver fat content (57.3%). Further supplementation with choline permitted normal liver fat values, while a supplement of folic acid and vitamin B_{12} had a partial favorable effect (groups 13 to 15). The use of higher levels of methionine may have reduced the liver fat further, but the danger of toxic effects would increase.

In additional studies, choline was used as the methyl donor in the transmethylase activity determinations. In these experiments, the *in vitro* synthesis of methionine was found to be slow and small in amount. In contrast, previous studies with the rat have shown choline to be a fairly active methyl source for the methylation of homocysteine (Williams, '51a; Sauberlich, '53). This is probably a reflection of the low choline oxidase activity of mouse liver. On the other hand, the transmethylase activity of the normal mouse, when betaine was employed, was higher than that reported for the rat (Sauberlich, '53; Kensler and Langemann, '54).

DISCUSSION

The present studies appear to demonstrate that the ability of homocystine to replace methionine in the nutrition of the mouse is related to the presence in the diet of folic acid, vitamin B_{12} and methyl compounds or precursors of methyl com-

pounds. The observation that homocystine, in the absence of a methyl source, required both folic acid and vitamin B_{12} to permit survival and limited growth would indicate that these vitamins are involved in the synthesis and transfer or utilization of methyl groups. Studies with other species seem to demonstrate that vitamin B_{12} aids in the synthesis of methyl groups, while folic acid is important in certain transfers of methyl groups (Stekol et al., '53; Smith, '54; Pfiffner and Bird, '56; Totter, '57).

The ability of dietary betaine and homocystine to permit survival and one-half of normal growth without the presence of either folic acid or vitamin B_{12} is in marked contrast to that observed with choline supplements. Apparently the requirement for folic acid is reduced or less essential when betaine is the source of methyl groups rather than choline for the methylation of homocysteine. This may be explained on the basis that the requirement for folic acid to maintain choline oxidase activity for the conversion of choline to betaine, as demonstrated in the rat and chick (Dinning et al., '51; Williams, '51b) would be avoided. Folic acid may still be required for the betaine-homocysteine transmethylase reaction as has been demonstrated in the chick (Dinning et al., '51). However, Young et al. ('55) found with the chick that folic acid was not necessary for the formation of choline from betaine and monomethylaminoethanol.

It is apparent from the present studies that the liver choline oxidase activity of the normal mouse is low when compared with that of the rat. This is in agreement with findings of Kensler and Langemann ('54). It is also apparent that a deficiency of folic acid in the diet produced a marked reduction in the choline oxidase activity. The stimulating effect of vitamin B_{12} on choline oxidase activity may be related indirectly to an influence on methyl synthesis, which in turn may stimulate choline oxidase production directly, or by sparing folic acid. In this respect it was noted that the presence of choline in the diet increased the liver choline oxidase activity, whereas betaine was without effect.

The low liver choline oxidase activity of the mouse may exert a protective effect on tissue choline and thus moderate or prolong the period before choline-deficiency effects would occur. However, the availability of the methyl groups of choline for methylation reactions in the mouse becomes very dependent upon the presence of an adequate supply of folic acid as the present studies indicate that this vitamin has also a role in the transmethylase activity. Folic acid has also been demonstrated to be involved in the transmethylase activity of the rat and chick (Dinning et al., '51; Williams, '51a; Sauberlich, '53).

The importance for the mouse of vitamin B_{12} in relation to this activity is less certain. In the absence of choline but in the presence of folic acid, vitamin B_{12} appeared to have a small effect. When choline was in the diet, vitamin B_{12} had some effect in the absence of folic acid, but not in its presence. This may well be a reflection of needs for methyl groups that could be furnished by *de novo* synthesis in the presence of vitamin B_{12}, but could not be furnished by choline except in the presence of folic acid.

