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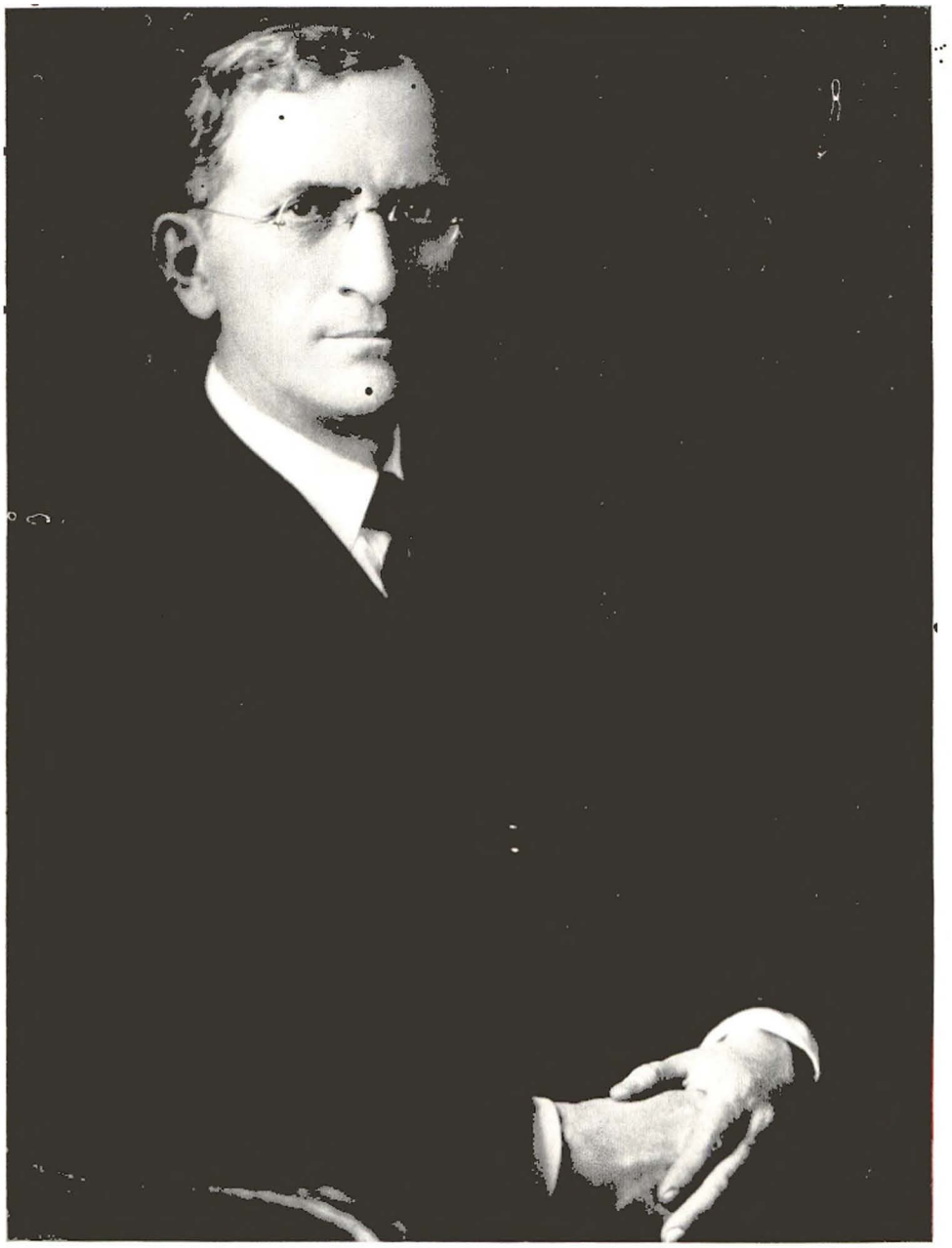
(1874 – 1929)



Sincerely
Jos. Goldberger

JOSEPH GOLDBERGER

(July 16, 1874 – January 17, 1929)

Joseph Goldberger was one of the great men of medicine. He is known especially to nutritionists for his work on pellagra. This work, however, constitutes only one phase of a brilliant career in medical research. His life was that of a completely unselfish man of the keenest intellect, driven by a determination to lessen suffering and premature death.

He was tall and well-built, with a sharp nose, wavy hair, and piercing brown eyes that looked straight through you from his rimless glasses with an inquiring and tolerant expression. To enter into an argument with him on a medical subject was an unforgettable experience. He was intolerant of expressions of opinion unless supported by evidence, while his own arguments were presented in logical sequence and based on proof. He thought and argued in much the same manner in which his experiments were planned and his reports written.

His papers present little in the way of theories or philosophy. As a rule, they usually gave briefly a reason for the particular experiment, considerable experimental detail, and a conclusion based on the evidence, in other words, cause and effect. One must look at the entire series of his writings on pellagra to see how his logical mind went from point to point in the massing of evidence that pellagra was not an infectious disease, that it was a dietary deficiency disease, was preventable by an unidentified dietary factor (which he named the P. P., or pellagra-preventive, factor), the distribution of this factor in many foods, its properties and that it probably belonged to the class of substances called vitamins. At the time of his death, he and I were engaged in attempts to further the characterization of the factor, to make concentrates

of it and possibly to isolate it. Altogether, his studies were reported in about 45 papers on pellagra published from 1914 to 1930. (The three 1930 papers were published after his death.) This series of papers constitutes the transition in our knowledge of pellagra from a disease of unknown etiology and epidemic proportions to an understanding of its cause and methods for its control.

Joseph Goldberger was born in Giralt, Hungary, on July 16th, 1874. He was brought to this country as a boy of seven by his parents, who opened a grocery store on Pett Street in New York City. His boyhood was spent in the crowded tenement area of New York. He had three brothers and three sisters. It was an economic struggle for Joseph's father, Samuel, to send him to college, but he began his college work toward an engineering degree at New York City College in 1890.

After two years in engineering school, where he stood high in his class, he changed his mind about his career and in 1892 entered Bellevue Hospital Medical College. He graduated with honors as one of the two top men in his class in the summer of 1895. He stood first in his intern examination and therefore had his choice of services at Bellevue. He elected the medical service with a view to research opportunities.

After his year of internship, ending in October 1896, he was appointed house physician for a period of six months. Following a few unsuccessful months as a general practitioner in New York City, he moved to Wilkes-Barre, Pennsylvania. It was there that his first medical paper was written in 1898, on the use of saline in shock from injuries.

Immediately after the beginning of the Spanish-American War in 1898, Goldberger applied for a commission as a medical officer in the Navy, but his application was rejected. Since his practice in Wilkes-Barre was something less than a financial success, he took the examination for a commission in the U. S. Public Health Service. In July 1899 he was notified that he stood first in the class.

His career with the Public Health Service began with a detail to the immigration station at Ellis Island, New York. He went from there to another quarantine station, and in 1902 requested that he be sent to Tampico, Mexico, in order to study yellow fever. In the course of his studies there, he contracted the disease.

Following his recovery, he went to Puerto Rico and then back to Mexico. During these years he acquired an extensive knowledge of yellow fever and mosquitoes. His work with yellow fever, his knowledge, enthusiasm for research, and his ability had so impressed M. J. Rosenau, Director of the Hygienic Laboratory (now the National Institutes of Health), that Goldberger was invited to join the small staff. There, in the Division of Zoology in association with Charles W. Stiles, he became engrossed in work on parasites. When the last epidemic of yellow fever broke out in the United States in 1905, Goldberger was sent to Mississippi to check upon suspected cases, and on his return he and Rosenau went to New Orleans and conducted further laboratory studies on the nature of the yellow fever infectious agent.

He fell in love with Mary Farrar, a New Orleans debutante and daughter of a prominent family. This led to family opposition on both sides. Joseph's orthodox parents objected as violently as Mary's socially prominent family in New Orleans. But in spite of these difficulties, they were married in the Farrar home in New Orleans on April 19th, 1906. They moved to Washington, where he resumed his research work at the Hygienic Laboratory.

His next research problem was concerned with typhoid fever in the District of Columbia and the pollution of the Potomac River. Typhoid was epidemic in Washington in 1906, although a new sand filter system had been installed in the water supply the year before. This was a serious health problem, and his meticulous survey of the entire Potomac led him to Cumberland, Maryland, and Staunton, Virginia, and gave him additional experience in medical field survey work. This was to be invaluable later in his pellagra studies, and at the

same time demonstrates the infinite care and detail with which he and Rosenau worked under great public pressure.

In 1907 he was sent to investigate an outbreak of dengue fever in Brownsville, Texas, with George McCoy, an authority on dengue. They both contracted the disease. The paper they wrote on their observations is one of the outstanding reports in this field. In 1915 McCoy became Director of the Hygienic Laboratory, and the association with Goldberger continued for the rest of his life.

Goldberger returned to his work with Stiles on parasites, and in the summer of 1909, at Woods Hole, he became acquainted with Ricketts, the great authority on typhus fever. In the fall of 1909, an outbreak of typhus in Mexico City resulted in his setting up a laboratory there in conjunction with John F. Anderson, where they succeeded in showing that typhus and Rocky Mountain spotted fever were two different diseases. They produced typhus in monkeys and were on the trail of the body louse as the transmitting agent when Nicolle's publication preceded theirs. Ricketts and Wilder were also working on this problem at that time and the three groups independently and almost simultaneously suspected the body louse. Following a bite by one of the lice, Goldberger contracted typhus in January 1910. Within a few weeks of this, Ricketts also contracted typhus and died.

In 1911 Goldberger and Anderson were able to prove that "Brill's disease," which had been found in New York City, was identical with typhus fever. During this same period (1910–1911), Goldberger was also doing some of the work fundamental to our understanding of measles. He and Anderson transmitted measles to monkeys, showed that it was caused by a filterable virus, and that infection was transmitted from nasal and throat secretions and not from skin scales.

In the fall of 1913 Goldberger was assigned to help with a problem of epidemic diphtheria in Detroit. He contributed importantly to our knowledge of diphtheria carriers. It was while on this detail that the Surgeon General, early in 1914, asked him to undertake an investigation of pellagra. This

problem occupied the rest of his life, and all prior study and experience were prologue. Having become an eminent research bacteriologist with wide experience in a variety of epidemic diseases, he was now assigned to study a new epidemic, believed to be infectious in nature. His training, experience, and logical mind soon led him to the conclusion that pellagra could not be an infectious disease.

Let us look for a moment at pellagra as Goldberger saw it in 1914. Endemic pellagra was first reported in the United States by Searcy in 1907. By 1909 it was recognized in 26 states, and the many thousands of cases and deaths alarmed health authorities.

In 1916 it was the second cause of death in South Carolina. Soon after its recognition, the American disease was found to be identical with the Italian disease, and an extensive review of the literature was made. The long history of the disease (from 1753) and the conclusion that it was caused by an unknown infectious agent, transmitted in an unknown manner, gave little reason for hope of solving the problem.

Goldberger tackled pellagra in characteristic fashion. He concerned himself with neither the literature nor current theories: he went out and observed the circumstances under which the cases were occurring. His first trip carried him, among other places, to the Methodist Orphan Asylum at Jackson, Mississippi, where pellagra afflicted 68 of 211 children. It is interesting to read the first paper Goldberger ever wrote on pellagra ("Etiology of Pellagra. The Significance of Certain Epidemiological Observations with Respect Thereto." Pub. Health Rep., 29: 1683, June 26th, 1914). Here, he calls attention to the fact that the employees of institutions never contracted the disease. He discusses this as follows: "To the writer this peculiar exemption or immunity is inexplicable on the assumption that pellagra is communicable." He also states, "The explanation of the peculiar exemption under discussion will be found in the opinion of the writer in a difference in the diet of the two groups of residents."

The fact that Goldberger himself had contracted yellow fever, dengue and typhus in the course of his research work must have forcefully impressed upon him the importance of this observation.

He then proceeded in his careful, methodical way to study the children in the Methodist orphanage, to observe differences in their living conditions. The one thing that impressed him most was the difference in the food supply. The little ones got milk (only 2 cases of pellagra among 25 children under 6 years of age). The older children were able to obtain food to supplement their diet (one case in 66 over 12 years of age). But the children of the middle group received no milk and were not old enough to add to their food (65 cases in 120). Examination of the diet of this group showed it to consist of biscuits, hominy grits, corn meal mush, sirup, gravy and fat pork, with little or no milk, meat or eggs.

This was the type of diet found wherever pellagra occurred in the United States. There was no pellagra in the Army, Navy or Philippine Scouts.

Goldberger proposed to test his idea by improving the diet principally by the addition of meat and milk for two years at both the Baptist and Methodist orphanges in Jackson. At the same time a similar study was started at the Milledgeville State Hospital at Milledgeville, Georgia. Within a few weeks, the cases recovered; no new cases occurred; and in 1915 there was only one case of pellagra in the orphanages. At the asylum, there were 32 cases of pellagra used as controls, with no change in diet, and 72 cases were given the improved diet. All of the latter recovered, while 15 of the controls had recurrences.

He now felt that the disease was dietary and that he could prevent it. To clinch the proof, however, he should be able to produce the disease in healthy men by use of the deficient diet. With the approval of Governor Earl Brewer of Mississippi, 12 convicts volunteered to submit to the test in return for pardons. As a control group, 80 convicts on the prison farm were kept under the same conditions except for diet.

The study was undertaken in the spring of 1915. The 12 convicts received the typical diet of the pellagrous south and the orphanages. Five of the eleven men who completed the study developed pellagra. Experts were called in to confirm the diagnosis, and the convicts were pardoned and released. The results were published under the title "Experimental Pellagra in the Human Subject Brought About by a Restricted Diet," (Pub. Health Rep., 30: 3336, Nov. 12, 1915), and the complete details of the study were later published as Hygienic Laboratory Bull. No. 120, Feb. 1920.

In spite of this study, the long-held theory that pellagra was infectious was so firmly rooted in the minds of many physicians that they were unwilling to accept the evidence. Having shown that pellagra could be prevented and cured by improving the diet, and could be produced by a poor diet, there was still one more experiment to be done. This must prove that the disease could not be transmitted from person to person. All attempts to transmit the disease to animals had failed, but there was still the possibility that man alone was susceptible.

Goldberger now tried to transmit the disease to volunteers — namely, himself, his wife, his good friend and close associate G. A. Wheeler, and several other friends. They injected themselves with blood from pellagrous patients, swabbed secretions from the nose and throat and applied them to their own, and swallowed pills and capsules of skin scales and excreta (Pub. Health Rep., 31: 3159, Nov. 17, 1916. So. Med. Jour., 10: 277, 1917). All of these attempts failed.

Goldberger now turned his attention more fully to the collection of data on pellagra as it occurred throughout the South. He gathered information on diets, incidence, age distribution, seasonal variation — all the evidence obtainable to help him find a pellagra-preventive diet that was practical for the South and its poor mill-village population. He had seen pellagra disappear from every institution that followed his dietary advice. It was clear that he could recommend meat and milk, but some means to provide them would have to be

found or a cheaper preventive discovered. Now he had to try to identify the dietary factor or factors responsible for pellagra prevention. He visited McCollum, Hess, Osborne, Mendel, Graham Lusk, and many others, and read widely in the nutrition literature. Foods were analyzed and attempts made to produce pellagra in experimental animals.

In 1920 he had reached the conclusion that the pellagra-preventive power of milk or meat was due to the correction of a deficiency of (1) amino acids, (2) mineral supply, (3) some unknown vitamin, or (4) some combination of these (Pub. Health Rep., 35: 648, 1920). In 1922 he published an interesting paper entitled "Amino Acid Deficiency Probably the Primary Etiological Factor in Pellagra" (Pub. Health Rep., 37: 462, 1922), in which he states that having excluded minerals and known vitamins, he must refer the role of the diet primarily to the amino acid supply, although the possibility of an unknown dietary essential is not excluded. This paper was published with his associate Tanner, and it is of interest that Tanner wrote him a letter, dated August 5, 1921, presenting the following data:

> The use of tryptophan was begun at the evening meal August 1 and has been continued since. On the third some slight improvement was noticed in the skin condition. Yesterday this improvement was more marked, and today the skin shows a condition which is nearly normal, except for an appearance of tenderness and loss of elasticity. The erythema has almost entirely disappeared from the backs of the hands. The lesions on the feet are no longer acutely erythematous but appear of very nearly normal color, and the especially erythematous patches present the appearance sometimes seen when blebs have healed without bursting. I might add that the improvement in this patient's skin condition has surpassed anything I have ever seen in a case of pellagra in an equal period of time. It might be well for you to see her as early as you find it convenient.

Now it was found that blacktongue in dogs was the analogue of human pellagra. The first paper on this subject points out the similarity to the Chittenden-Underhill syndrome in dogs described in 1917 (Pub. Health Rep., 37: 1063, May 5, 1922).

In 1925 Goldberger and Tanner found that dried brewer's yeast would prevent pellagra with little "cooperation" from the protein in the diet, and they now turned to the P-P factor

as the essential in the prevention of pellagra (Pub. Health Rep., 40: 54, 1925). He now began studies with rats as well as dogs, and almost simultaneously with Smith and Hendricks, observed that what was then known as vitamin B really consisted of at least two distinct factors — one heat-sensitive and the other heat-resistant, the heat-resistant factor being indistinguishable from the pellagra preventive. Having found that autoclaving yeast would destroy the antineuritic factor but not the P-P factor, he made a concentrate by adsorbing from the yeast with fuller's earth. He observed what he reported as "A Note on an Experimental Pellagra — Like Condition in the Albino Rat" (Pub. Health Rep., 41: 1025, 1926). He was careful not to assume that this was an analogue of human pellagra, although the rats had red, swollen and scaly paws. This condition was later shown to result from pyridoxine deficiency.

Now his work concentrated on testing all available, cheap foods for their pellagra-preventive value. One paper in 1928, the last year of his life, reports on 16 foodstuffs. This study was interrupted by the Mississippi flood of 1927, when probably 50,000 cases of pellagra occurred. Goldberger was able to recommend dried yeast and canned salmon to the Red Cross and other relief agencies for the control of the disease. The search for foods rich in pellagra-preventive value continued at full speed, with each test designed to yield as much information as possible about the factor. He was well aware that pellagra was a problem of poverty and that only improvement in economic conditions would eradicate the disease with knowledge available at that time, but he could see the great economic and social advantages to be gained if the cycle could be broken and the disease prevented long enough for the stronger, healthier people to help themselves by improving their own food supply.

Goldberger had many devoted assistants during his pellagra work — Waring, Willets, Tanner, Wheeler, Sydenstricker, to name a few of the closest. They all contributed wholeheartedly in one of the greatest pieces of medical re-

search this country has ever seen. Twenty-five years later, one is still unable to see the end of the far-reaching results of their contributions. It is interesting to speculate on what the prosperous, growing southern United States would be today if Goldberger's work had not shown the way to eradicate pellagra.

In the fall of 1928 he fell ill. Even as he weakened he still kept in touch with the laboratory work. We had begun to observe in our dogs a previously unseen condition, in which they suddenly died with a very yellow liver. He frequently discussed these dogs, which were later shown to have a riboflavin deficiency. Goldberger steadily declined to his death from a rapidly spreading carcinoma on January 17, 1929. His ashes were scattered on the waters of the Potomac in a simple ceremony on the afternoon of January 18, 1929.

An interesting and complete biography of Goldberger was published under the title *The Trail to Light* by Robert P. Parsons (Bobbs-Merrill Co.: Indianapolis and New York, 1943).

W. H. Sebrell

THE USE OF RADIOACTIVE PHOSPHORUS IN THE ASSAY OF VITAMIN D

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THREE FIGURES

(Received for publication August 2, 1951)

Numerous attempts have been made in recent years to improve the assay of vitamin D. Many of these methods have been chemical or physical in nature, but have not gained wide acceptance because of the serious limitation of questionable specificity. The inherent difficulties in vitamin D determination by physico-chemical or biological means have been reviewed by Bills ('47). Bourdillon et al. ('31) have estimated that nearly one-third of the total error in a vitamin D biological assay is due to the errors in assessing the various degrees of healing.

Biological methods, because of the specific nature of the responses, have been preferred, and considerable effort has been expended in attempts to improve the precision of the technique. One such approach has been described by Shue, Friedman and Tolle ('52) who used photographic methods for determining the extent and area of calcification in the line test, thus minimizing the customary subjective nature of this assay. Another approach, one which seemed to offer several advantages, was the description by Snyder, Eisner and Steenbock ('51) of the determination of vitamin D with radiophosphorus. This method, which measures the amount of radioactive phosphorus incorporated into bone as a function

of the amount of vitamin D administered, appeared to have the elements of an assay suitable for routine use. This paper describes our experience with the radiophosphorus procedure over a period of 18 months in which commercial multivitamin preparations containing vitamin D were tested. In addition, comparisons of the radiophosphorus and the U.S.P. "line test" methods were carried out simultaneously in several of these assays.

EXPERIMENTAL

Preparation and handling of the rats

Twenty-one-day-old rats, of the Sprague-Dawley Strain, 45 to 60 gm in weight, were used in these studies. This strain gave dose-responses which were considerably steeper and less variable than those obtained with another commercial strain. The question of strain variation should be examined if animals of other strains are used. On the day of receipt of the animals, the rats were randomized (Bliss, '52) and fed the following modified U.S.P. No. 2 vitamin D-deficient diet: Yellow corn, 76%; wheat gluten, 20%; calcium carbonate, 3%; sodium chloride, 1%; lysine monohydrochloride, 0.5%; and the following vitamins in milligrams per kilo of diet: Thiamine hydrochloride, 2; riboflavin, 4; niacin, 10; and calcium pantothenate, 4. Supplementing the diet with lysine and the vitamins listed reduced the time required for development of rickets by approximately 30%. The animals were fed this diet ad libitum during the 16-day depletion period. To insure ourselves that rickets developed in this period of time, 4 rats were examined by the line test technique (U. S. Pharmacopeia, Vol. XIV).

After rachitogenesis had been established, the rats were given orally, with a syringe fitted with a blunt edge No. 18 needle, the various vitamin D doses in 0.2 ml of corn oil. Forty-eight hours later each rat received, by intraperitoneal injection, 0.5 ml of physiological saline containing 20 to 25 μc of radioactive phosphorus. This amount of radioactivity is sufficient to give about 3000 counts/minute over the wrist of the rat at the time the experiment is terminated. During the

test period the rats were fed 10 gm of the diet daily for a total of 9 calendar days after the administration of vitamin D.

Determination of radioactivity

Equipment used in the determination of the radioactivity in the wrist of the rats is shown in figure 1. The Geiger-Muller counting tube (window thickness about 2 mg/cm^2) is held in place on an ordinary ring stand with burette clamps.

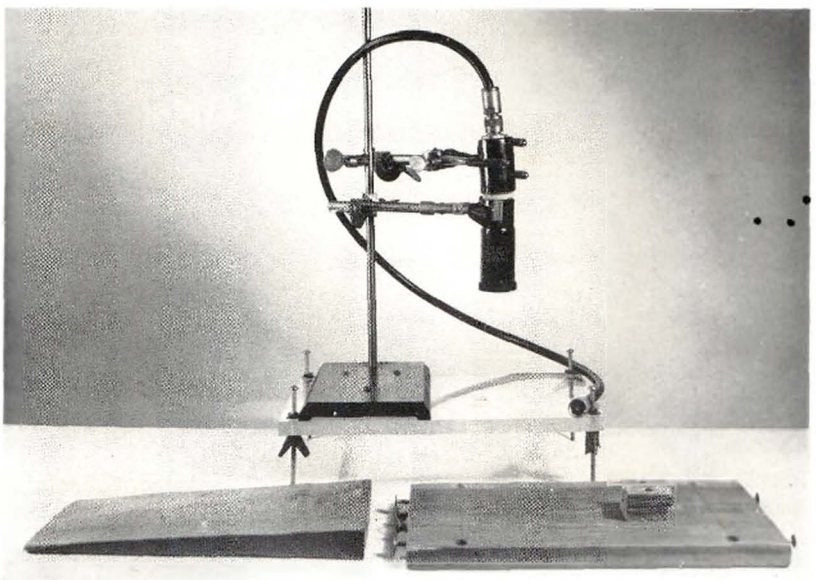

Fig. 1 Apparatus for measurements of radioactivity in the paw of rats.

The ring stand itself is fastened with screws to a piece of lucite 8 × 13 × 9/16 inches. A hole one-half inch in diameter is drilled through the plastic and the Geiger-Muller tube is centered over it. The hole acts as a collimator and eliminates all radiation other than that in the rat's wrist directly under the hole. A lucite thickness of 9/16 inch is sufficient to absorb completely the 1.7 Mev beta particle emitted by P^{32}.

The animal holder consists of a block of wood with a recess one-half inch wide and one-eighth inch deep mounted on a flat piece of board as shown in figure 1. In operation, the

animal, under anesthesia, is placed in position such that the right front paw rests in the recess on the raised block of wood. The paw is then held in position with a piece of cellophane tape; clean new tape is used for each animal. The position of the bone under the tape is then outlined with a red crayon. Care should be taken so that the cellophane tape covers all of the paw which will appear under the collimating hole. The animal is then placed under the tube stand in such a position that the red crayon mark appears in the center of the hole. The animal holder is held firmly against the collimating hole by pushing the wedge-shaped piece of wood under the animal holder far enough to raise two legs of the tube stand off the table. Under these conditions the distance between the Geiger-Muller tube and the wrist of the animal is kept constant. The total number of counts for two minutes is recorded.

U.S.P. line test

The "line test" was made directly on the bones of rats which had received P^{32} and had been assayed by the radioactive method. Separate groups of rats were used in assaying the samples by the "line test" only. At the higher dosages of vitamin D (20 to 48 units) the degree of healing was so great that the readings were indefinite. As experience was gained it was noted that the readings could be extended to a dosage range of three to 14 units.

RESULTS

Fourteen experiments have so far been performed. The results have been uniform in showing a definite log-dose response relationship between the amount of vitamin D injected and the C/M (counts per minute) measured. This relationship is substantially linear and similar from assay to assay as is illustrated by the data in figure 2. The standard deviation of individual readings about the average at any dose is consistently proportional to the average overall count for the assay.

When the bones of the same animals are read by the line test method, curves similar to the radiophosphorus curves are obtained. These results are shown in figure 2. The short dotted lines show the results of a standard U.S.P. line test assay run simultaneously with the radiophosphorus assay.

There is a relationship between the C/M reading obtained in the radiophosphorus assay and line test reading on the

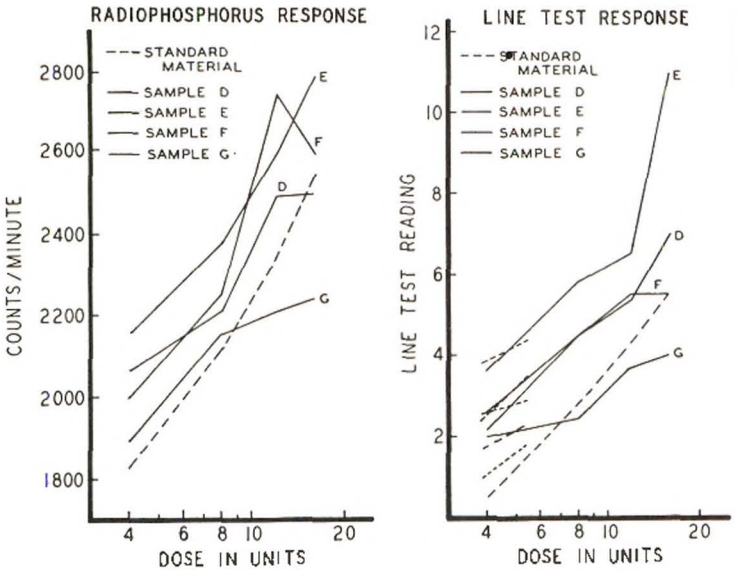

Fig. 2 Comparison of radiophosphorus and line test results. The curves on the right are line test readings on the radiophosphorus-treated animals shown at the left. The short dotted lines in the right figure are line test readings from a concurrent U.S.P. assay.

same animal. This is evident from the scatter diagram at the left in figure 3. The portion of figure 3 on the right shows the relationship between the average reading at each dose level as obtained by the two different methods. These lines connect points referring to the same sample.

Although Snyder et al. ('51) have reported a usable range of activity of 0.5 to 50 units in the radiophosphorus assay, we have limited our use to the range 4 to 16 units. Below two units and above 25 or 30 units less reliable results and a

flatter response curve were obtained. The reasons for this difference between our results and those of Snyder et al. are not apparent, but the differences themselves are of little consequence when compared to the usual rather restricted range of three to 8 units for the line test (fig. 2).

Tests were carried out to study the variability introduced into the results by different operators and by the placement

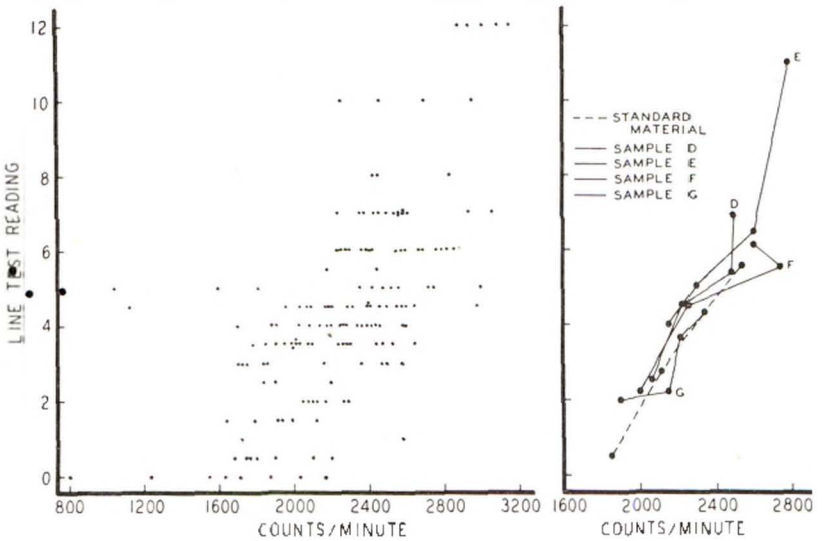

Fig. 3 Each dot in the left chart represents the c/m reading and the corresponding line test reading on an individual rat. In the chart on the right, each point represents an average c/m reading versus the average line test reading for a given dose. The lines connect, for each unknown tested, the points for successive doses of 4, 8, 12 and 16 units.

of the animal. The variability introduced by either of these factors is negligible compared to the inherent animal variability.

When the data of an assay are analyzed to obtain potencies of the unknowns tested, results are obtained similar to those in table 1. In order to obtain the maximum information about the response curve we have used a minimum of 4 dose levels so that a few more rats have been used than is usual in the standard line test assay. This resulted in a lower standard

error of the potency as obtained by the radiophosphorus method than that by the line test method. Preliminary investigations indicate that changing to a three-dose-level assay with the two-fold dose intervals will reduce the number of rats below that commonly used for the line test assays without increasing the standard error significantly above that of the line test assay. Indications are that further economies

TABLE 1

Results of simultaneous line test and radiophosphorus vitamin D assays

ASSAY METHOD	ASSUMED POTENCY	CALCULATED POTENCY	95% CONFIDENCE LIMITS	σ [1] POTENCY EXPRESSED AS % OF POTENCY	σ [1] SLOPE EXPRESSED AS % OF SLOPE	λ [2]
		Sample D				
Radio phosphorus	330 units	427	330–554	14	12	4.6
U.S.P. "line test"	per capsule	496	321–764	24	40	5.0
		Sample E				
Radio phosphorus	267 units	479	344–665	18	14	4.0
U.S.P. "line test"	per capsule	899	214–3773	105	54	3.4
		Sample F				
Radio phosphorus	267 units	394	286–537	17	14	3.8
U.S.P. "line test"	per capsule	443	205–956	47	62	3.1
		Sample G				
Radio phosphorus	384 units	312	228–428	17	15	3.7
U.S.P. "line test"	per gram	289	191–436	24	53	4.2

[1] σ = Standard deviation.
[2] λ = Slope/standard deviation.

in the number of rats injected at each dose level may be possible without a serious raising of the standard error. Higher values of λ are sometimes obtained with the line test method but these are based on values of slopes with much higher standard deviations than the slopes obtained by the radiophosphorus method. Thus the λ value for the line test method cannot itself be considered very reliable.

Agreement in final potencies, as obtained by the two methods, is good with the exception of Material E (table 1) where

the difference between the assumed potency and the actual potency is obviously very large. When a complete replicate of an assay is carried out results are obtained as shown in table 2.

TABLE 2

Comparison of potencies obtained in two different assays

ASSAY METHOD	ASSUMED POTENCY	FIRST ASSAY		SECOND ASSAY	
		Calculated potency	σ [1] Potency expressed as % of Potency	Calculated potency	σ [1] Potency expressed as % of Potency
		Sample H			
Radio phosphorus "Line test" on radiophosphorus rats	1660 units per ml	1689	13	2487	24
		1804	15	2023	16
U.S.P. "line test"		2800	18	2250	31
		Sample I			
Radio phosphorus "Line test" on radiophosphorus rats	1000 units per capsule	639	13	777	22
		668	19	668	19
U.S.P. "line test"		880	10	649	24
		Sample J			
Radio phosphorus "Line test" on radiophosphorus rats	200 units per gram	126	21	155	29
		181	21	204	16
U.S.P. "line test"		204	10	213	13

[1] σ = Standard deviation.

SUMMARY

The validity of the radiophosphorus procedure for vitamin D assay reported by Snyder, Eisner and Steenbock has been confirmed. The method has been compared against the line test in concurrent assays and has given potencies agreeing with those obtained with the U.S.P. standard line test. The radiophosphorus assay has been shown to possess several advantages over the U.S.P. "line test." The prime advantage

NIACIN-TRYPTOPHAN DEFICIENCY RESULTING FROM IMBALANCES IN AMINO ACID DIETS[1]

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The addition of threonine or phenylalanine to low-niacin, 9% casein diets, supplemented with 0.2% L-cystine causes a marked suppression in the growth rate of rats. Diets containing this amount of casein provide marginal levels of the above amino acids as well as of tryptophan. With this in mind, it was suggested (Hankes, Henderson and Elvehjem, '49) that tryptophan is spared for conversion to niacin when the rate of protein synthesis is limited by some other amino acid, such as threonine. However, upon the addition of threonine to the diet, tryptophan assumes the role of the most limiting amino acid and is drawn into protein at the expense of niacin formation, thus causing a deficiency of this vitamin.

