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1. J. Agr. & Food Chem. 4:418, 1956.
2. A.M.A., Council on Foods & Nutrition: J.A.M.A. 146:35, 1951.

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(May 28, 1865 – November 11, 1944)

In the study of beriberi and the story of its final classification as a disorder due to faulty diet, some of the important links in this chain of discovery were forged by Gerrit Grijns. This Dutch physician, on his arrival in Netherlands India [1] in 1892, was assigned to the laboratory just established for Dr. Chr. Eijkman.

The Pekelharing-Winkler Commission had been sent to its Far Eastern colony by the government of Holland with the instruction to determine the cause of beriberi, a disease which at that time claimed many victims, especially in prisons and barracks. The Commission inclined to the view that beriberi is an infection with a coccus which had been encountered repeatedly in the research. Although this was the favored hypothesis, the Commission was not satisfied that it had fully solved the problem, and therefore judged it necessary to continue the research. It was arranged therefore that the medical surgeon Eijkman, who had been the Commission's assistant during its stay in Netherlands India, should be exempt from further military service, and that a laboratory should be put at his disposal to enable him to continue the research on beriberi. It was to this laboratory that Grijns received his assignment in 1892.

Gerrit Grijns was born at Leerdam, Holland, on May 28, 1865, the son of Cornelis Dirk Grijns and his wife Janette Christina Adriana Maria Seret Grijns. Leerdam, famous for its manufacture of fine glass, is located 30 miles east of Rotterdam and 20 miles south of Utrecht; there Gerrit's father operated a large lumber business. The parents were very strict church people and raised their children accordingly.

[1] Now the independent Republic of Indonesia.

Young Gerrit attended the local public school. A contemporary playmate of his during these public school days was H. Colijn, later Premier of the Netherlands and leader of a well known political party. A brother of Gerrit, John Grijns, became a physician and practised in Utrecht for 40 years, and also became a member of the Provincial State Legislature. While attending the Gymnasium in Delft, Gerrit proved to be a very good student in mathematics and physics, and therefore was allowed to read other books during classes or do other things, provided he did not miss the answers to questions his teacher asked him.

After attending the Gymnasium at Delft, Gerrit began his medical studies at the University of Utrecht in 1885. As a student he had already occupied himself with scientific researches, and thus it was possible for him to offer his thesis and take his M.D. degree in 1891, before passing the final government examination ("arts-examen"), which, in Holland, is necessary in order to obtain the right to practice medicine. His thesis was written under the guidance of Engelmann and bore the title "Contribution to the Physiology of the Nervus Opticus."

In this investigation Grijns had to make use of a very sensitive galvanometer. In 1891 the method of constructing and of suspending such an instrument was not as perfect as it is now. Therefore, this investigator was obliged to make his observations during the night so as not to be disturbed by the vibrations caused by the day-time traffic outside the building. The subject of this research was deemed to be sufficiently important to warrant making the thesis available to a larger circle of readers, and therefore a German translation of it was published. The medical faculty in Utrecht rewarded its author by appointing him to the Donders Scholarship by which he was enabled, after passing the "arts" examination in March 1892, to study a "semester of physiology" with Carl Ludwig in Leipzig, Germany.

In September 1892, the young Doctor Grijns married Miss J. C. de Wilde, daughter of a sea captain who sailed to

Indonesia as well as other parts of the world. One can only speculate as to how much influence this young wife — daughter of a sea captain — exerted on her physician-husband to leave Holland for service as a medical surgeon in Netherlands India.

As has already been stated, his first assignment was to the laboratory recently established for Doctor Eijkman. It was an act of remarkably clear insight on the part of the Netherlands-Indian government, probably resolved on the basis of information from Holland, to detach Grijns to the Eijkman laboratory immediately on his arrival in Netherlands India. This laboratory had the august title "Laboratory of Bacteriology and Pathology" but consisted merely of two small rooms in the Military Hospital in Batavia. Grijns proved fully worthy of this appointment, and from his hand elaborate essays soon appeared on physiological, particularly tropical physiological subjects. One of these, dealing with the permeability of red blood cells, is now considered to belong to the classical researches on this subject.

In 1894, when the Lombok attack necessitated the mobilization of all available forces, this laboratory work had to be abandoned for the time being. After this was over, Grijns joined the expedition against the Atjeh pirates.

Reference was made above to the attitude taken by the Pekelharing-Winkler Commission on the question of the cause of beriberi, namely, that it was probably an infection caused by a particular coccus. This fact deserves some emphasis. Students of the history of bacteriology are aware that during the decade from 1880 to 1890 at least 22 definite diseases were proven to be due to specific microorganisms. It was natural, therefore, that this Commission should find it easy to believe beriberi to be an infection of some sort, and therefore the main problem in etiology was to identify the organism, and the proper treatment was disinfection of living quarters and related measures. One other possible cause was easy to entertain, namely, poisoning by some unknown agent. It will be noticed that these two ideas had a common feature, in that the cause was an outside positive agent of

some sort, either living (a microorganism) or non-living (a chemical). The idea that the cause could be the *absence* of some essential substance from the diet had been only hinted at or expressed in a vary vague way at the time. The researches of Lunin, published in 1881, had remained unnoticed. By 1896, however, Eijkman had succeeded in establishing beyond any doubt that *polyneuritis gallinarum* is caused by feeding chickens a diet consisting solely of overmilled (completely polished) rice. In his publications describing these experiments, however, he still expressed doubt as to whether a disease of such severity in man as beriberi could be explained so simply, especially since this explanation ran counter to the prevailing ideas that the two main causes of disease are infection and intoxication. At Eijkman's request, his friend Vorderman made a study of beriberi in the prisons of Java. This brought to light the interesting fact that beriberi very often occurred in prisons where rice polished by machinery formed the chief food (so much so indeed, that an imprisonment of longer than three months was often equal to capital punishment, as all convicts developed beriberi after such a period); whereas in prisons where the prisoners themselves husked the rice by hand, and thus obtained a product with a greater part of the silver layer (pericarp) still attached to the grain, practically no beriberi occurred. The conviction that a disease must be caused by some outside positive agent was so strong at the time, however, that even Eijkman could not easily abandon it. He was still inclined to the idea of a toxin. In particular he surmised the possible production in the intestinal canal of a toxic substance from food rich in carbohydrate (rice in this case); this toxin was supposed to act as a more or less specific poison on the nervous system while the pericarp (the so-called silver layer or silver skin) contained an antidote. This, then, was the "climate of scientific opinion" concerning beriberi into which Grijns came when, in 1896, Eijkman found it necessary for health reasons to leave for Europe where, soon after, he was appointed Professor of Hygiene at Utrecht. Grijns was appointed to

the laboratory in Batavia and expected to continue Eijkman's investigations.