SUMMARY

1. Weanling mice fed a methionine-free, choline-free basal diet, supplemented with homocystine, died or failed to grow when either folic acid or vitamin B_{12} was added. The addition of both vitamins permitted survival but little growth. Further supplementation with glycine, serine and threonine resulted in 50% of normal growth.

2. The addition of choline alone to the homocystine-supplemented basal diet permitted only slight growth, whereas about 50% of normal growth was obtained with betaine. The addition of folic acid in the presence of either choline or betaine resulted in nearly normal growth. The further addition of vitamin B_{12} had only a slight effect. Methionine alone permitted nearly normal growth which was improved slightly by the presence of choline, folic acid and vitamin B_{12}.

3. The liver choline oxidase activity was markedly reduced in mice fed the basal diet supplemented with homocystine. The

reduction in activity was largely prevented by dietary supplements of folic acid and partially with vitamin B_{12}. Choline was also effective, whereas betaine was ineffective.

4. Liver transmethylase activity was reduced when choline, folic acid, and vitamin B_{12} were omitted from the basal-homocystine diet. The activity was improved when folic acid and vitamin B_{12} were added to the diet. When choline was present in the diet, supplements of folic acid permitted normal activity while partial improvement was noted with supplements of vitamin B_{12}.

5. Extremely fatty livers were observed in mice fed the basal-homocystine diet, either in the presence or absence of folic acid and vitamin B_{12}. When choline or betaine was present, the liver fat was greatly reduced. Normal values were obtained when choline, folic acid and vitamin B_{12} were fed.

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EFFECT OF SATURATED FAT UPON ESSENTIAL FATTY ACID METABOLISM OF THE RAT [1]

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INTRODUCTION

Natural and processed fats vary greatly in the proportions of their non-essential and essential fatty acid contents. However, there is little information available concerning the requirements of essential fatty acids (EFA), such as linoleic and arachidonic acids, when these are diluted with dietary non-essential fatty acids.

Non-essential fatty acids, when fed with EFA, appear to stimulate better growth than do supplements of EFA alone. Greenberg et al. ('50, '51) found that EFA-deficient rats fed high levels of cottonseed oil in the diets grew better and had higher food efficiencies than those receiving EFA supplements alone. Thomasson [2] has observed that certain natural oils, or their fully hydrogenated products, do not support the growth of rats as well as does an optimum mixture of the two fats.

Different nutritive responses have been observed with rats fed high-fat diets differing widely in their EFA content. Barboriak et al. ('58) observed that rats fed high-fat diets, which were rich in EFA, did not grow as well as those receiving more

[1] Grateful acknowledgment is made to the Hormel Foundation and the National Dairy Council for supporting much of the work reported here.

[2] Personal communication, Dr. H. J. Thomasson.

saturated fats, which were poor in EFA. However, the EFA-rich diets showed less tendency to promote obesity in the rats than did the more saturated fats. Barki et al. ('50) found the optimum level of fat for growth of rats to be 10% for corn oil, which is rich in EFA, and 35% for butterfat, which is rich in non-essential fatty acids. These and other observations on the nutritive value of various fats (Cowgill, '45; Thomasson, '55; Dryden et al., '56) may be explained by differences in the ratios of non-essential fatty acids to EFA in the fats.

Tissue lipides appear to be affected by dietary fats according to their relative concentrations of non-essential and essential fatty acids. Dietary oils, rich in EFA, tend to lower plasma cholesterol levels, whereas, fats, low in EFA, have an opposite effect (Ahrens et al., '57; Avigan and Steinberg, '58; Bronte-Stewart et al., '56; Kinsell and Michaels, '55), and, in addition, promote increases in plasma trienes (Holman et al., '57). However Hegsted et al. ('57) suggested that the combined effects of saturated fatty acids and EFA are to lower plasma cholesterol levels in hypercholesteremic rats. These investigators have obtained data suggesting that oleic acid and other non-essential unsaturated acids, found in natural fats, promote elevated plasma cholesterol levels.