If the above explanation is correct, it should be possible to demonstrate this type of growth suppression regardless of the protein source in the diet, if the amino acid levels are properly adjusted. Also, any essential amino acid should be able to assume the role played by threonine in the casein ex-

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[2] The experimental data in this paper are taken from a thesis submitted by O. J. Koeppe in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate College of the University of Illinois. Present address, Department of Biochemistry, University of Minnesota, St. Paul, Minnesota.

periments if its level is varied in a range somewhat below the requirement for optimum growth. Data have been presented which show that imbalances involving threonine in acid-hydrolyzed casein diets and lysine or valine in appropriately supplemented zein diets, will cause the suppression of the rate of rat growth if the diets contain approximately 0.1% of DL-tryptophan (Henderson, Koeppe and Zimmerman, '53). The effects of varying levels of certain essential amino acids upon the growth of rats receiving diets containing only purified amino acids as a protein source have now been determined. Growth inhibition has resulted from raising the levels of threonine, lysine, isoleucine, or leucine, in appropriate purified amino acid diets containing from 0.10 to 0.11% of DL-tryptophan.

EXPERIMENTAL

Male weanling rats were obtained from the Biochemistry Division colony and from the Department of Dairy Science. Animals from these two sources gave comparable results. The care of the animals was similar to that previously reported (Henderson et al., '53). It should be reemphasized that the rats were placed on experimental diets on the day that their weight exceeded 45 gm.

The basal diet consisted of about 18% amino acids, 4% salts IV,[3] 2% niacin-free vitamin mixture (Henderson et al., '53), 5% corn oil, and sucrose to make 100%. The amino acid mixture used was similar to that used in the best purified amino acid diet reported by Ramasarma and co-workers ('49). The tryptophan level was lowered to 0.10 or 0.11% of the DL-form. All of the other essential amino acids, with the exception of the one being studied, were provided at levels equal to or greater than those recommended by Rose (Rose, '37; Womack and Kade, '44; and Rose and Womack, '46) for maximum growth of rats. The complete mixture had the following composition: L-lysine · HCl, 1.24%; L-histidine · HCl · H_2O, 0.54%; L-arginine · HCl, 0.75%; DL-tryptophan, 0.10–0.11%;

[3] Phillips and Hart, '35.

DL-phenylalanine, 0.90%; L-leucine, 0.80%; DL-isoleucine, 1.00%; DL-threonine, 1.00%; DL-methionine, 0.60%; DL-valine, 1.40%; DL-alanine, 1.54%; L-asparagine, 1.57%; L-cystine, 0.26%; glycine, 0.90%; L-glutamic acid, 2.00%; L-proline, 1.15%; DL-serine, 0.64%; and L-tyrosine, 1.80%; to give a total of 18.19% of the diet. In the experiment involving gelatin, the basal diet contained 10% gelatin; salts, vitamins, corn oil, and sucrose as shown above; and 4.74% of an amino acid mixture (footnote 1, table 4). The amino acid mixture for each experiment was ground in a ball mill overnight, then blended with the other ingredients by hand mixing.

RESULTS AND DISCUSSION

Threonine experiments

To show that threonine inhibition was in no way specifically associated with a protein or protein hydrolysate used in the diet, studies were undertaken with diets containing only purified amino acids as the nitrogen source. It did not prove difficult to demonstrate threonine inhibition with this type of diet, since the most desirable tryptophan level (0.1%) has been determined from the results of acid-hydrolyzed casein experiments (Henderson et al., '53). The results in table 1 show clearly that growth is suppressed when the threonine level is raised from 0.6 to 0.8% of the DL-form. The critical level above which inhibition occurs is apparently between 0.6 and 0.7% of DL-threonine, which is biologically equivalent to 0.30 to 0.35% of L-threonine assuming that the DL-form is inactive (Rose, '38). This value is in close agreement with the level of threonine provided by a 9% casein diet (0.34%) which is approximately 70% of the requirement for growth.

Lysine experiments

Table 1 shows data obtained when the lysine concentration was varied in amino acid diets. The results are very similar to those obtained with threonine. Inhibition occurred when

the lysine level was raised above about 0.5%. This is in close agreement with the critical level of lysine as determined by earlier experiments with zein diets (Henderson et al., '53) and is surprisingly low considering the amount required for growth (Rose, '37).

TABLE 1

Effect of varying levels of threonine or lysine on the growth of rats receiving purified amino acid diets containing 0.1% of DL-*tryptophan*

GROUP	NO. OF RATS	AMINO ACID	NIACIN	GROWTH IN 4 WEEKS
		%	*mg %*	*gm*
1	3	0.5% DL-threonine	None	15.3 ± 4.5 [1]
2	8	0.6% DL-threonine	None	29.9 ± 1.5
3	3	0.7% DL-threonine	None	21.0 ± 4.2
4	8	0.8% DL-threonine	None	10.5 ± 1.3
5	6	0.8% DL-threonine	2.5	63.3 ± 3.1
51	3	0.3% L-lysine	None	25.7 ± 4.7 [1]
52	3	0.4% L-lysine	None	29.3 ± 10.9 [2]
53	3	0.5% L-lysine	None	31.3 ± 6.4
54	3	0.6% L-lysine	None	11.7 ± 0.7
55	3	0.6% L-lysine	2.5	55.3 ± 5.9

[1] Standard error of the mean.

[2] One animal was not well and grew only 8 gm in 4 weeks.

Isoleucine experiments

Attempts were made to demonstrate isoleucine inhibition with purified amino acid diets (table 2). In the first experiment, the diets contained 0.10% of DL-tryptophan and varying levels of isoleucine. All of the groups receiving from 0.4 to 0.8% of DL-isoleucine grew very poorly. The addition of niacin to these diets for one week after the end of the regular experimental period indicated that the groups receiving 0.6, 0.7, and 0.8% of DL-isoleucine were definitely suffering from niacin deficiency, whereas the groups receiving 0.4 and 0.5% of DL-isoleucine were not growing, due to a lack of isoleucine. This indicated that the critical level of isoleucine was within the range of the levels being used in this experiment. The group receiving 0.8% of DL-isoleucine and added niacin

throughout the experiment grew well, indicating that the growth of the other groups was not being limited by the absence of some other essential nutrient.

In the next experiment, the level of DL-tryptophan was raised to 0.105%. The results again showed no isoleucine inhibition and were very similar to those obtained with diets containing 0.10%. However, when the level of tryptophan

TABLE 2

Effect of varying levels of isoleucine on the growth of rats receiving purified amino acid diets containing from 0.100 to 0.110% of DL*-tryptophan*

DL-ISOLEUCINE	GROWTH IN 4 WEEKS		
	0.100% DL-trypt.[1]	0.105% DL-trypt.[1]	0.110% DL-trypt.[2]
%	*gm*	*gm*	*gm*
0.4	5.7 ± 0.9		
0.5	6.0 ± 4.0	9.0 ± 0.6	20.7 ± 2.1[3]
0.6	8.7 ± 2.0	7.7 ± 1.2	16.2 ± 3.0
0.7	6.7 ± 1.5	7.3 ± 1.4	12.5 ± 2.3
0.8	9.7 ± 1.5	7.7 ± 4.1	11.3 ± 2.6
0.8 plus 2.5 mg % niacin	55.7 ± 2.6	52.7 ± 1.5	66.7 ± 6.0

[1] Three rats at each level of isoleucine.

[2] Six rats at each level of isoleucine.

[3] Standard error of the mean.

was raised to 0.11%, some isoleucine inhibition was demonstrated above 0.5 or 0.6% of DL-isoleucine.

Leucine experiments

Table 3 shows the results of studies involving leucine in purified amino acid diets. With 0.10% of DL-tryptophan in the diet, growth inhibition appeared above about 0.4% of L-leucine though the results were not striking. It was hoped that more satisfactory results might be obtained by using 0.11% of DL-tryptophan in the diet, since this level proved satisfactory in the demonstration of isoleucine inhibition. However, the figures in table 3 indicate that the results obtained with the two tryptophan levels were very similar.

The leucine and isoleucine experiments both gave somewhat inconsistent results and the "uninhibited" groups did not grow as well as might be expected. It may be said that these results are qualitatively but not quantitatively similar to those obtained with threonine and lysine. No explanation

TABLE 3

Effect of varying levels of leucine on the growth of rats receiving purified amino acid diets containing 0.10 and 0.11% of DL-*tryptophan*

L-LEUCINE	GROWTH IN 4 WEEKS	
	0.10% DL-trypt.[1]	0.11% DL-trypt.[2]
%	*gm*	*gm*
0.3	5.3 ± 0.8	
0.4	17.2 ± 1.7	19.7 ± 3.2[3]
0.5	16.2 ± 4.3	9.0 ± 1.2
0.6	6.8 ± 1.6	5.7 ± 1.5
0.7		9.0 ± 1.5
0.6 plus 2.5 mg % niacin	63.0 ± 2.6	
0.7 plus 2.5 mg % niacin		68.7 ± 3.2

[1] Six rats at each level of leucine.

[2] Three rats at each level of leucine.

[3] Standard error of the mean.

is offered for these differences or for the fact that isoleucine inhibition appears only when the diet contains 0.11% of DL-tryptophan.

Phenylalanine experiments

Four experiments were conducted in an attempt to obtain conditions to demonstrate phenylalanine inhibition of growth with amino acid diets. In all the tyrosine was present at 1.8% of the diet and tryptophan at 0.1%. In each experiment the results were comparable to those obtained in the first isoleucine experiments, i.e., the growth was so poor at all levels

of phenylalanine that the suppression at high levels was not pronounced. The marked growth stimulation from the addition of niacin demonstrated that a deficiency of this vitamin was developing. Further experiments must be completed using 0.11% of tryptophan and lower levels of tyrosine before it can be ascertained whether phenylalanine does or does not fit the generalizations which have been postulated.

Gelatin experiments

The growth suppression resulting from the addition of threonine to sucrose diets is not noted with dextrin-containing diets (Hankes et al., '48). On the other hand, it was noted that the addition of 6% of gelatin did result in niacin deficiency. This suggested that gelatin might contain factors other than threonine which were responsible for the deficiency. It had been noted that glycine, an amino acid present in large amounts in gelatin, would suppress growth when fed at a 2% level (Henderson et al., '47). Lyman and Elvehjem ('51) have obtained evidence that an amino acid mixture simulating 6% of gelatin is as effective as this level of the protein in inducing niacin deficiency with 9% casein-dextrin diets. To obtain further information about the pellagragenic effect of gelatin, this protein was fed at a 10% level with supplements of 0.1% of DL-tryptophan, suitable levels of essential amino acids known to be missing, and varying levels of threonine. It is evident from the results (table 4) that threonine at 0.5% caused growth suppression as with diets containing other proteins. Thus there seems to be nothing unusual about gelatin in this connection.

If the suggested mechanism (Hankes et al., '49; Henderson et al., '53) whereby amino acid imbalance results in niacin deficiency is correct, it might be expected that with diets where tryptophan is the most limiting amino acid, growth would occur as fast as the tryptophan supply would permit until the niacin stores were exhausted. At this time, growth would be arrested and some of the ingested tryptophan should again

become available for synthesis of the vitamin. Examination of the growth curves from some experiments shows that the animals grow at a rate of about 2 gm per day for the first week, then do not grow or may even lose weight during the subsequent three weeks. It is reasonable to assume that pyridine nucleotides are involved in the reactions by which tryptophan gives rise to niacin. This may account for the failure of the animal to resume growth when tryptophan becomes

TABLE 4

Effect of varying levels of threonine on the growth of rats receiving 10% gelatin diets supplemented with 0.1% of DL*-tryptophan and certain other essential amino acids* [1]

GROUP	NO. OF RATS	DL-THREONINE	NIACIN	GROWTH IN 4 WEEKS
		%	*mg %*	*gm*
G-1	3	0.2	None	14.3 ± 2.3 [2]
G-2	3	0.3	None	38.3 ± 2.7
G-3	3	0.4	None	37.3 ± 5.0
G-4	3	0.5	None	14.0 ± 4.6
G-5	3	0.5	2.5	58.7 ± 7.5

[1] The amino acid supplement provided the following proportions of the total diet: 0.47% of L-histidine·HCl·H_2O, 0.62% of L-lysine·HCl, 0.70% of DL-phenylalanine, 0.1% of DL-tryptophan, 0.25% of L-cystine, 0.55% of DL-methionine, 0.45% of L-leucine, 0.70% of DL-isoleucine, and 0.90% of DL-valine.

[2] Standard error of the mean.

available for niacin synthesis as a result of the reduced protein synthesis. It might be expected that the free tryptophan levels in the tissues would be reduced when this amino acid is most limiting. Microbioassays of tryptophan, threonine, leucine, and phenylalanine in tungstic acid filtrates of liver, muscle and plasma were made (Schurr et al., '50) by sacrificing animals at two-day intervals during the development of the deficiency. The levels of all of the amino acids measured, especially tryptophan and threonine, were somewhat lower than those of rats of comparable age being fed stock diet. However, there was no consistent change in concentration with time.

The loss of appetite, which results from the niacin deficiency, may contribute to the failure of most animals to resume growth when tryptophan supplies are no longer depleted by protein formation. In an effort to determine the influence of this factor, studies involving paired feeding were conducted. In these experiments the paired feeding was done on an individual basis, i.e., the food intake was restricted to that of the corresponding animal in the deficient group.

TABLE 5

Threonine growth suppression under paired feeding conditions

GROUP NO.	DIET	MANNER OF FEEDING	GROWTH IN 4 WEEKS[1]			
			Exp. 1	Exp. 2	Exp. 3	Average
			gm	*gm*	*gm*	*gm*
1	9% casein + 0.2% L-cystine	Ad libitum	26.2	23.2	26.4	25.3
2	Same as no. 1	Restricted to consumption of group 3	16.0	19.9	16.0	17.3
3	9% casein + 0.2% L-cystine + 0.1% DL-threonine	Ad libitum	11.6	19.0	11.9	14.2
4	9% casein + 0.2% L-cystine + 0.1% DL-threonine + 2.5 mg % niacin	Restricted to consumption of group 3	24.2	25.2	12.6	20.7
5	Same as 4	Ad libitum	77.8	88.4	92.8	86.3

[1] Five animals per group in each experiment.

The data for three experiments done in this way (table 5) demonstrate that reduced food intake occurs in the deficient group, and that the restriction of the control groups (2 and 4, table 5) decreased the growth rate to a point where the differences readily observed under conditions of ad libitum feeding largely disappeared. The observed lowered food intake as the deficiency progressed is not unexpected and might account for the failure of the animals to recover when protein synthesis is arrested by lack of niacin. Under these conditions, less tryptophan would be required for protein formation,

but also less is ingested so the deficiency is further aggravated so long as tryptophan is more limiting than other essential amino acids.

SUMMARY

It has been demonstrated that rats receiving purified amino acid diets containing 0.10–0.11% of tryptophan develop a niacin deficiency when all of the remaining essential amino acids are provided at levels approaching those required for optimum growth. If the leucine, isoleucine, valine, threonine or lysine content of the diet is reduced to some fraction of the usual requirement, such that the amino acid in question becomes more limiting than tryptophan, growth is improved indicating less severe niacin deficiency. These findings and others reported are in general agreement with the view that tryptophan serves as a source of niacin more effectively when a relative lack of some other essential amino acid is limiting its use for protein formation.

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NON-DELETERIOUS EFFECTS OF POLYOXYETHYLENE ESTERS IN THE NUTRITION OF RATS AND CATS [1]

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INTRODUCTION

Food technology, which has contributed materially to the improvement of food and food products, has frequently developed "unnatural" materials or chemical additives which after trial, test, and experience have been accepted and placed into common use if they contributed nutritional value or technological advantage, or both, and were safe. It is important to differentiate chemical additives on the basis of their effect nutritionally and the advantage they impart technologically.

Compounds, known as non-ionic emulsifiers, developed some time ago, have achieved signal success as additives to bread and other baked goods to improve texture and to assist in retaining softness and taste of the loaf.

Since these materials were added in amounts of less than 0.5% it is evident that they were not used to alter the nutritional value of the product, but to provide a technological advantage.

The chemical of principal interest in terms of bread technology is polyoxyethylene-8-monostearate (Myrj 45) [2] (here-

[1] The authors are indebted to the Atlas Powder Company, Wilmington, Delaware, for a grant in aid to support this work and for the polyoxyethylene compounds used in this study.

[2] Produced by the Atlas Powder Company, Wilmington, Delaware.

after referred to as POE-8-monostearate). This preparation is a partial ester of stearic acid and mixed polyoxyethylene diols. The synthesis of this class of compounds is illustrated as follows:

$$R \cdot COOH + n(CH_2)_2O \rightarrow RCOO\cdot(CH_2 \cdot CH_2O)_n\text{-}CH_2\cdot CH_2OH.$$

In the case of POE-8-monostearate, stearic acid is esterified to a polymer chain containing an average of 8 ethylene oxide units. As prepared commercially, however, one obtains a preparation which is a mixture of free "polyol," monoester, and diester; also polymer esters containing less and more than 8 mols of ethylene oxide are present. As used in bread no further alteration of the molecule is evident as the result of processing.

Although these chemicals are not naturally-occurring substances in foodstuffs, they are readily hydrolyzed in the alimentary tract by lipolytic enzymes to yield the "polyol" and the fatty acid, the latter, of course, being identical with the normal product of fat digestion.

Polyoxyethylene-8-monostearate is a lipophilic, water insoluble ester with distinct surface active properties. Therefore some question properly arises as to the pharmacological effect due to this "surfactant." The question also arises whether fatty acids other than stearic, when esterified with the POE fraction, would influence physiological action. The laurate esters of this series are of particular interest here since lauric acid esters of other types fed at high levels are known to cause undesirable physiological effects, particularly in the absence of other fatty acid esters. Since the "surfactant activity" of POE-8-ester is related to its free OH group which makes the compound more polar, POE-8-diesters were prepared which have no free OH group. Lauric acid, stearic acid, and oleic acid were used to illustrate the different types of fatty acids.

The present study was designed, therefore, to observe and compare in rats and cats the effects of polyoxyethylene-8-mono- and di-stearates, mono- and di-laurates, and mono- and di-oleates, and the corresponding polyoxyethylene polymer

containing 8 mols of ethylene oxide with respect to (a) growth, (b) food consumption, (c) unfavorable physiological effects, and (d) the histopathology produced after long term feeding.

EXPERIMENTAL

Sprague-Dawley rats were used in this experiment because of previous excellent experience with this strain with respect to uniformity of growth response and general health. Male rats, approximately 21 days of age, were selected since reproduction studies were not planned, and because male rats have a more prolonged and continuous growth; furthermore there is no influence of estrus on growth or food intake.

The rats were housed in individual screen-bottom cages and kept in an air-conditioned room maintained at a temperature of 76 to 78°F. with a relative humidity of 50 to 55%. Fresh water was provided daily and the animals were fed ad libitum. The animals were weighed at regular intervals and examined daily when food or water was given. Food consumption data were obtained for periods of two to 5 days throughout the experimental period. When the rats had attained nearly constant levels of body weight so that food consumption data could better reflect caloric maintenance requirements, each rat was placed in a metabolism cage for several periods of two and three days and food consumption and fecal output were measured. After collection, the feces were dried at 100°C. for 24 hours before weighing. Food spillage, which was at a minimum, was recorded and deducted from the total to obtain net food intake.

The basal diets used were of the so-called "synthetic type," the composition of which is given in table 1. It will be noted that diets C-1 and C-2, used for the rats, were alike except that C-2 contained 6% more lard than diet C-1. The caloric values therefore of diets C-1 and C-2 were 4.61 and 4.91 Cal./gm respectively. Diet C-3 was a high-fat diet, designed and used with considerable success in this laboratory for the growth of cats. This diet has the high caloric density of

5.3 Cal./gm and is appropriately supplemented with high levels of vitamins.

As indicated before, this experiment was intended to observe the "surfactant" effects of several polyoxyethylene fatty acid esters and diesters, each compound tested having approximately the same chain length and content of polyoxyethylene polymer, i.e., 8 mols of ethylene oxide in poly-

TABLE 1

Composition of basal diets fed (a) to rats alone (controls) and supplemented with test polyoxyethylene esters (experimental) as indicated in table 2, and (b) to cats as shown in table 7

COMPONENT	BASAL DIETS		
	Fed to rats		Fed to cats
	C-1	C-2	C-3
	%	%	%
Crude casein (B_3F)[1]	20.0	20.0	30.0
Vitaminized casein[2]	5.0	5.0	10.0
Vitaminized corn oil[3]	5.0	5.0	5.0
Lard	12.0	18.0	25.0
Amijel[4]	51.9	45.9	...
Sucrose	...	...	21.9
Salts IV[5]	4.0	4.0	5.0
Ruffex	2.0	2.0	...
Choline chloride·HCl	0.1	0.1	0.1
Whole liver powder	...	...	3.0
	100.0	100.0	100.0

[1] B_3F feeding grade casein — Borden Company, 350 Madison Ave., New York, N. Y.

[2] B complex vitamins and vitamin K were mixed with B_3F grade casein so that a level of 5% supplies each kilogram of diet (C-1 and C-2)[3] with the following quantities: thiamine·HCl 5 mg, riboflavin 5 mg, pyridoxine·HCl 5 mg, menadione 5 mg, niacin 50 mg, calcium pantothenate 50 mg, biotin 0.5 mg, folic acid 0.5 mg, inositol 100 mg, and *p*-aminobenzoic acid 100 mg. (Diet C-3 contains twice the above levels of vitamins.)

[3] Abbott's Halibut liver oil, Drisdol, and α-tocopherol acetate were added to corn oil in such amounts that a 5% quantity of corn oil added to the diet supplied 30,000 I.U. of vitamin A, 100 μg of D_2, and 100 mg of α-tocopherol acetate per kilogram of diet.

[4] A grade of dextrinized starch prepared by the Corn Products Refining Co., New York, N. Y.

[5] Salt mixture of Hegsted et al., J. Biol. Chem., *138:* 459, 1941, modified to contain cobalt ($CoCl_2 \cdot 6H_2O$, 0.6 gm).

meric form esterified to 1 or 2 mols of different fatty acids. The compounds studied were polyoxyethylene mono- and di-stearate, polyoxyethylene mono- and di-oleate, and polyoxyethylene mono- and di-laurate. The above noted POE mono- and di-esters were added at a level of 6% to diet C-1 at the expense of lard.

During the course of this experiment, considerable increased interest was evidenced concerning POE monostearate (Myrj 45). This compound for some time had been used as an emulsifying agent in bread and thus had become the subject of a controversy as to whether or not it should be included in a new Federal Standard for bread. Since more data were desired on the chronic effect of the long term feeding of POE monostearate, several additional groups, with controls, were included in the experimental series to enlarge the scope of the study and the number of animals receiving POE monostearate. These additional groups were as follows: (1) a group fed diet C-1 to which the POE moiety derived from POE monostearate was supplied at a level of 6% in place of lard and its control group II fed diet C-1 and, (2) a group fed diet C-2 which contained POE monostearate at a level of 10% in place of lard, and a corresponding control group III, fed diet C-2.

A summary of the various experimental groups with the number of rats started in each group and the experimental period for each is given in table 2.

Since a considerable experience has been developed in this laboratory on the nutrition of the cat, it was decided to extend the studies on POE monostearate to this animal species. This seemed particularly desirable because of the fact that the cat appears to be more readily susceptible than other species to nutritional inadequacies or influences imposed upon it. This we have learned through unfortunate experiences in developing "artificial diets" for nutritional studies with cats. In this study the cats were placed on the experimental regimen as soon after weaning as possible, but

not until they had been de-wormed and treated with 100,000 units of procaine penicillin given intramuscularly to eliminate any possible infection.

Since the cat seems to thrive on a high fat diet, POE monostearate was incorporated into the diet, C-3, at a level of 20% at the expense of lard. The animals were kept in individual screen-bottom cages of the size commonly used for

TABLE 2

Summary of experimental groups of rats fed various polyoxyethylene (POE) esters

GROUP	DIET USED		NUMBER OF RATS PER GROUP	LENGTH OF EXPERIMENTAL PERIOD
	Basal mixture [1]	Test supplement		
				days
Control I	C-1		9	500
Experimental: E-1	C-1	+ 6% POE-8-monostearate (Myrj 45)	9	500
Experimental: E-5	C-1	+ 6% POE monolaurate	9	507
Experimental: E-6	C-1	+ 6% POE dilaurate	9	505
Experimental: E-7	C-1	+ 6% POE distearate	9	508
Experimental: E-8	C-1	+ 6% POE monooleate	9	513
Experimental: E-9	C-1	+ 6% POE dioleate	9	508
Control II	C-1		6	470
Experimental: E-2	C-1	+ POE moiety	9	492
Control III	C-2		10	367
Experimental: E-3	C-2	+ 10% POE-8-monostearate (Myrj 45)	12	418

[1] For detailed composition of these mixtures see table 1.

rabbits, and housed in the same quarters as the rats, with temperature and humidity controlled. Food and water were given ad libitum. Food consumption was recorded routinely and the cats were weighed at regular intervals of approximately one week.

Any animal that died during the course of the experimental period was autopsied and examined thoroughly for any evidence of gross pathology. Tissues from such animals were not preserved for histological study, however, since the influ-

ence of body decomposition prevents adequate interpretation of the results.

At the termination of each experimental period the animals were killed by the intraperitoneal administration of a lethal dose of nembutal. The spleen, liver, lungs, testes, thyroid, adrenal, kidney, intestine, urinary bladder, heart vessels and heart muscle were removed, weighed where indicated, and placed at once in 10% neutral formalin. The following were weighed in all cases: carcass, liver, kidney, adrenal, testes.

The organs and tissues after fixing were embedded in 58°C. paraffin, cut in serial section, fixed and stained with Harris' hematoxylin according to the method of Putt as used in the Yale University Department of Pathology.

Duplicate slides of serial sections of each organ and tissue were prepared, marked, and coded. One set of the sections was submitted to Dr. John R. McCoy, Rutgers University, who examined them for evidence of pathology. Since identification of each slide was by numerical code only, the experimental group of each animal was unknown to the pathologist.

RESULTS

An examination of table 3 reveals that although the experimental period of most groups extended over 500 days, a substantial portion of the life span of the rat, a high degree of survival was obtained. We have used the term *survival index* in order to give a fairer picture of survival than merely "per cent survived," since a number of animals in each group died just a short time prior to arbitrary termination of the experiment. These results confirm those on rats given in the report by the Food Protection Committee of The Food and Nutrition Board of the National Research Council on "The Safety of Polyoxyethylene Stearates for Use as Intentional Food Additives" ('53).

It is particularly worthy of note (table 3, E-2 under Control II), that the group fed "polyol 8" at a level of 6% in the diet (equal to about 10.8% of POE monostearate) showed no significant difference from the controls, either with re-

spect to survival or growth. Also, group E-3 which was given POE monostearate at a level of 10% actually had a better survival history than the corresponding control group, despite the fact that the experimental period was somewhat longer.

The growth data obtained (table 3) attest conclusively to the excellence of the basal diets and the lack of significant depression or stimulation of growth as the result of adding any of the "polyol" esters or the "polyol" itself. All groups had the usual or normal rapid growth during the first 6 months on the diet and continued to grow, with few exceptions, until the time the experiment was terminated.

Food consumption data and fecal excretion in the various groups of rats (table 4, group E-2 under Control II) revealed that as the result of the lowered caloric density of the diet containing 6% polyol, animals in this group consumed more food per 100 gm of body weight; this was also true of the animals fed the diet containing 6 or 10% POE monostearate (groups E-1 and E-3, table 4). Strangely, no difference of significance could be observed with respect to the other POE esters fed at the 6% level, with the exception of POE dilaurate (group E-6) in which case the food intake in grams/100 gm of body weight was greater than for the controls (Control III). We do not attach any significance to the differences in mean food intakes shown in column 6 of table 4 other than these comments relating to caloric densities of the respective rations.

The organs selected for weighing were the liver, kidneys, adrenals, and testes, because they best indicate physiological and anatomical abnormalities. Marked atrophy or hypertrophy of the liver is quickly evident from the organ weight, just as evidence of gross metabolic abnormality, such as "fatty liver," is apparent as the result of gross inspection. Marked atrophy of the testes is suggested in a group, if the weight of testes is significantly less than the normal weight of kidney. An animal that has been continually exposed to

TABLE 3

Growth and survival of rats fed basal food mixtures alone[1] (controls) or supplemented with test polyoxyethylene esters

EXPERIMENTAL GROUP, DIET FED, AND PERIOD OF FEEDING	RAT NO.	BODY WEIGHT AT START, END, AND AFTER APPROXIMATE TIME INTERVAL INDICATED							PERIOD OF EXPERIMENT TOTAL[2]	SURVIVAL INDEX[3]
		Start	1 Mo.	2 Mo.	4 Mo.	8 Mo.	1 Year	End		
		gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*days*	%
Control I	55	69	206	340	410	475	582	417	492 D	98.4
Diet C-1 only	56	64	...	...	...	...	...	149	17 D	3.4
	57	55	186	310	370	427	487	478	497 D	99.4
Experimental	58	60	204	326	320	362	415	434	500 S	100
period: 500 days	59	76	251	370	380	380	402	512	500 S	100
	60	71	231	384	426	521	590	586	500 S	100
6/24/51–11/5/52	61	64	209	275	300	382	475	512	500 S	100
	62	53	175	308	360	425	525	381	448 D	89.6
	63	65	226	326	400	462	565	596	500 S	100
Group E-1	19	74	...	...	...	...	...	164	17 D	3.4
Diet C-1 + 6%	20	60	195	290	370	425	424	429	500 S	100
POE-8-monostearate	21	67	208	280	310	370	398	356	500 S	100
(Myrj 45)	22	61	225	...	...	...	...	279	57 D	11.4
	23	78	245	342	400	495	522	494	500 S	100
Experimental	24	70	241	360	450	480	535	566	500 S	100
period: 500 days	25	66	174	262	310	360	434	478	500 S	100
6/24/51–11/5/52	26	70	245	358	412	518	578	604	500 S	100
	27	79	259	342	385	450	554	460	390 D	78
Group E-5	1	64	120	220	350	407	420	429	507 S	100
Diet C-1 + 6%	2	48	140	240	330	348	350	333	507 S	100
POE monolaurate	3	74	235	338	410	451	480	476	507 S	100
	4	71	238	366	436	408	444	363	507 S	100
Experimental	5	59	205	300	378	420	465	494	507 S	100
period: 507 days	6	82	...	...	...	...	...	83	4 D	0.8
	7	70	249	362	461	497	540	357	498 D	98.2
6/24/51–11/12/52	8	75	237	336	380	421	465	528	507 S	100
	9	70	218	280	406	371	405	454	507 S	100
Group E-6	10	76	240	314	364	401	425	486	505 S	100
Diet C-1 + 6%	11	70	204	294	372	432	470	460	505 S	100
POE dilaurate	12	54	201	300	351	437	465	322	498 D	98.6
	13	61	201	280	347	396	470	496	505 S	100
Experimental	14	68	226	330	391	400	...	406	306 D	60.6
period: 505 days	15	74	...	...	...	...	...	131	16 D	3.2
	16	60	230	332	406	477	525	583	505 S	100
6/24/51–11/10/52	17	82	284	380	441	452	463	472	401 D	79.4
	18	47	170	297	301	350	468	468	505 S	100
Group E-7	28	69	228	332	394	457	535	537	508 S	100
Diet C-1 + 6%	29	65	202	300	384	454	422	403	508 S	100
POE distearate	30	57	...	...	...	...	...	57	2 D	0.4
	31	73	242	354	424	529	300	348	508 S	100
Experimental	32	70	240	380	391	456	547	534	508 S	100
period: 508 days	33	78	216	322	...	...	...	322	65 D	12.8
	34	63	174	274	347	406	393	260	492 D	96.9
6/24/51–11/13/52	35	76	246	340	407	536	584	573	508 S	100
	36	58	170	304	395	442	482	403	508 S	100
Group E-8	37	63	...	...	...	...	...	102	8 D	0.2
Diet C-1 + 6%	38	78	263	370	447	471	570	528	513 S	100
POE monooleate	39	63	232	344	392	490	540	578	513 S	100
	40	67	210	330	391	452	498	532	513 S	100
Experimental	41	85	223	346	399	482	518	541	513 S	100
period: 513 days	42	76	232	346	407	451	495	535	513 S	100
	43	78	255	370	424	471	432	543	513 S	100
6/24/51–11/13/52	44	78	224	346	397	409	480	370	513 S	100
	45	65	206	306	371	451	572	581	513 S	100
Group E-9	46	68	194	312	371	387	364	328	411 D	80.9
Diet C-1 +6%	47	70	180	264	304	371	445	478	508 S	100
POE dioleate	48	70	224	340	376	440	538	578	508 S	100
	49	71	197	300	375	436	535	508	506 D	99.6
Experimental	50	80	237	380	464	527	585	632	508 S	100
period: 508 days	51	62	221	...	...	...	...	256	36 D	6.9
	52	71	225	330	391	436	483	514	508 S	100
6/24/51–11/13/51	53	75	242	340	406	507	395	375	385 D	75.8
	54	67	207	318	423	456	484	516	308 S	100
Control II	64	69	239	302	460	584	651	650	457 D	97.2
Diet C-1 only	65	82	254	317	461	570	608	590	470 S	100
	66	62	215	328	472	582	642	634	470 S	100
Experimental	67	61	219	330	482	609	216	704	470 S	100
period: 470 days	68	67	215	270	394	431	460	338	467 D	99.4
7/31/51–11/12/52	69	66	214	338	451	545	550	528	461 D	98.1
Group E-2	70	57	188	300	460	579	626	635	492 S	100
Diet C-1 + 6%	71	63	232	310	426	468	476	408	492 S	100
POE moiety	72	61	238	280	409	560	545	538	458 D	93
	73	76	200	248	375	460	530	488	492 S	100
Experimental	74	72	206	380	385	396	535	462	426 D	86.6
period: 492 days	75	71	242	340	482	564	602	605	447 D	90.9
	76	61	185	280	391	514	571	592	492 S	100
7/31/51–12/4/52	77	65	197	279	401	542	626	591	492 S	100
	78	72	214	280	422	540	594	550	492 S	100
Control III	97	76	214	350	354	405	...	390	304 D	82.8
Diet C-2 only	98	93	266	388	460	516	603	603	367 S	100
	99	92	266	347	444	514	613	613	367 S	100
Experimental	100	87	184	316	380	432	482	482	367 S	100
period: 367 days	101	82	256	365	444	470	484	484	367 S	100
	102	56	214	245	...	...	...	227	109 D	29.7
2/14/52–2/16/53	103	96	270	353	...	...	...	351	96 D	93.2
	104	59	244	325	454	479	571	571	367 S	100
	105	77	254	370	474	...	...	408	181 D	49.3
	106	80	242	270	368	350	...	275	259 D	70.6
Group E-3	1A	47	190	322	406	466	507	535	418 S	100
Diet C-2 + 10%	2A	51	198	295	406	472	511	520	418 S	100
POE-8-monostearate	3A	52	210	326	430	495	581	610	418 S	100
(Myrj 45)	4A	52	210	316	380	490	521	530	418 S	100
	5A	54	198	332	390	430	469	495	418 S	100
Experimental	6A	48	193	321	387	439	486	485	418 S	100
period: 418 days	7A	52	206	332	391	450	465	478	418 S	100
	8A	53	188	312	380	453	500	515	418 S	100
5/23/53–7/15/53	9A	48	185	315	386	472	500	527	418 S	100
	10A	52	182	...	...	...	...	250	56 D	13.4
	11A	47	189	295	...	...	...	365	105 D	25.1
	12A	50	200	295	391	450	...	460	280 D	67

[1] For detailed composition of these basal mixtures see table 1.