It had become clear to students of the problem at that time, that beriberi in man and polyneuritis gallinarum in birds were caused by food consisting exclusively of polished rice and could be prevented or cured by feeding unpolished rice. How could this difference between polished and unpolished rice be explained? Grijns — and this is his imperishable merit — faced the question with a completely unbiassed mind and in a number of carefully thought out experiments, progressing logically step by step, came nearer and nearer to the core of the matter. He was able to exclude successively various possibilities, and particularly the idea that the presence of carbohydrate is essential for the causation of polyneuritis gallinarum. He also showed that the disease could be produced by feeding the bird exclusively on meat provided it had been heated long enough at 120°C.

Grijns was also able to show that the disease could not be the result of a lack of salt, fat or protein, and finally came to the conclusion that polyneuritis gallinarum, and by analogy most probably human beriberi, was due to a deficiency — a partial hunger — or relative lack of certain still unknown substances which can easily be destroyed by moist heat, and which cannot be omitted from food without serious harm to the nervous system. These substances (later called *vitamins* by Casimir Funk in 1912) which are present in the silver layer of rice, Grijns (1901) also found to be present in other articles of food, notably *katjang idjoe,* one of the legumes. These researches were carried out over a period of more than three years and were finished in March 1900. They were recorded in a masterly essay which was published in 1901.

The demonstration by Grijns that katjang idjoe prevents and cures polyneuritis gallinarum led to the tests by Hulshoff Pol of the value of this legume as a preventive and curative agent in human beriberi. In his first trials Hulshoff Pol fed 150 gm daily to 31 patients who had suffered from beriberi for a long time. All of these patients improved but still

suffered from paralyses. This experiment led to the following conclusions: (1) a distinction should be made between beriberi itself and its effects, namely, the degeneration of nerves and resultant paralyses; (2) the disease was curable through the administration of katjang idjoe, but the resultant paralyses could be cured only through the slow processes of regeneration. Hulshoff Pol also tested the prophylactic action of this legume in an insane asylum containing 300 inmates who were housed in 12 pavilions. The inmates in three of the pavilions received 150 gm of legume daily instead of one meal of rice. The inmates of three other pavilions were fed 300 gm daily of different kinds of vegetables in addition to the usual amount; these were served partly raw and partly cooked. The inmates of three other pavilions served as controls and were fed only the customary rice ration. In addition, an attempt was made to control the possible variable of infection by having the inmates of the remaining three pavilions fed the customary diet and nothing else but having their living quarters thoroughly disinfected with a three per cent solution of carbolic soap. In light of our modern knowledge, the incidence of beriberi in the various pavilions was what we would expect. The value of the legume was obvious. The results with inmates fed various vegetables were inconclusive. The experiment was therefore extended and varied in certain respects until it finally covered a period of over three years. No case of beriberi occurred in the pavilions which used the diet of rice and katjang idjoe, while in those where rice alone was fed 211 cases occurred during the same period. This experiment demonstrated that beriberi must be due to some deficiency in the diet, since, as Vedder has commented, "it is hardly conceivable that if the rice were toxic that the disease could be prevented simply by the addition of another vegetable to the diet. If we administer an amount of arsenic or other known poison sufficient to produce an intoxication, we do not expect that these effects will be counteracted in the least by the administration of beans or any other food." This whole experiment, so significant for

human nutrition be it remembered, was suggested by the Grijns observation that katjang idjoe prevents and cures polyneuritis gallinarum. Grijns, therefore, was the first to set forth clearly what is justly to be called a deficiency disease and to study systematically the properties and distribution of a vitamin.

Not all of the doctors in Netherlands India possessed such a scientific mind as Grijns. As a result the new food-science was by no means readily accepted, much less applied in practical fashion. Grijns had to overcome many difficulties before he could get these new ideas applied to the food of the prisoners. When at last he did succeed, beriberi disappeared forever from the Netherland Indies prisons.

The work of Eijkman and Grijns paved the way directly for the experimental studies of "ship beriberi" and scurvy by Axel Holst. In his first publication on this subject in the Journal of Hygiene (1907) Holst himself states: "My starting point has been the excellent researches of the Dutch authors Eijkman and Grijns on the so-called polyneuritis gallinarum. Doctor Grijns most obligingly showed me his experiments during my stay in Batavia in 1902:" . . . "It seemed to me that a continuation of the experiments of Eijkman and Grijns might throw some light upon the possible unwholesomeness of the articles of food, which I have mentioned in connection with ship beriberi." Later, Holst and Frolich transferred their investigations to mammalia and cleared up the etiology of scurvy.

While in Netherlands India Grijns did not occupy himself solely wtih laboratory work. He arrived in Batavia in 1892. During the following year he gave lessons in Anatomy at the Stovia, the school for native doctors. After his return to Batavia in 1896 following the military assignments, he taught Physiology and Ophthalmology. Later still he also taught Pathological Anatomy and Medical Jurisprudence. For certain of his courses he published a textbook on Ophthalmology.

From 1902 to 1904 Grijns was on leave to Europe because of ill health. While in Europe he did not indulge in much

leisure either, but worked in the laboratories of Went (botany) and Zwaardenmaker (physiology).

In 1904 he returned to Batavia where he was once more appointed subdirector of the laboratory. During the next few years he and de Haan saw an opportunity to apply the newly developing knowledge in immunology to the old question whether polyneuritis gallinarum and human beriberi do involve an infection in some subtle way. They applied the complement fixation technique to blood samples from birds exhibiting the polyneuritis and from beriberi patients. All of the results were negative. No deviation of complement was ever obtained. As a result, these workers were not in favor of the infectious theory of origin of the disease. Their paper was published in 1910.

In 1912 Grijns succeeded de Haan as Director of the Laboratory. Reference has already been made to the primitive nature of the original quarters, a couple of rooms in the Military Hospital. Grijns now had a chance to develop plans for building a spacious and well equipped laboratory. Unfortunately he was hardly able to reap any benefit from it himself, for, soon after it was opened in 1917, he returned to his native country.