These and other observations indicate that non-essential fatty acids can exert a marked influence on the function and utilization of EFA. A series of experiments was planned to study the effects of widely differing ratios of saturated fat to EFA upon the growth, dermal conditions and tissue polyunsaturated acids of rats.

EXPERIMENTAL

Young male Sprague-Dawley rats,[3] weighing 40 to 55 gm, were maintained on the experimental diets for 5 and 7 weeks in experiments I and II, respectively. The animals were housed individually, or in groups of two, in metal screen-bottom cages. Fresh diets were supplied and food intakes were

[3] Holtzman Rat Corporation, Madison, Wisconsin.

recorded three times weekly. Both food and water were offered ad libitum. Body weights and dermal scores were measured at weekly intervals.

The experimental diets varied only in their fatty acid composition and sucrose content. The ethyl esters from corn oil [4] were used in experiment I and ethyl linoleate [5] was used in experiment II as the source of EFA. Isocaloric amounts of completely hydrogenated coconut oil [6] (HCO) were included in the EFA-deficient diets. The unsaturated esters were fed in the form of urea-inclusion compounds to prevent oxidation (Holman and Ener, '54) and equivalent quantities of urea were incorporated in the HCO diets. All diets had the following percentage composition: casein [7] 18, urea 3, α-cellulose [7] 4, salt mixture [8] 4, glucose-vitamin mixture 2. Vitamins were supplied in milligrams per 100 gm of diet as follows: thiamine, 7.3; riboflavin, 3; pyridoxine·HCl, 3; nicotinic acid, 6; calcium pantothenate, 7; choline, 130; inositol, 132; *p*-aminobenzoic acid, 60; ascorbic acid, 50; biotin, 0.05; folic acid, 1.1; vitamin B_{12}, 0.2; menadione, 0.6; vitamin A palmitate, 0.5; calciferol, 0.01; α-tocopherol, 10.0. The water-soluble vitamins were mixed with glucose and the fat-soluble vitamins were sprayed on to the diet from an ether solution, 0.5 ml per 100 gm diet. All diets were refrigerated until needed. The variable components of the experimental diets are shown in table 1.

In experiment I all rats were fed diet A-1 upon arrival at the laboratory. They were maintained on this diet for 10 days and then randomly distributed into the 4 groups shown in table 1. The animals in experiment II were immediately distributed into the indicated groups upon arrival.

The rats were sacrificed by ethyl ether anesthesia. Their carcasses and organs were weighed, immediately frozen in

[4] Mazola.

[5] Prepared from safflower oil by low temperature and urea crystallizations and vacuum distillation on a Podbielniak still. Alkali isomerization showed this ester to contain more than 95% linoleate.

[6] "Hydrol" obtained from Durkee's Famous Foods, Chicago, Illinois.

[7] Nutritional Biochemicals, Cleveland, Ohio.

[8] Wesson salt mixture, Nutritional Biochemicals.

dry ice and maintained at −20°C until further analyses could be made. Each heart was macerated in 25 ml of 60% ethanol made 1N with respect to KOH. The mixture was allowed to saponify overnight at room temperature and then brought to boil for 15 minutes. After removal of the unsaponifiable portions with petroleum ether (b.p. 30 to 60°C) the heart digests were acidified with 6N HCl and the fatty acids recovered by