[2] "D" indicates the animal died; "S" indicates it was sacrificed.

[3] Survival Index = $\frac{\text{Actual survival (days)}}{\text{Experimental period (days)}} \times 100$.

TABLE 4

Food consumption and stool weights of rats fed basal food mixtures[1] (a) alone (controls) and (b) supplemented with test polyoxyethylene (POE) esters (experimental groups)

GROUP AND DIET USED	NUMBER OF RATS	COLLECTION PERIOD	RAT BODY WEIGHT		FOOD INTAKE		STOOL WEIGHT PER RAT PER DAY
			Start	End	Per rat per day	Per 100 gm body weight	
		days	*gm*	*gm*	*gm*	*gm*	*gm*
Control I: C-1 only	8	4	433	433	12.2	2.81	0.93
E-1: C-1 + 6% POE-8-monostearate	7	2	428	440	15.2	3.50	1.00
E-5: C-1 + 6% POE monolaurate	8	3	429	430	11.2	2.60	1.00
E-6: C-1 + 6% POE dilaurate	8	2	416	420	13.8	3.29	0.80
E-7: C-1 + 6% POE distearate	7	4	471	473	13.3	2.82	0.94
E-8: C-1 + 6% POE monooleate	8	4	469	466	12.6	2.69	0.90
E-9: C-1 + 6% POE dioleate	8	4	447	447	11.5	2.58	0.82
Control II: C-1 only	6	4	565	569	16.6	2.93	1.07
E-2: C-1 + 5% POE moiety	8	4	508	519	21.4	4.18	1.59
Control III: C-2 only	7	6	458	446	11.5	2.54	0.71
E-3: C-1 + 10% POE-8-monostearate[2]	9	2	495	495	15.5	3.13	...
E-3: C-1 + 10% POE-8-monostearate[2]	9	2	519	519	16.1	3.09	...

[1] For detailed composition of these basal diets see table 1.

[2] With these 9 rats there were two separate periods during which body weights and food intakes were measured. They were 75 days apart. The differences were not significant. Stools were not collected.

stress, either metabolic or physical, reflects this through hypertrophy of the adrenal, just as lack of adrenal function results in adrenal atrophy. An inspection of table 5 will make it clear that there is no significant deviation from the normal weights of organs between controls and experimental groups. In view of the fact that there were 6 instances of adrenal weights in excess of 60 mg, some renal hypertrophy might be inferred. It might be noted in passing, that with respect to adrenal weights and kidney: testes weight ratios, the group of rats receiving 10% POE monostearate in their diet were most consistently in the "normal range." No significance is attached to this, however. Despite the fact that the diets used contained an appreciable amount of fat and were fed for long periods of time, the livers at autopsy were firm, non-friable, and normal in color, showing no gross evidence of "fatty livers."

Of special interest to us were the experimental results obtained with cats. Although POE monostearate is quite unpalatable, a level of 20% was used in the diet fed the cats in order to observe its effects at abnormally high concentrations. Despite this high level, the cats ate the experimental ration quite satisfactorily. The growth results obtained on both the control and experimental diets were good and equal to those obtained in previous experience with cats in this laboratory (table 6). These animals, both control and experimental, were in excellent physical condition when brought to autopsy.

When one calculates from the data in table 7 the caloric intake per kilo of body weight of cat, estimating the caloric value of the experimental diet to be about 4 cal./gm compared to 5.3 cal./gm for the control (diet C-3), one finds that the caloric intakes per kilo of body weight were quite similar (60.8 for experimental animals vs. 66.6 for the controls).

Since little information is available in the literature on the organ weights of adult healthy cats raised under laboratory conditions, a basis of comparison is difficult. It is quite

TABLE 5

Weights of the body and of various organs of rats fed basal food mixtures alone (controls) or supplemented with test polyoxyethylene (POE) esters (experimental)

EXPERIMENTAL GROUP, DIET FED, AND PERIOD OF FEEDING	RAT NO.	WEIGHT				
		Body	Liver	Kidneys	Adrenals	Testes
		gm	*gm*	*gm*	*mg*	*gm*
Control I	58	434	12.9	3.30	45.4	2.65
Diet C-1 only	59	512	12.7	3.00	33.2	3.49
	60	586	18.7	3.96	49.0	3.22
Experimental	61	512	13.1	3.00	43.8	3.21
period: 500 days	63	596	21.1	3.46	54.6	3.40
Group E-1	20	429	13.4	3.53	38.8	2.90
Diet C-1 + 6%	21	356	15.6	2.78	44.0	2.80
POE-8-monostearate	23	494	20.2	3.60	41.2	3.27
(Myrj 45)	24	566	16.7	2.93	50.6	2.97
Experimental	25	478	18.9	2.46	33.0	2.26
period: 500 days	26	604	20.0	3.60	37.0	2.58
Group E-5	2	333	10.5	2.0	59.2	3.06
Diet C-1 + 6%	3	476	11.3	2.51	52.6	3.22
POE monolaurate	4	363	10.9	2.24	48.2	2.89
	5	494	13.9	2.52	57.2	3.13
Experimental	8	528	14.5	2.84	41.9	3.47
period: 507 days	9	454	12.5	2.56	60.4	2.83
Group E-6	10	486	12.0	2.36	43.8	3.28
Diet C-1 + 6%	11	460	15.8	3.17	45.2	3.11
POE dilaurate	13	496	14.2	2.64	47.2	2.93
Experimental	16	583	15.6	4.57	59.8	2.84
period: 505 days	18	468	18.6	3.51	48.6	3.27
Group E-7	28	537	12.3	2.95	44.8	4.40
Diet C-1 + 6%	29	403	12.8	2.36	67.4	2.79
POE distearate	31	348	11.3	2.16	42.3	2.95
	32	534	13.4	2.41	59.7	2.99
Experimental	35	573	9.5	2.08	64.2	3.23
period: 508 days	36	403	14.7	2.42	59.0	2.16
Group E-8	38	528	12.4	2.25	50.0	2.27
Diet C-1 + 6%	39	578	16.9	2.61	45.8	2.76
POE monooleate	40	532	16.3	2.52	53.0	2.79
	41	541	13.3	2.40	62.5	2.67

TABLE 5 *(continued)*

EXPERIMENTAL GROUP, DIET FED, AND PERIOD OF FEEDING	RAT NO.	WEIGHT				
		Body	Liver	Kidneys	Adrenals	Testes
		gm.	*gm.*	*gm.*	*mg.*	*gm.*
Experimental	42	535	12.4	2.13	46.2	2.53
period: 513 days	43	543	13.3	2.32	59.0	3.06
	44	370	7.6	1.98	48.8	2.29
	45	581	11.6	2.64	55.2	2.79
Group E-9	47	478	12.1	1.90	52.2	2.42
Diet C-1 + 6%	48	578	14.9	2.54	51.6	3.07
POE dioleate	50	632	26.0	3.58	57.4	3.38
Experimental	52	514	13.2	2.97	44.2	3.37
period: 508 days	54	516	21.6	3.01	52.4	3.20
Control II	65	590	18.1	3.88	49.2	7.68 [2]
Diet C-1 only	66	634	19.9	4.27	59.6	4.40
Experimental period: 470 days	67	704	28.1	4.38	56.4	3.15 [3]
Group E-2	70	635	13.8	2.94	34.8	4.10
Diet C-1 + 6%	71	408	14.3	3.71	55.6	3.64
POE moiety	73	488	15.4	3.53	54.4	3.30
	76	592	14.6	2.55	46.4	3.99
Experimental	77	591	13.9	3.33	51.8	5.07
period: 492 days	78	550	12.3	3.28	62.4	3.03
Control III	98	603	18.2	3.15	40.0	3.40
Diet C-2 only	99	613	18.9	3.44	42.0	3.20
	100	482	14.9	2.28	57.4	3.27
Experimental	101	484	15.6	3.32	86.8	2.41
period: 367 days	104	571	14.6	2.57	50.0	3.18
Group E-3	1A	535	14.8	3.19	46.2	3.30
Diet C-2 + 10%	2A	520	14.6	3.12	43.4	3.20
POE-8-monostearate (Myrj 45)	3A	610	15.6	3.35	47.9	3.51
	4A	530	13.8	3.46	43.4	3.20
Experimental	5A	495	15.3	3.10	49.4	3.21
period: 418 days	6A	485	13.6	3.17	38.1	3.20
	7A	478	12.8	2.40	39.2	3.20
	8A	515	14.8	3.40	48.0	3.38
	9A	527	13.7	3.30	48.0	3.40

[1] For detailed composition of these mixtures see table 1.

[2] One testicle very large and bloated.

[3] Testicles showed evidence of hemorrhage.

TABLE 6

Growth response of (a) cats fed a basal food mixture alone (controls) as compared with (b) cats fed the same diet supplemented with 20% polyoxyethylene-8-monostearate (Myrj 45)

GROUP AND DIET	CAT NO. SEX	START OF EXP. Date	START OF EXP. Wt.	WEIGHT AT INTERVALS OF APPROX.: 1 Mo.	2 Mo.	4 Mo.	6 Mo.	8 Mo.	END OF EXP. Date	END OF EXP. Wt.	PERIOD ON EXP. DIET
			gm	*gm*	*gm*	*gm*	*gm*	*gm*		*gm*	*days*
Control IV group	5 ♀	7/8/52	1048	1654	2500	2940	2900	3025	7/10/53	3060	367
Diet C-3 only	6 ♀	7/8/52	1495	2020	2980	2410	2700	2700	7/10/53	2830	367
	7 ♂	7/8/52	1325	1804	3125	....	....	....	11/30/52	3950	145 [1]
	8 ♀	7/8/52	1028	1452	2460	....	....	....	12/2/52	2540	148 [1]
	9 ♀	7/8/52	1095	1430	2100	2280	2400	2370	7/10/53	2210	367
Group E-4	1 ♀	7/8/52	1138	1436	1874	2350	2400	2300	7/10/53	2320	367
Diet C-3 + 20%	2 ♂	7/8/52	1308	1723	2444	3075	....	....	12/1/52	2540	147 [1]
POE-8-mono-	3 ♀	7/8/52	1163	1834	2442	2680	3200	3225	7/10/53	3155	367
stearate	4 ♀	7/8/52	1145	....	....	....	....	....	8/13/52	767	36 [2]
(Myrj 45)	4A ♂	8/20/52	1140	1780	2170	3325	3700	4000	7/10/53	4030	324
	10 ♂	8/20/52	1136	1360	1640	2150	2570	3250	7/10/53	3613	324

[1] Between 11/26/52 and 12/1/52 all the cats in the colony were infected to some degree with viral pneumonia. Therapy with penicillin 100,000 μ/day I.M. and terramycin oral 100 mg, 2 times per day aided materially in the recovery of all cats except no. 2 ♂, 7 ♂, and 8 ♀.

[2] *Cat no. 4 ♀ literally died of inanition since it refused to eat the diet.*

TABLE 7

Food consumption of (a) cats fed a basal diet alone [1] (controls) as compared with (b) cats fed the same diet supplemented with 20% polyoxyethylene-8-monostearate (Myrj 45)

PERIOD OF OBSERVATION		GROUP E-4; DIET C-3 + 20% MYRJ 45						GROUP CONTROL IV; DIET C-3 ONLY			
		Cat no. 1		Cat no. 4A		Cat no. 10		Cat no. 6		Cat no. 9	
Start	No. days	Weight	Food intake	Weight	Food intake	Weight	Food intake	Weight	Food intake	Weight	Food intake
		gm	*gm/day*	*gm*	*gm/day*	*gm*	*gm/day*	*gm*	*gm/day*	*gm*	*gm/day*
3/6/53		2300		4000		3250		2700		2370	
3/13/53	7	2300	35.4	4100	53.1	3275	52.6	2590	40.0	2400	35.0
3/20/53	7	2300	42.6	4100	45.9	3225	47.0	2690	34.6	2310	24.0
3/27/53	7	2280	36.6	4050	46.0	3275	47.1	2600	36.3	2450	27.4
4/10/53		2260		4025		3400		2600		2300	
4/17/53	7	2230	31.1	4125	56.9	3500	60.0	2650	22.9	2330	34.6
4/24/53	7	2200	39.7	4000	58.9	3500	60.9	2530	38.0	2250	18.6
5/1/53	7	2170	30.6	4100	56.3	3500	51.1	2610	30.6	2400	32.9
6/19/53		2260		4100		3700		2600		2300	
6/29/53	7	2320	53.4	4100	55.1	3600	53.4	2660	31.1	2260	30.6

[1] For detailed composition see table 1.

TABLE 8

Body and organ weights of (a) cats fed a basal food mixture alone [1] (controls) and (b) cats fed the basal mixture supplemented with 20% polyoxyethylene-8-monostearate (Myrj 45)

GROUP AND DIET	CAT NO. SEX	WEIGHT						
		Body	Heart	Liver	Spleen	Adrenals	Kidneys	Thyroids
		gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
Control IV group	5 ♀	3060	13.3	59.3	6.8	0.391	17.8	0.258
Diet C-3 only	6 ♀	2830	12.1	43.4	3.4	0.484	22.0	0.249
	9 ♀	2210	8.0	33.5	3.7	0.370	10.6	0.310
	12 ♀ [2]	3069	12.3	73.8	11.0	0.447	24.9	0.388
Group E-4	1 ♀	2320	12.6	61.6	9.4	0.274	16.8	0.126
Diet C-3 + 20%	3 ♀	3155	12.8	74.0	6.9	0.454	25.2	0.450
Polyoxyethylene-8-	4A ♂	4030	13.6	72.2	7.9	0.402	21.8	0.189
monostearate (Myrj 45)	10 ♂	3613	12.8	77.0	10.2	0.364	24.6	0.182

[1] For detailed composition of diet C-3 see table 1.

[2] Cat no. 12 ♀ was an adult when placed on control diet C-3 on 10/20/52 but included in the experimental series in order to supply additional data on organ weights in control animals. This cat received the control diet for 263 days.

evident, however, that there is no material difference between the control and experimental cats as can be seen from the figures presented in table 8.

Gross pathology — general observations — rats

Certain generalizations can be drawn concerning the gross pathology seen in the rats that died during the course of the experiment. Most, if not all, of these animals, both control and experimental, had mild to severe signs of respiratory infection prior to death, with mottled and hemorrhagic lungs at autopsy. Many older animals brought to autopsy shortly before or at the termination of the experiment had cystic spots on the liver due to parasitic invasion. In view of a previous report (Food Protection Committee, '53) of the presence of bladder stones in animals fed non-ionic emulsifiers, the urinary bladder of each animal brought to autopsy was examined for evidence of such pathology. Of all animals studied (100 rats and 11 cats), bladder concretions were found in only two animals as indicated below, one being in the control group (I), the other in experimental group E-9 (POE dioleate).

Incidence of gross and microscopic pathology by groups:

(In the following summaries, the figures indicate the number of animals involved.)

Control I, diet C-1 only —

Gross: hemorrhagic and congested lungs, 4; hypertrophied testes, 1; concretion in bladder, 1; cystic kidney, 2; parasites in liver, 2.

Microscopic: adrenal cortical hyperplasia, 1; chronic interstitial nephritis in kidney, 2; spleen lymphoid hyperplasia, 2; focal parenchymal hepatitis in liver, 1; liver vacuolization, 1.

POE monostearate (Myrj 45), as supplement to diet C-1 (group E-1) —

Gross: hemorrhagic and congested lungs, 3; enlarged thyroid, 1.

Microscopic: chronic interstitial nephritis, 2; spleen lymphoid hyperplasia, 1.

POE monolaurate, as supplement to diet C-1 (group E-5) —

Gross: severe overgrowth of teeth, 1; cyst in liver, 1; congested lung, 2.

Microscopic: numerous hyaline tubule casts in kidney, 1; few hyaline tubule casts, 1; chronic interstitial nephritis, 1; spleen lymphoid hyperplasia, 4; focal parenchymal hepatitis, 2.

POE dilaurate, as 6% supplement to diet C-1 (group E-6) —

Gross: cystic spots on liver, 1; hemorrhagic lungs, 1; large tumor, 1 (fibrosarcoma, Wt. = 128.6 gm).

Microscopic: focal parenchymal hepatitis, 3.

POE distearate, as a 6% supplement to diet C-1 (group E-7) —

Gross: cataract in left eye, 1; severe dermatitis, 2; hemorrhagic kidneys, 1.

Microscopic: few hyaline tubule casts in kidney, 2; spleen lymphoid hyperplasia, 1; liver vacuolization, 1; thyroid-colloid follicular retention, 2.

POE monooleate, as 6% supplement to diet C-1 (group E-8)—

Gross: cyst in left kidney, 1; cyst in liver, 1; hair ball in stomach, 1.

Microscopic: occasional hyaline tubule cast in kidney, 5; focal parenchymal hepatitis, 2; liver vacuolization, 1; thyroid-colloid follicular retention, 1; chronic interstitial nephritis, 4.

POE dioleate, as 6% supplement to diet C-1 (group E-9) —

Gross: cystic spots on kidney, 1; cyst on liver, 1; congested right testis, 1; mottled lung, 1; concretion in bladder, 1.

Microscopic: few hyaline tubule casts in kidney, 2; liver vacuolization, 3; thyroid-colloid follicular retention, 1.

Control II, diet C-1 only —

Gross: hemorrhagic lungs, 2; parasites in liver, 1.

Microscopic: numerous hyaline tubule casts in kidney, 1; spleen lymphoid hyperplasia, 2; focal parenchymal hepatitis, 1; liver vacuolization, 1.

Polyol moiety, as 5% supplement to diet C-1 (group E-2) —

Gross: markedly enlarged heart, 1; congested lungs, 1.

Microscopic: adrenal cortical hyperplasia, 1; numerous hyaline tubule casts in kidney, 2; spleen lymphoid hyperplasia, 6; focal parenchymal hepatitis in liver, 2; liver vacuolization, 1; testes neoplasia, 1.

Control III, diet C-2 only —

Gross: hemorrhagic and congested lungs, 2; cystic spots on liver, 2.

Microscopic: excessive hematoidin deposition, 3; acute bronchopneumonitis, 2; numerous hyaline tubule casts in kidney, 4; chronic interstitial nephritis, 1; colloid follicular retentions in thyroid, 1; proteinaceous sediment, 1.

POE monostearate (Myrj 45), as 10% supplement to diet C-2 (group E-3) —

All the tissues examined from the 9 rats sacrificed in this group were found to be normal except as noted below:

Gross: at the time of autopsy all rats appeared to be in excellent physical condition except for two animals that exhibited signs of respiratory infection which were confirmed at autopsy by the finding of lung congestion.

Microscopic: focal parenchymal hepatitis, 2; eosinophilic perivascular cupping in liver, 4; mononuclear perivascular cupping in liver, 1; spleen reticulum cell hyperplasia, 5; focal chronic interstitial nephritis, 4; glomerular and tubular dilatations, 1; proteinaceous sediment and granular casts in bladder, 2; cystic degeneration of epithelium of bladder, 3; acute pneumonitis, 2; early acute pneumonitis, 1; subendothelial myocarditis, 1.

Gross and microscopic pathology — cats

The cats, when brought to autopsy, were excellent specimens, in fact, some of the best that have ever been raised in this laboratory. On gross inspection no differences could be detected between the control animals and those fed the Myrj 45. Tissue slices were prepared as previously indicated from the following organs: heart, urinary bladder, small intestine, liver, spleen, kidney, gall bladder, adrenal, thyroid, thymus, lungs, heart vessels, and ovary or testes, respectively.

All of the above tissues were judged normal except in the animals noted below.

Controls, diet C-3 only —

Liver perivascular cupping (mixed leucocytic and lymphocytic), 1; spleen reticulum cell hyperplasia, 1; slight chronic interstitial nephritis, 2; colloid follicular retentions in thyroid, 3.

Experimentals, diet C-3 + 20% Myrj 45 (POE monostearate) —

Spleen reticulum cell hyperplasia, 1.

DISCUSSION

Based on the observations made during the course of this study, no reasonable doubt remains in the authors' minds concerning the safety of the polyoxyethylene stearates even when fed in quantities constituting up to 20% of the diet under the conditions of these experiments. Naturally, the nonnutritive character of the "polyol" moiety, and the unpalatability of all of the POE compounds make it impractical to use them in foods for man at any but very low levels even if one wished to do so.

While it is generally unwise to evaluate the response of a human being to a substance and draw sweeping conclusions applicable to man from studies done with experimental animals, it is important to point out that in instances too numerous to mention individually, tests made with experimental animals have proven to be useful guides in human nutrition. Certainly, in the present experiment the test substance was fed at many times the level that might reasonably be encountered in practical human nutrition.

From the report of the Food Protection Committee, Food and Nutrition Board of the National Research Council, it is estimated that on a 600 gm. moisture-free diet, containing substantial amounts of bread and rolls, sweet rolls, doughnuts and ice cream in which are present the amounts commonly used by the baking industry, the consumption of POE stearate (Myrj 45) might be of the order of 0.1% and under very unusual circumstances as much as 0.6%. "For example, on a dry weight basis, a 2% diet for the rat compared with 0.1% for man would represent a safety of 20 to 1." *(p. 4)*. By comparison, in the experiments here reported, Myrj 45 at levels of 6, 10 and 20% represent comparable safety factors of 60, 100 and 200 to 1, respectively. Since no definite value of the safety factor has been arbitrarily established by any official agency, the Food and Drug Administration for example, it is not possible to say whether these values tested in our experiments are to be judged as adequate. In our opinion they are.

Although it might very well be argued that the number of animals in each specific experimental group is relatively small, it should be pointed out that the total number of control and experimental animals is quite large if one combines the several control groups and remembers that after hydrolysis in the alimentary tract each of the POE compounds tested yields the same polyoxyethylene diol with an average chain length of 8 mols of ethylene oxide plus fatty acids which would presumably be handled in the same manner as the mixed fatty acids derived from a natural fat or oil.

It must be remembered also, that Myrj 45 (POE monostearate) is not a pure monostearate, but rather an equilibrium mixture of equimolar concentrations of mono- and di-esters and the corresponding free diols. With this in mind one might well add to the total of rats from other groups the experimental group (E-2) fed the 6% polyoxyethylene moiety derived from Myrj 45.

It is difficult to establish a theoretically complete control for the experimental groups because of the fact that the calorie yield of the various POE compounds tested is less than that of a comparable weight of lard which was replaced gram for gram. Therefore the *exact* balance of nutrients in the control and experimental diets cannot be achieved. This, plus the variable effects of palatability contributes to the difficulty of arriving at a judgment, when comparing mortality and growth data of the control and experimental groups, particularly when no differences are discernible. If there are any true differences they are so slight as to be undetectable even under the extreme conditions of our experiments.

In evaluating the data on gross and micropathology in long-term experiments such as ours, it is difficult to differentiate pathology due to the presence or absence of a specific factor in the diet (the variable of interest) from that due to the normal "attrition of life" over a relatively long period of time. We believe it is very significant that in these experiments one cannot point to any pathology in the experimental groups that was not also found in similar degree in the control groups. In other words, the presence of the test polyoxyethylene ester in the diet exerted no demonstrable influence.

If the POE stearates in the diet exert a harmful effect at the 6% level, then one might expect that feeding at the 10% level would result in a proportionately greater damage. No such differences were observed, however. In fact, the rats that received the 10% level were perhaps the finest group from the standpoint of physical appearance, growth and extent of any pathology.

SUMMARY

Polyoxyethylene mono- and di-stearates, mono- and di-oleates, and mono- and di-laurates were fed to groups of rats at a level of 6% of the diet. Polyoxyethylene-8-monostearate (Myrj 45) was fed to rats and cats at levels of 10% and 20% of the diet, respectively. The polyoxyethylene moiety of Myrj 45 was fed to rats at a dietary level of 6%. These experiments were of the long-term type extending over one and a half years for the rats and over one year for the cats.

At the end of the experiment the animals were autopsied and the spleen, liver, lungs, testes, thyroid, adrenal, kidney, intestine, urinary bladder and heart were removed for preparation of tissue sections and histological examination for possible evidence of pathology.

There were no significant differences between the groups fed the control diets and those given the same diets supplemented with these various polyoxyethylene esters.

It is concluded that under the experimental conditions employed, no reasonable doubt exists concerning the safety of the polyoxyethylene stearates even when fed at levels up to 20% of the diet. The low palatability of these compounds together with the fact that the polyoxyethylene moiety has no nutritive value renders impractical their usefulness at anything but low levels.

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THE NUTRITIVE VALUE OF THE PROTEINS OF BEEF EXTRACTED WITH DIFFERENT SOLVENTS, AND OF EGG, MILK AND WHEAT GERM FOR THE GROWING RAT [1, 2]

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TWO FIGURES

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The effectiveness of particular foods in performing the varied and specific functions of proteins has been investigated extensively, and the feasibility of improving the quality of inferior proteins by supplementation with amino acids or other proteins has been demonstrated. The influence of certain methods of processing also has been examined. Inasmuch as reports concerning the nutritive values of proteins have emanated from many laboratories employing different experimental conditions, it seemed pertinent to evaluate, in a single laboratory by more than one method, certain proteins that are included frequently in the diets of human subjects. Beef, egg, milk and wheat germ were studied, alone and in combination. Criteria were employed that reflect the utilization of nitrogen, namely, growth, nitrogen efficiency, nitrogen balance, and deposition of nitrogen in the liver and carcass. During these investigations, interesting observations were made with respect to the influence of certain solvents upon the nutritive value of beef.

[1] Contribution No. 180, Department of Home Economics, Kansas State Agricultural Experiment Station, Manhattan.

[2] A preliminary report appeared in Federation Proceedings, *12*: 411, 1953.

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EXPERIMENTAL PROCEDURE

Ten male rats of the Sprague-Dawley strain, weighing 50 to 55 gm, were assigned to almost all groups. Nitrogen metabolism was measured during the last 7 days of the 28-day experimental period. Urine was collected on acid-washed filter papers which were transferred daily to a 20% hydrochloric acid solution, and at the end of the balance period, individual solutions and cage washings were removed by suction. Carmine was used as the fecal marker. Feces were placed in 20% hydrochloric acid, heated in a steam bath, sieved, and diluted. The animals were anesthetized with sodium pentothal 24 hours after the balance test terminated. Livers were weighed and treated in the same manner as feces. Carcasses were covered with concentrated hydrochloric acid, allowed to stand for 7 days, and prepared for analysis. Nitrogen was determined by the Arnold-Kjeldahl-Gunning method with mercuric oxide as the catalyst. Gains in nitrogen by livers and carcasses were estimated by subtracting from the final content the initial concentrations which were, respectively, 57 mg and 1.385 gm.

All proteins were fed in the dry fat-free state. Beef was prepared by two methods. For the initial phase of the experiment, raw chuck taken directly from frozen storage was dehydrated and defatted simultaneously with ethylene dichloride by an azeotropic process under the direction of Ezra Levin of the VioBin Corporation of Monticello, Illinois. The procedure, which involved refluxing the meat for three hours with the solvent (Levin and Lerman, '51), was believed to remove fat almost completely without appreciable loss of protein. Responses to beef prepared by this method differed strikingly from those obtained in an earlier investigation (Clark, Wilmeth, Harrison and Vail, '55) in which ether-extracted beef was fed. Therefore, raw chuck was ground and dried before a fan at room temperature. The dried beef then was ground finely and percolated with ether in a Soxhlet apparatus for approximately 8 hours. Whole egg powder

extracted with acetone,[4] freshly prepared nonfat milk powder, and wheat germ dehydrated and defatted with ethylene dichloride were tested also.

As estimated by the Wisconsin Alumni Research Foundation, employing the method of Henderson and Snell ('48), each gram of ash-fat-moisture-free beef contained, in milligrams: histidine 32.8, isoleucine 28.0, leucine 76.6, lysine 120.8, methionine 23.5, cystine 8.8, phenylalanine 58.6, tyrosine 39.5, threonine 39.9, tryptophan 12.0, and valine 60.4. Egg, milk, and wheat germ contained 20.1, 11.7, and 5.8 mg, respectively, of methionine and 18.9, 3.0, and 3.7 mg of cystine per gram.

Each protein was incorporated in a basal low-nitrogen ration which contained 20% of fat from Crisco. In preliminary tests nitrogen balances and gains in weight were almost identical whether 20% Crisco, 20% lard, 10% Crisco plus 10% butterfat, or 10% lard plus 10% butterfat was used in a diet containing 1.8% of nitrogen from beef. The rations contained 2% of roughage,[5] 5% of a mixture of vitamins and dextrin, 5% of salt mixture (Richardson and Hogan, '46), and dextrin to total 100%. Vitamins were supplied in milligrams per kilogram of diet as follows: α-tocopherol acetate 100, ascorbic acid 100, biotin 0.1, choline chloride 1000, folacin 1, inositol 1000, niacin 50, p-aminobenzoic acid 1000, calcium pantothenate 20, pyridoxine hydrochloride 4, riboflavin 6, thiamine hydrochloride 4, and vitamin B_{12} 0.03. Biotin was increased when egg powder was included. One drop of Navitol with Viosterol was administered weekly. All diets were offered ad libitum.

RESULTS AND DISCUSSION

Data pertaining to the response of rats fed the test proteins are presented in table 1. Daily gains in body weight of 5.0 gm or more were induced by ether-extracted beef, egg, or laboratory chow. The diet supplying 2.0% of nitrogen from beef, equivalent to 237 mg of nitrogen per day, permitted almost

[4] By the Seymour Packing Co., of Topeka, Kansas.

[5] Ruffex.

TABLE 1

Average daily gains in weight and nitrogen in a 28-day period, and nitrogen metabolism in 7 days of rats fed beef dehydrated and defatted with ether or ethylene dichloride, eggs, milk, or wheat germ

N IN DIET	DAILY INTAKE			DAILY GAIN IN			N/7 DAYS/100 CM²			AV. BODY WT.[1]
	Nitrogen	Methionine	Cystine	Body wt.	Liver N	Carcass N	In diet	In urine	Balance	
%	mg	mg	mg	gm	mg	mg	mg	mg	mg	gm
Beef extracted with ether										
0.9	57	0.8	0.4	1.0	0.2	31	190	43	106	81
1.2	104	1.5	0.7	2.1	1.4	55	357	96	187	103
1.6	169	2.2	1.0	3.6	4.1	96	553	149	317	143
1.8	203	3.3	1.9	4.3	4.7	119	656	170	407	161
2.0	237	3.1	1.5	5.1	5.0	141	708	174	446	175
2.4	293	4.0	1.9	5.4	5.4	143	797	107	600	187
Ether-extracted beef (1.6% N) plus DL-methionine[2]										
(0)	180	2.5	1.1	3.9	4.1		583	159	326	148
(20)	188	4.8	1.2	4.1	4.3		569	117	367	153
(40)	187	7.1	1.2	4.2	4.0		567	125	350	159
Beef extracted with ethylene dichloride										
0.9	53	0.7	0.4	0	0.2	11	242	113	69	58
1.2	61	0.8	0.4	0.3	0.5	15	311	138	119	62
1.5	92	1.2	0.7	1.0	0.9	31	400	197	138	77
1.8	129	1.6	0.9	1.3	1.4	41	502	269	160	85
2.1	156	1.9	1.1	1.8	2.0	56	592	331	189	99
2.4	181	2.3	1.3	2.0	2.6	63	678	363	243	104
3.0	262	2.9	1.6	2.9	4.0	86	964	564	310	121
Laboratory chow										
3.9	589			5.5	8.1	150	1619	724	531	196
Egg										
0.8	60	0.9	1.1	0.9	0.4		270	51	148	75
1.2	96	1.5	1.8	1.8	1.0		393	64	246	98
1.6	167	2.6	3.1	3.7	3.3		566	107	357	144
2.0	232	3.8	4.4	5.3	5.8		662	130	438	185
2.4	258	4.0	4.7	5.6	7.4		740	151	481	190
Milk										
1.6	148	2.4	0.7	2.6	2.5		540	139	282	121
2.0	203	3.3	1.1	3.8	4.4		673	170	368	151
2.4	254	4.3	1.3	4.8	6.0		835	238	426	171
Wheat germ										
1.6	129	1.1	0.9	1.8	2.2		506	220	188	100
2.0	202	1.7	1.4	3.1	3.8		683	310	263	130

[1] In 7-day balance period.

[2] To provide 0, 20 or 40 mg of nitrogen per 100 gm of diet.

optimal growth. Supplementation of a diet yielding 1.6% of nitrogen with sufficient DL-methionine to provide 20 or 40 mg of additional nitrogen per 100 gm of diet did not improve growth, although the intake of sulfur-containing acids was higher than when 2.0% of nitrogen was available from beef alone. Nitrogen balance was increased significantly, however, by either 20 or 40 mg % of methionine nitrogen and livers were distinctly heavier but not richer in nitrogen. Fortification of a diet yielding 1.2% of nitrogen from beef with 30 mg % of methionine stimulated growth but did not affect the livers. The failure of methionine to accelerate growth contrasts with the report of Hoagland, Ellis, Hankins and Snider ('48) who observed substantial gains in weight when either methionine or cystine was added to the same percentage of nitrogen from beef. The discrepancy in results may be attributable to a sparing effect upon methionine of the higher concentrations of choline and vitamin B_{12} in the present study, to strain differences in methionine requirement (Rutman, '53), or to other experimental variables.

Growth of all rats fed beef treated with ethylene dichloride and providing from 0.3 to 3.3% of nitrogen was unsatisfactory. The greatest daily gain elicited by the commercial sample was 2.9 gm when 3.0% of nitrogen was fed. The rats gained almost twice as much when ingesting the same quantity, 181 mg, of nitrogen from ether-extracted beef as from the ethylene-dichloride-treated material. The regressions of nitrogen balance on intake in response to graded levels of nitrogen from beef extracted with ether or ethylene dichloride are depicted in figure 1. The respective equations were — $\hat{Y} = 0.656\ X - 31$ and $\hat{Y} = 0.327\ X + 3$.