In 1919 he taught Tropical Hygiene in the Colonial Institute at Amsterdam. In 1920 he was for a time Conservator in Professor Eijkman's laboratory in Utrecht, and in 1921 he was called to the position which he held until 1935, namely, Professor of Animal Physiology at the Agricultural University of Wageningen. Although he was actually provided with only a small part of the assistance that had been his in prospect, here again he was able to carry out worthwhile investigations, notably on vitamin E. He published several papers dealing with reproduction and fertility in rats.

His family consisted of his wife, the sea captain's daughter, and two sons, one of whom became an engineer, the other a physician. Because of his own preoccupation with his books and scientific journals the training of the children was left to their mother.

In 1910 Grijns suffered a chlorea infection (laboratory infection) which he diagnosed himself. Afterwards he had very sensitive intestines, and was very careful with his food. In 1912 he had another chance for an official furlough but did not take it. In 1917 he would have gone, but the trip would have had to be made across the United States of America and his government would not give him permission to do this. He therefore resigned and returned to Holland.

During his career he received many medals and honors, notably that of being made a Commander in the Order of Orange-Nassau. In 1938 he was honored with the chairmanship of the Third International Congress for Tropical Medicine and Malaria which was held in Amsterdam. In 1935 he retired from his professorship at the Agricultural University of Wageningen, having reached the age of 70 years, the retirement age in the Netherlands. After his retirement he worked for several years in the Laboratory of Hygiene in Utrecht; these years were made more difficult for him by the Parkinson disease which developed at this time.

Grijns died November 11, 1944, before the end of the second World War. He wished to be cremated but this was prevented by the German invasion. He was buried in Utrecht without any ceremonies; this was in accord with his simple character. No statue has been erected in his memory. However, in the government museum for the history of National Science, Steenstraat la Leiden, Holland, one can find a collection of all the diplomas, medals, honors, portraits, etc., which he received, and his scientific notes, as well as letters of correspondence with other scientists, as, for instance, Robert Koch who believed beriberi to be an infectious disease.

In this biography the chief object has been to point out the great merits of Grijns in the development of the science of nutrition; more particularly in establishing clearly the deficiency disease concept.

I was priviledged to be one of his students during 1917 to 1923. I remember how Doctor Grijns related to us in detail the experiments that led to the correct interpretation of

Doctor Eijkman's earlier work. He was an inspiring teacher, able investigator and well loved by all of us.

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COMPARATIVE METABOLISM OF PHYTATE AND INORGANIC P^{32} BY CHICKS AND POULTS

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(Received for publication November 26, 1956)

The nutritional role of phytin has interested biochemists for many years due to its widespread occurrence in cereals and other foods and feedstuffs of plant origin. The possible adverse effect of phytin on utilization of other minerals has been widely discussed (Harris, '55). More recently the availability of phosphorus from this source for growth and bone formation has received attention. Students of this problem seem to be agreed that there is much less efficient utilization of phytate than of conventional sources of inorganic phosphate (Lowe et al., '39; Krieger et al., '40; Krieger and Steenbock, '40; Heuser et al., '43; McGinnis et al., '44; Singsen et al., '47; Singsen, '48; Gillis et al., '49, '53). Avian species have been shown to be particularly inefficient users of phytin.

Since radioactive phosphorus has become generally available to investigators, an additional, and possibly more precise, method is at hand to study the manner in which phytate phosphorus is metabolized by animals. In particular, it is easier to study the immediate fate of administered phosphorus in the digestive tract and its metabolic dispersal in the blood and other tissues.

The only report on the use of radioactive phytin which has come to our attention is that by Singsen, Matterson and Kozeff ('50) who described its administration to turkey poults. After feeding a diet containing 1% labeled calcium

phytate for a period of two weeks, they found that P^{32} had entered the bones, indicating at least a limited utilization. Its retention was increased by supplying vitamin D. No comparisons were made with inorganic radioactive sources of P^{32}. The amount of radioactivity administered and the percentage of the dose retained were not detailed, although, presumably, 1% labeled phytate in the diet constituted a high level of dosage.

The work reported herein was carried out for the purpose of comparing P^{32}-labeled phytate with P^{32}-labeled inorganic orthophosphate as sources of phosphorus for chicks and poults. The influence of vitamin D on the utilization of both types of phosphorus was also investigated. *In vitro* studies were also made in an effort to determine whether or not chemical exchange occurs between phytin phosphorus and phosphorus from an inorganic source.

EXPERIMENTAL

Preparation of P^{32}-labeled calcium phytate

Phytate was isolated as the calcium salt from kernels of corn which carried P^{32}. The corn was kindly prepared for our use by Dr. J. D. Sayre of the Ohio Experiment Station, who accomplished the radioactive labeling by injecting a solution of P^{32} directly into the corn stalks as the grain approached maturity. The phytate was isolated from the mature, dry grain by the general method described by Singsen et al. ('50), but modified to increase the extraction time with 0.5 N HCl from three hours to 18 hours. One kilogram of corn yielded about 15 gm of P^{32}-labeled calcium phytate. The phytate was recrystallized from hot 20% acetic acid just prior to use in the feeding experiments to remove traces of inorganic phosphorus which may result from breakdown of phytin. The samples of radioactive phytate used in the separate studies with chicks and poults were prepared at different times from different batches of corn.

For comparison with phytate, disodium phosphate appeared to be the most practical source of P^{32}-labeled inorganic orthophosphate readily obtainable. Since it was administered as a solution in tracer quantities, solubility was not considered to be a factor in these studies.

Radioactivity measurements. The calcium phytate, obtained as described above, as well as the solution of radioactive disodium phosphate used in these studies were assayed for radioactivity. The phytin was dissolved in acetic acid and diluted for counting. Aliquots were evaporated to dryness on copper planchets and counted, in triplicate, in a windowless gasflow counter. The radioactivity in the materials fed experimentally was as follows: In the chick experiment, calcium phytate — 5.10×10^7 counts/min./gm; disodium phosphate solution — 6.59×10^6 counts/min./ml. In the turkey poult experiment: calcium phytate — 7.53×10^7 counts/min./gm; disodium phosphate solution — 4.275×10^6 counts/min./ml.