TABLE 1

Dietary variables used in experiments I and II

GROUP	SUCROSE	FAT CALORIES	CORN OIL ETHYL ESTERS	ETHYL LINOLEATE	HYDROGENATED COCONUT OIL
	%	%	%	%	%
			Experiment I		
A–1	68	2.4	—	—	1
A–2	68	2.4	1	—	—
A–3	59	21.6	—	—	10
A–4	59	21.6	1	—	9
			Experiment II		
B–1	68	2.4	—	—	1.0
B–2	68	2.4	—	0.5	0.5
B–3	59	21.6	—	—	10.0
B–4	59	21.6	—	0.5	9.5
B–5	44	47.6	—	—	25
B–6	44	47.6	—	0.5	24.5

three 100-ml extractions with petroleum ether. The extracts were combined, dried over sodium sulfate and the solvent evaporated, *in vacuo,* to approximately 5 ml. Ten milliliters of redistilled methanol were added and the total volume was again reduced to 5 ml to remove residual petroleum ether. The solution of fatty acids was then made up to a final volume of 10 ml with methanol and used for further analyses. All operations were carried out under an atmosphere of nitrogen. Liver samples were treated in a similar manner.

Polyunsaturated fatty acids were analyzed by a modification of the methods of Herb and Riemenschneider ('53) and Holman ('57).

RESULTS

In Experiment I (see table 2) it was found that rats fed EFA-free diets containing 1 or 10% of HCO showed equivalent body weight gains and dermal scores. However, control animals receiving 1% of CO and 9% of HCO showed significantly greater body weight gains than the group receiving 1% of CO alone. In the second experiment groups of rats were fed diets containing 1 to 25% of HCO or 0.5% of ethyl linoleate plus 0.5 to 24.5% of HCO. In this study the growth of EFA-deficient rats was inhibited by feeding high levels of saturated fat. Since the group receiving 25% of HCO (B-3) had the highest food intake, this inhibition could not have been a reflection of a reduced intake of fat calories. Rats receiving 0.5% of linoleate did not show such a growth inhibition when 0.5 to 24.5% of HCO was added to the diet.

Although the food intake of the linoleate-fed groups was equal to, or less than, that of the groups fed HCO, the caloric efficiencies remained high in the EFA-supplemented rats. However, the caloric efficiencies of EFA-deficient rats were reduced as the HCO content of the diets were increased. In each comparison between groups fed the same level of fat, the EFA-supplemented groups had higher body weight gains and higher caloric efficiencies. These results, summarized in table 2, demonstrate that EFA are necessary for the proper utilization of fat calories by the rat.

In addition to the biological evaluations used in these studies the changes in the concentration of tissue polyunsaturated fatty acids was used as another criterion of essential fatty acid deficiency. Rieckehoff et al. ('49) found that the polyenoic acids from heart ventricle varied the most in response to changes in dietary EFA. In our studies there was a general increase of heart polyenoic acids in all groups, as the HCO content of the diet was increased (see table 3). Dienoic acids derived from exogenous fat, and higher polyenoic acids synthesized by the rat (endogenous polyunsaturated acids) were both increased by high intakes of non-essential fatty acids

TABLE 2

Effect of saturated fat on EFA deficiency in rats

GROUP	DIET [1]	NO. OF RATS	WEIGHT [2] GAINS	TOTAL FOOD INTAKE	CALORIC [2] EFFICIENCY	TERMINAL [2,3] DERMAL SCORES
			gm	*gm/rat*	*gm gain/100 Cal.*	
			Experiment I, 5 week study			
A–1	HCO, 1%	6	128.3 ± 7.9	—	—	3.2 ± 0.6
A–2	CO, 1%	5	124.0 ± 7.3	—	—	0.0
A–3	HCO, 10%	5	117.2 ± 8.2	—	—	2.9 ± 0.6
A–4	HCO, 9%; CO, 1%	5	164.2 ± 7.4	—	—	0.0
			Experiment II, 7 weeks study			
B–1	HCO, 1%	8	166.5 ± 9.7	569	7.86 ± 0.32	3.6 ± 0.3
B–2	HCO, 10%	8	153.9 ± 3.8	549	6.72 ± 0.09	3.5 ± 0.3
B–3	HCO, 25%	8	144.9 ± 5.1	620	5.29 ± 0.67	2.1 ± 0.2
B–4	HCO, 0.5%; EL, 0.5%	8	185.8 ± 3.2	582	8.61 ± 0.26	0.0
B–5	HCO, 9.5%; EL, 0.5%	7	182.1 ± 7.1	571	8.04 ± 0.83	0.0
B–6	HCO, 24.5%; EL, 0.5%	5	169.6 ± 15.4	473	7.74 ± 0.55	0.0

[1] Hydrogenated coconut oil, corn oil and ethyl linoleate are abbreviated as HCO, CO and EL in the above table.