Retardation of growth could not be ascribed to a deficit of known vitamins. When a diet containing 1.8% of nitrogen was fed, growth was neither accelerated nor retarded by offering the vitamin supplement separately, by reducing to one-half or doubling the concentration of all vitamins, or by increasing certain vitamins singly. Growth was depressed

slightly but nitrogen balance was not altered when choline was doubled or tripled. The short soft sparse hair of the rats, their slow growth, and the absence of specific lesions suggested a lack of methionine or cystine. Therefore, the ration supplying 1.8% of nitrogen from ethylene-dichloride-extracted beef was supplemented with DL-methionine or L-cystine or both in quantities to supply from 10 to 60 mg of additional nitrogen per 100 gm of diet. The concentrations of other amino acids in the diets were unchanged. Inclusion

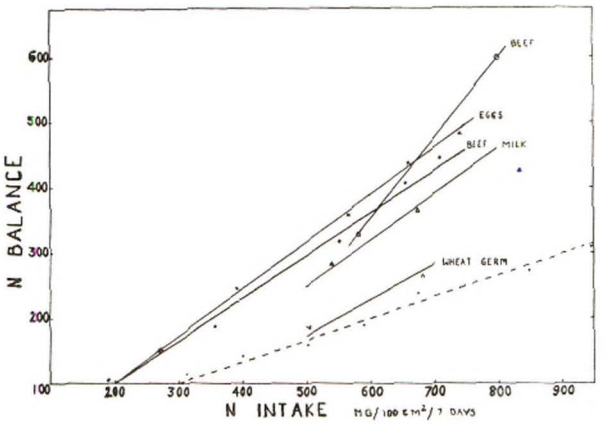

Fig. 1 Regression of nitrogen balance (mg/100 cm²/7 da) on intake when diets containing beef dehydrated and defatted with ether (solid line) or ethylene dichloride (dashed line), egg, milk, or wheat germ were fed to growing rats.

of as little as 10 mg % of nitrogen from methionine distinctly improved growth, nitrogen balance, and storage of nitrogen in the tissues. A gain in weight of 4.0 gm, three times as great as from the unsupplemented diet, was attained with 30 mg % of methionine-nitrogen, and the quantities of nitrogen in livers and carcasses increased. Growth was not accelerated further by larger increments of the amino acid. From analysis of covariance, it was concluded that the change in nitrogen balance with added methionine-nitrogen was quadratic, the optimum supplement being approximately 50 mg of methionine-nitrogen per 100 gm of this diet.

The growth response to 30 mg % of additional nitrogen was the same whether methionine or cystine was fed, although 40 mg of nitrogen from cystine was superior to the same quantity from methionine. Various combinations of the two amino acids were comparable to either methionine or cystine alone.

Supplementation of a diet containing 1.5% of nitrogen with 30 mg % of nitrogen from methionine alone or 40 mg % from a mixture of methionine and cystine was effective. Nitrogen efficiency was doubled, daily gain tripled, and nitrogen retention improved distinctly. A comparable improvement occurred when methionine was added to a diet supplying 2.5% of nitrogen from ethylene-dichloride-extracted beef; and when a diet contributing 3.0% of nitrogen from beef was fortified with 40 mg % of methionine-nitrogen, the daily gain in response to 283 mg of dietary nitrogen was 5.5 gm. Although nitrogen balance was improved by enrichment of the latter diet, it was not superior to that of rats fed 1.8% of nitrogen with a similar supplement. Thus, when supplemented adequately with either methionine or cystine, ethylene-dichloride-extracted beef permitted excellent growth, as rapid as that associated with ether-extracted beef, egg, or laboratory chow.

The findings relative to the influence of the azeotropic method of extraction upon the nutritive value of beef were unexpected since excellent results have been obtained in several laboratories from other animal and plant products prepared with ethylene dichloride by the same procedure. The process has been used successfully with whole eggs (Mitchell and Block, '46), corn germ (Mitchell and Beadles, '44; Beeson et al., '47), coconut and sunflower seed meal (Mitchell, Hamilton and Beadles, '45), and peanut flour (Mitchell, Hamilton and Beadles, '49). Furthermore, wheat germ dehydrated and defatted commercially with this solvent under the same conditions as beef yielded essentially the same results in the present experiment as did wheat germ that was not defatted. That the treatment should affect beef adversely but not eggs is indeed puzzling. Apparently beef

reacts differently than other proteins to a particular procedure for dehydration and defatting.

The original observation concerning the quality of ethylene-dichloride-extracted beef was confirmed with three separate lots of beef. In an attempt to elucidate the problem, (1) a sample of dehydrated beef was divided into two portions which were washed thoroughly with ethylene dichloride or with ether; (2) the concentrations and availability of methionine and cystine were ascertained; and (3) other extractants were tested.

The weight gains and nitrogen balances of rats fed 1.8% of nitrogen from dehydrated beef washed with ethylene dichloride were almost identical with those resulting from treatment with ether. The effects associated with the commercial process therefore could not be attributed entirely to exposure of the meat to ethylene dichloride. Certain dissimilarities existed between the two methods in which ethylene dichloride was employed. For example, beef prepared in our laboratory was dried to a relatively low moisture content before it was exposed to the solvent for approximately one hour, whereas the azeotropic method, in which dehydration and defatting occurred simultaneously, involved refluxing for two or three hours in the presence of approximately one pound of water per two pounds of solvent. The solubility of methionine in water is three times as great at 75° as at 25°C. Temperature was not an important factor, however, since comparable results were obtained with beef extracted commercially at 37 or 75°C. Also, the nutritive value of ether-extracted beef was not altered by being cooked to an internal temperature of 80 or 112°C. before being dried and extracted (Clark et al., '55), nor was availability of the essential amino acids reduced by roasting (Kuiken and Lyman, '48). Autolysis of tissues would be minimized by the azeotropic process and favored by separate dehydration and defatting.

On the basis of ash-fat-moisture-free material, beef prepared commercially with ethylene dichloride contained 21.6 mg of methionine and 10.0 mg of cystine per gram, whereas

the values for two samples of ether-extracted beef were 26.5 and 23.5 mg of methionine, and 12.5 and 8.8 mg of cystine. To determine the relative rates of liberation of the sulfur-containing acids under the action of pancreatin, a sample of beef extracted commercially with ethylene dichloride and one treated with ether were submitted to Dr. B. L. Oser, Director of Food Research Laboratories,[6] for in vitro digestibility studies. The procedure employed was essentially that described by Melnick and Oser ('49). Each sample was treated with the enzyme according to the scheme shown in figure 1 of that paper. The methionine and cystine contents of the resulting hydrolysate were determined microbiologically by the method of Steele, Sauberlich, Reynolds and Baumann ('49), using *Leuconostoc mesenteroides* P-60 as the test organism. From each gram of beef (uncorrected for ash, fat, and moisture) that was treated with ether and with ethylene dichloride, respectively, 17.78 and 20.06 mg of methionine, and 8.3 and 7.6 mg of cystine were liberated. Using these values to represent 100%, the release of microbiologically available methionine after 120 hours of in vitro digestion occurred to a significantly greater extent, 67.7 vs. 32.0%, in beef treated with ether than in that treated with ethylene dichloride. On the other hand, the difference in digestion rates as seen from the liberation of cystine was not striking (55.0 vs. 45.9%) and probably was not significant.

To ascertain the influence of other solvents, the VioBin Corporation generously supplied samples of beef extracted with heptane, propylene, or trichloroethylene. That heptane produced results similar to ether, propylene to ethylene dichloride, and trichloroethylene intermediate between them is apparent from figure 2. Hydration of propylene-treated beef did not improve it, but supplementation with methionine was beneficial. Therefore, a given solvent may exert a deleterious effect upon only a specific product and under certain conditions. Generalizations concerning the influence of any

[6] Long Island City, N. Y.

processing method upon the nutritive value of foods cannot be made safely without experimentation.

It is evident from data in table 1 that the voluntary intakes of nitrogen from beef, egg, and milk were generally similar and greater than from wheat germ, when the same percentage of nitrogen was included in the diet. Probably the mechanisms

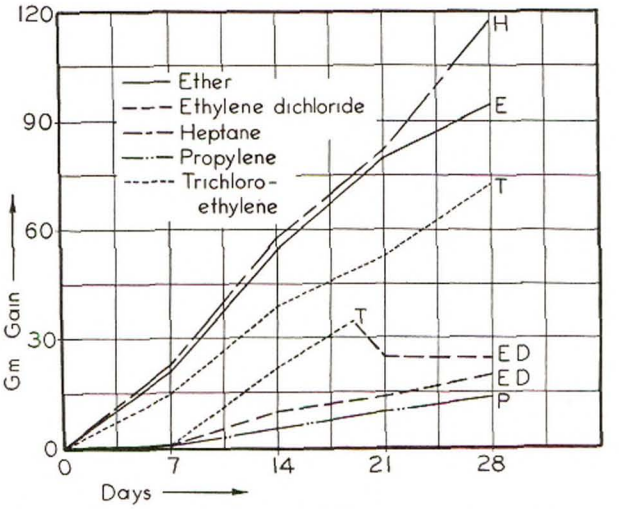

Fig. 2 Gains in body weight of rats fed beef extracted with different solvents.

controlling appetite are sensitive not only to calories (Hegsted and Haffenreffer, '49) but also to the amino acid composition of the diet (Frazier et al., '47) in relation to the requirements of the organism for maintenance and growth.

The regression of daily gain on nitrogen intake was significantly larger ($P < 0.001$) for egg than for any of the other proteins. The regression coefficients for milk, beef, and wheat germ did not differ significantly from each other, using the 5% level as the line. Expressing gain as Y and nitrogen intake as X, $\hat{Y}$ equalled for: egg, $24.48\,X - 498.14$; beef, $19.96\,X + 81.93$; milk, $20.34\,X - 350.84$; and wheat germ, $17.62\,X - 475.47$.

Hoagland and Snider ('46) reported that dried skim milk was equivalent to beef but inferior to eggs in promoting

growth of rats. The deficiency of sulfur-containing amino acids for this species can be overcome by supplementing milk with methionine or cystine (Block, '49; Henry and Kon, '53). When wheat germ supplied 10.0% of protein in the diet, Jones and Widness ('46) concluded that it was equal to skim milk powder, but less efficient than whole egg, and Beeson, Lehrer and Woods ('47) reported a slightly lower protein efficiency ratio for wheat germ than for egg.

Inasmuch as beef (Hoagland et al., '48; Mitchell and Block, '46), milk (Henry and Kon, '53), and wheat germ (Oser, '51) contain suboptimal amounts of the sulfur-containing amino acids for the growing rat, it seemed pertinent to compare the concentrations of these acids in the materials that were tested. Therefore, the quantities of nitrogen supplied by methionine and cystine in the various diets were included in table 1. Methionine contributed approximately 67% of the total nitrogen derived from sulfur-acids in beef, 45% in eggs, 75% in milk, and 55% in wheat germ. Excellent growth resulted when rats consumed daily from beef 4.0 mg of methionine-nitrogen and 1.9 mg of cystine-nitrogen, equivalent to 43 mg of methionine and 16 mg of cystine. The ration providing 1.6% of nitrogen from beef, including 2.5 mg of methionine-nitrogen and 1.1 mg of cystine-nitrogen, induced a gain of 3.9 gm daily. Nevertheless, supplementation of this diet with methionine to increase the total intake of nitrogen as sulfur-acids to 6.0 or even to 8.3 mg per day failed to stimulate growth. The rats ingested approximately the same total amount of methionine and cystine from milk as from beef in the various diets. The gain resulting from 8.2 mg of nitrogen from the sulfur-acids of eggs was approximately the same as when beef contributed either 4.6 or 5.9 mg of nitrogen from this source. Since excellent growth occurred in response to a smaller quantity of nitrogen from methionine and cystine when beef was fed instead of egg, care should be exercised in using egg as a standard of comparison for other proteins.

From an average daily intake of food supplying 293 mg of nitrogen as beef, weanling rats that gained 5.4 gm daily

during a 4-week interval consumed on the average the following quantities of amino acids, in milligrams per day: histidine 60, isoleucine 55, leucine 140, lysine 220, methionine 43, cystine 16, phenylalanine 11, tyrosine 72, threonine 73, tryptophan 22, and valine 110. Undoubtedly some of these quantities were higher than necessary for optimal growth.

A rapidly growing rat not only synthesizes new tissues constantly, but also must reconstruct others simultaneously. Therefore requirements for growth and maintenance are closely interwoven. Nevertheless, evidence concerning total nitrogen balance should be useful in assessing the comparative worth of proteins, since it reflects the overall protein metabolism of the animal. All metabolic data during the 7-day interval (table 1) were expressed in terms of surface area ($12.54 \times W^{0.6}$). Since two lots of ether-extracted beef were fed, two regression equations which differed significantly from each other ($P < 0.001$) were depicted in figure 1, i.e., $\hat{Y} = 0.656\,X - 31.2$ and $\hat{Y} = 1.228\,X - 384.6$. The second lot was fed over a narrow range and the urinary nitrogen of rats fed 2.4% of nitrogen was low. Other equations for regression of nitrogen balance on intake were: for egg, $\hat{Y} = 0.718\,X - 44.0$; for milk, $\hat{Y} = 0.706\,X - 103.1$; and for wheat germ, $\hat{Y} = 0.541\,X - 95.9$. Those for egg and milk were homogeneous and significantly higher than for beef ($P < 0.01$) or wheat germ ($P = 0.06$). Fecal nitrogen was considerably higher when milk was ingested instead of other proteins.

In general, an increment in hepatic nitrogen of 1 mg paralleled a gain in body weight of 1 gm. The animals stored little nitrogen in the livers when 1.2% or less of nitrogen was included in the ration, whereas the livers were large and proportionately high in nitrogen when 2.4% of nitrogen was ingested from egg. The rats that consumed the commercial ration also had large livers with a high concentration of nitrogen.

To obtain additional information concerning the worth of beef proteins as a dietary supplement, rations yielding 1.6% of nitrogen were prepared in which beef contributed 0.4, 0.8, or 1.2% of nitrogen, and egg, milk, or wheat germ provided the remainder. Responses to these mixtures were compared with each other and with those in which each protein supplied 1.6% of nitrogen. In table 2, each protein present in a given ration is designated by the first letter of its name. In certain diets, equal parts of nitrogen from two or more sources were used; in others, one protein supplied 0.4% and another 1.2%, the relationship being expressed by the ratio 1 : 3 or 3 : 1. For example, in diet BW 1 : 3, beef yielded one-fourth (0.4% N) and wheat germ three-fourths (1.2% N) of the total amount of nitrogen.

In table 2, the groups are arrayed in order according to the adjusted nitrogen balances. Growth responses approximated those typical of either beef or egg alone when equal parts of nitrogen were derived from: beef and egg; beef and milk; egg and milk; beef, egg, and milk; or egg and wheat germ. A mixture of 1.2% nitrogen from beef and 0.4% from wheat germ induced a daily gain of 2.8 gm in contrast to 1.8 gm from wheat germ alone. A combination of equal parts of egg and wheat germ was as valuable as egg or beef alone, and twice as useful as the same percentage of nitrogen from wheat germ only. Gain in body weight generally paralleled the quantity of methionine and cystine that was ingested, and gains of 3.5 gm or more were associated with intakes of 3.5 to 4.6 mg daily of nitrogen from sulfur-containing amino acids.

In respect to adjusted nitrogen balance, the experimental groups could be separated into 5 divisions that differed significantly from each other. With 125 degrees of freedom, the least significant difference was 21.5 mg. The mixture containing beef and wheat germ in a 1 : 3 ratio was least efficient, being inferior to one in which each protein yielded 0.8% of nitrogen. All combinations of beef and milk were as satisfactory as if 1.6% of nitrogen were derived from either pro-

TABLE 2

Average daily gains in weight and hepatic nitrogen during 28 days, and nitrogen metabolism in 7 days of rats fed 1.6% of nitrogen from mixtures of beef, egg, milk, and wheat germ

DIET [1]	DAILY INTAKE			DAILY GAIN IN		N/7 DAYS/100 CM²				AV. BODY WT.[2]
	Nitrogen	Methionine	Cystine	Body wt.	Liver N	In diet	In urine	Balance	Adj. balance	
	mg	*mg*	*mg*	*gm*	*mg*	*mg*	*mg*	*mg*	*mg*	*gm*
BW 1:3	157	1.6	1.1	2.5	2.6	532	204	224	249	119
BW	151	1.7	1.0	2.8	3.0	528	181	251	279	125
BM 1:3	159	2.5	0.8	2.9	3.0	578	151	305	291	123
BM	166	2.6	0.9	3.5	2.9	547	153	287	299	142
BM 3:1	173	2.5	1.0	3.4	3.9	557	164	296	301	144
BW 3:1	157	2.0	1.0	3.1	3.1	541	159	291	308	134
BEMW	164	2.2	1.5	3.3	2.9	577	139	342	330	133
BEM	187	2.7	1.8	4.0	4.2	591	119	365	341	150
EW	160	1.9	2.0	3.7	3.9	584	124	365	346	142
BE	170	2.5	2.1	3.7	4.1	567	124	354	350	145
EM	166	2.6	0.9	3.5	2.9	573	153	385	375	142

[1] Designated by letters indicating the protein(s) present in the ration (B, beef; E, egg; M, milk; W, wheat germ). When equal parts were not contributed by each component, the ratio between them is expressed.

[2] In 7-day balance period.

tein alone. Also, a mixture of beef and wheat germ in a 3:1 ratio was equivalent to beef alone. The most favorable balance resulted from the ration containing equal parts of milk and egg. The proteins were ranked differently when adjusted nitrogen balance was used as a criterion instead of gain in weight.

Gains in hepatic nitrogen were related more closely to increase in body weight than to nitrogen balance. When beef and milk contributed equal amounts of nitrogen, the increment was intermediate between the values typical of the individual proteins, whereas the combination of beef, egg, and milk was superior to egg or milk alone and almost equal to beef in increasing the content of nitrogen in the liver.

SUMMARY

The nutritive value of beef for growing rats may be reduced markedly by simultaneous dehydration and defatting with ethylene dichloride or propylene under certain conditions. The availability of methionine was decreased significantly by such treatment. Growth was improved by adding methionine or cystine. This reaction is peculiar to beef since the same procedure has been shown to exert no deleterious effect on several other proteins. From the nutritional standpoint, ether, heptane, and trichloroethylene were satisfactory solvents. The importance of testing the influence of technological processes upon the nutritive value of specific foods was emphasized.

The regression of daily gain in body weight on intake of nitrogen was significantly greater for egg than for beef, milk, or wheat germ, which were essentially the same. Excellent growth resulted from a diet containing 2.4% of nitrogen from beef, from which the rats ingested on the average 43 mg of methionine and 16 mg of cystine daily. Quantities of other essential amino acids also were estimated. The regressions of nitrogen balance on intake per 100 cm^2 of surface area were homogeneous for egg and milk, and significantly higher than for beef or wheat germ. Egg, but not beef, supplemented

milk effectively as reflected by nitrogen retention, whereas either egg or beef improved wheat germ.

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STUDIES ON THE EFFECT OF SODIUM BISULFITE ON THE STABILITY OF VITAMIN E[1]

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Carlson and associates ('49) reported the development of a syndrome in chicks which had been fed a purified diet containing "alpha-protein" (isolated soybean protein). Tremors, partial or complete paralysis, and a type of hemorrhage, which was not typical of vitamin K deficiency, were observed. In later work conducted at this laboratory, the hemorrhage was observed to be accompanied frequently by edema. The edemic symptoms, except possibly for somewhat more hemorrhage, were similar to those of exudative diathesis first described by Dam and Glavind ('38), and reported by these authors ('39) to be caused by vitamin E deficiency.

The diet used in the experimental work of Carlson and associates ('49) contained one milligram of α-tocopherol per 100 grams, a quantity found by Hill, Norris and Heuser ('44) to be sufficient to prevent the development of vitamin E deficiency in chicks fed a purified diet containing casein and gelatin as sources of protein. Subsequently it was learned that "alpha-protein" contains variable amounts of sodium bisulfite as a consequence of manufacturing procedure. Sodium bisulfite is known to split thiamine into biologically inactive components. Thus, the tremors and paralysis may have been caused by thiamine deficiency due to the presence of

[1] The research work reported in this paper was conducted in the nutrition laboratories of the Department of Poultry Husbandry.

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sulfite in the "alpha-protein." However, these symptoms are also characteristic of encephalomalacia, the other form of vitamin E deficiency in chicks. The exudative diathesis appeared, however, to be caused by vitamin E deficiency.

Studies, therefore, were undertaken to determine means of preventing the development of exudative diathesis in chicks fed purified diets containing commercial "alpha-protein" and to ascertain the effect of sodium bisulfite added to a diet containing "alpha-protein" freed of the original bisulfite by washing. The results of these studies are presented in this report.

EXPERIMENTAL

Day-old, Single-Comb White Leghorn male chicks obtained from a local hatchery were used in this investigation. The chicks were identified with numbered wing bands at the time they were placed on experiment. All lots were confined in battery brooders provided with wire screen floors to prevent consumption of feces. Feed and water were provided ad libitum. Each chick was weighed individually at the start of the experiments and weekly thereafter until the experiments were ended.

A purified basal diet containing purified "alpha-protein" was fed in most of the experiments. The composition of this diet is given in table 1. In several of the first experiments commercial "alpha-protein" was used. The composition of the basal diets containing commercial "alpha-protein" was similar to that of the purified basal diet.

The commercial "alpha-protein" was found on analysis to contain from 0.6 to 1.2% of sulfite as sodium bisulfite. The sulfite was reduced to 0.04% or less by washing three times with water for 30 minutes with constant stirring, decanting off the water and drying.

Evidence of destruction of vitamin E by sulfite

In table 2 are presented the results of a number of typical experiments showing that exudative diathesis developed in

chicks fed the basal diet containing commercial "alpha-protein." With one exception, only experiments in which no symptoms of paralysis or tremors were observed are reported because at the time the work was done a clear differentiation had not been made between thiamine deficiency and encephalomalacia. In work with D-α-tocopheryl acetate,

TABLE 1

Composition of basal diet

	gm		mg
Cerelose	64.83	$FeSO_4 \cdot 7H_2O$	54.0
Purified alpha-protein [1]	23.5	$MnSO_4 \cdot 4H_2O$	48.0
Ground cellophane	3.0	KI	3.0
Soybean oil	2.5	$CuSO_4 \cdot 5H_2O$	1.5
Dicalcium phosphate	1.9	$ZnCl_2$	1.0
Ground limestone	1.2	$CoCl_2 \cdot 6H_2O$	0.2
Sodium chloride	0.6	Niacin	5.0
DL-Methionine	0.6	Mixed tocopherols	4.0 [2]
Dibasic potassium phosphate	0.5	Ca pantothenate	2.0
Cod liver oil (3000 A,		Menadione	1.0
400 D per gm)	0.5	Thiamine HCl	1.0
Glycine	0.3	Riboflavin	1.0
Magnesium sulfate	0.25	Pyridoxine HCl	1.0
Choline chloride	0.2	Folic acid	0.2
		Biotin	0.02
		Cobalamin	0.002

[1] The Glidden Co., Chicago.

[2] Composed of at least 2 mg D-α-tocopherol with the remainder chiefly D-γ-tocopherol.

paralytic symptoms were observed but these occurred for the most part in the last week of the experiment and did not appear to have any material effect on growth.

The exudative diathesis was prevented and growth was increased by including 6% of dried brewers' yeast in the diet. Patrick and Morgan ('43) observed that yeast prevented the development of vitamin E deficiency in the chick but found that the yeast was ineffective after extraction with hot Skellysolve B. Purification of the "alpha-protein" and the substitution of starch for the cerelose in the basal diet also prevented the occurrence of exudative diathesis. The latter

treatment, however, failed to promote an increase in growth, presumably because it did not affect the thiamine content of the diet or increase the supply of unidentified growth factors, shown by Miller ('51) to be present in dried brewers' yeast when it was included in the diet containing purified "alpha-protein." The substitution of D-α-tocopheryl acetate for mixed tocopherols likewise prevented the development of exudative diathesis whereas in work not reported herein

TABLE 2

Effect of various dietary treatments in preventing development of exudative diathesis

TREATMENT	AV. WT. AT 4 WKS.	MORTALITY	CASES OF DIATHESIS
	gm	%	%
Basal diet [1]	226 (77) [3]	26.0	58.9
+ 6% dried brewers' yeast	277 (96)	8.6	0.0
Vitamin E acetate [2] substituted for vitamin E alcohol	231 (42)	6.7	0.0
Starch substituted for cerelose	229 (14)	6.7	0.0
Purified "alpha-protein" substituted for commercial "alpha-protein"	259 (15)	0.0	0.0

[1] Similar to basal diet, table 1, except that commercial "alpha-protein" was used in place of purified "alpha-protein."

[2] D-α-Tocopheryl acetate, 2, 4 and 8 mg per 100 gm of diet to 3 lots of chicks.

[3] Numbers in parentheses indicate surviving chicks.

because of the development of thiamine deficiency, increasing the alcohol form from 4 to 10 mg per 100 gm of diet failed to prevent the occurrence of exudative diathesis.

The results of these experiments indicated indirectly that the sulfite in the commercial "alpha-protein" accelerated the destruction of the alcohol form of vitamin E. An experiment, therefore, was undertaken to determine if the observed effects were definitely caused by the presence of sulfite in "alpha-protein" or by the development of a substance by the sulfite destructive of vitamin E, which was removed by purification.

Graded levels of sodium bisulfite were added to the basal diet to ascertain this and also to determine the level of sodium bisulfite to use in future experimental work. The sodium, calcium and chlorine content of all the diets was held constant by means of sodium chloride, calcium chloride and calcium carbonate. This precluded variations in the quantity of these elements from influencing the development of vitamin E deficiency. In order to prevent thiamine deficiency each chick

TABLE 3

Effect of $NaHSO_3$ on incidence of vitamin E deficiency

TREATMENT [1]	AV. WT. AT 6 WKS.	CASES	
		Encephalomalacia	Exudative diathesis
	gm	*no.*	*no.*
Basal diet	401 (14)[2]	0	0
+ 0.1% $NaHSO_3$	342 (10)	5	3
+ 0.2% $NaHSO_3$	209 (1)	10	2
+ 0.3% $NaHSO_3$	... (0)	12	0
+ 0.4% $NaHSO_3$	.. (0)	11	0

[1] Sodium content of all diets, 0.2%.

[2] Numbers in parentheses indicate survivors; 15 chicks per lot at start.

in this and subsequent experiments was injected intramuscularly with 0.25 mg of thiamine twice during the first week and with 0.3 mg once a week thereafter.

The results showed that the inclusion of sodium bisulfite in the basal diet promoted the development of both forms of vitamin E deficiency in the chick, and that as the level of the sulfite was increased the number of cases of encephalomalacia increased and the incidence of exudative diathesis disappeared. This indicated that encephalomalacia is the more acute form of vitamin E deficiency in the chick and exudative diathesis the more chronic form. The encephalomalacia was recognized by the presence of muscular incoordination, head retractions and convulsions and by edema and by histo-

logical changes in the cerebellum compatible with this form of vitamin E deficiency.[4]

In this experiment, the inclusion of sodium bisulfite in the basal diets accelerated the development of oxidative rancidity. The degree of rancidity appeared to be correlated with the quantity of sodium bisulfite supplied. Therefore, in further experimental work, organoleptic studies were made of the development of rancidity in the feed.

TABLE 4

Effect of $NaHCO_3$ and certain other substances on incidence of vitamin E deficiency

TREATMENT [1]	AV. WT. AT 4 WKS.	CASES		RANCIDITY
		Encephalomalacia	Exudative diathesis	
	gm	*no.*	*no.*	
Basal diet	275 (15)[2]	0	0	?
Basal diet + 0.2% $NaHSO_3$	136 (14)	15	0	+++
+ 0.2% $NaHCO_3$	185 (15)	7	7	+++±
+ 0.6% $NaHCO_3$	252 (14)	1	9	+++
+ 2% lecithin	182 (13)	7	6	+++±
+ 0.25% cephalin	235 (15)	1	5	++
+ 0.125% methylene blue	177 (15)	0	0	0
Starch substituted for cerelose + 0.2% $NaHSO_3$	159 (4)	8	4	++++
Casein + gelatin substituted for alpha-protein + 0.2% $NaHSO_3$	116 (11)	9	0	++++

[1] The sodium content of the first 4 diets was 0.24, 0.28, 0.33 and 0.43% respectively, that of the remainder 0.28%.

[2] Numbers in parentheses indicate survivors; 15 chicks per lot at start.

Measures counteracting the destructive effect of sulfite

Since Dam and Glavind ('42), Bird ('43) and Dam ('44) reported that high levels of soluble salts, particularly sodium salts, increased the tendency for exudative diathesis to develop, an experiment was conducted in which sodium bicarbonate was combined with sodium bisulfite, thereby increasing the quantity of sodium in the diet. The results of this experiment are presented in table 4. They confirmed the findings

[4] Autopsies including histological examination of the brains of chicks showing symptoms of encephalomalacia were kindly conducted by Malcolm Peckham, D.V.M., New York State College of Veterinary Medicine.

obtained in the previous experiment that the addition of sodium bisulfite to the basal diet promoted the development of vitamin E deficiency in the chick. Observations on the development of oxidative rancidity in the feed indicated that the degree of rancidity at the end of the experiment was approximately equal in all diets to which sodium bisulfite was added.

The addition of sodium bicarbonate together with the sodium bisulfite appeared to counteract the effect of the sulfite to some extent, with the result that the development of encephalomalacia was prevented for the most part and the incidence of exudative diathesis increased. At the highest level of sodium bicarbonate (0.6%) growth was almost equal to that of the chicks supplied the basal diet in spite of the fact that more than half of the chicks developed symptoms of exudative diathesis. Increasing the sodium content by means of sodium bicarbonate tended, therefore, to prevent the development of the more acute form of vitamin E deficiency and to favor the development of the more chronic form. The results both of this experiment and the preceding one indicate, therefore, that the quantity of vitamin E available at the time the feed is consumed by the chicks determines the type of vitamin E deficiency which develops. They do not support the hypothesis of Dam ('44) that sodium salts favor the development of exudative diathesis because of their propensity to accumulate in the tissues.

The effect of dried brewers' yeast and starch in preventing the development of vitamin E deficiency in chicks fed a basal diet containing commercial "alpha-protein," and the results of Olcott and Mattill ('36a, '36b), Patrick and Morgan ('43) and Swift, Rose and Jamieson ('42) suggested the possibility that the phospholipids in these materials acted as antioxidants. Therefore, a supply of crude soybean lecithin [5] was fractionated according to customary procedures into a lecithin fraction and a cephalin fraction. These fractions were fed to

[5] Supplied through the courtesy of Joseph Eichberg, American Lecithin Company, Woodside, L. I., New York.

chicks in a final experiment to determine if they would aid in preventing vitamin E deficiency. In this experiment methylene blue was also used for this purpose since it had been found previously by Pappenheimer, Goettsch and Jungherr ('39) and by Dam and associates ('51) to be effective.

The results of the experiment are presented in table 4. They showed that the lecithin fraction had a slight effect in preventing the development of vitamin E deficiency. This was made evident both by the better growth and by the fact that fewer acute and more chronic cases of deficiency were observed. The cephalin fraction, however, was much more effective. When it was included in the basal diet, growth was approximately normal and only one case of encephalomalacia and 5 cases of exudative diathesis were observed. The rancidity in the feed was also reduced to some extent. Methylene blue completely prevented the development of vitamin E deficiency in the chicks and rancidity in the feed supply, but the level supplied retarded chick growth materially. This amount thus appeared to be slightly toxic.

Relation of the destructive effect of sulfite to fat

Since, in a work reported by Dam ('44), the omission of fat from the diet or the feeding of low-fat diets appeared to prevent the development of vitamin E deficiency in the chick, an experiment was conducted in which a fat-free basal diet was fed minus mixed tocopherols and sodium bisulfite and with these two substances alone and in combination. The results of this experiment are presented in table 5. Growth was approximately normal in all lots of chicks, but three cases of exudative diathesis were observed in the lot fed the fat-free diet without mixed tocopherols and sodium bisulfite and one case was observed in the lot fed the fat-free diet plus sodium bisulfite and minus mixed tocopherols. None occurred in the lots supplied the fat-free diet with the mixed tocopherols, or with both sodium bisulfite and mixed tocopherols. It appeared from these results, therefore, that the effect of

sodium bisulfite in promoting the destruction of vitamin E was an indirect one, and occurred through its effect in accelerating the development of oxidative rancidity in the fat in the diet.

This was confirmed by supplying diets containing unsaturated fat, hydrogenated fat and rancidified unsaturated fat. The results are also presented in table 5. When the basal diet contained unsaturated fat, sodium bisulfite either with

TABLE 5

Effect of lack, type and condition of fat on incidence of vitamin E deficiency

TREATMENT	AV. WT. AT 5 WKS.	CASES		RANCIDITY
		Encephalomalacia	Exudative diathesis	
	gm	*no.*	*no.*	
No fat	351 (15)[1]	0	3	0
+ vitamin E alcohol	356 (15)	0	0	0
+ 0.2% $NaHSO_3$	342 (15)	0	1	0
+ vit. E alcohol + 0.2% $NaHSO_3$	372 (15)	0	0	0
Unsaturated fat + 0.2% $NaHSO_3$	163 (1)	13	1	++++
+ vitamin E alcohol	... (0)	15	4	+++±
Hydrogenated fat + 0.2% $NaHSO_3$	390 (15)	0	0	0
+ vitamin E alcohol	383 (15)	0	0	0
Rancidified fat + 0.2% $NaHSO_3$	207 (15)	1	3	+++
+ vitamin E alcohol	375 (15)	0	0	++±

[1] Numbers in parentheses indicate surviving chicks; 15 chicks per lot at start.

or without mixed tocopherols caused severe vitamin E deficiency, and the feed supply became very rancid during the course of the experiment. When the unsaturated fat was replaced with hydrogenated fat, the growth of the chicks was normal, vitamin E deficiency was prevented and no rancidity developed in the feed supply either in the diet containing sodium bisulfite or the one containing sodium bisulfite plus mixed tocopherols. When rancidified fat was supplied, growth was subnormal, a few cases of vitamin E deficiency were observed and the odor of rancidity was observed in the feed supply throughout the course of the experiment in the lot given sodium bisulfite. When mixed tocopherols were supplied

together with sodium bisulfite, growth was normal, no vitaman E deficiency developed and the rancid odor of the feed supply was slightly less than that of the feed without mixed tocopherols.

The inclusion of 0.2% of sodium bisulfite in the basal diet produced no direct toxic effects. This was revealed when the sulfite was added to the fat-free diet, with or without mixed tocopherols, and to the experimental diet containing hydrogenated fat, with or without mixed tocopherols. Under these dietary treatments, no mortality occurred and the rate of growth was good.