All samples of blood, bone ash and excreta collected in the experiments were also counted in triplicate in the windowless gas-flow counter. The bone ash and excreta were first dissolved in acid, diluted, plated and dried on copper planchets. The expressions for radioactivity found in the tissues and excreta and recorded in tables 2 and 3 have been corrected for decay of P^{32}.

Experiments with chicks. One-day-old White Leghorn male chicks were divided into two groups of 20 each. One group was given a vitamin D-deficient chick diet (table 1) while the other group received the same diet plus a supplement of 50 International Chick Units of vitamin D per 100 gm of diet. After 5 weeks on this dietary regime, 8 chicks of approximately equal weight were selected from each dietary group and placed in wire metabolism cages. Four chicks from the vitamin D-deficient group and 4 from the normal group received doses of radioactive calcium phytate during a two-day period. The remaining 4 normal and 4 vitamin D-depleted chicks received doses of radioactive inorganic phosphorus at the same time. The 8 chicks selected to receive inorganic

phosphate were given 4 separate doses of 1 ml each of a solution of $Na_2HP^{32}O_4$ carrying 6.59×10^6 counts/min./ml at the time the first dose was administered. Two doses were administered approximately 6 hours apart on the first day and the dosing was repeated twice on the second day at the same time interval. The 8 chicks selected to receive radioactive calcium phytate were given 4 separate doses of 127 mg

TABLE 1

Basal diets used for chicks and poults

INGREDIENT	VIT. D-DEFICIENT CHICK DIET	VIT. D-DEFICIENT POULT DIET
	%	%
Yellow corn meal	67.5	30.95
Soybean meal	25.0	50.0
Crude casein	5.0	
Corn gluten meal		10.0
Alfalfa meal		3.0
Dried brewers' yeast		2.0
Methionine		0.1
Salt	0.5	0.5
Tricalcium phosphate	2.0	
Dicalcium phosphate		2.25
Limestone		1.10
	per lb.	*per lb.*
$MnSO_4 \cdot H_2O$	100 mg	120 mg
Vitamin B_{12}	5 μg	10 μg
Riboflavin	1.5 mg	3 mg
Niacin	10 mg	10 mg
Calcium pantothenate	3 mg	5 mg
Choline chloride	454 mg	454 mg

each of this material (6.46×10^6 counts/min. at the time of the first dose). The $Na_2HP^{32}O_4$ solution was placed in the chicks' crops by means of a pipet. The calcium phytate was weighed into gelatin capsules and inserted directly into the chicks' crops. During the two-day dosing period the chicks continued to receive the same diets as previously, ad libitum.

Forty-eight hours after administering the initial dose of radioactive phosphorus from the two sources, blood samples were collected from the wing vein of each chick. The blood

was heparinized, centrifuged, and aliquots of plasma were plated and dried for radioactivity counting as described above. After collection of the blood samples the chicks were sacrificed and both tibiae removed. All excreta were collected during the experimental period. The tibiae from each chick were freed from adhering flesh and ashed. The ash was prepared for counting by dissolving in hydrochloric acid and diluting suitable aliquots with distilled water. The excreta were similarly ashed, dissolved in hydrochloric acid and diluted with distilled water.

TABLE 2

Results of chick study; radioactivity measurements made after dosing with equivalent quantities of isotope carrier

DIETARY HISTORY	P^{32} CARRIER [1]	RADIOACTIVITY OF TOTAL PLASMA	RADIOACTIVITY OF 100 MG TIBIA ASH	% DOSE RETAINED/ 100 MG TIBIA ASH	% DOSE EXCRETED
		av. counts/ min.	*av. counts/ min.*[2]		
Vitamin D-deficient	Ca phytate	3.2×10^3	6.0×10^3	.025	76.6
Vitamin D-deficient	$Na_2HP^{32}O_4$	1.1×10^5	2.95×10^5	1.180	23.2
Vitamin D-adequate	Ca phytate	9.6×10^3	2.20×10^4	0.09	56.5
Vitamin D-adequate	$Na_2HP^{32}O_4$	9.6×10^4	2.57×10^5	1.040	18.5

[1] A total of 4 doses of isotope carrier were administered to all chicks over a two-day period. The initial dose was administered 48 hours and the final dose approximately 18 hours before the chicks were sacrificed.

[2] Corrected to a standard body weight of 200 gm.

The radioactivity of the blood is shown in table 2, expressed as counts/min./total plasma. Since the normal chicks were heavier than the vitamin D-deficient chicks and would, therefore, contain more blood, the radioactivity in the normal chicks would necessarily occupy a greater volume. The quantity of total plasma was arrived at by assuming the blood to be 9% of the body weight and the plasma to comprise 70% of the blood. The radioactivity of the blood plasma of normal chicks which received inorganic P^{32} was 10 times greater than that of normal chicks which received a com-

parable quantity of phytin P^{32}. The vitamin D-deficient chicks showed an even greater disparity in the utilization of phytin and inorganic phosphorus. The radioactivity of the plasma of the vitamin D-deficient chicks was approximately 34 times as great after administration of inorganic P^{32} as was found following the administration of phytin P^{32}. It is of interest to note that the plasma of the vitamin D-deficient chicks contained slightly more inorganic P^{32} than the plasma of vitamin D-adequate chicks. This indicates no impairment in the utilization of the readily available inorganic phosphate due to vitamin D-deficiency. This is in marked contrast to the profound effect which vitamin D has been observed to have on the absorption of calcium (Keane, Collins and Gillis, '56).

The corrected counts/min./100 mg of tibia ash as well as the percentage dose retained per 100 mg of tibia ash are also presented in table 2. Since the normal chicks were heavier than the vitamin D-deficient chicks, these values have also been corrected to a standard body weight of 200 gm. These data show that chicks receiving the normal vitamin D-adequate diet retained 11 times as much radioactivity per unit of tibia ash following administration of inorganic phosphorus as they retained from phytin phosphorus. Chicks which received the vitamin D-deficient diet retained 47 times as much radioactivity per unit weight of tibia ash when the source of phosphorus was sodium phosphate rather than calcium phytate. The data on tibia ash also reflects the fact that the vitamin D-deficient chicks utilized inorganic phosphate as efficiently as chicks having received an adequate amount of vitamin D.