[2] Mean values ± the standard error of the mean as calculated by using the formula: $S\bar{x} = \sqrt{\frac{\Sigma(\bar{X} - \bar{x})^2}{N(N-1)}}$

[3] Severity of scaliness of tail is graded 0–4, feet 0–3, and roughness of coat as 0–2. Dermal scores above are average values of the sum of these three measurements.

from HCO. This indicates that the feeding of high-fat diets favors the accumulation of tissue polyenoic acids in heart muscle. Since the rat is unable to synthesize dienoic acids, i.e. linoleate, heart dienoic acids are of exogenous origin, being derived from the diet or from dienoic acid reserves accumulated prior to the experiment.

The pattern of polyunsaturated acids was quite different in the EFA-deficient and linoleate-supplemented groups. All EFA-deficient rats had equally high concentrations of trienoic acids in their hearts. In contrast, the increase of saturated fat intake from 0.5 and 24.5% of the diet caused more than a 20-fold increase in trienoic acids in the hearts of rats fed linoleate. In all comparisons between groups fed the same level of fat, rats had the highest concentration of heart dienoic acid, (linoleic acid) when fed the EFA supplement. Both EFA-deficient and linoleate-fed rats receiving 1% of fat had similar concentrations of tetraenoic acids (arachidonic acid) in their hearts. This suggests that the HCO-fed groups were not markedly depleted of their arachidonic acid stores although they did accumulate high concentrations of trienoic acids during this short-term study. High levels of dietary fat promoted the accumulation of 100% more arachidonic acid in the hearts of linoleate-fed rats. This occurred even though the total food intake, and therefore linoleate intake, was essentially the same for both the 1% and 10% fat, EFA-supplemented groups (see table 3). Both dietary linoleate and high-fat intakes tended to promote an accumulation of pentaenoic and hexaenoic acids in the rat's heart.

The data in table 4 show the relative amounts of endogenous fatty acids found in the hearts of rats fed different levels of dietary fat. In both EFA-deficient and control groups the total endogenous polyenoic acids were increased by feeding higher levels of dietary fat. Nevertheless, the relative amounts of individual endogenous polyenoic acids remained constant in the EFA-deficient rats. This suggests that the increased levels of dietary fat had little effect on the polyunsaturated fatty acid metabolism of EFA-deficient rats. In rats fed mini-

TABLE 3

Effect of saturated fat on polyunsaturated acids of the heart after 7 weeks on experimental diets

GROUP	DIET	HEART POLYENOIC ACIDS (MG/100 GM)[1,2]				
		Dienoic	Trienoic	Tetraenoic	Pentaenoic	Hexaenoic
B-1	1% HCO	82.9 [7] ± 15.5	162.6 ± 15.2	132.1 ± 23.0	20.1 ± 2.8	15.5 ± 1.9
B-2	10 % HCO	91.9 [8] ± 8.8	176.9 ± 15.5	193.7 ± 13.5	20.9 ± 3.3	7.5 ± 3.2
B-3	25% HCO	130.0 [7] ± 13.0	197.3 ± 22.2	195.1 ± 19.8	24.8 ± 3.3	35.6 ± 4.7
B-4	0.5% HCO + 0.5% EL	121.1 [6] ± 17.0	2.6 ± 1.3	139.1 ± 31.4	35.1 ± 8.1	13.3 ± 2.4
B-5	9.5% HCO + 0.5% EL	501.7 [7] ± 67.3	25.6 ± 5.5	393.0 ± 20.6	58.4 ± 4.6	17.8 ± 3.2
B-6	24.5% HCO + 0.5% EL	289.3 [5] ± 17.6	55.2 ± 8.1	384.6 ± 12.6	54.0 ± 3.2	40.7 ± 4.1

[1] Includes standard error of the mean.