The use of purified casein and gelatin as the source of dietary protein promoted encephalomalacia to a greater degree than that observed when purified "alpha-protein" was used. Therefore, the catalytic effect of sodium bisulfite on the development of oxidative rancidity in unsaturated fat and in promoting vitamin E deficiency in chicks was not due to a peculiar characteristic of purified "alpha-protein."

The substitution of starch for cerelose in the basal diet containing purified "alpha-protein" plus sodium bisulfite had little effect in preventing the development of vitamin E deficiency in chicks contrary to the results obtained with commercial "alpha-protein." Growth of the chicks fed the starch diet, however, was somewhat better than of those fed the cerelose diet. Moreover, several cases of exudative diathesis were observed in the chicks fed the former diet whereas vitamin E deficiency in the chicks fed the latter diet was limited entirely to encephalomalacia. A probable explanation of the discrepancy is that when starch was substituted for cerelose in the work with commercial "alpha-protein," the effective sulfite content of the protein was less than that supplied by 0.2% sodium bisulfite.

DISCUSSION

The unsaturated fats (soybean oil and cod liver oil), containing linoleic and linolenic acid, included in the basal diet used in this investigation are particularly susceptible to

autoxidation. The results of the experimental work showed that the inclusion of sodium bisulfite in the basal diet increased the rate of oxidation of the unsaturated fats. The effect of the sulfite appeared similar to that observed by Waterman, van Vlodrop and Pfauth ('48) when unsaturated fat is heated to 180 to 220°C. under high sulfur dioxide pressure. This treatment promoted the development of conjugated double bonds in the fat and made it more reactive to oxygen. In the research work described herein, the presence in the basal diet of the salts of iron, manganese and copper, minerals known to catalyze autoxidation, probably made high temperature and high sulfur dioxide pressure unnecessary. The development of conjugated double bonds in fatty acids which display unsaturation of the methylene interrupted type during the incorporation of molecular oxygen has also been demonstrated by Farmer, Koch and Sutton ('43) by means of spectrophotographic measurements.

The initial product formed by the autoxidation of fatty acids which have interrupted methylene unsaturation is a hydroperoxide (Noller, '51). Preceding the formation of this product an intermediate free radical is created by the abstraction of hydrogen. However, as long as the feed supply contains vitamin E alcohol, the free radicals are reconverted to fatty acids by abstraction of hydrogen from the vitamin. During this process, the induction period of fat autoxidation, vitamin E is transformed into the stable tocopheryl quinone which is inactive biologically. At the end of the induction period, active oxygen uptake begins and complete rancidification of the unsaturated fatty acids takes place.

The results obtained in these studies indicated that the catalytic effect obtained from sodium bisulfite was related to the presence of iron, manganese and copper salts in the diet. The change in the symptoms of vitamin E deficiency from those of the acute form to the chronic, less severe form and the increased growth which occurred on adding sodium bicarbonate to the basal diet was probably due to the conversion of the highly soluble sulfates of iron, manganese and

copper into the relatively insoluble carbonates, thus reducing the overall pro-oxidant effect of the diet.

The rapid destruction of the chick's reserves of vitamin E together with the vitamin E in the feed supply was undoubtedly due to the absorption of the more reactive fatty acid molecules from the digestive tract. It seems improbable that this was due to the absorption of sulfite, since Heimberg, Fridovich and Handler ('54) have presented evidence indicating the existence in the liver and kidney of various mammals of an enzyme which catalyzes the oxidation of sulfite to sulfate. Previously, Schmidt and Clark ('22) had shown that sulfite is oxidized to sulfate in vivo.

The effect of cephalin in delaying the destruction of vitamin E and of methylene blue in preventing it, may be due to the fact that these substances under the experimental conditions supplied hydrogen to the unsaturated fatty acids more readily than vitamin E or replaced that lost by vitamin E. Thus, both the fatty acid molecules and that of vitamin E are maintained intact.

Methylene blue, however, is not an unsaturated fat antioxidant when used alone. In order to provide hydrogen for reconverting the fatty acid free radicals to fatty acids, some of the methylene blue in the chick diet would have to be reduced to the leuco form. The possibility that methylene blue destroyed the relationship between sodium bisulfite and the mineral catalysts in the diet by combining with the latter is also suggested by the results obtained in this work with sodium bicarbonate and by the report of Tappel ('54).

Whether or not the increased capillary permeability present in exudative diathesis and encephalomalacia is due to the harmful effect of absorbed fatty acids with conjugated double bonds or to the direct effect of vitamin E deficiency, cannot be determined from the evidence. The increased capillary permeability cannot be caused by the products of rancidification of the unsaturated fatty acids, since it has been found by Dam ('44) that thoroughly rancid fat does not promote the development of vitamin E deficiency in the chick. Also, in this work,

no vitamin E deficiency was observed in chicks fed a diet adequate in vitamin E, but containing fat which had been subjected to considerable rancidification.

The failure to obtain oxidative rancidity in the feed, and vitamin E deficiency in the chicks, when the diet contained hydrogenated fat was obviously due to the fact that oxidation of the fat could not take place to any great extent and thus the presence of sulfite was without effect in increasing the rate of oxidation. It could not be due to the presence of vitamin E in the hydrogenated fat since the quantity used, approximately 3 mg per 100 gm of diet according to Lange ('50), was not sufficient in view of results not reported here which showed that the addition of 12 mg of vitamin E acetate per 100 gm of diet failed to prevent the development of vitamin E deficiency when the diet contained 0.2% of sodium bisulfite.

The results obtained in this work, in agreement with evidence reported by Mattill and Golumbic ('42) and by Dam ('44), showed that actively developing oxidative rancidity in unsaturated fats is directly concerned in the destruction of vitamin E. They also indicated that one of the functions of vitamin E is the maintenance of the integrity of unsaturated fatty acid molecules. Dam and associates ('51) presented evidence of this by showing that in vitamin E deficiency peroxidation of the depot fats occurs in the chick, and Dam and Granados ('52) have proposed that the basic function of vitamin E is to prevent the autoxidation of unsaturated fats.

SUMMARY

Evidence has been obtained in studies with chicks that the inclusion of sodium bisulfite in a purified diet containing iron, manganese and copper salts accelerates the development of oxidative rancidity in unsaturated fat and increases the incidence and severity of vitamin E deficiency. Both encephalomalacia and exudative diathesis were observed. Encephalomalacia appeared to be the more acute form and exudative diathesis the more chronic form of vitamin E deficiency.

The results showed, in agreement with evidence reported by other workers, that actively developing oxidative rancidity in unsaturated fat is directly concerned in the destruction of vitamin E and indicated that one of the functions of the vitaman is the maintenance of the integrity of unsaturated fatty acid molecules.

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IN VITRO DIGESTIBILITY STUDIES ON MODEL PEPTIDES HEATED WITH GLUCOSE[1,2]

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FOUR FIGURES

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In earlier work from this laboratory (Davis, Rizzo and Smith, '49; Mader, Schroeder and Smith, '49) it was shown that dry proteins which had been heated in the presence of reducing sugars showed a decrease in the nutritive value of the protein as determined by growth of rats and by nitrogen balance in dogs. In view of the fact that commercial heat processing as used in the preparation of evaporated milk and milk powder does not diminish the nutritive value of the protein constituents (Schroeder, Iacobellis and Smith, '53), a further study was made of the role of water and of fat in the Maillard reaction (Schroeder, Iacobellis, Lees and Smith, '53); the presence of water prevented the loss of nutritive value of the protein under heat treatment in the presence of sugar, when assayed by the nitrogen balance method with dogs.

Inasmuch as the first studies showed a marked increase in fecal nitrogen in those periods when the heated protein was fed, *in vitro* digestion experiments were carried out with crystalline trypsin and chymotrypsin (Schroeder, Iacobellis, Lees and Smith, '53). These studies demonstrated again the

[1] The data in this paper are taken from the dissertation submitted by Michael Iacobellis for the degree of Doctor of Philosophy, Wayne University, February 1954.

[2] Aided by a grant from the American Dairy Association.

protective action of water and the actual enhancement of nutritive value of the heated proteins, an observation strongly suggested by the early *in vivo* studies.

It is apparent that environmental conditions regulate the type and extent of interaction between amino compounds and carbohydrates. In order to more closely approach an explanation of the mechanism of the alteration of the nutritive value of protein heated with reducing sugars, model peptides were synthesized and the influence of water and pH have been studied (Schroeder, Iacobellis and Smith, '55). Sufficient evidence is at hand to suggest that browning and the Maillard reaction are essentially independent phenomena; whereas browning appears to be due to the effect of pH on carbohydrates, the interaction of carbohydrates with amino compounds, the so-called Maillard reaction, is limited and occurs in alkaline solutions and in the dry state. Recent studies (Iacobellis, '54) have produced evidence that glyconyl peptides are formed in this interaction.

Inasmuch as the results of the *in vitro* digestion experiments upon proteins were so closely parallel to those from the intact animals, similar *in vitro* experiments have been carried out with the same peptide-sugar mixtures used in the chemical studies (Schroeder, Iacobellis and Smith, '55) in order to relate these later more critical studies to the earlier nutritional experiments. The results are the basis of the present report.

EXPERIMENTAL

Glycyl-L-leucine and glycyl-DL-valine were autoclaved at 15 pounds pressure for 30 minutes with glucose, in the dry state, and in the presence of water. All samples (including an unheated peptide-glucose mixture) were weighed out in calibrated NPN tubes. The ratios of the amino compound to glucose were 1:1 and 1:3 on a molar basis. The total volume in those samples heated in an aqueous medium was 25 ml; these were autoclaved unbuffered as well as in phosphate buffer at pH 9.7. All samples after autoclaving were brought

to the 50 ml mark with water and aliquots were taken for the determination of total nitrogen, amino nitrogen and for paper chromatography. Other samples of the autoclaved peptide-glucose mixtures to be used in the digestion experiments were buffered with 0.2 M KH_2PO_4 and the pH adjusted to 7.6 with sodium hydroxide; pancreatin was then added, the volume brought to 50 ml and the mixture incubated at 37°C. with frequent shaking. In these proteolysis experiments a modification of the methods of Lowry and Thiessen ('50) and Melnick and Oser ('49) were used. Aliquot samples were removed at various time intervals for amino nitrogen determination and for paper chromatography which were the criteria used to follow the digestion.

The total nitrogen was determined by the micro Kjeldahl method, while the amino nitrogen was estimated by the method of Pope and Stevens ('39) as modified by Schroeder, Kay and Mills ('50). The chromatographic method used is described in detail in a previous study (Schroeder, Iacobellis and Smith, '55).

The detailed results of the chemical determination for peptides autoclaved with glucose in the dry state and in the presence of water have been reported previously (Schroeder, Iacobellis and Smith, '55); it is apparent that only in those samples of amino compounds which had been autoclaved with glucose in the dry state and in alkaline buffers was there an appreciable decrease in amino nitrogen as well as browning. On the other hand, the amino compounds autoclaved in an unbuffered solution (in which the pH drops progressively with heating) showed browning but no decrease in amino nitrogen. Total nitrogen remained constant in all of the heated mixtures, indicating that no loss of nitrogen had occurred during the heating process.

Preliminary studies of the proteolysis of peptides heated with and without glucose in the dry state and in the presence of water had shown that water had an inhibitory effect on the interaction of amino compounds and carbohydrates. This had been observed in earlier experiments with milk products.

The results of the present *in vitro* digestibility studies are shown in figures 1, 2, 3 and 4. It is evident that the *rate* of amino nitrogen liberation is the same in practically all samples; no difference was observed between the enzyme activity in the peptide-glucose mixtures autoclaved dry and those in an aqueous medium, as may be seen in figures 1 and 3. However, in figures 2 and 4, which show the total *quantity* of amino nitrogen liberated from the samples at different time intervals, one can see that the *in toto* available amino nitrogen present in the dry autoclaved and in the samples autoclaved in an alkaline buffer is lower than that in either the unautoclaved mixtures or in those autoclaved in an unbuffered aqueous medium. Crystalline egg albumin which had been reacted comparably with glucose gave similar results when subjected to proteolysis by trypsin and chymotrypsin.

DISCUSSION

The hypothesis has been previously advanced (Schroeder, Iacobellis and Smith, '55) that browning and the Maillard reaction occur independently of one another, the extent of each being determined by the conditions under which the reactions are allowed to take place.

In the light of the present *in vitro* digestibility studies, a more adequate interpretation of the previous investigations of heated proteins in intact dogs (Schroeder, Iacobellis and Smith, '53; Schroeder, Iacobellis, Lees and Smith, '53) can be made in those instances where an apparent decrease in nutritive value of the protein was observed. This decrease must be attributed to a diminution in the quantity of the substrate available to the proteolytic enzymes, rather than to a decrease in the rate of enzyme activity, an interpretation supported by the observed high fecal nitrogen excretions. Furthermore, it was observed early in these continuing studies (Schroeder, Mader and Smith, '49) that the *in vivo* biological values of the heated proteins were uniformly high. This would signify that whatever amino nitrogen was enzymatically released and absorbed was well utilized. In order, then, to

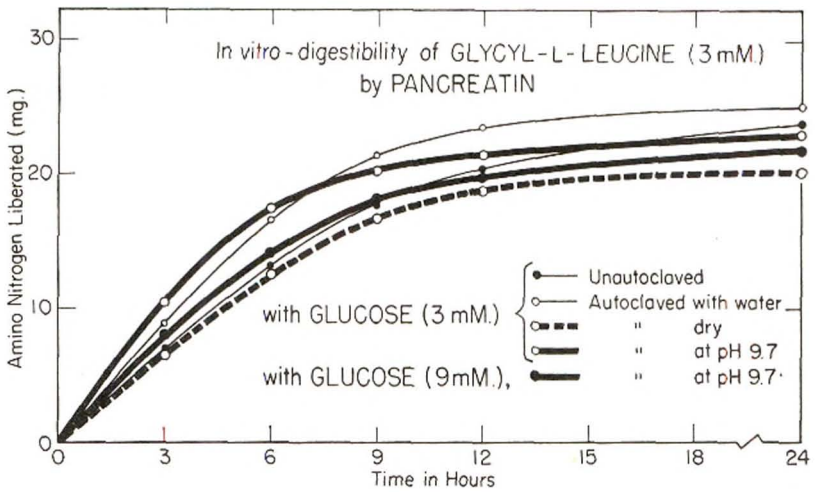

Fig. 1 Liberation of amino nitrogen from glycyl-L-leucine by pancreatin *in vitro*.

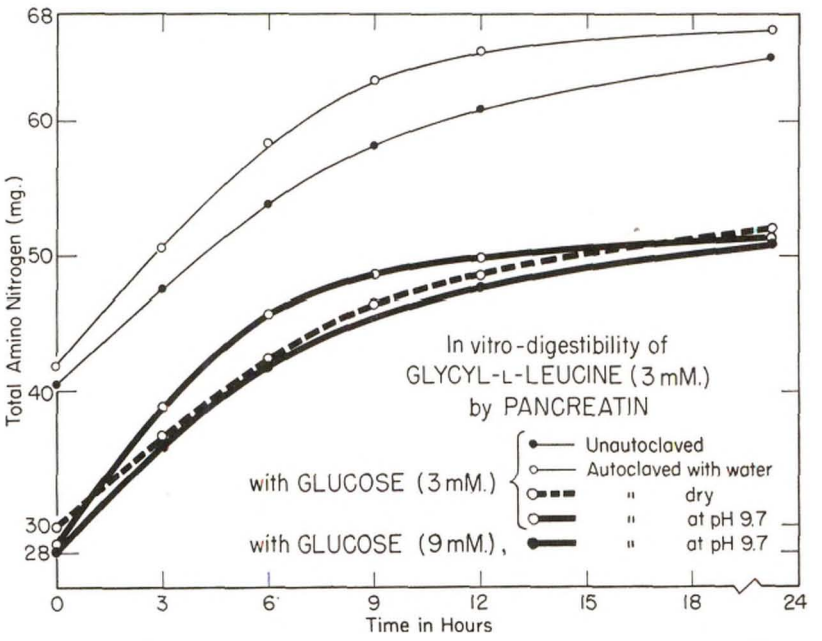

Fig. 2 Total amino nitrogen present during *in vitro* digestion of glycyl-L-leucine by pancreatin.

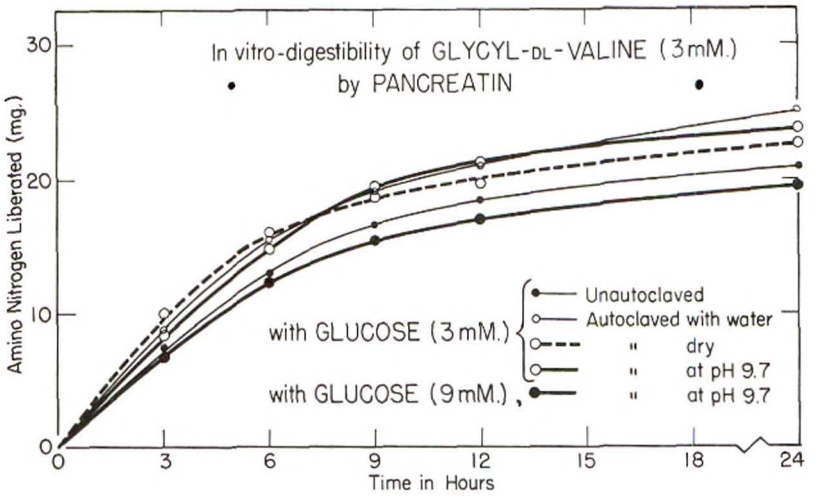

Fig. 3 Liberation of amino nitrogen from glycyl-DL-valine by pancreatin *in vitro*.

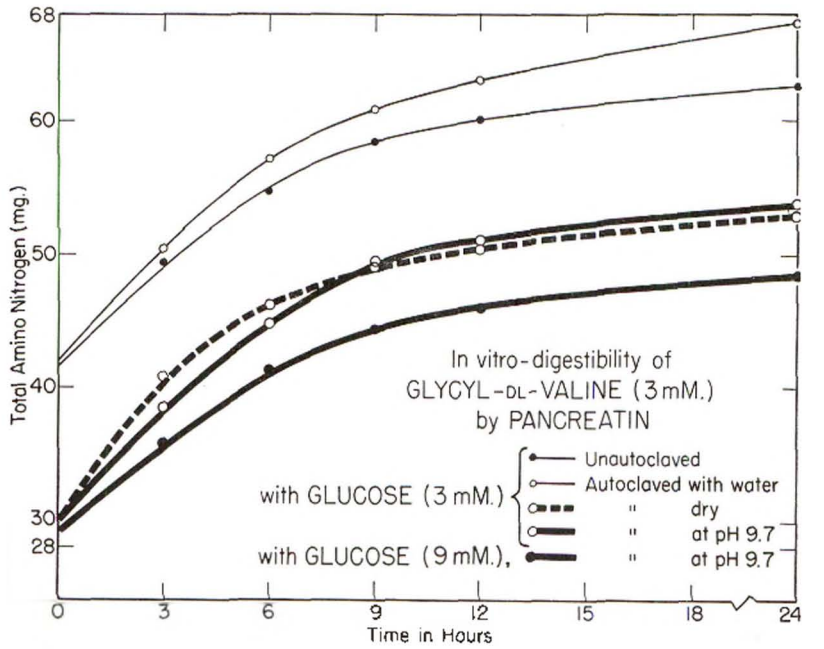

Fig. 4 Total amino nitrogen present during *in vitro* digestion of glycyl-DL-valine by pancreatin.

achieve a nitrogen balance *in vivo,* one must supply an additional amount of digestible protein nitrogen to compensate for that which is rendered unavailable whenever the conditions of the heating permit the interaction between the amino compound and the carbohydrate.

The present *in vitro* digestibility study supplements and supports the findings which previously had been secured through nitrogen balance studies on intact animals, digestibility studies with crystalline proteases and chemical studies based on total and amino nitrogen as well as chromatography. It appears then that browning and the Maillard reaction are independent processes. When amino compounds are heated in the presence of reducing sugars, browning *may* occur. However, the interaction of these compounds, the Maillard reaction, takes place only in the dry state or if the solution becomes alkaline. In this event, the available amino nitrogen is reduced and the reaction yields compounds not susceptible to hydrolysis by ordinary proteolytic enzymes. Further enzyme studies on these compounds are in progress to account on the basis of chemical combination for the observed decreases in proteolysis whenever conditions optimal for the interaction of amino compounds and carbohydrates are employed.

SUMMARY

Peptide-glucose mixtures have been autoclaved dry, with water unbuffered, and in aqueous buffers at pH 9.7 and the resulting products digested with pancreatin.

The progress of the enzyme action, based upon the liberation of amino nitrogen, indicates that the decrease in nutritive value of proteins heated with reducing sugars in the dry state or at an alkaline reaction, arises from the formation of chemical bonds which are resistant to hydrolysis by digestive enzymes.

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GERMFREE CHICKEN NUTRITION

II. VITAMIN INTERRELATIONSHIPS [1]

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TWO FIGURES

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Nutrition studies with germfree animals should give an estimate of the importance of the contribution of the intestinal microflora to the nutrition of the host. Although the germfree chicks of Cohendy ('12) grew poorly (they hardly tripled their hatch weight at 6 weeks), they grew at a rate comparable to that of chicks reared under similar, non-sterile conditions when fed the same sterilized crude diets. Balzam ('37) obtained better growth when sterilized crude diets were used; again the growth rate of the germfree chicks was comparable to that of the conventional control chicks. Similar results were obtained by Luckey et al. in 1948 and Reyniers et al. ('49a and '50), using semi-synthetic diets. The latter workers also reported egg production and hatch from germfree chickens ('49b). Such data indicate that chicks can utilize the ingredients of both crude and semi-synthetic diets without contribution from living intestinal microorganisms either to help digest natural foodstuffs or to synthesize any required nutrient. They also suggest that there are no unknown nutrients required for normal growth, reproduction and good general appearance, available to the chick via

[1] These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and the University of Notre Dame, NR 131-067.

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"intestinal synthesis,"[3] other than those present either in the semi-synthetic diets or in tissue stores of the chick or both. Such conclusions from these relatively short experiments are substantiated by the maintenance of a colony of germfree rats at the Lobund Institute through 6 generations.

Balzam's second experiment was designed to study "vitamin B" deficiency with the yeast omitted from his practical-type diet. Both the germfree and conventional chicks developed polyneuritis and died within 6 to 10 days. This indicated that there had been no intestinal synthesis of thiamine to offset the acute deficiency of thiamine in the food.

This report presents a study of the effect of individual vitamin deficiencies upon the metabolism of B-vitamins in germfree and conventional chicks. The concentration of vitamins in the liver and cecal contents of chicks fed a complete diet is compared with similar data obtained from chicks deficient in thiamine, riboflavin, niacin, biotin, folic acid and vitamin K. A detailed discussion of growth, clinical symptoms and morphological changes are presented elsewhere (Reyniers et al., '54).

METHODS

The methods used to obtain and maintain chicks in the complete absence of all demonstrable living microorganisms are described by Reyniers et al. ('49a). White Wyandott Bantam chicks reared in the jar system were used in the first thiamine experiments. All other experiments were performed with standard size, single comb, White Leghorn chicks in the standard Reynier's units. The germfree chicks were shown, by accepted bacteriological techniques of visual and cultural examination, to be entirely free from living microorganisms. No evidence of virus activity was seen.

The germfree chicks were watered after the hatch debris had been cleared away, and were fed 24 to 72 hours after

[3] The term intestinal synthesis, as applied herein and by nutritionists generally, implies the synthesis of a material by intestinal microorganisms and the subsequent action of that material on the host, usually by absorption and utilization.

hatch was completed. The germfree chicks fed deficient diets were maintained in units separate from chicks fed any other diet. Thus there was almost no possibility for dust or other debris to supply any of the vitamin under study. All birds were maintained on one-half inch mesh wire screen and fed and watered ad libitum. The conventional chicks were placed in a commercial chick starter battery and fed and watered as nearly as possible at the same time as the germfree birds. The deficient conventional chicks were housed in the same room with other chicks being fed complete diets.

The composition of the complete diet as made and as fed is given in table 1. Each deficiency was studied by omitting a single vitamin from this formula. The diet of Ansbacher ('40) was used for some of the vitamin K experiments.

Analyses were performed on moribund, freshly dead or decapitated chicks using methods given previously (Reyniers et al., '50).

RESULTS

The germfree White Wyandott chicks failed to grow when fed the thiamine-deficient diet; when fed the complete diet they doubled their initial weight in two weeks. Both conventional and germfree birds fed deficient diets showed opisthotonos in about one week and died at the same time (8 to 14 days). Analyses of these birds (table 2) revealed an interesting phenomenon. Thiamine in the cecal contents decreased during the deficiency in germfree and conventional chicks. The concentration of thiamine (wet basis) in the cecal contents was about 4 times greater than in the liver of dying and freshly dead deficient chicks — even in the germfree birds. The rectal contents of deficient chicks contained a concentration of thiamine about 20 times higher than the livers. Evidently the birds die while they are excreting what appear to be physiological amounts of the vitamin needed to save their lives. The intraperitoneal injection of therapeutic amounts of thiamine produced a remarkable change in the condition of these chicks, even when treated while in the moribund condition.

In the first experiment with White Leghorn chicks fed the low thiamine diet, the chicks (one germfree and 10 conventional) all died within 10 days. In the second experiment two of the three chicks died in 10 days and 4 of the 10 conventional chicks died in 10 days. All chicks were sacrificed for examination and analysis at this time.

TABLE 1

Composition of complete diet

CONSTITUENT	AMOUNT	EFFECT OF STEAM STERILIZATION	
		Before	After
	per 100 gm	*per 100 gm*	*per100 gm* [1]
Cornstarch, gm	53		
Cellu-flour, gm	6		
Corn oil,[2] gm	7	7.8	6.2
Salts L-II,[3] gm	5		
Casein (Labco), gm	25	23.6	23.2
L-cystine, gm	.4		
Glycine, gm	.5		
L-arginine, gm	.3		
Vitamin A, I.U.	600	...	400
Vitamin D, I.U.	100	...	...
Vitamin E (pure), mg	10	...	5
Vitamin K, mg	1	...	...
Ascorbic acid, mg	100	...	80
Thiamine HCl, mg	5	2.76	0.394
Riboflavin, mg	2	1.32	0.93
Ca pantothenate, mg	10	3.61	3.04
Nicotinamide, mg	10	9.76	8.31
Choline Cl, mg	200	...	194
Pyridoxine HCl, mg	2	...	1.6
i-inositol, mg	100	...	...
Biotin, mg	0.05	0.050	0.035
Folic acid, mg	2	0.36	0.25
Vitamin B_{12}, μg	2	1.23	0.99

[1] Average data from 6 of the diets used, except for the values for protein, vitamin A, vitamin E, vitamin C, pyridoxine and choline which were obtained by extrapolation from data on other semi-synthetic diets.

[2] Mazola.

[3] The salt mixture contains the following salts, in grams per kilogram: $CaCO_3$, 18.0; $CaHPO_4$, 3.3; K_2HPO_4, 13.5; Na_2HPO_4, 12.0; NaCl, 3.0; KI, 0.045; $MgSO_4 \cdot 7H_2O$, 4.5; $MnSO_4 \cdot 4H_2O$, 0.75; Fe $(C_6H_5O_7)2$, 4.5; $CuSO_4 \cdot 5H_2O$, 0.23; $CoCl_2 \cdot 6H_2O$, 0.03; $ZnSO_4 \cdot 7H_2O$, 0.06; $Na_2B_4O_7 \cdot 10H_2O$, 0.03; and $AlK(SO_4)_2 \cdot 10H_2O$, 0.045.

TABLE 2

Thiamine in bantam chicks at 11 ± 3 days

Micrograms per gram on wet basis

DIET	MICROBIAL STATUS	LIVER			CECAL CONTENTS			RECTAL CONTENTS		
		No.	Av.	(Range)	No.	Av.	(Range)	No.	Av.	(Range)
Deficient [1]	Germfree	3	1.2	(0.17–3.04)	7	4.9	(2.45–8.98)	1	29.1	
	Normal	4	2.2	(1.71–3.23)	2	7.96	(6.41–9.50)	3	40.3	(29.3–60.9)
Complete	Germfree	2	5.6	(pooled)	2	7.1	(pooled)			
	Normal	4	4.10	(2.41–8.20)	4	23.9	(9.90–53.4)	1	55.5	

[1] The deficient diet had less than 1 μg of thiamine/100 gm of diet as determined by chemical analysis.

TABLE 3

Interaction of vitamins in germfree and conventional chicks[1]

DIET		COMPLETE				THIAMINE LOW				FLAVIN LOW			
		Liver		Cecal contents		Liver		Cecal contents		Liver		Cecal contents	
		A	B	A	B	A	B	A	B	A	B	A	B
Thiamine,	N	9	13			3	9	2	3	8	5	3	2
μg/gm	M	3.99	3.00			0.824	0.647	0.953	0.97	3.27	3.86	1.70	1.94
	σ	0.723	1.27			0.830	0.388	0.0255	0.0734	1.09	0.710	2.56	0.0374
Riboflavin,	N	*10*	13	3	10	3	8	1	3[2]	8	5		5
μg/gm	M	21.6	23.3	10.9	7.38	17.7	25.0	13.5	4.43	7.1	12.5		3.3
	σ	2.06	7.25	7.57	2.38	5.45	1.97			4.98	2.98		0.040
Niacin,	N	8	14	9	13	3	6	1		8	5		
μg/gm	M	109	108	12.1	37.2	69.3	104	64.8		86	123		
	σ	9.9	45.1	6.7	18.7	16.1	140			21.3	32.9		
Pantothenic	N	5	10	9	12	3	7	1		8	5		2
acid,	M	66.4	48.0	5.47	7.74	47.6	45.0	28.4		57.7	47.3		8.0
μg/gm	σ	10.4	9.9	2.40	3.72	18.9	12.9			27.92	13.3		3.30
Pyridoxine,	N	10	14										
μg/gm	M	10.2	9.8										
	σ	4.4	3.8										
Biotin,	N	13	18	9	13	3	6	1	3[2]	7	5	3	3
μg/gm	M	5.14	4.31	0.241	0.488	4.47	4.70	318	0.15	4.3	4.0	2.4	0.15
	σ	1.0	2.1	0.182	1.59	1.27	1.68			1.18	0.574	0.373	0.0648
Folic acid,	N	12	18	7	13	3	7	1	3[2]	8	5		4
μg/gm	M	4.41	3.27	2.29	1.82	3.7	2.96	3.0	0.35	5.2	2.8		1.1
	σ	1.26	1.15	0.46	0.94	1.95	1.33			2.70	1.52		0.575
Vit. B_{12},	N	13	18	7	2								
μg/gm	M	323	171	14.2	18.4								
	σ	146	169										
Dry weight,	N	10	12	8	13		2		2	1			
%	M	25.9	29.8	13.5	21.8		26.1		18.3	28.4			
	σ	1.55	1.32	1.97	2.86		5.13		0.725				

DIET		NIACIN LOW				FOLIC LOW				VITAMIN K LOW			
		Liver		Cecal contents		Liver		Cecal contents		Liver		Cecal contents	
		A	B	A	B	A	B	A	B	A	B	A	B
Thiamine,	N	7	10	1		9	10			8	6		
μg/gm	M	4.94	4.26	0.464		2.49	2.12			4.52	4.84		
	σ	0.894	0.734			0.564	0.816			0.413	4.50		
Riboflavin,	N	7	10	6	6	9	11	2	4	7	6	5	5
μg/gm	M	34.7	22.7	6.2	9.4	34.9	19.3	5.4	4.6	16.1	10.9	3.6	2.6
	σ	17.7	10.1	1.04	2.14	7.37	5.79	1.48	1.50	4.75	3.91	0.400	0.775
Niacin,	N	7	10	5	10	9	11	1	3	8	6	8	6
μg/gm	M	145	110	14.6	37.2	104	87	7.8	8.1	130	141	25.0	19.1
	σ	3.94	22.9	3.80	20.6	30.9	40.8		2.30	16.0	15.9	3.00	5.06
Pantothenic	N	7	10	1	5	9	11		1	8	6	4	5
acid,	M	54	51	15.2	11.7	19.3	13.7		2.9	19.6	17.9	16.2	3.88
μg/gm	σ	8.24	9.70		1.84	2.48	6.77			4.30	12.2	6.30	0.794
Pyridoxine,	N	1	10			2	4	2	4	8	6		
μg/gm	M	8.8	11 8			22.9	13.9	1.2	2.4	10.1	9.3		
	σ		3.51			1.23	4.71	0.0348	1.20	1.69	1.50		
Biotin,	N	7	10	2	2	9	11			8	6	4	4
μg/gm	M	5.1	6 5	0.21	0.38	4.15	3.49			4.9	3.7	0.35	0.49
	σ	2.55	0.0308		0.340	1.47	1.79			1.63	0.843	0.160	0.285
Folic acid,	N	7	10	6	7	12	14	9	13	8	6	5	5
μg/gm	M	2.9	2.3	2.1	0.74	3.60	4.15	0.55	1.22	5.0	3.4	1.05	0.67
	σ	0.743	0 842	0.490	0.208	1.64	0.339	0.123	0.358	1.57	1.55	0.414	0.0837
Vit. B_{12},	N	1	11			12	14	7	7	7	6		
μg/gm	M	20	222			189	200	11.3	1708	266	252		
	σ		240			19.9	97.4	5.07	1013	2.24	190		
Dry weight,	N		4			6	12	5	6	4	5	1	5
%	M		25.2			26.3	24.9	14.4	18.0	27.3	27.0	17.4	23.5
	σ		1.45			1.97	1.97	4.03	4.06	1.04	1.31		6.79

[1] The number of White Leghorn chicks analyzed is given by N, the arithmetical average by M and the standard deviation by σ. Data under columns A were taken from germfree birds while column B denotes conventional birds fed the same sterilized diet. All chicks were about 4 weeks of age except the thiamine deficient birds which were 10 days old.

[2] Pooled samples.

Analysis of tissues of White Leghorn chicks (table 3) would indicate a very similar vitamin content in the livers of germfree and conventional, thiamine-deficient chicks. The one exception noted is the low level of niacin in the livers of the germfree thiamine-deficient chicks. It is interesting that thiamine-deficient germfree and conventional chicks have a similar quantity of thiamine in their livers and cecal contents.