The percentage of the dose of P^{32} excreted per chick is also presented in table 2. The data on excretion in this experiment are of limited value since the chicks were sacrificed before the digestive tract would normally have voided all of the unabsorbed and re-excreted phosphorus. It might also be expected that the soluble sodium phosphate would be more rapidly absorbed and partially eliminated via the kidneys, than would the phytate phosphorus. Consequently, at the time

the chicks were sacrificed the non-utilized fraction of sodium phosphate would be more completely present in the excreta than the non-utilized phytin phosphorus. The data in table 2 are of qualitative interest since they show that excretion of P^{32} is much greater immediately after ingestion of phytin than after ingestion of sodium phosphate.

Experiment with turkey poults. One-day-old Beltsville White turkey poults of mixed sexes were divided into two groups of 20 each. One group was fed the vitamin D-deficient poult ration shown in table 1 while the other group received a supplement of 180 International Chick Units of vitamin D in 100 gm of the same diet. After 11 days on this experimental regime, the poults in the first group exhibited definite symptoms of vitamin D-deficiency. At this time 12 poults of approximately the same weight were selected from each group and placed in wire metabolism cages. After placing the poults in metabolism cages, feed was withheld for a period of three hours prior to administration of the isotope although water was supplied ad libitum. Six normal and 6 vitamin D-deficient poults were then each given a 2 ml solution of $Na_2HP^{32}O_4$ containing a total of 8.55×10^6 counts/min. At the same time 6 normal and 6 vitamin D-deficient poults were each given an equivalent dose of isotope from P^{32}-labeled calcium phytate. The isotope carriers were placed in the crops of the poults in exactly the same manner as described above for chicks. After dosing the poults were allowed the same diets as previously ad libitum.

Forty-eight hours after administration of the radioisotope blood samples were collected and plasma obtained by the same procedure as in the experiment with chicks. The poults were sacrificed and both tibiae removed and prepared for ashing. All excreta were collected during the experimental period. The blood, bone ash and excreta were handled and counted in the same manner as described in the chick experiment.

The results obtained are presented in table 3. The radioactivity of the blood is again expressed on the basis of

counts/min./total plasma. The radioactivity of the blood of normal poults which received inorganic phosphorus was 17 times greater than that of normal poults which received the phytin phosphorus. In the case of poults which were deficient in vitamin D, there was 28 times as much radioactivity in the blood plasma of those which had received inorganic phosphorus as there was in the plasma of those which had received phytin phosphorus.

The corrected counts/min./100 mg of tibia ash as well as the percentage dose retained per 100 mg of tibia ash are presented in table 3. Since the normal poults were heavier

TABLE 3

Results of turkey poult study; radioactivity measurements made 48 hours after a single dosing with equivalent quantities of isotope carrier

DIETARY HISTORY	P^{32} CARRIER	RADIOACTIVITY OF TOTAL PLASMA	RADIOACTIVITY OF 100 MG TIBIA ASH	% DOSE RETAINED/ 100 MG TIBIA ASH	% DOSE EXCRETED
		av. counts/ min.	*av. counts/ min.*[1]		
Vitamin D-deficient	Ca phytate	7.14×10^2	4.36×10^3	0.058	47.7
Vitamin D-deficient	$Na_2HP^{32}O_4$	2.00×10^4	3.66×10^5	4.27	26.3
Vitamin D-adequate	Ca phytate	1.23×10^3	6.56×10^3	0.087	30.3
Vitamin D-adequate	$Na_2HP^{32}O_4$	2.11×10^4	4.41×10^5	5.16	6.7

[1] Corrected to a standard body weight of 135 gm.

than the vitamin D-deficient poults all values were corrected to a standard poult weight of 125 gm. Since the percentage of phosphorus in a given quantity of the bone ash of the poults was the same, for all practical purposes, regardless of the state of vitamin D nutrition, a given weight of ash from the different groups, in this case 100 mg, would contain the same amount of phosphorus. These values in effect, therefore, refer to specific activity, or counts per minute per unit weight of phosphorus. The data in table 3 show that normal poults retained 59 times as much activity following the administration of P^{32} in disodium phosphate as resulted

from the administration of P^{32} in calcium phytate. The vitamin D-deficient poults made even less use of phytin. They retained 74 times as much radioactivity in the bone ash following administration of disodium phosphate as they retained following administration of calcium phytate.

The percentage of the dose of radioactive phosphorus excreted by the poults in the various groups is shown in table 3. It was found that normal poults excreted, within 48 hours, about 4.5 times as much P^{32} from phytin as from disodium phosphate. Poults deficient in vitamin D also excreted a larger proportion of the phytin phosphorus than the inorganic phosphorus. However, as compared with the normal poults their excretion of inorganic phosphorus was also much higher. Due to this high output of inorganic phosphorus the disparity between excretion of phytin and inorganic phosphorus was not so great for vitamin D-deficient poults in this study. It appears that, at least in this experiment, vitamin D-deficiency interfered to some extent with the utilization of inorganic phosphorus by the poult.

Chemical exchange studies, in vitro. Since it has been known for some time that simple chemical exchange occurs between certain inorganic phosphates, it was of interest to determine whether exchange may occur between inorganic phosphorus and the phytin molecule. Singsen et al. ('50), have leaned heavily toward the exchange theory to explain the apparent utilization of phytin phosphorus by poults. If such a reaction takes place in the digestive tract, appearance in the tissues of P^{32} from phytin may not be wholly ascribed to net utilization of this source of phosphorus. Conversely, the administration of P^{32}-labeled inorganic phosphate along with a diet containing substantial amounts of phytin might yield deceptively low values for the uptake of inorganic P^{32} due to its exchange with the phytate molecule.

In a preliminary experiment to explore the possibilities of exchange, 140 mg of non-radioactive calcium phytate were dissolved in 8 ml of 25% acetic acid. Two milliliters of solution containing $Na_2HP^{32}O_4$ were added and the solution al-

lowed to stand at room temperature, with occasional stirring, for 24 hours. The calcium phytate was reprecipitated by heating the solution in a water bath and filtering while hot. The precipitate was washed once with hot 8% acetic acid, three times with water and three times with 95% ethanol. The precipitate was dried by passing a stream of air through it for one hour and then placing overnight in a vacuum desiccator. When completely dry the weight was found to be 56 mg. It was then dissolved in 4 ml of 25% acetic acid, diluted 1:10 with distilled water and aliquots plated out for radioactivity counting. The results are shown in table 4.