[2] Figures in superscript represent number of hearts analyzed individually.

TABLE 4

Effect of saturated fat on the relative concentrations[1] of endogenous polyenoic acids (E.P.)[2] found in the heart tissues after the 7-week experimental period[3,4]

GROUP	DIET	TRIENOIC/E.P.	TETRAENOIC/E.P.	PENTAENOIC/E.P.	HEXAENOIC/E.P.	TOTAL ENDOGENOUS POLYENOIC ACIDS
						mg/100 gm
B-1	1% HCO	48.3[7] + 2.9	40.0 ± 1.8	6.50 ± 0.75	5.22 ± 0.77	316.0 ± 59.3
B-2	10% HCO	44.3[8] ± 4.6	49.2 ± 2.2	5.24 ± 0.64	1.84 ± 0.78	396.5 ± 22.0
B-3	25% HCO	43.6[7] ± 2.16	43.1 ± 1.60	5.46 ± 0.81	7.93 ± 0.64	452.8 ± 43.6
B-4	0.5% HCO + 0.5% EL	1.05[6] ± .60	68.9 ± 5.95	16.92 ± 1.24	6.59 ± 0.24	208.6 ± 42.6
B-5	9.5% HCO + 0.5% EL	5.43[7] ± 1.44	80.1 ± 1.65	11.85 ± 2.23	3.68 ± 0.66	490.7 ± 21.1
B-6	24.5% HCO + 0.5% EL	9.98[5] ± 1.27	72.3 ± 1.0	10.09 ± 0.34	7.65 ± 0.75	534.6 ± 21.1

[1] Relative concentration of the endogenous polyenoic acids was calculated according to the formula:

$$\% \text{ of endogenous polyenoic acids} = \frac{\text{Specific polyenoic acid}}{\text{Total E. P.}} \times 100$$

[2] Endogenous polyunsaturated acids (E.P.) synthesized by the rat. These include trienoic, tetraenoic, pentaenoic and hexaenoic acids.

[3] Data include standard error of the mean.

[4] Superscripts refer to the number of hearts analyzed individually.

mal amounts of linoleate, however, increasing the intake of HCO promoted a 7-fold increase in heart trienoic acids. Normally the EFA-supplemented rat has little trienoic acid in its tissues. The data in tables 3 and 4 suggest that increasing the ratio of saturated fat to linoleate in the diet caused a metabolic shift similar to that seen with EFA-deficient rats. Longer term studies, or the feeding of higher ratios of HCO: linoleate, might be expected to cause additional increases in trienoic acid. The relative amounts of endogenous tetra- and pentaenoic acids were 70 and 140% greater respectively in linoleate-fed groups than in the EFA-deficient groups.

The relative concentrations of the individual endogenous fatty acids of livers from groups fed 1% of HCO were almost identical to those found in the heart tissues of this same group.

DISCUSSION

In agreement with the studies of Greenberg et al. ('50, '51) non-essential fatty acids have been found to stimulate the growth of EFA-deficient rats provided minimal quantities of linoleate are fed to the animal. However, EFA-deficient rats are unable to utilize calories from saturated fat for growth in the absence of supplements of EFA. Thus, esesntial fatty acids appear to be necessary for the proper utilization of fat calories. Although other unsaturated, non-essential fatty acids, i.e., oleic and linolenic, were not tested in these studies it seems unlikely that these would have a similar function. The non-essential trienoic acids were significantly higher than dienoic acids (linoleic) in the heart and liver tissues of EFA-deficient rats (see table 3). Mead ('57) found that fat-deficient mice accumulate significant concentrations of palmitoleic acid in their organ and depot fats. The depot fats from rats fed low-fat diets, or diets low in EFA, contain oleic acid as a major component in addition to elevated levels of palmitoleic acid (Spadola and Ellis, '36; Hilditch, '56). Nevertheless, these three unsaturated, non-essential fatty acids, palmitoleic, oleic, and eicosatrienoic acid (Mead, '57), did not allow EFA-deficient rats to utilize their fat calories for growth.