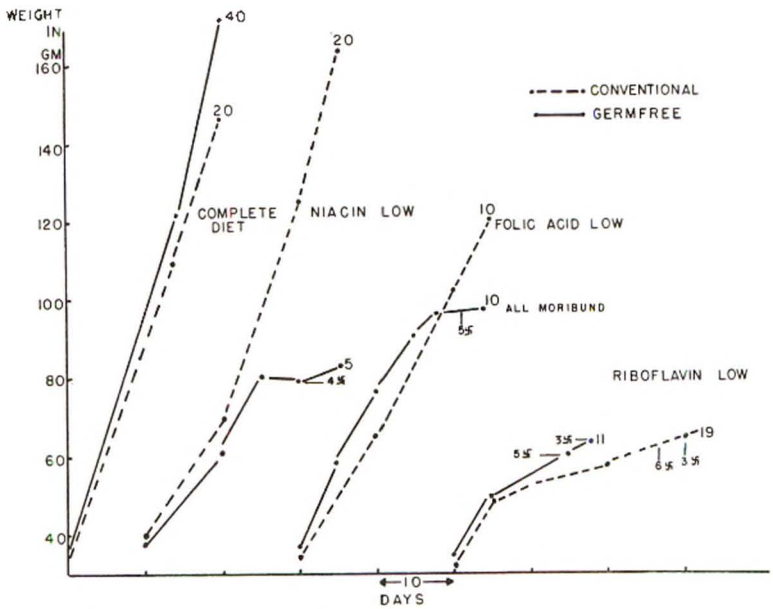

Fig. 1 Growth curves of White Leghorn chicks fed semi-synthetic diets. The number of chicks which finished the experiment is given at the end of the curves indicating the average growth obtained for 2 to 5 experiments. The numbers preceding the swastikas indicate the death of deficient chicks.

When these data are compared to those taken from chicks fed adequate thiamine it is seen that the thiamine-deficient chicks had only about one-quarter as much thiamine in the livers. Other B-vitamins were in concentrations comparable to those of chicks receiving the complete diet. The analyses on cecal contents of one germfree chick indicated high B-vitamin concentrations in the absence of thiamine. The biotin value particularly needs confirmation.

When riboflavin was omitted from the diet a more acute deficiency was evidenced in the germfree birds than in conventional birds as shown by the mortality (fig. 1) of the germfree birds at two weeks, while the conventional birds did not start to die until the 5th week. The mortality data summarized in these two experiments indicate a more severe deficiency developed in the germfree chicks. Analysis of the liver and cecal contents of these birds (table 3) showed no striking difference between the germfree and conventional birds with the possible exception of the biotin level in germfree cecal contents. The germfree birds may have somewhat less riboflavin and niacin in their livers than is found in conventional chicks. The deficient birds have less riboflavin in the livers than was found in chicks fed the complete diet.

In two experiments wherein niacin was omitted from the diet, the germfree chicks lost weight after the second week and died at about 4 weeks while the conventional birds continued to gain with little mortality exhibited (fig. 1). These data may indicate a somewhat higher dietary requirement of the germfree chicks for niacin. The riboflavin and niacin content of the livers of germfree chicks deficient in niacin was somewhat higher than either that of the conventional "deficient" chicks or the germfree chicks receiving dietary niacin. The amount of niacin in the cecal contents was quite normal. This is of special interest since the germfree birds were presumably dying from a niacin deficiency. The folic acid content of the liver decreased somewhat in both groups of chicks receiving the low-niacin diet. The vitamin B_{12} content of the liver of one niacin-deficient-germfree bird was markedly reduced when compared with the chicks in all other groups.

Two experiments with the folic acid-low diet gave very similar results. In the first experiment the red blood cell counts of two chicks in each group were 1.2 and 1.7×10^6/ml for germfree birds and 1.2 and 2.0×10^6/ml for the conventional chicks. The combined growth data (fig. 1) indicate a faster initial growth in the germfree chicks followed by decreased growth rate after the first week and death at three

weeks. The germfree chicks were much weaker than the conventional birds after two weeks. Vitamin analyses indicated that the concentration of vitamin B_{12} was possibly low in the germfree chick livers while the riboflavin and pyridoxine contents were somewhat high. Pantothenic acid was low in the livers of both germfree and conventional chicks when compared to that of chicks receiving the complete diet. Folic acid in the livers was unaffected while the amount

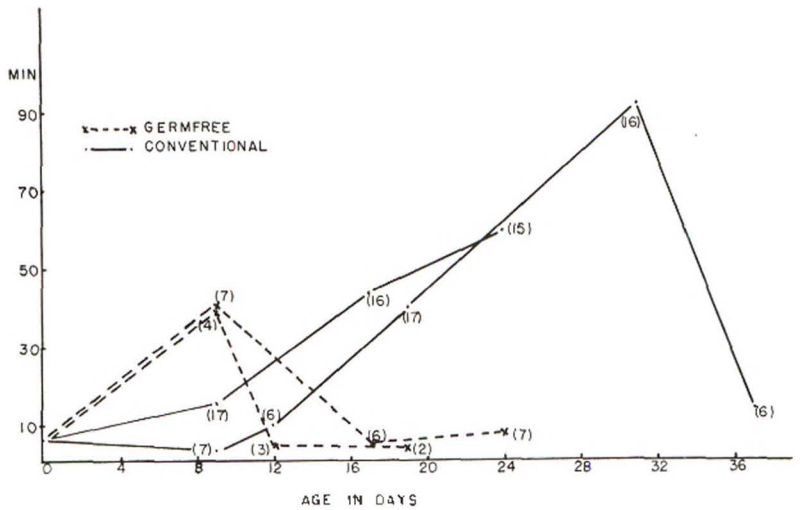

Fig. 2 Whole blood clotting time for White Leghorn chicks fed the diet of Ansbacker in two experiments. The numbers of chicks used to obtain each average are indicated in parentheses. Some of the 10 chicks examined to obtain data at hatch were taken from the shell after pipping.

of folic acid in germfree cecal contents was decreased. The concentration of vitamins in cecal contents was generally low. The remarkably high values for vitamin B_{12} obtained in conventional chicks is of interest.

A vitamin K deficiency was not at first observed by feeding germfree birds the synthetic type diet with vitamin K omitted. The reason became apparent later: the clotting time of whole blood of germfree birds, fed vitamin K low diets, often returns to normal before conventional birds become deficient

(fig. 2). B-vitamin analyses indicated that chicks fed the synthetic-type vitamin K-low diet had less pantothenate and possibly less riboflavin in their liver with definitely less riboflavin in cecal contents than birds fed the control diet. The pantothenate values of cecal contents were high in germfree and low in conventional birds fed the vitamin K-low diet.

DISCUSSION

Despite the small numbers of chicks used, the uniformity of the results allow certain conclusions. The main pattern seen in these vitamin interrelationships is the similarity in the reactions of the germfree and control chicks. Vitamin metabolism, as read from analyses of liver and cecal contents, in the pure physiological reactions of the germfree chicks does not appear to differ greatly from the metabolism in conventional chicks, with all their potential reactions via the intestinal microorganisms. Thus the concentrations of both thiamine and riboflavin in the livers of germfree and conventional chicks are low when the chicks are moribund from a deficiency of thiamine and riboflavin respectively, while liver folic acid and niacin values are unaffected when the chicks are fed diets deficient in folic acid and niacin respectively.

A second general relationship is seen for both germfree and conventional chicks: only one vitamin change is noted in the deficiencies of thiamine, riboflavin and niacin while changes in several vitamin concentrations are seen in folic acid and vitamin K-deficiency states. In conventional chicks the diet low in vitamin K lowered cecal concentration of riboflavin, niacin and pantothenate. Germfree chicks fed the low-vitamin K diet showed a lower cecal riboflavin concentration and a higher concentration of niacin, pantothenate, and possibly folic acid. Since the conventional chicks showed a difference from the simple physiological action seen in the germfree birds, it would be of interest to correlate changes in cecal flora with the omission of vitamin K. The folic acid-

deficient diet appears to increase the cecal concentration of vitamin B_{12} and decrease cecal niacin concentration in conventional chicks without affecting either of these vitamins in the ceca of germfree chicks. Dietary folic acid has been shown by Moore et al. ('46) to have a greater affect on the intestinal flora than the addition of antibiotics or sulfa drugs.

The phenomenon of deficient germfree chicks excreting relatively normal concentrations of the vitamin which is desperately needed by the tissues indicates that the excretion threshold cannot be varied greatly to help the economy of the tissues. It is noted that, on a dry basis, cecal or rectal contents often contain greater concentrations of B-vitamins than the diet. Fecal vitamin excretion may represent a relatively less efficient absorption of dietary vitamins (relative to the quantity of dietary fat, carbohydrate and protein absorbed simultaneously), or an excretion of the vitamin from the tissues into the intestinal lumen in excess of the products of "intestinal synthesis." It is emphasized that these results are not affected by the synthetic action of the intestinal microorganisms since data from germfree and conventional chicks are similar. The net effect of the microflora may well be zero with microbiological synthesis and destruction of a given nutrient occurring at an equal rate. Some action of the intestinal microflora may account for the less acute riboflavin, niacin, and folic acid deficiencies obtained in conventional chicks than was found in germfree chicks fed the same steam-sterilized diets; however, the possibility of dust carrying small amounts of vitamins was not eliminated. These data do not allow an accurate quantitative estimate of the contribution of the microflora: they do indicate that critical experimentation is needed.

Apparently the action of the microflora has been overestimated in the mechanism of the action by which chicks overcome the deficiency of and requirement for vitamin K. The results from the two germfree experiments show that young chicks can overcome the need for dietary vitamin K in the complete absence of any living microorganisms.

SUMMARY

Germfree and conventional White Leghorn chicks exhibited similar metabolism of and qualitative requirements for individual B-vitamins. The deficiencies of thiamine, riboflavin, niacin and folic acid were apparently somewhat more acute in germfree than in conventional chicks. Excreta (as measured from cecal and rectal contents) of deficient birds contain appreciable quantities of the vitamin which would save their lives if injected or given orally. The presence of these vitamins in excreta cannot be attributed to microbial synthesis in the germfree birds.

Germfree chicks have been found to recover spontaneously from a vitamin K deficiency.

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EFFECT OF PROTEIN ON THE TOXICITY OF HEXACHLORONAPHTHALENE [1]

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INTRODUCTION

The highly chlorinated naphthalenes are of interest in nutrition because they are capable of inducing hyperkeratosis or X-disease in cattle. This was first demonstrated by Bell ('52) and has been confirmed by several other groups of workers (Sikes and Bridges, '52; Olafson et al., '52; Hoekstra et al., '54). Bovine hyperkeratosis simulates many of the features of vitamin A deficiency, and cattle poisoned with highly chlorinated naphthalenes exhibit a prompt and almost complete disappearance of vitamin A in the circulating blood (Hansel et al., '51; Copenhaver and Bell, '54). Poisoning from chlorinated naphthalenes in humans, described as cable rash, cable itch, or chloracne, is reported in the literature (Engel and Bell, '53).

The possibility of animal or human exposure to highly chlorinated naphthalenes and the need for further investigation of the malnutrition implications of poisoning by these compounds led to these studies. Furthermore, it seemed desirable to explore the possibilities of developing a small animal assay for the determination of chlorinated naphtha-

[1] A preliminary report of this study was made at the annual meeting of the Federation of American Societies for Experimental Biology, Federation Proceedings, Volume 13, Number 1, Part 1, p. 456, 1954.

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lenes since simple isolation procedures for these compounds had not been developed and the results obtained by colorimetric analysis (Engel et al., '53) needed confirmation in animal tests.

PROCEDURE

In preliminary trials it was established that hexachloronaphthalene,[4] when injected subcutaneously or when fed to weanling rats, produced a measurable liver enlargement and an increase in liver fat. Consequently, the routine procedure adopted was to administer the compounds to weanling rats by either of these routes. When the compound was added to the diet a petroleum ether (30 to 60°C. B.P.) solution was mixed into the dry ingredients; when injected a 2.5% corn oil solution was used.

The animals used were weanlings of both sexes, 40 to 55 gm in body weight, of the Holtzman strain rat.

The basal diet used to establish an assay procedure was low in protein and had the following percentage composition: casein, vitamin-free, 9.0; sucrose, 86.7; salts (Salmon, '47), 4; and L-cystine, 0.3. It was supplemented with the following in milligrams per kilogram: thiamine, 5; pyridoxine, 5; riboflavin, 10; calcium pantothenate, 20; niacin, 40; inositol, 200; choline chloride, 2,000; and alpha tocopherol acetate, 50. Vitamins A and D were supplied as Quadrex at a level to supply 40,000 and 5,000 I.U., respectively, per kilogram of diet. The vitamin supplement was mixed into the basal ingredients in a small portion of the sucrose component.

The animals were placed on the following feeding schedule: three grams of the diet were fed to each rat the first day and the feeding rate was increased one gram daily to a maximum of 7 gm per rat daily. The animals were housed in individual cages with screen bottoms. The experimental periods varied from one to 4 weeks. At autopsy the livers were weighed, dried at 90°C. to constant weight, ground, and extracted

[4] The hexachloronaphthalene used was reported by the supplier to be 90 to 95% hexachloro-derivative of naphthalene. The remaining 5 to 10% represented the penta- and hepta-derivatives of naphthalene.

with anhydrous ethyl ether for 16 hours. The ether extracted residue was analyzed for total nitrogen by the Kjeldahl procedure.

The influence of dietary protein on the toxicity of hexachloronaphthalene was evaluated by comparing the 9% casein basal diet with the same diet in which the casein level was increased to 18 or 27% at the expense of sucrose. The effect of substituting 20% of Crisco for an equal amount of sucrose and the deletion of the vitamin A and D supplement was also investigated.

RESULTS AND DISCUSSION

The results of some preliminary trials indicated a marked effect on liver fat (ether extractable material) when the basal diet contained 400 p.p.m. of hexachloronaphthalene. Two weanling rats fed the basal diet alone for three weeks had an average of 15.9% liver fat, whereas two littermates fed the same diet containing 400 p.p.m. of hexachloronaphthalene for three weeks had livers that averaged 40.8% in fat. The controls gained an average of 57 gm, whereas the treated animals gained only 2 gm. The treated animals each consumed an average of 35 mg of hexachloronaphthalene during the three-week period.

Two rats each received 30 mg of hexachloronaphthalene in corn oil by subcutaneous injection. At the end of two weeks their livers averaged 41.7% fat. Two control animals receiving a similar injection of corn oil alone had liver fat values of 20.1%. This was repeated with similar amounts administered intraperitoneally with resultant liver fat values of 41.1% and 20.1% for treated and control animals, respectively. These results established clearly that 30 to 35 mg of hexachloronaphthalene per rat resulted in severe liver changes in two to three weeks.

The experiments summarized in table 1 were designed to establish minimum toxic levels from subcutaneous administration of the hexachloronaphthalene and to explore the possible influence of diet modifications. In all cases the animals were sacrificed for liver analysis after one week.

TABLE 1

Influence of hexachloronaphthalene on body weight and liver fat under various dietary conditions

(Hexachloronaphthalene in corn oil was injected subcutaneously at the rate of 4 mg daily, starting on day 1, until indicated total dose was reached. The control animals each received an equal amount of corn oil alone for the first 4 days. One-week experimental period)

TRIAL NO.	DIET	HEXACHLORONAPHTHALENE INJECTED	AV. BODY WT. GAIN	NO. RATS	LIVER ETHER EXTRACT
		mg	*gm*		% *(dry basis)*
1	Basal	0	7	2	12.1
		4	4	3	32.8
		8	5	3	35.6
		16	5	3	31.4
	Vitamins A and D omitted	0	6	3	12.5
		4	5	3	34.0
		8	4	3	31.3
		16	6	3	32.7
2	Vitamins A and D omitted	0	6	1	19.8
		4	6	2	28.0
		8	4	2	25.5
		16	6	2	32.5
	Vitamins A and D omitted, 20% Crisco added	0	7	2	13.7
		4	8	2	27.9
		8	7	2	31.1
		16	5	2	25.3
3	Vitamins A and D omitted	0	2	2	12.9
		4	1	2	24.7
		8	0	2	23.9
		16	—3	2	26.8
	Vitamins A and D omitted	0	4	2	10.7
		4	3	2	14.8
		8	3	2	17.4
		16	4	2	18.8
4	Vitamins A and D omitted	0	5	5	10.2
		8	6	5	27.0
	Vitamins A and D omitted, 18% casein added	0	12	5	11.9
		8	12	5	21.4

The noteworthy effect from hexachloronaphthalene treatment was the increase in liver fat. This was evident from a single 4 mg treatment and higher levels did not consistently produce further increases in liver fat. The ratio of liver weight to body weight and the percentage of liver dry matter increased while the concentration of liver nitrogen decreased in the animals receiving hexachloronaphthalene. These changes are generally observed and have been reported by others in short-term experiments involving liver fat changes. The data have not been included in table 1 for the sake of brevity.

Omitting the vitamin A and D supplement from the diet failed to influence the effect of the hexachloronaphthalene on liver fat (trial 1). Neither was any detectable change noted when 20% of the sucrose in the diet was replaced by Crisco (trial 2).

The substitution of 9 or 18% of dietary sucrose with casein significantly reduced the toxicity of hexachloronaphthalene as evidenced by a reduction in liver fat. Considering only the lowest treatment level; i.e. 4 mg per rat, the substitution of casein for 9% of sucrose in the diet decreased liver fat to 14.8% as against a value of 24.7% on the control diet (trial 3). Likewise, the increase in liver fat from 10.2 to 27.0% from the 8 mg treatment on the low-casein diet represents an increase of 160%, contrasted with an increase of only 80% (from 11.9 to 21.4%) in animals receiving 18% of added dietary casein (trial 4).

From these experiments it was evident that the protein effect should be investigated further, particularly under conditions where smaller amounts of hexachloronaphthalene are administered. Also, it seemed likely that the protein effect might be more pronounced if the toxic material were included in the ration, a condition under which only minute amounts of the toxic material would be presented to the tissue at any one time for detoxification.

This was investigated in the experiment that is summarized in table 2. There is clearly an increase in liver size, liver fat,

TABLE 2

Body weight and liver composition as influenced by hexachloronaphthalene under high and low dietary protein
(Seven rats per treatment; two-week experimental period; values following ± are standard deviations)

Dietary casein (%)	9	27	9	27	9	27	9	27	9	27	CONFIDENCE LIMITS	
Hexachloronaphthalene in diet (p.p.m.)	0		10		25		50		100			
Hexachloronaphthalene average consumed per rat (mg)	..	..	.86	.86	2.20	2.20	3.60	3.68	6.60	7.15	5% Level	1% Level
Body weight gain (gm)	20±3	32±3	19±3	32±5	23±3	36±4	15±4	34±4	15±5	31±8	5	6
Final body weight (gm)	63±4	75±5	70±7	83±8	70±6	83±8	61±6	82±8	64±4	76±9		
Liver composition:												
Fresh weight (% of body weight)	5.0±.4	4.5±.4	5.4±.6	4.9±.5	5.8±.6	5.9±.7	7.0±.4	6.3±.7	7.7±.5	7.0±.7	.6	.8
Dry matter (%)	29.7±.9	28.8±.4	30.1±.6	28.9±.8	31.3±.7	29.2±.8	38.6±2.5	30.6±1.1	42.1±2.2	33.5±2.0	1.5	2.0
Ether extract (%, dry basis)	18.5±4.0	9.5±1.3	16.8±4.6	7.9±2.0	22.5±3.9	12.0±2.4	43.1±6.6	16.6±3.9	51.4±5.7	28.3±7.4	5.0	6.7
Ether extract (mg/100 gm rat)	274±79	123±22	273±87	112±28	412±100	208±67	1181±225	324±120	1667±182	672±233	156	208
Nitrogen (%, dry basis)	9.9±.5	12.2±.2	9.7±.5	10.1±1.1	9.1±.7	10.3±1.2	7.2±.6	9.8±1.2	7.3±.8	9.3±1.0	.9	1.3
Nitrogen (mg/100 gm rat)	145±12	158±12	156±13	156±15	163±10	175±21	196±21	186±15	237±25	214±15	18	23

and an absolute increase in liver fat and nitrogen per unit of body weight with increasing levels of hexachloronaphthalene in the diet. A very marked effect on these liver components was observed when the hexachloronaphthalene in the low-protein diet was increased from 25 to 50 p.p.m. In contrast, the increase in liver fat was considerably less apparent in the presence of high dietary protein even at the treatment level of 100 p.p.m.

It is also evident that there was a significant difference in liver composition between high- and low-protein-fed animals in the absence of hexachloronaphthalene. However, the protective effect of protein against the compound can be seen if the increase in liver fat concentration from a given increment of the toxic material is compared under conditions comparable with respect to liver fat. For example, increasing the hexachloronaphthalene in the high-protein diet from 50 to 100 p.p.m. increased liver fat concentration from 16.6 to 28.3%, a difference of 11.7%. In contrast, increasing the compound in the low-protein diet from 0 to 50 p.p.m. increased liver fat from 18.5 to 43.1%, a difference of 24.6%. Thus, the 50 p.p.m. increment under the low-protein regime resulted in an increase in liver fat concentration more than twice as great as that observed under the high-protein regime. Test for significance by the Fisher "t" test revealed that $t_{01} = 3.24$ ($P = < 0.01$).

The increase in liver nitrogen per unit of body weight with increasing concentration of dietary hexachloronaphthalene is interpreted as evidence of true liver hypertrophy induced by the toxic material. Liver hypertrophy is also indicated by the significant increase in liver fresh weight and dry matter content with increases in hexachloronaphthalene in the diet.

The rats made significantly better weight gains on the high-protein diet than on the basal diet. Under the low-protein dietary condition there was some evidence of a growth depression when the diet contained 50 or 100 p.p.m. of hexa-

chloronaphthalene. A similar growth depression was not observed in the animals receiving the high-protein diet.

The data regarding liver ether extract (dry basis) were subjected to an analysis of variance. The effects of hexachloronaphthalene and protein were highly significant ($P = < 0.01$). The interaction between hexachloronaphthalene and protein was also highly significant ($P = < 0.01$), which is an indication that the protective effect of protein was a real one.

From these results it is apparent that weanling rats will tolerate considerably greater concentrations of hexachloronaphthalene in the diet under adequate protein conditions than under limiting dietary protein conditions if liver fat accumulation can be considered a reasonable criterion of toxicity. Whether or not the elevation of protein, rather than the depression of carbohydrate (fat precursors), is responsible for the protective effect has not been established. However, since substitution of carbohydrate with fat was without influence, it seems likely that the protection was mediated through protein.

The results (table 2) indicate that the weanling rat may be useful in the bioassay for highly chlorinated naphthalenes. Nearly a three-fold increase in liver fat (milligrams liver fat per 100 gm rat) was observed by increasing the hexachloronaphthalene from 25 to 50 p.p.m. in the diet.

Liver damage from the feeding of highly chlorinated naphthalenes to rats has been reported by Bennett and associates ('38). These workers used a diet of Purina dog chow supplemented with lettuce, eggs, milk, and cod liver oil. Under their conditions a mixture of penta- and hexachloronaphthalene induced a liver enlargement of 30 to 40% when the animals received 100 mg of the compound as a daily intake. This is a rather excessive dosage when the present results establish severe liver changes and liver enlargement of equal magnitude on a total of 3.6 mg of hexachloronaphthalene consumed over a two-week period (an average daily intake per rat of approximately 0.25 mg).

If it can be assumed that the physiological mechanisms that are brought into play in disposing of the highly chlorinated naphthalenes are the same in the rat and the bovine, the present results would indicate that cattle in a poor state of protein nutrition might be much more susceptible to hyperkeratosis-induction by these agents than animals on a liberal protein intake. High-protein diets in the treatment of human chloracne resulting from exposure to highly chlorinated naphthalenes, might also be indicated from these results.

SUMMARY

The administration of as little as 3 to 4 mg of hexachloronaphthalene, total dose, over a one- or two-week experimental period, resulted in liver hypertrophy and the accumulation of liver fat in rats if the animals were fed a 9% casein diet. Increasing the casein in the diet to 18 or 27% at the expense of sucrose afforded a significant protection against liver fat accumulation both in the presence and in the absence of the highly chlorinated naphthalene.

The protective effect of dietary protein against hexachloronaphthalene-induced fatty livers was expressed by a much more gradual increase in liver fat in animals receiving a high-protein diet contrasted with a rapid increase in animals receiving a 9% casein diet.

Substitution of 20% of Crisco for an equal amount of casein in the diet, or omitting the vitamin A and D supplement, had no influence on the liver changes induced by hexachloronaphthalene.

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SOME OBSERVATIONS ON THE METABOLISM OF VITAMIN B_{12} BY JAMAICAN CHILDREN

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The following work was undertaken as part of an investigation of the nutritional status of children in Jamaica, B.W.I. A paper by Mackay, Patrick and Stafford describing this investigation is in preparation. A dietary survey had indicated that these children had a low intake of animal protein and it was thought possible that they might be deficient in vitamin B_{12}. The following experiments were carried out to investigate this possibility.

The experiments included (1) the determination of the plasma levels of vitamin B_{12} in children who had received supplements of the vitamin by mouth and in unsupplemented children; (2) an attempt to "saturate" two children by repeated intravenous administrations of vitamin B_{12}; (3) determination of the plasma levels of vitamin B_{12} and the percentage of excretion of the vitamin by children to whom a test dose of the vitamin had been administered.

The children were almost all of mixed African descent and ranged in age from 8 to 15 years. Equal numbers of boys and girls were used whenever possible.

METHODS

Crystalline vitamin B_{12} (Merck) was used as a standard in all assays and this material was used for the intravenous injections of the children. The dietary supplementation was carried out by administering to each child daily, except

Saturday and Sunday, a troche containing 100 μg of vitamin B_{12} to be taken by mouth. Assays for the vitamin B_{12} content of urine and plasma were carried out by the use of *Lactobacillus leichmanii* 4797 according to the method of Skeggs et al. ('50). A total of at least 10 test tubes containing graded amounts of the solution to be assayed were used in each determination. Urine samples were diluted with water to an appropriate degree and then used directly in the assay. Eight experiments on the determination by this method of B_{12} added to normal urine gave recoveries of 94 to 104%. The amounts of B_{12} added were from 0.01 to 0.10 μg per 100 ml of urine. Two methods were used for the determination of vitamin B_{12} in blood. The first method was used only to follow the changes in the concentration of the vitamin in the plasma after a test dose had been administered intravenously. The method consisted simply of withdrawing 0.2 ml of blood from the finger tip by means of a blood pipette, delivering it into 2.0 ml of 0.9% saline, centrifuging, removing the supernatant, washing the cells with one more 2.0 ml portion of saline and making the extracts to 10.0 ml. The plasma solution was then used directly in the assay as described by Skeggs et al. ('50). The procedure included autoclaving the plasma in the medium, a procedure which, according to Scheid and Schweigert ('51), liberates most of the vitamin B_{12} bound to protein. Six experiments on the determination by this method of B_{12} added to plasma gave recoveries of 94 to 107%. The amounts of B_{12} added were from 0.1 to 0.8 μg per 100 ml of plasma. This method was not of sufficient sensitivity to measure accurately the B_{12} content of normal sera and for this purpose the method of Rosenthal and Sarett ('52) was employed. This procedure involves removal of the plasma protein after bound vitamin B_{12} is liberated by boiling in an acetic acid buffer. A few determinations of the vitamin B_{12} activity of whole blood or red blood cells were carried out and these were done simply by adding an appropriate amount of water (usually 100 volumes) and using the hemolysate directly in the assay procedure.

RESULTS AND DISCUSSION

Thirty-eight children from the same village were divided into two groups, comparable with respect to age, sex and economic condition. Each child of the first group received dietary vitamin B_{12} supplementation over a period of 9 months. The average total consumption of B_{12} per child was 13.9 mg. The second group of children received placebos for the same length of time. Determinations of the vitamin B_{12} concentration of the serum of each child were carried out during the last month of the supplementations. The average concentration of vitamin B_{12} in the serum of the unsupplemented children was 0.021 μg $\pm$ 0.010 μg per 100 ml, with a range of 0.008 to 0.043 μg per 100 ml. The corresponding figures for the supplemented group were 0.033 $\pm$ 0.020 μg per 100 ml with a range of 0.007 to 0.90 μg per 100 ml. Thus the supplemented children had significantly higher levels ($P = 0.034$) of serum B_{12}. It is interesting to note the comparatively small increase in B_{12} activity of the plasma which occurred as a result of such massive B_{12} supplementation in the diet. This is in agreement with the findings of Chow et al. ('51) and Smith ('52) that almost all of an oral dose of vitamin B_{12} administered to rats is eliminated in the feces and very little is retained. The values found in the group of unsupplemented children were very similar to those reported by Rosenthal and Sarett ('52) for healthy American adults.

An attempt was made to "saturate" two children by repeated intravenous injections of vitamin B_{12}. Each child received 1.5 μg of B_{12} per kilogram of body weight by intravenous injection on the morning of the first, 5th, 8th, 12th and 15th days of the experiment. Blood was withdrawn just *before* each injection for determination of its B_{12} content and urine was collected for two periods after the injection: zero to 5 hours and 5 to 7 hours. The average results of this experiment are given in table 1. The vitamin B_{12} activity in the whole blood of the children before the injection seemed significantly higher than that in the plasma of normal children quoted above and indeed it was found that the inclusion of protein in the as-

say always produced some vitamin B_{12} activity. It is doubtful whether this activity represents actual vitamin B_{12} or is a non specific effect of the protein. In any case it seems clear that the resting levels of B_{12} in the blood were significantly raised by the injections but there was no corresponding increase in the excretion of B_{12} after each test dose. Only a very small percentage of the B_{12} was excreted in the urine following the 5th hour after the injection and it was found in this and other experiments that 90 to 95% of the total amount of B_{12} excreted was excreted within the first two hours after the injection. The pattern of urinary excretion of B_{12}

TABLE 1

Vitamin B_{12} activity in blood and urine of children after repeated injections of vitamin B_{12}

NO. OF INJECTIONS	B_{12} ACTIVITY IN WHOLE BLOOD	PERCENTAGE OF THE DOSE EXCRETED IN THE URINE	
	μg %	*0–5th hour*	*5th–7th hour*
0	0.041	..	..
1	0.070	38	0.05
2	0.13	43	0.12
3	0.10	41	0.10
4	0.12	38	0.15
5	0.12	33	0.15

found in these experiments seems different from that reported by Chesterman et al. ('51) by being somewhat more rapid and more complete. This is probably due to the fact that in these experiments the vitamin B_{12} was administered intravenously whereas Chesterman administered the B_{12} intramuscularly.

Preliminary experiments indicated that practically all of the vitamin B_{12} activity found in the blood after an intravenous injection of B_{12} was in the plasma. Apparently, little if any of the injected B_{12} entered the cells of the blood. Plasma levels of vitamin B_{12} and percentage excretion after a test dose of the vitamin were followed in two groups of children. Each of the first group of 5 children was judged to be failing in growth by reference to the Wetzel grid (Wetzel, '44). Each

of the second group of children was judged of normal development by the same method. After the initial tests each child of the first group received dietary B_{12} supplementation over a period of 9 months. The average total consumption of B_{12} over this period was 14.9 mg per child. The tests were then repeated on each child of this group. The test dose was 1.5 µg of B_{12} per kilogram of body weight administered intravenously. Table 2 presents the average results obtained, together with results obtained by the same method from three normal adults.

From these data it appears that:

(a) there was no significant difference in the amounts of vitamin B_{12} excreted in the urine by the three groups of children.

(b) the "resting" vitamin B_{12} activity of the plasma of the retarded children was raised by the administration of the dietary supplements of B_{12}.

(c) the test dose of B_{12} caused consistently higher plasma B_{12} levels in the retarded children after they had received vitamin B_{12} dietary supplementation, but the *rate* of removal of B_{12} from the plasma appeared to be substantially unaltered.

(d) although the "normal" children showed higher plasma B_{12} levels after the test dose than did the "retarded" children, the differences are of doubtful significance.

(e) most of the injected B_{12} is removed from the plasma within two hours and this is in accord with the pattern of urinary excretion of B_{12}. However, a small proportion of the injected B_{12} appears to remain in the plasma for some time, there being very little decrease in B_{12} activity in plasma from the 4th to the 6th hour, although the B_{12} level at this time is higher than the level before the injection of B_{12}.

SUMMARY

1. A group of 19 Jamaican children who had received dietary vitamin B_{12} supplementation over a period of 9 months

TABLE 2

Vitamin B_{12} levels in plasma and percentage excretion after a test dose (1.5 mg per kilogram of body weight of vitamin B_{12})

GROUP	NO. IN GROUP	PLASMA B_{12} LEVELS (μG PER 100 ML OF BLOOD)						PERCENTAGE OF DOSE EXCRETED IN URINE	
		Before test dose	Time after injection of test dose					Time after injection of test dose	
			10 min.	*1 hour*	*2 hours*	*4 hours*	*6 hours*	*0–5th hour*	*5th–7th hour*
Normal adults	3	0.02	0.38	0.19	0.14	0.11	0.09	50	0.03
Normal children	6	0.03 ± 0.01	0.40 ± 0.06	0.17 ± 0.03	0.12 ± 0.04	0.10 ± 0.03	0.09 ± 0.02	40 ± 6	0.03
Retarded children	5	0.03 ± 0.01	0.35 ± 0.07	0.14 ± 0.03	0.11 ± 0.02	0.08 ± 0.02	0.08 ± 0.01	37 ± 6	0.03
Retarded children receiving dietary supplementation of B_{12}	5	0.06 ± 0.02	0.43 ± 0.13	0.19 ± 0.03	0.17 ± 0.05	0.13 ± 0.04	0.13 ± 0.04	35 ± 6	0.03

had significantly higher plasma B_{12} concentrations than did a control group. The control group of children had plasma B_{12} concentrations which were very similar to those reported for normal American adults by Rosenthal and Sarett ('52).

2. An attempt to saturate two children by repeated intravenous injections of vitamin B_{12} resulted in higher "resting" plasma levels of the vitamin. The percentage of the vitamin which was excreted after each test dose did not, however, rise significantly.

3. Plasma levels and urinary excretion of vitamin B_{12} after a test dose of the vitamin were compared in (a) children of retarded growth, (b) the same children after dietary B_{12} supplementation, (c) children with normal growth. No marked differences were observed between the groups in the urinary excretion of B_{12} or in the rate of removal of B_{12} from the plasma. The "resting" level of vitamin B_{12} in the plasma was, however, raised by the B_{12} supplementation.

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ANEMIA IN ALASKAN ESKIMOS

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THREE FIGURES

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Over a considerable area of Alaska, the Eskimo population is moderately anemic by the usual standards. The present study was designed to characterize this anemia, and determine its origin.