TABLE 4

Results of in vitro studies on the possibility of phosphate exchange between calcium phytate and inorganic phosphate

NON-LABELED PHYTATE USED	RADIOACTIVITY ADDED AS $Na_2HP^{32}O_4$	Ca PHYTATE RECOVERED	RADIOACTIVITY AFTER EXCHANGE	% APPARENT EXCHANGE
	counts/min./mg phytate	*mg*	*counts/min./mg phytate*	
(Experiment 1)				
140 mg	1.53×10^4	56	6.57×10^3	43
(Experiment 2. First recrystallization)				
1141 mg	1.03×10^3	542	3.4×10^2	33
	(Second recrystallization)			
		161	3.6×10^2	35

In a second experiment a similar plan was followed except that the calcium phytate was recrystallized to a constant specific activity in order to be certain that all inorganic phosphorus was removed. One thousand one hundred and fourteen milligrams of non-radioactive calcium phytate were dissolved in 50 ml of 25% acetic acid. Three milliliters of $Na_2HP^{32}O_4$ were added and the solution allowed to stand at room temperature for 24 hours. The calcium phytate was then reprecipitated, washed and dried as previously described. The weight was found to be 542 mg. It was then dissolved in 30 ml of 25% acetic acid, a portion was diluted 1:10 with

25% acetic acid and radioactivity measurements made. The calcium phytate was found to contain 3.4×10^2 counts/min./mg.

The remainder of the solution was again treated to precipitate the calcium phytate which was washed and dried as described above. The weight was found to be 161 mg. This material was dissolved in 20 ml of 25% acetic acid and aliquots plated out for radioactivity measurement. The calcium phytate was found to contain 3.6×10^2 counts/min./mg. This was considered a constant specific activity within experimental error.

The data obtained in the exchange studies are presented in table 4 and indicate that P^{32} from $Na_2HP^{32}O_4$ exchanged quite readily with phosphorus from calcium phytate *in vitro*. We do not wish to draw the inference that these observations can be extrapolated to the surviving animal organism. Precise biological experiments would be required to establish the quantitative aspects, if any, of the exchange mechanism *in vivo*. In a qualitative way, however, the data do strongly suggest the probability of phosphate exchange between the phytin molecule and inorganic sources.

DISCUSSION

In the work detailed in this report the primary interest was a comparison of the effectiveness of phytate versus soluble inorganic orthophosphate as a dietary source of phosphorus for chicks and poults. In view of the effect of vitamin D on phytin utilization the comparison was made with both normal and vitamin D-deficient birds. Criteria of utilization were the concentrations of the radioisotope in blood and bones 48 hours after an initial dose followed by three succeeding doses in chicks, and 48 hours after a single dose in the case of poults. Data on excretion of the isotope were obtained to supplement the other findings, although, due to time limitations, these are of limited value.

Previous feeding experiments with phytin have been handicapped by high mortality in young chicks and poults when phytin was the only source of dietary phosphorus. Due to

the high mortality it has not been posible to make reliable comparisons between phytin and other materials as the sole source of phosphorus. A feeding experiment of this nature requires a minimum of two or three weeks time when growth and calcification are the measures of biological utilization. Short-term experiments with isotope dosing should provide more reliable data of a directly comparative nature.

In the work described in this report there was a much higher P^{32} content in blood and bones from chicks and poults given radioactive disodium phosphate than in birds given radioactive calcium phytate. This held true regardless of the previous history of the birds with respect to vitamin D nutrition. These data confirm earlier feeding experiments with unlabeled phytate which showed qualitatively that the young chick cannot make effective use of this source of phosphorus for growth and bone formation (Gillis et al., '49).

In drawing conclusions from the present work concerning the utilization of phytin and inorganic P^{32}, the data on retention of P^{32} in bones constitute probably the most useful and reliable statistic. The bone ash data indicate that chicks which received adequate vitamin D utilized calcium phytate approximately 10% as effectively as they utilized disodium phosphate. This value is corroborated by the values for retention in the blood plasma also. This rate of utilization also agrees well with the survival time of chicks fed phytin as the sole source of supplementary phosphorus in a low-P diet compared with that of chicks fed low levels of inorganic phosphorus (Gillis et al., '49). It is suggested, therefore, that on the basis of all available evidence, phytin phosphorus is utilized approximately 10% as effectively as inorganic orthophosphate by normal chicks under average dietary conditions.

Normal turkey poults made much poorer use of phytin than did chicks. It is well established that the requirement of poults for phosphorus is higher than that of the chick (Bird et al., '54). The specific activity of P^{32} in the bone ash indicates that less than 2% as much radioactivity was retained by

poults for calcification from phytin as from disodium phosphate. On the basis of these data it is suggested that calcium phytate should be considered valueless, for practical purposes, as a source of phosphorus for young turkey poults.

The effect of vitamin D on utilization of phytin phosphorus in these experiments confirms and extends the observations previously reported. In both chicks and poults vitamin D-deficiency resulted in negligible utilization of phytin phosphorus as measured by retention of P^{32} in bone ash. The phytin P^{32} retained by the deficient birds was in the order of 1 to 2% of that retained from disodium phosphate by deficient birds.

The utilization of inorganic phosphate was not affected significantly by vitamin D deficiency in the chick. In fact, both blood and bone ash showed slightly more inorganic radioactivity in the vitamin D-deficient chick than in the normal chicks. On the other hand, the turkey poults' utilization of inorganic phosphate seemed to be slightly, though significantly ($P < 0.05$) impaired by a deficiency of vitamin D. The vitamin D requirement, as well as the phosphorus requirement, of the poult is higher than for the chick. It is our opinion that the primary function of vitamin D is in calcium absorption (Keane, Collins, and Gillis, '56). In this case calcium absorption in the poults may have been interferred with to such an extent that retention of phosphorus was indirectly affected, although the effect was small.

CONCLUSIONS

Normal young chicks utilized P^{32}-labeled calcium phytate only about one-tenth as effectively as inorganic orthophosphate, $Na_2HP^{32}O_4$. Normal young turkey poults made only negligible (less than 2%) use of calcium phytate compared with a disodium phosphate.

In the presence of a deficiency of vitamin D, neither chicks nor poults made significant use of phytate phosphorus. However, the chicks' utilization of the inorganic source of isotope was not impaired by vitamin D deficiency. In poults, vitamin

D deficiency had a slight, but apparently significant, adverse effect on utilization of inorganic phosphorus.