Provided EFA-supplements were given, the increased intake of saturated fat, from 1 to 25%, did not alter the ability of the rat to utilize calories. The high intake of food by the group receiving 25% of HCO (table 2) may be a reflection of the animal's need for calories since dietary fat could not be assimilated for growth by these rats.

Rieckehoff et al. ('49) demonstrated that, in long-term studies, the EFA-deficient rat accumulates trienoic acids in its heart and is depleted of its arachidonic acid stores. Furthermore, EFA-deficient rats showed a preferential deposition of dietary polyunsaturated acids in their hearts. The present studies demonstrate that the EFA-deficient rat accumulates more polyunsaturated acids in its heart when the intake of saturated fat is increased. Such an increase of polyenoic acids in the heart may be due to: (1) a "sparing" action of saturated fat on the metabolism of tissue polyenoic acids; (2) a mobilization of polyenoic acids from other tissues to the heart muscle; (3) a stimulatory effect of high levels of dietary fat, *per se,* on the synthesis of polyenoic acids. Mead et al. ('56) found that fat-deficient mice metabolized stearate and linoleate at a significantly higher rate than did normal mice. The results reported here may be due, to a large extent, to a "sparing" effect of saturated fat on the metabolism of polyunsaturated acids in EFA-deficient rats. More specific studies are necessary to establish the mechanism by which dietary saturated fats allow higher levels of polyenoic acids to accumulate in the heart tissue of rats.

Various methods have been found to promote the onset of EFA deficiency in rats. Peifer and Holman, ('55, '56) and Holman and Peifer ('56) demonstrated that dietary cholesterol, diabetes, and hypothyroidism accelerate the onset of EFA deficiency in rats. The accumulation of trienoic acids, typical of an EFA deficiency, due to an increased intake of saturated fat by linoleate-fed rats suggests that saturated fats also promote the onset of an EFA deficiency. As the content of saturated fat of the diet was increased there was an apparent increase in the EFA requirements of the rat. These studies

emphasize the necessity of considering the ratio of saturated fat: EFA rather than the EFA content alone, when judging the adequacy of a diet. Such an imbalance may be responsible for some of the differences in the biological effect of dietary fats observed by Barki et al. ('50), Thomasson ('55) and Bronte-Stewart et al. ('56).

SUMMARY

Two different experiments were conducted using male weanling rats fed fat-free diets supplemented with 1 to 25% of fat. When completely hydrogenated coconut oil (HCO) was the only source of fat, the intake of high-fat diets inhibited growth and reduced caloric efficiencies. When fed diets containing EFA, as 1% of corn oil or 0.5% of ethyl linoleate, increased intakes of HCO allowed greater growth and did not alter the caloric efficiencies of the rats. The ability of EFA-supplemented rats to utilize calories was greater than that of EFA-deficient rats at each level of fat intake.

High intakes of HCO promoted the accumulation of polyunsaturated acids in the hearts of both EFA-supplemented and EFA-deficient rats. EFA-deficient rats fed all levels of HCO had equally high concentrations of trienoic acids in their hearts. Rats fed EFA had significantly lower heart trienoic acid content but showed a 20-fold increase in these polyenoic acids when 24.5% of HCO was added to the diet.

These studies demonstrate that essential fatty acids are required for the proper utilization of fat calories. Furthermore, high ratios of saturated fat: EFA promote the onset of EFA-deficiency symptoms in the rat.

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