METHODS

All blood samples were taken from a finger tip. Hemoglobin was determined as oxyhemoglobin in 0.06 M ammonium hydroxide either with the Beckmann spectrophotometer (laboratory studies) or the Klett-Summerson photoelectric colorimeter (field studies). In the former method, standardization was by determination of total iron (Scott, '45). The extinction coefficient at 540 mμ was $E^{1\%}_{1\,cm} = 8.23$, assuming the iron content of hemoglobin to be 0.339%. The Klett-Summerson method was calibrated by the Beckmann method. Pipettes were calibrated with mercury. Hematocrit was determined by the Van Allen method, except that the Van Allen tubes were sealed with cement ("Pyseal") to avoid leakage. Serum iron was determined by the method of Burch et al. ('48) except that the volume of serum was 0.06 ml and the volume of isoamyl alcohol 0.15 ml. Copper was determined on 0.2 ml of serum after wet ashing, by the diethyldithiocarbamate method (Kirk, '50). Cell diameter was measured on stained smears with an ocular micrometer.

In a therapy experiment, adults were selected who had hemoglobin levels less than 14 gm/100 ml (men), or 12 gm/100 ml (women), who from recent x-rays did not have active tuberculosis, and who were not pregnant. These individuals were divided into three groups, each of which was asked to take one of the following daily for 6 weeks: 0.65 gm of ferrous sulfate, 0.66 gm of calcium lactate, or 100 mg of ascorbic acid. Each person was given a definite number of pills, and asked to return for more when his supply was gone. If he did return, it was assumed that the pills had been consumed.

RESULTS

The distribution of hemoglobin values of 715 Eskimo men is shown in figures 1 and 2. These men were members of the National Guard, and were between the ages of 17 and 50. In figure 1 the geographic distribution is shown. Each hemoglobin value in this figure is the average of 20 or more determinations, and the standard deviations of these averages ranged from 0.65 to 1.30. These people were divided into a northern and a southern group. Hemoglobin levels on 297 northern Eskimo men averaged 14.91 gm/100 ml with a standard deviation of 1.06, as measured about November 15th. Hemoglobin levels of 418 southern Eskimo men averaged 14.60 gm/100 ml with a standard deviation of 1.19 as measured about December 10th. By the same method the mean hemoglobin level of 110 white soldiers stationed near Anchorage was 15.76 gm/100 ml with a standard deviation of 1.23. Analysis of these results showed that the age of the subjects bore no relation to the hemoglobin level, and that tuberculosis (as suspected from x-ray diagnosis at this time) was not a significant factor. The values from any one locality were uniform, and low mean values were therefore not the result of severe anemia in a few individuals in an otherwise normal population. The lowest value found in these 715 men was 10.9 gm per 100 ml.

Further studies were concerned with 4 villages in southwestern Alaska, located within 60 miles of one another. Two

of these (villages 1 and 2) were selected because the results on National Guard personnel from these places indicated low mean hemoglobin levels, while in men from the other two, high

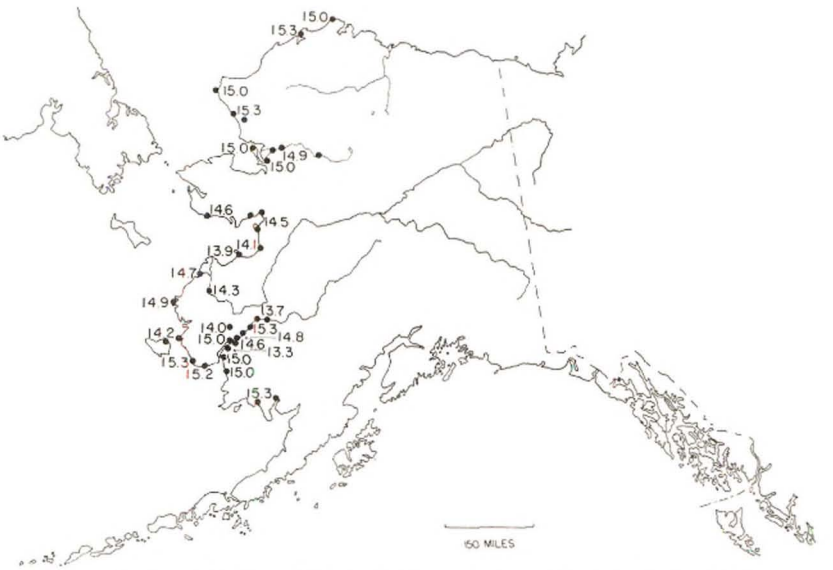

Fig. 1 Geographic distribution of mean hemoglobin levels of Eskimo men.

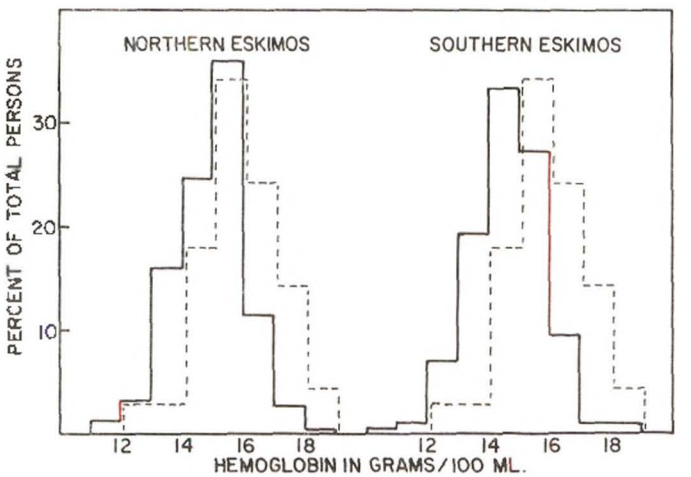

Fig. 2 Distribution of hemoglobin levels of Eskimo men. The dotted lines represent distribution of hemoglobin levels of a white soldier population in Anchorage.

TABLE 1

Hemoglobin levels of Eskimos in selected villages

	VILLAGE 1	VILLAGE 2	VILLAGE 3	VILLAGE 4
National Guard personnel:				
December 10	13.41 ± 0.14 (23)[1]	13.54 ± 0.22 (13)	14.96 ± 0.28 (17)	15.47 ± 0.39 (15)
February 1	13.85 ± 0.22	13.86 ± 0.30	14.84 ± 0.29	...
March 15	13.31 ± 0.17	...	...	13.33 ± 0.32 (5)
Adult males[2]	13.39 ± 0.12 (44)	13.89 ± 0.17 (39)	14.63 ± 0.19 (40)	12.83 ± 0.25 (19)
Adult females	11.39 ± 0.25 (39)	12.67 ± 0.31 (35)	13.14 ± 0.21 (33)	11.96 ± 0.19 (37)
School children	12.37 ± 0.18 (33)	12.00 ± 0.15 (28)	13.21 ± 0.17 (37)	11.79 ± 0.14 (30)
Pre-school children	10.97 ± 0.37 (19)	...	11.88 ± 0.46 (10)	...
Population	160	139	154	150

[1] Mean and standard error of the mean in gm/100 ml. Figures in parentheses are number of people tested. Unless otherwise specified, hemoglobin levels in villages 1, 2 and 3 were determined about February 1; in village 4, about March 15.

[2] Includes National Guard personnel.

mean levels had been found. A survey of the hemoglobin levels of individuals in the 4 villages was made with the results shown in table 1. In villages 1, 2 and 4, low mean hemoglobin levels were found in all age groups. The variation of hemoglobin level with age in these three villages is shown in figure 3. In village 3, all age groups had higher hemoglobin levels.

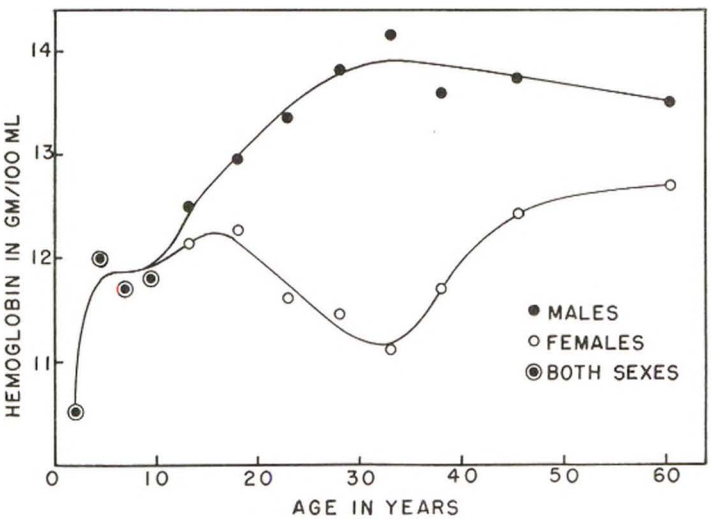

Fig. 3 Hemoglobin levels in Eskimos from selected villages at various ages.

For hematological study, groups of adults in villages 1, 2 and 4 were selected. The bases of selection and the results are shown in table 2. In some cases, adults with hemoglobin levels above 14 gm/100 ml (men) or 12 gm/100 ml (women) were not included, but so few people were excluded by these criteria, that the results differed little from unselected averages. The hematological data from villages 1 and 2 were very similar. In men, the anemia was moderate, with only a slight tendency toward microcytosis. In many women, the same situation was found. However, 15% of the women had hemoglobin levels below 10 and 6% had levels below 8 gm/100 ml. In all women with hemoglobin levels below 10, the anemia was definitely

TABLE 2

Hematology of adult Eskimos

	VILLAGE 1			VILLAGE 2		VILLAGE 4	
NUMBER TESTED	Females, selected [1]	Males, selected	Males, National Guard	Females, selected	Males, unselected [2]	Females, selected	Males, selected
	16	10	23	10	26	10	9
Mean age, years	27.2	48.2	28.2	34.0	28.2	33.5	37.1
Hemoglobin, gm/100 ml	11.4 ± 0.3	13.0 ± 0.2	13.3 ± 0.2	10.0 ± 0.7	12.7 ± 0.2	10.6 ± 0.3	12.7 ± 0.3
Red cells, millions/mm^3	4.41 ± 0.08	4.66 ± 0.17	4.83 ± 0.07	4.00 ± 0.11	4.61 ± 0.08	3.92 ± 0.11	4.66 ± 0.13
Hematocrit, %	33.7 ± 0.6	38.6 ± 1.3	39.7 ± 0.6	30.1 ± 1.3	38.8 ± 0.7	33.3 ± 0.9	40.1 ± 0.7
Mean corpuscular volume, μ^3	76.6 ± 1.4	83.9 ± 3.7	82.6 ± 0.4	75.2 ± 2.7	85.4 ± 1.6	85.3 ± 2.9	86.2 ± 1.6
Mean corpuscular hemoglobin, $\mu\mu$g	25.9 ± 0.7	28.2 ± 1.3	27.6 ± 0.5	24.9 ± 1.6	27.8 ± 0.6	27.4 ± 1.3	27.4 ± 0.6
Mean corpuscular hemoglobin concentration, %	33.9 ± 0.8	33.7 ± 0.7	33.5 ± 0.5	32.0 ± 1.5	32.7 ± 0.5	32.0 ± 0.5	31.8 ± 0.4
Cell diameter,[3] μ	6.93 ± 0.08	7.09 ± 0.10	7.26 ± 0.07	7.02 ± 0.08	7.17 ± 0.04	7.60 ± 0.10	7.44 ± 0.16
Serum inorganic iron, μg/100 ml	23.2 ± 4.6 [4]	29.9 ± 3.7 [4]	51.5 ± 5.9 [5]	27.5 ± 7.3 [4]	28.1 ± 2.9 [4]	52.6 ± 5.7 [5]	71.0 ± 6.7 [5]

[1] Persons who had active tuberculosis, who were pregnant, or who had hemoglobin levels above 14 gm/100 ml (men) or 12 gm/100 ml (women) were not included in the selected groups. All values are the mean and standard error of the mean.
[2] Included all males between the ages of 16 and 50 who did not have active tuberculosis.
[3] None of the subjects showed significant anisocytosis.
[4] Determined about February 1. Serum iron levels in village 3 at this time were 41.7 ± 8.0 (16 women) and 36.4 ± 7.6 (10 men).
[5] Determined about March 15.

microcytic and hypochromic. Village 4 differed from villages 1 and 2 in that there was little tendency toward microcytosis in either men or women. Serum iron levels in all villages were low. A low serum iron level appears to be a rather general phenomenon in Alaskan Eskimos and Aleuts, as shown in table 3. Serum copper was slightly elevated when compared with that of Anchorage whites.

The experiment in therapy was conducted in village 2. At the end of 6 weeks there was virtually no change in any of the three groups in hemoglobin level, cell count, hematocrit,

TABLE 3

Serum inorganic iron and serum copper of Alaskan Eskimos, Aleuts and whites
Values in μg/100 ml

	NUMBER OF DETERMINATIONS	INORGANIC IRON [1]	COPPER [1]
Northern Eskimos, male	44	80 ± 30	114 ± 45
Northern Eskimos, female	43	72 ± 35	157 ± 61
Southern Eskimos, male	40	61 ± 31	125 ± 33
Aleuts, male	38	89 ± 45	123 ± 24
Aleuts, female	37	79 ± 52	136 ± 24
Anchorage whites, male	50	112 ± 40	102 ± 37
Anchorage whites, female	49	98 ± 37	104 ± 34

[1] Mean and standard deviation.

cell size, cell hemoglobin content or cell diameter. The significant data are shown in table 4. One woman on iron therapy showed an increase in hemoglobin from 5.6 to 10.8 gm/100 ml, but no others changed significantly. Six out of 14 people on iron therapy showed a marked increase in serum iron level and the mean increase in this group appeared to be significant. However, during the same period there was an increase in serum iron in several individuals in villages 1 and 2. In village 1, this may have been due to an increased consumption of ptarmigan, which were generally eaten in March but unavailable earlier. Dietary changes in village 2, if they occurred, are not known.

DISCUSSION

Village 1 consisted of 160 people who lived in small frame or log houses. There were 36 houses with a total of 47 rooms. The mean floor space per family was 227 square feet or 51 square feet per person. Water was obtained from a river and fuel was wood cut on the day it was needed. Almost every family had a dog team, and all had boats with outboard motors. The estimated mean cash income was $1,170 per family per year. Sanitation was poor, but the people and houses were generally clean.

TABLE 4

Changes in hemoglobin, mean cell volume, and serum iron due to dietary supplementation

	TYPE OF SUPPLEMENTATION		
	Ferrous sulfate	Ascorbic acid	Calcium lactate
Number of subjects:			
Male	10	9	7
Female	4	2	2
Change [1] in:			
Hemoglobin, gm/100 ml	0.36 ± 0.48	−0.25 ± 0.22	0.26 ± 0.53
Mean cell volume, μ^3	−0.7 ± 3.1	−2.5 ± 2.8	2.0 ± 2.4
Serum inorganic iron, μg/100 ml	28.6 ± 11.2	11.8 ± 9.0	6.9 ± 5.5

[1] Mean and standard error of the mean.

In village 1, 65% of the adults had lived in or near the village all their lives and may have been related. The others were not so related, and some of these were part white.

In January, the inhabitants of village 1 were living on a diet composed of wheat flour, seal oil, fish and sugar. Individual intakes of food were not obtained. People did not have meals as such but ate as they pleased during the day from sour-dough pancakes, dried king salmon (*Oncorhynchus tschawytscha* Walbaum), uncooked frozen whitefish, and tea. Candy was a common food item. The fish was often dipped in seal, cottonseed or corn oil. Meat in the diet was welcome but unobtainable at this time. During the fall mink and

muskrat had been eaten, but at the time of this study rabbits were very scarce and ptarmigan not available.

All of the earlier inhabitants of village 2 with one exception were reported to have died in an epidemic in 1901. The present villagers came from widely scattered areas, with no common genetic background. Otherwise village 2 resembled village 1 in most respects. The only difference in diet was the consumption of uncooked pike (*Esox lucius* L.) in place of whitefish. Ptarmigan was available but seldom eaten. Village 2 was located only 6 miles from village 1.

Village 3 was located about 30 miles from village 2. The food resources of village 3 were not so limited as those in villages 1 and 2. In the spring seal was available, and in the fall, both moose and seal were eaten. During mid-winter however, neither was available to any extent. In villages 1 and 2, blackfish (*Dallia pectoralis* Bean) was eaten only as a last resort, but here it was the preferred fish. It was eaten whole and usually uncooked. Ptarmigan were available but not usually eaten.

Village 4 was about 20 miles distant from village 1 and differed from the others in several respects. Thirty-eight per cent of the adult inhabitants were descendants of a man and woman who moved there about 1870, while the origins of the rest were widely scattered. Families were larger than in other villages but the housing was much more adequate. Vegetable gardens were kept by several families, and vegetables, meat and fish were canned by some for winter use. Local potatoes were a common article of food during the winter. Moose were killed in the fall, and beaver was available during the trapping season. Most of the inhabitants spoke English, and had higher cash incomes than was the case elsewhere.

The results on National Guard personnel presented above showed that the hemoglobin levels of Alaskan Eskimo men were significantly lower than those of whites. The results on three of the 4 villages studied showed that the population of these communities as a whole had low hemoglobins. Al-

though all individuals in these villages might not be considered anemic by strict clinical standards, the low mean values combined with small standard deviations led to the conclusion that wide-spread moderate anemia was present in these areas.

In considering the possible cause of these low hemoglobin levels, it appeared that a racial or genetic explanation was possible. The evidence against this was as follows: (1) A considerable number of inhabitants of villages 1 and 2 were born in village 3, and yet the hemoglobin values of these were not higher than the average. (2) The hemoglobin level of residents of part white ancestry in these villages was not above average. (3) Atttempts to correlate hemoglobin level with known family relationships were unsuccessful. For example, the man in village 1 who had the highest hemoglobin level (15.4 gm/100 ml) had a mother, brother, and several sisters in village 2, but their hemoglobin levels were less than average for village 2.

Although diphyllobothriasis does occur in this area, it appeared to bear no relation to hemoglobin level. In February, stool examinations showed the prevalence of tapeworm in the population to be: village 1, 4%; village 2, 13%; and village 3, 4%. The hemoglobin levels of those persons with tapeworm did not differ from the average, and they did not show macrocytosis nor eosinophilia.

That the low hemoglobin level was nutritional in origin appeared indicated by the following facts: (1) The diets of these people were very restricted at the time of study. (2) School-children, all of whom received a hot breakfast, had more nearly normal hemoglobin levels than adults. The breakfast consisted of a hot dish of soup, beans, rice, or oatmeal. In addition crackers or bread, vitamin preparations and dried fruit were served in some cases. (3) A notably high hemoglobin level was observed in National Guard personnel from village 4 in December, which was in contrast to the hemoglobin levels of the rest of the population measured the following March. Most of these men, when tested in December, had but recently returned from working on the Pribilof

Islands, where they ate a high quality diet typical of most diets in the United States. Only a few of these same men could be reinvestigated in March, but of those that were reexamined, hemoglobin levels had fallen from 15 or 16 gm/100 ml to 12 or 13 gm/100 ml. Arguing against a nutritional origin of the low hemoglobin levels are the facts that the diet in village 3 did not appear to differ enough from those in villages 1 and 2 to explain the difference in hemoglobin level, and the difference found in the diet in village 4 had no discernible effect on hemoglobin level.

That iron deficiency was a factor in the low hemoglobin levels was indicated by the following observations: (1) In many cases, the anemia was microcytic and hypochromic. In persons with severe anemia in villages 1 and 2 (all were women) the red cells were definitely microcytic and hypochromic. In the same villages, cells of 50% of the women and 27% of the men were microcytic (mean cell volume $< 80\ \mu^3$). Five per cent of the men and none of the women showed macrocytosis (mean cell volume $> 100\mu^3$). (2) The serum iron level was uniformly low. (3) Iron therapy had an effect on serum iron level. On the other hand, the lack of effect of iron therapy on hemoglobin level or on cell size argues against this explanation. Furthermore, the diet was composed primarily of fish and enriched flour and, while low in ascorbic acid and calcium, should have had a fair iron content. The iron intake on any combination of available foods could scarcely have been less than 10 mg per day. The availability of this dietary iron is of course unknown.

It appeared, however, that iron deficiency was not a sufficient explanation for the low hemoglobin levels observed. The evidence supporting this view was as follows: (1) Many individuals with moderate anemia did not show microcytosis or hypochromia, and a few tended toward macrocytosis. (2) Anemia in women, who would be expected to have a higher iron requirement than men, was more typical of iron deficiency than that in men. Mean cell volume in 41 women studied had a low but significant positive correlation with hemoglobin

level ($r = 0.357$) but this was not the case in 73 men ($r = 0.098$). Iron thus appeared to be a limiting factor for only part of the population. (3) Even after allowance was made for lack of strict control in the iron therapy experiment, there was so little effect of iron supplementation that iron did not appear to be a limiting factor in all cases. (4) In village 4, anemia in women was as severe as in villages 1 and 2, but the red cells were more nearly normal in size and hemoglobin content. Thus iron deficiency appeared to be of less consequence in village 4.

The simplest explanation consistent with these results appeared to be that iron deficiency and another factor were responsible for low hemoglobin levels found in Eskimos. The second factor may cause the wide-spread, moderate normocytic anemia, while in those cases where iron requirement is high, an iron deficiency anemia is superimposed. However, no direct evidence of a second factor has as yet been obtained.

SUMMARY

A moderate anemia occurs in Eskimos in a considerable area of Alaska. The anemia is often microcytic and hypochromic, but a considerable proportion of people, mostly men, have a moderate normochromic, normocytic anemia. Severe anemia was found only in women, and was definitely microcytic and hypochromic. Serum inorganic iron levels were uniformly low in mid-winter. Iron therapy over a 6-week period was effective in increasing serum iron in some cases, but was not effective in raising hemoglobin level except in one severely anemic woman.

The data suggest that iron deficiency and some other factor are responsible for this condition.

ACKNOWLEDGMENT

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RELATION OF THIAMINE TO REPRODUCTION IN THE RAT[1]

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For many years it has been known that thiamine deficiency in the rat suppresses the estrous cycle and results in gonadal hypofunction. As early as 1922 Evans and Bishop reported the immediate cessation of estrous cycles in rats placed on vitamin B-deficient diets. Ueno ('34) and Coward, Morgan and Waller ('42) have mentioned briefly the occurrence of reproductive disturbances in rats deficient in vitamin B or B_1, but no studies on reproduction during B_1-deficiency have been made in which purified diets, otherwise nutritionally complete according to present standards, have been used. The present communication reports (1) the effects of improved thiamine-deficient diets on reproductive performance, (2) the relation of inanition to the reproductive disturbances of thiamine-deficient rats and (3) the relation of the ovarian hormones, estrone and progesterone, to such reproductive disturbances.

EXPERIMENTAL

Normal female rats of the Long-Evans strain, 90 to 100 days of age and averaging 225 gm in body weight, were bred with normal males and placed on the thiamine-deficient diet

[1] Presented in part before the 18th Annual Meeting of the American Institute of Nutrition, April, 1954 (Nelson and Evans, 1954b).

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from the day of breeding to the end of gestation.[2] Other groups of animals were placed on the deficient diet for 6 to 22 days before breeding and continued on the same diet throughout the gestation period. Control groups received the thiamine-deficient diet for two or three weeks before breeding and then were given the thiamine-supplemented diet throughout gestation. Ten to 12 animals were used for each group and all experiments were repeated at least once. Vaginal smears were examined daily during gestation for the presence of erythrocytes, the sign of implantation; the rats were weighed at regular intervals. Autopsies were carried out a few hours after parturition had taken place, unless weight loss or other signs indicated that resorption had occurred; in such cases the autopsies were usually performed on day 22 or 23 of gestation. At autopsy the uterus was examined to determine whether the implantation sites were normal or in the process of resorption. All animals that died before the end of the gestation period were likewise autopsied and the fetuses examined for viability or signs of beginning resorption. In order to differentiate between the effects of the deficiency on placental and fetal development and those effects on the maternal organism presumably unrelated to pregnancy,[3] rats dying before parturition were considered to have litters if the dead fetuses found at autopsy exhibited no signs of resorption; these fetuses are recorded in the tables as dead young.

Two thiamine-deficient diets, differing principally in the level of vitamin supplementation, were used in this study. Since few differences in groups receiving these two diets were noted, the data have been combined. The basal thiamine-deficient diet was composed of 24% alcohol-extracted casein, 64% sucrose, 8% hydrogenated cottonseed oil,[4] and 4% salts [5];

[2] The day that sperm is found in the vaginal smear is called the day of breeding and is considered to be day zero.

[3] The rate of death for unbred rats of the same age given the thiamine-deficient diet was similar to that observed for pregnant rats.

[4] Crisco or Primex.

[5] Salts no. 4 of Hegsted et al., ('41).

the level of vitamin supplementation per kilogram of diet was 300 μg biotin, 5 mg 2-methyl-1,4-naphthoquinone, 5 mg pyridoxine HCl, 5.5 mg pteroylglutamic acid, 10 mg riboflavin, 20 mg niacin, 50 mg D-calcium pantothenate, 400 mg inositol, and 1.0 gm choline chloride. In the second thiamine-deficient diet the levels of all vitamins except choline were doubled, 50 μg crystalline vitamin B_{12} added per kilogram diet, and 0.5% L-cystine substituted for an equivalent amount of sucrose. Control animals were given one of the above diets supplemented with 5 or 10 mg of thiamine HCl per kilogram of diet. All rats received weekly a fat-soluble vitamin mixture containing 800 I.U. vitamin A, 115 chick units vitamin D,[6] 6 mg synthetic DL-alpha-tocopherol, and 650 mg corn oil.[7]

RESULTS

Table 1 summarizes the reproductive performance of rats placed on the thiamine-deficient diets for varying periods of time. When the deficient diets were started on the day of breeding all rats littered but 21% of the young were dead. The living young averaged only 4 gm in birth weight in comparison with control values of 6 gm or more. The mothers gained an average of 14 gm in body weight during gestation in contrast to the average gain of 114 gm for thiamine-supplemented controls. Starting the deficiency 6 to 8 days before breeding accentuated the deleterious effects, resulting in a higher incidence of dead young, a lower birth weight of living young, and a maternal weight loss of 33 gm during gestation. In addition, 26% of the mothers died during the latter half of the gestation period.

When the thiamine-deficient diets were given still earlier before breeding, 11 to 15 days, 89% of the animals resorbed. Such animals exhibited an early onset of the placental sign, erythrocytes in the vaginal smear, on days 10 to 11 rather than the normal time of appearance, on days 12 to 13. More-

[6] Furnished by high-potency Sardilene (Booth Fisheries, Seattle, Washington) containing 15,000 I.U. vitamin A and 2,500 units vitamin D per gram.

[7] Mazola.

over, the resorption of the products of conception progressed so rapidly that only minute traces of implantation sites could be found at the end of gestation. The maternal weight loss was severe, averaging 76 gm. When the deficient diets were started as early as 16 to 22 days prior to breeding, more than half of the animals failed to show any signs of implantation, either by the placental sign or by implantation sites at au-

TABLE 1

Effect of thiamine deficiency on reproduction in the rat

DEFICIENCY PRIOR TO BREEDING	RATS BRED	WT. CHANGE DURING GESTATION	ONSET VAGINAL EBC	IMPLANTATIONS	RESORPTIONS OF IMPLANTATIONS	LITTERS OF IMPLANTATIONS	MATERNAL MORTALITY	YOUNG	
								Av. wt.	Dead
days	*no.*	*gm*	*day*	*%*	*%*	*%*	*%*	*gm*	*%*
Thiamine-deficient throughout gestation									
0	32	+ 14	12.3	100	0	100	3	4.0	21
6–8	31	— 33	12.8	100	3	97	26	3.1	56
11–15	30	— 76	10.6	93	89	11	7	2.0	21
16–22	32	— 87	9.1	44	100	0	53		
Thiamine-supplemented throughout gestation									
16–22	22	+ 114	13.0	100	0	100	0	6.5	6

topsy. All animals that implanted exhibited an exceedingly early onset of vaginal erythrocytes, day 9, indicating very early fetal death and resorption. Maternal mortality during the latter half of the gestation period was 53%, the highest observed in any of the deficient groups.

The young from mothers receiving the thiamine-deficient diets were small in size and weight but showed no specific signs of thiamine-deficiency. No congenital abnormalities were observed in the 650 young from these mothers except for one instance of umbilical hernia.

The use of the second thiamine-deficient diet, which contained increased vitamin supplementation and additional cystine, appeared to have no beneficial effects on reproductive performance except for a reduced incidence of dead young.

Comparison of food restriction and absence of dietary thiamine

To determine the relation of undernutrition or inanition to reproductive disturbances in animals given the thiamine-deficient diets, 4 successive experiments using pair-fed controls were carried out. A deficiency period of 9 to 12 days prior to breeding was chosen as the one most likely, in accordance with the preceding experiments, to result in approximately 50% litters. Deficient animals were paired in groups of three the day of breeding on the basis of body weight and length of deficiency. One-third of the animals were continued on the thiamine-deficient diet and their food intake measured daily throughout gestation; one-third received the thiamine-supplemented diet in the same amount as was consumed by a corresponding deficient rat on the same day of gestation; and the remaining rats received the thiamine-supplemented diet ad libitum throughout gestation. In one experiment a high level of thiamine, 10 mg per kilogram of diet, was used for the pair-fed control group and in another experiment all the crystalline vitamins were doubled and vitamin B_{12} added to the diet for the pair-fed group. Five rats per group were used in each experiment.

In the first three experiments all rats were autopsied at the termination of gestation, either by parturition or by maternal death. Table 2 presents the combined data for these experiments. As expected, the thiamine-deficient group had approximately 50% litters with a high incidence of dead young and subnormal birth weights for the living young. The mothers lost an average of 58 gm during the gestation period, and maternal mortality was 27%. The average daily food intake was only 5.2 gm per rat. The reproductive performance of the pair-fed control group receiving the thiamine-supplemented diet throughout gestation was similar to that of the thiamine-deficient group. Pregnancy was maintained to a slightly greater extent, 71%; there were fewer dead young and slightly heavier birth weights for the living young; the

maternal weight loss was the same, but maternal mortality was higher, 60%. The average daily food intake was also slightly higher because of earlier maternal death in this group than in the thiamine-deficient group. These results are in marked contrast to the normal reproductive performance of paired rats receiving the thiamine-supplemented diet ad libitum throughout the gestation period.

TABLE 2

Relation of food restriction to reproduction of thiamine-deficient rats[1]

EXP.[1] GROUP	RATS BRED	AV. DAILY FOOD INTAKE	WT. CHAGE DURING GESTATION	IMPLANTATIONS	RESORPTIONS OF IMPLANTATIONS	LITTERS OF IMPLANTATIONS	MATERNAL MORTALITY	YOUNG	
								Av. wt.	Dead
	no.	*gm*	*gm*	*%*	*%*	*%*	*%*	*gm*	*%*
Autopsied at parturition or death									
Thiamine-deficient	15	5.2	— 58	100	47	53	27	3.0	90
Thiamine-supplemented pair-fed	15	5.6	— 53	93	29	71	60	3.6	62
Thiamine-supplemented ad libitum	10	17.7	+ 115	100	0	100	0	6.2	0
Autopsied day 13 of gestation									
Thiamine-deficient	5	5.8	— 34	100	40	60	..	..	..
Thiamine-supplemented pair-fed	5	5.8	— 36	100	60	40	..	..	..
Thiamine-supplemented ad libitum	5	15.1	+ 46	100	0	100	..	..	..

[1] All groups averaged 9–12 days of deficiency prior to breeding.

In the 4th paired-feeding experiment all animals were autopsied 13 days after breeding to determine whether pregnancy was maintained in the pair-fed control animals for the first half of gestation. Table 2 shows that living young were found in 40% of the thiamine-deficient rats and in 60% of the pair-fed control animals. The loss of maternal body weight was again the same for the thiamine-deficient and the pair-fed control groups.

Effect of ovarian hormones in the absence of dietary thiamine

The reproductive disturbances observed in animals given the thiamine-deficient diet have characteristics similar to those previously observed in animals given a protein-free diet (Nelson and Evans, '53) or a vitamin B_6-deficient diet (Nelson and Evans, '51), namely the early onset of vaginal erythrocytes and a rapid resorption of implantation sites. In addition, unbred rats maintained on the thiamine-deficient diet exhibited an early loss of the estrous cycle, likewise observed in animals given either the protein-free or vitamin B_6-deficient diets. These characteristics suggested the possibility of similar hormonal deficiencies in the three dietary deficiencies. Previous studies have demonstrated that daily injection of estrone and progesterone will maintain pregnancy in animals on a protein-free diet (Nelson and Evans, '54a) and on a vitamin B_6-deficient diet (Nelson, Lyons and Evans, '51), as well as in rats hypophysectomized and oophorectomized after breeding (Lyons, '43). Therefore, these synthetic hormones were tested both singly and combined in thiamine-deficient rats.

For these experiments a deficiency period of 11 to 15 days prior to breeding was used to insure the occurrence of fetal death and resorption in 80 to 100% of the deficient rats not receiving the hormones. All rats were autopsied 13 days after breeding in order to avoid the maternal mortality previously observed during the latter part of gestation. Subcutaneous injections of the hormones or of the solvent, sesame

oil, were given from day three to day 12 of gestation. Ten to 12 animals were used for each experimental group.

Table 3 shows that only 18% of the untreated thiamine-deficient rats had living young at autopsy on the 13th day of gestation. The early onset of vaginal erythrocytes on day 10 and the maternal weight loss of 42 gm are in agreement

TABLE 3

Maintenance of pregnancy in thiamine-deficient rats with ovarian hormones[1]

HORMONE DOSAGE	RATS BRED	WT. CHANGE DURING GESTATION	ONSET VAGINAL RBC	IMPLANTATIONS	RESORPTIONS OF IMPLANTATIONS	LITTERS OF IMPLANTATIONS	IMPLANT SITES PER RAT	LIVING FETUSES PER LITTER[2]
per day	*no.*	*gm*	*day*	*%*	*%*	*%*	*no.*	*no.*
None[3]	50	— 42	10.4	100	82	18	9.6	10.6 (9)
Estrone 1–3 μg	34	— 43	10.3	100	65	35	8.9	8.6 (12)
Progesterone 4–6 mg	20	— 40	11.5	100	20	80	10.3	7.1 (16)
Estrone plus progesterone[4]	23	— 40	11.8	100	4	96	11.2	10.1 (22)

[1] All groups averaged 11 to 15 days of deficiency prior to breeding. Injections were given subcutaneously from days 3 to 12 and the rats were autopsied day 13 of gestation.

[2] The number of litters on which the average is based is given in parentheses.

[3] Half of these animals were injected with the solvent, sesame oil.