In vitro studies indicated that radioactive inorganic phosphate may exchange with phosphate in the phytin molecule.

LITERATURE CITED

BIRD, H. R., H. J. ALMQUIST, W. W. CRAVENS, F. W. HILL AND J. MCGINNIS Revised Jan. 1954 Nutrient requirements for poultry. Nat'l. Acad. Sci. — Nat'l. Res. Council Publication 301.

GILLIS, M. B., L. C. NORRIS AND G. F. HEUSER 1949 The effect of phytin on the phosphorus requirements of the chick. Poultry Sci., *28:* 283–288.

———— 1953 Phosphorus metabolism and requirements of hens. Ibid., *32:* 977–984.

HARRIS, R. S. 1955 Phytic acid and its importance in human nutrition. Nutrition Rev., *13:* 257–259.

HEUSER, G. F., L. C. NORRIS, J. MCGINNIS AND M. L. SCOTT 1943 Further evidence of the need for supplementing soybean meal chick rations with phosphorus. Poultry Sci. *22:* 269–270.

KEANE, K. W., R. A. COLLINS AND M. B. GILLIS 1956 Isotopic tracer studies on the effect of vitamin D on calcium metabolism in the chick. Ibid., *35:* 1216–1222.

KRIEGER, C. H., R. BUNKFELDT AND H. STEENBOCK 1940 Cereals and rickets. X. The availability of phytic acid phosphorus. J. Nutrition, *20:* 7–14.

KRIEGER, C. H., AND H. STEENBOCK 1940 Cereals and rickets. XII. The effect of calcium and vitamin D on the availability of phosphorus. Ibid., *20:* 125–132.

LOWE, J. T., H. STEENBOCK AND C. H. KRIEGER 1939 Cereals and rickets. IX. The availability of phytin-P to the chick. Poultry Sci., *18:* 40–44.

MCGINNIS, J., L. C. NORRIS AND G. F. HEUSER 1944 Poor utilization of phosphorus in cereals and legumes by chicks for bone development. Ibid., *23:* 157–159.

SINGSEN, E. P. 1948 The phosphorus requirements of the chicken with special reference to the availability of phytin phosphorus. Storrs Agr. Exp. Sta. Bull. 260.

SINGSEN, E. P., L. D. MATTERSON AND H. M. SCOTT 1947 Phosphorus in poultry nutrition. III. The relationship between the source of vitamin D and the utilization of cereal phosphorus by the poult. J. Nutrition, *33:* 13–26.

SINGSEN, E. P., L. D. MATTERSON AND A. KOZEFF 1950 Phosphorus in poultry nutrition. IV. Radioactive phosphorus as a tracer in studying the metabolism of phytin by the turkey poult. Poultry Sci., *29:* 635–639.

EFFECTS OF GAMMA RADIATION ON CERTAIN WATER-SOLUBLE VITAMINS IN RAW GROUND BEEF [1]

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The effects of irradiation on the nutritive value of a product must be established before sterilization by radiation can become an important method for preserving food. Very few quantitative studies have been conducted to determine the nutritive changes brought about by irradiation sterilization of food. No quantitative studies have been reported concerning the effect of irradiation on vitamins in which higher animals were employed as the test organisms.

From available data, it is evident that the destructive effect of irradiation on a particular nutrient depends to a large degree on the medium in which the nutrient is suspended. Proctor and O'Meara ('51) and Huber ('50) reported that ascorbic acid in orange juice was much less radiosensitive than it was in a 0.25% oxalic acid solution. Similarly, Markakis et al. ('51) showed that vitamin B_{12} in milk was much less radiosensitive than it was when irradiated as the sole

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solute. Also, Kung et al. ('53) showed that when various products were irradiated by sterilizing doses, the extent of vitamin destruction varied greatly. For instance, the destruction of vitamin A was three-fold greater in butter than it was in margarine at all levels of irradiation employed. It appears from these findings that each food product of major importance will need to be studied to ascertain the effects of irradiation on its nutritive quality.

In view of the lack of information on the effects of irradiation on vitamins, the present studies were undertaken.

EXPERIMENTAL

Fresh, raw, ground beef was selected as the test material for the irradiation studies. The meat was sealed in number 2 tin cans, frozen, and shipped in dry ice to Arco, Idaho, for irradiation. A portion of the beef shipped to Arco, Idaho, was not irradiated. This portion is referred to as the non-irradiated control sample. Another portion, hereafter referred to as the master control, was stored in a freezer (— 8°C.) at Auburn. When the irradiated and the control meat were returned (in dry ice) to Auburn, they were placed in the freezer with the master control sample, where they remained until used for experimental purposes. Three different shipments of beef received a gamma radiation dose of 3.0, 3.0, and 3.2 megarep,[2] respectively. Some of the beef in the second shipment received an irradiation dose of 4.0 megarep.

Weanling rats of the Sprague-Dawley strain were used as test animals for biological assays. After an initial depletion period for the particular vitamin under study, the rats were grouped uniformly with respect to sex and weight and housed individually on raised-wire screens in an air conditioned room. Food and water were given ad libitum. Food consumption and weekly weight gain records were kept for each rat during the assay period, which was 4 weeks in all cases.

[2] Megarep = one million roentgen equivalent physical.

The basal diet used for the biological assay of riboflavin and pyridoxine contained 71% sucrose, 20% methanol-extracted casein, 4% lard, 4% salts,[3] 1% cod liver oil; and was fully supplemented with all vitamins, except the vitamin under study. Vitamins were added at the following levels in milligrams per kilogram of diet: thiamine, riboflavin, and pyridoxine, 6 each; inositol, 1,000; calcium pantothenate, 30; niacin, 25; 2-methyl-1, 4-napthoquinone, 5; alpha-tocopherol, 50; alpha-tocopherol acetate, 50; choline chloride, 2,000; vitamin B_{12}, 0.03; and folacin, 2. For rat growth assays, raw undried beef was incorporated in the diets. The diets were mixed weekly, placed in glass jars and stored in a refrigerator (4°C.). Fat and protein were kept constant in all diets by decreasing the level of lard and casein as beef was added. The beef (irradiated and nonirradiated) contained 60% water, 20% fat (12-hour ether extraction) and 16% protein (Kjeldahl Nitrogen $\times$ 6.25).