[4] One group of 11 rats received injections of 0.5 μg estrone plus 4 mg progesterone; the other group was given daily 1 μg estrone plus 4 mg progesterone.

with observations on other thiamine-deficient groups with similar periods of deficiency prior to breeding but autopsied at the end of gestation (see table 1). The daily injection of *1, 2, or 3 μg of estrone* resulted in living young in 35% of the animals; only 8% of the corresponding uninjected group had living young. When *4 or 6 mg of progesterone* were given daily, pregnancy was maintained in 80% of the animals. The 6 mg level was more beneficial, resulting in

maintenance of pregnancy in 90% of the group. The number of living young per litter averaged 7.1, being slightly below normal values of 9 or more per litter. The combination of *0.5–1.0 μg of estrone and 4 mg of progesterone* maintained pregnancy in 96% of the animals; the number of living young per litter was normal, namely, 10.1 young. Food intake was measured for one group injected with estrone and progesterone, the intake averaging 5.0 gm daily per rat in comparison with a daily average of 5.9 gm for uninjected animals matched in body weight. Thus, maintenance of pregnancy in thiamine-deficient rats by the combination of hormones was not due to any increase in food intake. It may be noted that the loss in maternal body weight during the first 13 days of gestation was approximately 40 gm for all groups, regardless of the outcome of pregnancy.

DISCUSSION

The data presented in this study show that thiamine is essential for normal reproductive performance in the rat. Instituting the vitamin deficiency for only short periods before breeding, namely one to three weeks, resulted in marked reproductive disturbances as shown by subnormal birth weight or death of the young, early fetal death and resorption, or failure of implantation.

The failure of pair-fed controls to exhibit normal reproductive performance demonstrates that decreased food intake is an important factor in the reproductive upsets produced by the thiamine-deficient diet. All studies reported by other investigators have likewise concluded that undernutrition was concerned in the gonadal hypofunction resulting from this vitamin deficiency, e.g. Drill and Burrill ('44). The decrease in food intake during pregnancy was considerably greater than that observed in any other dietary deficiency previously studied in this laboratory. The thiamine-deficient rats averaged only 29% of the food intake of thiamine-supplemented controls eating ad libitum throughout gestation. This value may be contrasted with the average of 56% for

animals on a protein-free diet (Nelson and Evans, '53) or on a vitamin B_6-deficient diet (Nelson and Evans, '51), the average of 69% for pantothenic acid deficiency (Nelson and Evans, '46), and of 76% for pteroylglutamic acid deficiency (Nelson and Evans, '49). In all of these dietary deficiencies with a higher average food intake it has been possible to eliminate food restriction as a cause for the reproductive disturbances observed with the deficient diets. The necessity of increasing the dietary protein level to 30% to insure normal reproductive performance in pair-fed controls for rats on the protein-free diet indicates, however, the possibility of an induced protein deficiency when food consumption is less than 56% of the normal intake during pregnancy.

The maintenance of pregnancy in thiamine-deficient rats by the synthetic ovarian hormones, estrone and progesterone, demonstrates the inhibition of maternal sex hormone production in this vitamin deficiency. This inhibition may be due to (1) inadequate pituitary production and secretion of the gonadotrophic hormones (follicle-stimulating hormone, interstitial-cell stimulating or luteinizing hormone, and lactogenic or luteotrophic hormone); (2) inadequate ovarian production and secretion of estrin and progestin with normal gonadotrophic stimulation; (3) inadequate placental production and secretion of gonadotrophins. It is also possible that increased destruction or excretion of pituitary, placental or ovarian hormones may occur.

The sex hormonal inadequacies of thiamine-deficient rats are similar to those of rats given a protein-free or vitamin B_6-deficient diet and to those of rats hypophysectomized and oophorectomized after breeding in that pregnancy can be maintained in all 4 conditions by the daily injection of both estrone and progesterone. Measurement of food intake has demonstrated that the maintenance of pregnancy by the combination of hormones in thiamine-deficiency, as well as in protein deficiency, is not due to an increased food intake but, on the contrary, is associated with a slightly decreased food intake. The thiamine-deficient rats further resemble

protein-deficient animals in that pregnancy can be maintained in either deficiency by progesterone alone, thus indicating the presence of some circulating estrin in the majority of animals on either dietary regimen. In contrast, progesterone alone is ineffective in maintaining pregnancy in vitamin B_6-deficient rats.

Animals maintained on the three deficient diets during pregnancy differ markedly in their response to estrone. The injection of moderate levels of estrone, 1 to 3 μg, maintained pregnancy in 60 to 80% of the protein-deficient animals but in only 35% of the thiamine-deficient animals. It has previously been suggested (Nelson and Evans, '54a) that such beneficial effects of estrone may probably be interpreted as an indirect effect of the hormone in stimulating the pituitary production and secretion of luteotrophin which, in turn, stimulates the ovarian production of progestin. The limited effects of estrone in thiamine-deficient rats demonstrate a response, whether by the pituitary or by the ovary, that the vitamin B_6-deficient rat does not exhibit when injected with the 1 μg level. Thus, the sex hormonal inadequacies of rats given thiamine-deficient diets are slightly greater than those of animals on a protein-free diet but somewhat less than those of rats maintained on a vitamin B_6-deficient diet.

SUMMARY

Reproduction has been studied in adult female rats placed on thiamine-deficient diets the day of breeding or for one to three weeks prior to breeding. Instituting thiamine deficiency on the day of breeding or one week prior to breeding resulted in 97 to 100% litters; 21 to 56% of the young were found dead and the living young were subnormal in birth weight. The mothers gained only slightly or lost weight during the gestation period and 3 to 26% died during the latter half of pregnancy. Increasing the deficiency period to 11 to 22 days before breeding resulted in 89 to 100% resorptions or in failure of implantation. Marked losses in maternal body

weight and increased maternal mortality also occurred with the increased length of deficiency.

The failure of pair-fed controls to exhibit normal reproductive performance demonstrated that restriction in food intake was related to the reproductive disturbances produced by the thiamine-deficient diet. The food intake for thiamine-deficient animals averaged 29% of that for ad libitum controls, a degree of food restriction greater than that observed for any other dietary deficiency previously studied during pregnancy.

The synthetic ovarian hormones were tested for maintenance of pregnancy during the first 13 days of gestation in thiamine-deficient rats with 11 to 15 days of deficiency prior to breeding. Daily injection of 1 to 3 μg of estrone resulted in living young in 35% of the animals, whereas only 18% of the uninjected rats had living young. Injection of 4 to 6 mg of progesterone resulted in living young in 80% of the animals, and the combination of 0.5 to 1.0 μg of estrone with 4 mg of progesterone maintained pregnancy in 96% of the thiamine-deficient rats. Pregnancy was maintained by this combination of hormones notwithstanding the usual marked reduction in food intake.

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THE TOXICITY OF CORN GERM TO THE MEAL WORM, TENEBRIO MOLITOR[1,2]

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INTRODUCTION

During an investigation of the nutritive value of plant proteins for the mealworm, *Tenebrio molitor,* a growth inhibition was observed in larvae fed basal diets containing whole ground corn. Unlike the amino acid imbalance inducible in rats by a corn-casein-sucrose ration (Krehl, '49), additions of tryptophan and nicotinic acid failed to stimulate growth in this insect. Additional supplementation of the corn rations with high quality protein or specific amino acids and vitamins known to be limiting in maize was also without beneficial effect on *Tenebrio.* In further contrast to findings with vertebrates (Krehl et al., '46; Mitchell, Hamilton and Beadles, '49) it was observed that the larvae grew at increased rates on diets containing autoclaved corn. In view of the general interest in corn as a food for man and other animals, a study was made of this phenomenon.

The relatively small food requirement of *Tenebrio* made it possible to feed those regions of the unprocessed corn seed that could be obtained by manual dissection. This technique led to the discovery of a heat labile, extractable toxic substance in the germ or embryo region of the kernel. Since poor larval development persisted in the presence of those

[1] The data reported in this paper were taken from a thesis submitted by the senior author to the Graduate College of the University of Illinois in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[2] Supported in part by a grant from the United States Public Health Service.

nutrients which usually benefit animals fed large amounts of corn, and since Fraenkel and Stern ('51) demonstrated that *Tenebrio* larvae were unable to substitute dietary tryptophan for nicotinic acid, it would appear that the toxicity of corn germ to the mealworm was not related to any corn-induced growth retardation previously reported.

EXPERIMENTAL

Ten first-instar *Tenebrio* larvae were reared on three grams of diet as described by Fraenkel, Blewett and Coles ('50). The diets were ground to 30 mesh and the experiments were run in duplicate. Each feeding trial was performed under conditions of controlled temperature and humidity, usually 30°C. and 70%, respectively. The animals were weighed at regular intervals and the average weight was obtained by dividing the total weight of the larvae in each vial by the number of animals weighed. This procedure does not lend itself readily to statistical analysis, but since the weight differences which formed the basis of the experimental findings were considerably greater than those in vertebrate studies, the use of the group weighing method was justified. In addition, the time gained by this procedure permitted the execution of feeding trials with as many as 150 variables per experiment.

All diets described in this paper were fortified with the micronutrients necessary for the growth of *Tenebrio* larvae at levels three times in excess of the minimum requirement for normal growth and development (Fraenkel, Blewett and Coles, '50). These included, per gram of diet: McCollum-Davis 185 salts mixture, 20 mg; cholesterol, 10 mg; choline Cl, 500 µg; i-inositol, 250 µg; nicotinic acid,[3] 50 µg; thiamine HCl, 25 µg; Ca pantothenate, 25 µg; riboflavin, 12.5 µg; pyridoxine HCl, 12.5 µg; L-carnitine, 5 µg; folic acid, 2.5 µg; biotin, 0.5 µg. The term "basal" as used in this paper refers to such additions.

[3] Additional doses of nicotinic acid are designated as "extra" nicotinic acid.

The corn used in this study was a yellow commercial hybrid assaying 9.9% protein (N × 6.25). Dissected germ from such corn contained 28% protein after de-fatting. Labco vitamin-free casein [4] from the specific batch tested in the Rutgers collaborative amino acid determination (Rutgers University, '46-'50) was used. Wilson's B protein [5] is prepared commercially from acetone-extracted mammalian nerve tissue.

Dissection of the kernel into its components, namely germ, bran, starchy endosperm and horny endosperm, was facilitated by soaking the kernel in water overnight at room temperature. Most of the feeding trials to be discussed were performed with only two kernel regions, the germ, and the residue of the kernel. This residue is termed germ-free corn in the text and consisted of the bran and endosperm. Excision of the germ from the germ-free residue was accomplished with a scalpel after soaking overnight at 4°C. or by dissection of the dry, unsoaked kernel.

The ground germ was de-fatted in a soxhlet extractor with ethyl ether or hexane for 48 hours. In one experiment the whole ground kernel and germ-free residue were also de-fatted. All autoclaving operations were carried out on the dry, ground product at 15 pounds pressure, 121°C., for 30 minutes.

RESULTS

Series I. Diets not fortified with protein

The nutritive value of various raw and autoclaved cereals for the mealworm is presented in table 1, experiment 1. Leaving the question of the relative biological value of the maize proteins for future discussion, it is still evident that larval growth and survival was extremely poor on the raw corn regimen. Autoclaving the corn produced a 7-fold increase in the body weight, corn being the only cereal so improved.

In experiment 2, table 1, the presence of a growth inhibitor in the germ region of the kernel was demonstrated. The addi-

[4] The Borden Co., New York, N. Y.

[5] The Wilson Co., Chicago, Ill.

tion of 27% of germ, bran, starchy or horny endosperm to basal diets containing 70% of autoclaved corn resulted in 85% mortality and depressed weight gain in the case of the heated corn-plus-germ ration. None of the other fractions retarded growth to a greater extent than 27% of whole raw corn. Borrow et al. ('48) reported that the addition of corn

TABLE 1

Effect of whole corn and corn germ on Tenebrio larvae fed natural diets without supplementary protein

(All tests started with 20 animals per test)

EXP. NO.	DIET	NUMBER OF LARVAE SURVIVING [1]	AVERAGE WEIGHT
			mg
1	Whole wheat basal [2]	18	32.7
	Heated whole wheat basal	19	35.4
	Barley basal	19	30.6
	Heated barley basal	20	25.7
	Oats basal	20	35.9
	Heated oats basal	20	30.1
	Rye	19	28.7
	Heated rye basal	19	24.9
	Corn basal	11	1.8
	Heated corn basal	16	14.0
2	Corn basal	7	3.2
	Heated corn basal	13	12.9
	77% Heated corn + 27% corn basal	15	6.4
	77% Heated corn + 27% starchy endosperm basal	16	5.8
	77% Heated corn + 27% horny endosperm basal	15	15.9
	77% Heated corn + 27% bran basal	13	15.0
	77% Heated corn + 27% germ basal [3]	3	3.4
3	90% Germ-free corn + 7% germ basal	19	19.6
	90% Germ-free corn + 7% heated germ basal	19	43.2
4	93% Whole wheat basal [4]	19	33.0
	87% Whole wheat + 6% germ basal	18	15.3
	87% Whole wheat + 6% heated germ basal	19	47.6

[1] In experiment 1 the larvae were weighed at 7 weeks, in experiments 2 and 4 at 8 weeks and in experiment 3 at 9 weeks.

[2] All diets contain micronutrient mixture described on page 166.

[3] Germ in experiment 2 only, not de-fatted.

[4] Four per cent ether-extractable corn fat added to diets of experiment 4.

bran to diets marginal with respect to niacin or niacin precursors, inhibited the growth of rats. With *Tenebrio*, however, the bran ranked high in nutritive value among the kernel tissues tested.

Experiments 3 and 4 of table 1, showed the ability of raw, de-fatted corn germ to inhibit growth when fed at a level similar to that occurring in the maize seed. The addition of raw germ, but not autoclaved germ, to diets containing

TABLE 2

Effect of germ supplements on Tenebrio larvae fed semi-purified diets

(All tests started with 20 animals per diet)

EXP. NO.	DIET	NUMBER OF LARVAE SURVIVING [1]	AVERAGE WEIGHT
			mg
5	Casein (20%) + glucose basal [2]	20	64.4
	Casein (20%) + glucose + 3% germ basal	14	25.5
	Casein (20%) + glucose + 3% heated germ basal	17	48.0
6	Wilson's "B" (20%) + glucose basal	19	45.4
	Wilson's "B" (20%) + glucose + 5% germ basal	15	19.4

[1] Survival and weight recorded after 9 weeks.

[2] Diets of this table contain casein or Wilson's "B" protein, 20%; glucose, 77%; micronutrients, 3%. Additions at the expense of glucose.

wheat, the natural food of this insect, or germ-free corn, produced lowered rates of weight gain in the larvae.

Additional experiments with diets containing no supplementary protein showed that out of 5 yellow and one white variety tested, all could be markedly improved by autoclaving. Lime treated corn, which stimulated the growth of rats reared on niacin- and tryptophan-deficient diets (Laguna and Carpenter, '51), failed to improve weight gain in *Tenebrio*.

Series II. Studies with semi-purified diets

The unequivocal demonstration of toxicity on the part of corn germ depended on the ability of this material to check growth on diets whose composition was better defined

than the crude rations presented in table 1. Furthermore, the relatively poor rates of development on heated corn diets in which the presumed inhibitor was to a greater or lesser extent inactivated, pointed to numerous unanswered questions regarding both the relative digestibility and the biological value of the nutrients in the raw and autoclaved maize rations. In table 2 it is shown that the addition of 3 to 5% raw, de-fatted germ inhibited growth on semi-purified diets containing no other corn or corn fractions. It can also be seen that in the absence of raw germ the casein-glucose [6] and Wilson's B protein-glucose basal rations exhibited digestibility and food value of a high order as evidenced by the good growth rates on these diets. The demonstration of the toxicity of corn germ, therefore, was not dependent upon the presence of a large amount of corn protein or upon a treatment which increased the digestibility of the ration.

Series III. Studies with germ-free corn and germ

A necessary adjunct to the experiments described in series II, was the determination of the nutritive value of whole corn and germ-free corn with basal diets containing an adequate supply of high quality protein. Table 3, experiment 7, shows that in the presence of 20% casein, growth was significantly better on diets containing autoclaved corn or germ-free corn than on raw corn. When 6% germ was added to the ration containing germ-free corn a depression in the growth rate occurred which in this case, could be entirely alleviated by autoclaving the germ.

The inclusion of autoclaved germ, but not raw germ, in basal diets containing germ-free corn was shown to exert a beneficial effect on the rate of larval development in experiment 3 of table 1. It would appear from these data that

[6] Since these experiments were run, a change in the purity of the vitamin-free casein has been noted with the result that *Tenebrio* no longer develop at optimal rates on the casein-glucose-micronutrients ration. The toxic nature of corn germ has been demonstrated a sufficient number of times with casein purchased before 1953 to warrant presentation of the data in table 2.

inactivation of the toxic principle in the embryo tissue permitted the protein of high biological value which is present in the germ (Mitchell and Beadles, '44; Jones and Widness, '46) to supplement the zein-rich proteins of the endosperm.

TABLE 3

Effect of lysine, tryptophan and casein on Tenebrio larvae fed corn and germ-free corn

(All tests started with 20 animals per diet)

EXP. NO.	DIET	NUMBER OF LARVAE SURVIVING [1]	AVERAGE WEIGHT
			mg
7	20% Casein + 77% corn basal [2]	9	29.3
	20% Casein + 77% heated corn basal	18	39.5
	20% Casein + 77% germ-free corn basal	20	63.5
	20% Casein + 71% germ-free corn + 6% germ basal	19	43.0
	20% Casein + 71% germ-free corn + 6% heated germ basal	20	67.3
8	97% Corn basal	9	7.1
	96% Corn + .23% L-lysine and L-tryptophan basal	14	13.8
	97% Heated corn basal	16	37.4
	96% Heated corn + .23% L-lysine and L-tryptophan basal	18	50.1
	97% Germ-free corn basal	19	21.3
	96% Germ-free corn + .23% L-lysine and L-tryptophan basal	15	80.5
	90% Germ-free corn + .23% L-lysine and L-tryptophan + 6% germ basal	17	41.2

[1] In experiment 7 the larvae were weighed after 8 weeks; in experiment 8 after 9 weeks.

[2] All diets contain micronutrients described on page 166.

That this is apparently the case is evident from experiment 8, table 3, where it is shown that 0.23% of L-lysine and 0.23% of L-tryptophan improved the growth rate in a highly significant manner. This improvement was clearly manifest when autoclaved corn or germ-free corn was present in the ration, but to a much lesser extent in the case of whole corn or its equivalent in the form of raw germ plus germ-free maize.

The data of experiments 5, 7 and 8 indicated that the harmful effect of germ was not always completely reversible by autoclaving. In experiment 5, growth on the diet containing heated germ was still somewhat inferior to that on the control without germ. In experiments 7 and 8, the addition of casein or lysine and tryptophan to autoclaved corn resulted in a growth rate significantly below that sustained by these supplements in the presence of germ-free corn. No doubt exists, however, concerning the beneficial nature of the heat treatment on the food value of the germ.

In other feeding trials it was observed that amounts of L-lysine and tryptophan up to 1% of the diet, and "extra" nicotinic acid at the level of 200 µg/gm could not reverse the effect of the toxic material in the germ. Other amino acids such as threonine and methionine (Sure, '53) and valine and isoleucine (Sauberlich, Chang and Salmon, '53) had no beneficial influence on the growth of larvae receiving corn or germ-free corn basal diets fortified with lysine and tryptophan.

Rosen, Huff and Perlzweig ('47), working with rats, and Sundaram and Sarma ('53) using an insect, demonstrated that adequate levels of pyridoxine were required for proper utilization of dietary tryptophan. The possibility that the corn germ inhibitor functioned through an antagonism to pyridoxine was eliminated when it was observed that massive doses of this vitamin over and above that already present in the B-vitamin mixture were devoid of any beneficial effect on larvae fed basal rations containing either corn plus casein or corn plus lysine and tryptophan.

Series IV. Partial purification of the growth inhibitor and miscellaneous observations

Although the experiments with the semi-purified diets, which contained virtually no lipid, made it highly unlikely that the toxicity of corn to *Tenebrio* was caused by an unusual sensitivity of the larvae to products of rancidity, further proof was needed in the form of de-fatted corn diets. It

can be seen in table 4 that de-fatted corn and whole-fat corn were equally poor in food value. This failure of the larvae to grow normally on ether-extracted corn products also furnishes strong evidence that the inhibitory capacity of corn to *Tenebrio* was not due to the action of residual organic insecticides which might have been translocated from the root, leaf, or silk to the kernel.

TABLE 4

Effect of whole corn, de-fatted corn and acid extracts of raw germ on Tenebrio larvae

(All tests started with 20 animals per diet)

DIET	NUMBER OF LARVAE SURVIVING [1]	AVERAGE WEIGHT
		mg
97% Corn basal [2]	12	12.3
97% Corn, de-fatted [3] basal	18	11.0
87% Corn, de-fatted + 10% casein basal	18	36.0
87% Heated corn, de-fatted + 10% casein basal	19	68.8
85% Heated corn, de-fatted + 10% casein + 1.6% H_2SO_4 extract of raw, de-fatted germ [4] basal	17	42.1

[1] Weighed at 8 weeks.

[2] All diets contain micronutrients described on page 166.

[3] Extracted 48 hours with ethyl ether in soxhlet apparatus.

[4] Extracted 72 hours at 2°C. with 0.25 N H_2SO_4, followed by dialysis and lyophilization.

In an effort to corroborate the results of the nutritional studies with the dissected kernel fractions, attempts were made to prepare toxic extracts of corn germ. Table 4 also shows that extraction of germ at 2°C. with 0.25 N H_2SO_4, followed by dialysis and lyophilization of the extract, resulted in the concentration of a toxic fraction which inhibited larval growth at the 1.6% level. Other experiments have shown that this fraction may be rendered inactive by autoclaving at 15 pounds pressure for 30 minutes. Extraction of germ with water, 70% ethanol, 0.1 N NaOH, or detergent-bisulfite mixtures (Foster, Yong and Yui, '50) yielded fractions which were non-toxic to the larvae.

Through the courtesy of Dr. D. W. Woolley we have tested a concentrate of the niacin antagonist which he isolated from corn (Woolley, '46). No adverse effect on larval growth was observed following the feeding of this substance.

A close correlation has been observed between the toxicity of a given sample of corn and the interval between harvest and feeding. In general, the shorter the interval, the more toxic the corn and the germ removable from the corn by dissection. Storage of the kernels at 4°C. resulted in a greater retention of toxicity than storage at room temperature.

A survey of corn germ commercially available demonstrated a direct relation between toxicity to the mealworm and the rigor of industrial de-germing processes. In several experiments germ from commercially milled corn has been shown to be non-toxic to the larvae. Hand dissection of kernels from a given batch of maize which yielded inactive germ on commercial milling, demonstrated the presence of germ deleterious to larval growth in the dissected seed. It would appear, therefore, that the sulfite steeping and steam tempering operations which characterize the wet and dry milling processes respectively, inactivate the *Tenebrio* inhibitor.

DISCUSSION

The data presented fail to indicate the mode of action of the substance toxic to the mealworm, although certain causes for the growth inhibition may definitely be excluded from consideration. Thus, the solubility, heat lability, and resistance to niacin-tryptophan therapy of the inhibitor are such as to exclude those substances which have been implicated in the "pellagragenic" action of corn. Included in this category is indole-3-acetic acid (Kodicek, Carpenter and Harris, '47) as well as those niacin antagonists actually tested in this study, namely corn bran (Borrow et al., '48) and the anti-niacin prepared by Woolley ('46).

The ability of germ to limit the growth of this insect was demonstrated in the presence of high levels of the known growth factors for *Tenebrio,* as well as in the presence of 4 dif-

ferent sources of protein, namely wheat, corn, casein, and Wilson's "B." In view of the fact that lysine, tryptophan, "extra" niacin, and pyridoxine, as well as various combinations of amino acids (Sure, '53; Sauberlich, Chang and Salmon, '53) were also without beneficial effect on larval growth, no evidence exists for the contention that the addition of raw germ to the diet served to check larval growth by the imposition of an amino acid imbalance. Anderson and co-workers ('51) reported that very high levels of crystalline amino acids, in the vicinity of 4% of the diet, could induce an imbalance in rats and chicks receiving adequate levels of vitamins. However, the analyses of Block and Bolling ('51) show that the inclusion of 7% of de-fatted corn germ in a given ration increases the total dietary level of any given amino acid by not more than 0.2%, a quantity considerably below any reasonable figure for the imposition of an imbalance on the diets fed to *Tenebrio*.

In contrast to toxicities resulting from an accumulation of the products of fat rancidity, it has been observed that the inhibitory capacity of corn germ decreased in storage. The toxicity of germ was manifest on diets virtually devoid of lipid, see tables 2 and 4, indicating that the fatty fraction of the seed did not participate in the retardation of larval growth. Since the inhibitory property of corn germ was demonstrated on diets containing glucose as the major source of carbohydrate, it is unlikely that any block of starch utilization was involved in this inhibition.

The non-dialyzability, sensitivity to heat, and instability during storage of the material extractable by 0.25 N H_2SO_4 suggests that the toxic factor is in some manner associated with the protein fraction of the germ. Jones, Divine and Gersdorff ('42) demonstrated that substantial changes occur in the physical, chemical and biological properties of corn protein during storage, which may explain the decrease in the toxicity of germ with time. It is also interesting to note that Block and Bolling ('44), working with rats, reported a greater protein efficiency for "hot" expeller process corn

germ than for the "cold" solvent extracted material, further indicating that a protein or protein-bound factor may participate in the inhibition of larval development.

The effect of raw germ on the growth of higher animals remains a problem for further study. Since man does not consume unheated corn, it is unlikely that the mealworm inhibitor plays a role in the causation of pellagra or kwashiorkor. It is possible, of course, that domestic animals, which consume large amounts of raw maize, may prove sensitive to this factor. On the other hand, should additional investigations show that the ability of raw corn germ to inhibit growth does not extend beyond the Class Insecta, then some evidence exists for the view that plants evolve specific substances whose function is protection against attack by insects (Fraenkel, '54; Lipke, Fraenkel and Liener, '54).

SUMMARY

The larva of the mealworm, *Tenebrio molitor,* has been shown to undergo a growth inhibition following the inclusion of unheated corn germ or whole ground corn in basal diets containing high levels of the known growth factors for this organism. Additions of lysine, trypotphan and high quality protein failed to improve the rate of larval development, although autoclaving the germ or whole maize resulted in a marked increase in the rate of weight gain. Corn from which the germ was removed by manual dissection was shown to be of high nutritive value to the insect after supplementation with lysine and tryptophan.

The addition of 3 to 7% raw de-fatted germ to a variety of diets of high food value to the mealworm produced marked reductions in the growth rate. Extraction of unheated corn germ with 0.25 N H_2SO_4 resulted in the concentration of a non-dialyzable, heat labile toxic substance which inhibited larval growth at the 1.6% level. The relation of the corn germ inhibitor to the nutrition of vertebrates and insects is discussed.

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NICOTINIC ACID METABOLISM IN THE RHESUS MONKEY

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Determination of the metabolites of nicotinic acid as excreted in the urine of the rhesus monkey has not been reported. Such studies are necessary to understand the metabolism of nicotinic acid in this species of animal. Nicotinic acid deficiency was produced in rhesus monkeys by several workers (Chick and Hume, '20; Harris, '37; Cooperman et al., '46). It was also observed by Tappan et al. ('52) that this deficiency could be corrected by feeding either 10 mg of nicotinic acid or 4 gm of tryptophan per week. This observation indicated that tryptophan was possibly converted to nicotinic acid in the animal although the conversion ratio was very low.

The principal urinary excretory products of nicotinic acid metabolism in mammals are N-methyl nicotinamide (NMN) (Najjar and Wood, '40; Huff and Perlzweig, '43) and 1-methyl-3-carboxylamide-6-pyridone (Knox and Grossman, '47). If the administration of tryptophan increases the urinary excretion of these products in the urine of rhesus monkeys, as in rats (Rosen et al., '46) and man (Holman and de Lange, '50), it might be suggested that tryptophan is converted into nicotinic acid in this animal. Increased excretion of quinolinic acid following the administration of tryptophan has also been reported in man (Sarett, '50), the dog (Singal et al., '48) and other animals (Henderson et al., '49). It has been suggested that quinolinic acid is a precursor of nicotinic acid and is an intermediate in the conversion of tryptophan to nicotinic acid in man (Sarett, '51). The present investigation, therefore, was undertaken to study

the urinary excretion of the metabolites of nicotinic acid in rhesus monkeys when fed a natural diet alone and also when supplemented with nicotinic acid, sulfaguanidine or tryptophan.

EXPERIMENTAL AND RESULTS

Four female rhesus monkeys varying in weight between 1.9 and 2.0 kg were housed in individual cages. They were fed ad libitum a diet consisting of crushed wheat, 64; crushed gram,[1] 20; casein, 12; calcium carbonate, 3; and common salt, 1%. The daily food consumption of the animals was noted. Each of the animals was fed in addition 50 mg of ascorbic acid per day and two drops of a concentrate of vitamins A and D twice a week. One hundred grams of the diet contained 10 mg nicotinic acid, 10 mg nicotinuric acid, 31 mg trigonelline, 296 mg tryptophan [2] and 18 mg quinolinic acid. The diet did not contain NMN or 6-pyridone. With this diet all the animals grew normally.

The urine of each animal was collected in a bottle containing 2 ml of concentrated hydrochloric acid. Nicotinic acid and amide, nicotinuric acid, NMN, trigonelline and 6-pyridone were estimated by the methods described previously (Chattopadhyay et al., '53). Quinolinic acid was estimated after autoclaving urine in normal sulfuric acid at 20 pounds pressure for one hour and estimating the nicotinic acid formed by the chemical method. Quinolinic acid values were calculated by multiplying the increase of nicotinic acid by 5.8 as described by Sarett ('51).

After estimating the daily normal excretion of these metabolites each of the animals was fed 1 gm of sulfaguanidine mixed with 100 gm of diet for 5 days and the metabolites of nicotinic acid excreted in the 24-hour urines were estimated on the 6th day.

After the above studies were completed each of the animals was fed daily 100 mg of nicotinic acid in the morning for 4

[1] Gram *(cicer arietinum)*, a leguminous seed commonly consumed in India and other countries as a decoction with rice.

[2] Estimated microbiologically using *Lactobacillus arabinosus*.

days and the 24-hour urinary excretion of the metabolites of nicotinic acid were estimated on the 5th day.

Seven days after the discontinuation of the feeding of nicotinic acid the urinary excretion of the metabolites of

TABLE 1

Twenty-four-hour excretion of metabolites of nicotinic acid by rhesus monkeys

MONKEY NO.	SUPPLEMENT	NICOTINIC ACID AND AMIDE	NICOTINURIC ACID	NMN	6-PYRIDONE	QUINOLINIC ACID	TRIGONELLINE	DIET CONSUMED
		mg	*mg*	*mg*	*mg*	*mg*	*mg*	*gm*
1	none	0.40	0.88	0.27	2.2	5.39	4.04	97
2	none	0.45	0.84	0.31	1.8	5.91	3.80	92
3	none	0.58	1.40	0.25	3.9	5.27	3.27	98
4	none	0.62	1.23	0.29	3.0	5.92	3.62	89
1	sulfaguanidine [1]	0.49	0.86	0.21	2.0	3.65	3.48	90
2	sulfaguanidine	0.45	0.79	0.17	1.8	3.88	3.48	95
3	sulfaguanidine	0.56	1.00	0.14	2.9	4.23	3.82	90
4	sulfaguanidine	0.63	1.02	0.20	3.4	3.65	3.51	95
1	nicotinic acid [2]	23.70	11.10	3.18	25.0	36.50	3.78	90
2	nicotinic acid	22.00	8.36	3.37	26.7	33.06	3.82	90
3	nicotinic acid	18.7	10.40	3.32	33.3	29.00	3.30	80
4	nicotinic acid	16.9	7.81	3.38	35.1	27.14	3.51	78
1	tryptophan [3]	0.46	0.74	1.82	29.2	11.83	3.49	92
2	tryptophan	0.46	0.76	2.34	31.3	12.00	3.59	88
3	tryptophan	0.57	1.13	2.59	23.52	12.76	3.30	78
4	tryptophan	0.51	1.04	2.00	32.80	11.83	3.51	80

[1] One gram for 5 days.

[2] One hundred milligrams for 4 days.

[3] One gram for 4 days.

nicotinic acid came to the normal level. One gram of DL-tryptophan was mixed with 100 gm of diet which was fed to the monkeys for 4 days and 24-hour urine was collected on the 5th day for the estimation of different metabolites of nicotinic acid. The results are given in table 1.

DISCUSSION

Rhesus monkeys excrete in the urine nicotinic acid and amide, nicotinuric acid, NMN, 6-pyridone and trigonelline.

After the administration of nicotinic acid, the urinary excretion of all of these substances except trigonelline is considerably increased. The principal metabolic end products of nicotinic acid in monkeys are, therefore, 6-pyridone, NMN and nicotinuric acid. As in rabbits, trigonelline is not a metabolic end product of nicotinic acid in monkeys. After the administration of tryptophan, urinary excretion of 6-pyridone and NMN is increased considerably. This indicates that monkeys can convert tryptophan into nicotinic acid, which is further metabolized into 6-pyridone and NMN. Administration of sulfaguanidine only slightly decreases the NMN excretion. The excretion of other metabolites of nicotinic acid remains unaffected. It seems that intestinal flora help only slightly, if at all, in the intestinal synthesis of nicotinic acid.

Quinolinic acid excretion is considerably increased after the administration of nicotinic acid and tryptophan. The formation of quinolinic acid by decarboxylation of nicotinic acid seems to be a complex and difficult metabolic process. It is more probable that quinolinic acid is formed from dietary tryptophan after the feeding of nicotinic acid. Tryptophan is converted into quinolinic acid and nicotinic acid. When the supply of nicotinic acid is in excess, dietary tryptophan is possibly converted only into quinolinic acid and no nicotinic acid is formed from it. In experiments with fasted rats it has been found [3] that after administration of nicotinic acid no increased excretion of quinolinic acid takes place.

SUMMARY

Nicotinic acid and amide, nicotinuric acid, NMN, 6-pyridone, trigonelline and quinolinic acid were estimated in the urine of rhesus monkeys after they were fed sulfaguanidine, nicotinic acid and DL-tryptophan.

Sulfaguanidine produced only a slight diminution in the excretion of NMN. The excretions of other metabolites of

[3] Unpublished data from this laboratory.

nicotinic acid remained unchanged. Nicotinic acid produced an increased elimination of NMN, 6-pyridone and nicotinuric acid. The excretions of 6-pyridone and NMN increased considerably after the administration of tryptophan. Quinolinic acid excretion increased after the administration of both nicotinic acid and tryptophan.

NMN and 6-pyridone are the principal metabolic end products of nicotinic acid in the monkey. These animals can also convert tryptophan into nicotinic acid. When the diet contains sufficient nicotinic acid the dietary tryptophan is converted principally into quinolinic acid and in other cases tryptophan is converted into both quinolinic acid and nicotinic acid.

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