Riboflavin and niacin were determined microbiologically by a modification of the AOAC-USP collaborative study ('46), using *Lactobacillus casei* for the riboflavin and *Lactobacillus arabinosus* 17-5 for the niacin determinations. Basal medium number IV as given by Sauberlich and Baumann ('46) was used for the determination of all three above listed vitamins. Pyridoxine and inositol were determined microbiologically with *Saccharomyces carlsbergensis* 4228 as the test organism, by the method of Atkin et al. ('43).

Tryptophan determinations were made employing *Pediococcus pentosaceus* as the test organism and medium XI as given by Steele et al. ('49), supplemented with synthetic citrovorum factor (leucovorin).

RESULTS AND DISCUSSION

Riboflavin. Results from a rat growth assay for the riboflavin content of irradiated and nonirradiated beef are summarized in table 1. These data indicate that riboflavin in

[3] Salmon, W. D., J. Nutrition, *33:* 155 (1947).

beef was fairly resistant to irradiation; only about 8% was destroyed by the irradiation process. No appreciable loss of riboflavin in the beef occurred during shipping to and from Arco, Idaho, as evidenced by the assay values obtained on the nonirradiated sample and the master control sample.

TABLE 1

Biological assay of riboflavin in beef as measured by growth of riboflavin-depleted rats [1]

(28-day assay period)

DIET	RIBOFLAVIN ADDED TO DIET	NO. OF RATS	AV. DAILY FOOD INTAKE	AV. WT. GAIN	RIBOFLAVIN CONTENT OF BEEF (wet basis)
	μg/kg		*gm*	*gm*	*μg/gm*
Basal	0	8	4.9	8	..
Basal	250	8	5.9	19	..
Basal	500	8	6.0	33	..
Basal	750	8	6.9	49	..
Basal	1000	8	7.1	57	..
Basal	6000	8	14.7	154	..
Basal + 5% irradiated beef [2]	250	8	6.8	30	2.7
Basal + 5% nonirradiated beef	250	8	7.0	31	2.8
Basal + 5% nonirradiated MC [3]	250	4	6.7	31	2.8
Basal + 10% irradiated beef [2]	250	8	7.5	37	2.2
Basal + 10% nonirradiated beef	250	8	7.5	41	2.8
Basal + 10% nonirradiated MC [3]	250	4	7.4	36	2.0
Basal + 20% irradiated beef [2]	250	8	9.7	57	2.4
Basal + 20% nonirradiated beef	250	8	10.3	64	2.9
Basal + 20% nonirradiated MC [3]	250	4	9.4	60	2.7
Basal + 20% irradiated beef [2]	6000	8	16.9	169	..

[1] Weanling male and female rats were depleted of riboflavin for a two-week period prior to being placed on experimental diets.

[2] Processed in April, 1955 (3.0 megarep).

[3] Master control beef. This beef was not shipped to Arco, Idaho.

Dosages of gamma radiation of approximately 3.0 megarep destroyed about 10% of riboflavin in beef, according to results from 5 microbiological assays (table 2). Results from the individual assays indicated from 8 to 11% destruction of riboflavin. No detectable loss of riboflavin occurred during a 6-month period of storage in a freezer.

From these results, it appears that riboflavin in beef is much more resistant to the effects of irradiation than riboflavin in raw milk. Kung et al. ('53) found that gamma radiation dosages of 0.48 and 0.96 megarep destroyed 30 and 40% of the riboflavin (3.95 μg/ml) in milk, respectively. Other vitamins in milk were also shown to be very radiosensitive. Ascorbic acid, tocopherols and vitamin A in milk were destroyed to a greater extent than riboflavin.

TABLE 2

Microbiological assays for riboflavin, pyridoxine, niacin and inositol in irradiated and nonirradiated beef

SAMPLE	VITAMIN CONTENT OF BEEF (wet basis)			
	Riboflavin	Pyridoxine	Niacin	Inositol
	μg/gm	*μg/gm*	*μg/gm*	*mg/gm*
Irradiated beef [1]	1.76	1.70	40.0	8.43
Nonirradiated beef [1]	1.91	2.32	40.8	8.48
Nonirradiated MC [1]	1.91	2.28	42.0	...
Irradiated beef [2]	1.82	1.97	43.6	8.37
Nonirradiated beef [2]	2.15	2.55	40.1	8.30
Nonirradiated MC [2]	2.05	2.61	39.7	...
Irradiated beef [3]	1.79	2.07	48.1	8.53
Nonirradiated beef [3]	1.82	2.81	50.7	8.41
Nonirradiated MC [3]	2.15	2.83	46.9	...

[1] Processed in February, 1955 (3.0 megarep).
[2] Processed in April, 1955 (3.0 megarep).
[3] Processed in June, 1955 (3.2 megarep).

Pyridoxine. Data obtained from a rat-growth assay for pyridoxine in beef from shipment number 3 are presented in table 3 (experiment A). These results indicate that pyridoxine in beef was more radiosensitive than riboflavin. Approximately 24% of pyridoxine was destroyed by the irradiation (3.2 megarep). Data indicate that the master control sample of nonirradiated beef contained slightly more pyridoxine than the other control sample. Since the values obtained by microbiological assays (table 2) did not substantiate this finding, this small difference was considered to be within experimental error.

TABLE 3

Biological assay of pyridoxine as measured by the growth of weanling male rats[1]
(28-day assay period)

DIET	VITAMIN B_6 ADDED TO DIET	NO. OF RATS	AV. DAILY FOOD INTAKE	AV. WT. GAINS	VITAMIN B_6 CONTENT OF BEEF (wet basis)
	μg/kg		*gm*	*gm*	*μg/gm*
Experiment A (no penicillin)					
Basal	0	8	5.3	16	..
Basal	100	8	6.3	35	..
Basal	200	8	8.0	50	..
Basal	500	8	11.1	106	..
Basal	750	8	13.0	138	..
Basal	6000	8	17.3	176	..
Basal + 5% irradiated beef[2]	0	8	9.2	42	2.2
Basal + 5% nonirradiated beef	0	8	9.9	46	2.8
Basal + 5% MC[3]	0	4	10.0	51	3.0
Basal + 10% irradiated beef	0	8	12.6	70	2.2
Basal + 10% nonirradiated beef	0	8	13.7	88	3.0
Basal + 10% MC	0	4	13.6	91	3.2
Basal + 20% irradiated beef	0	8	17.2	124	2.3
Basal + 20% nonirradiated beef	0	8	17.8	140	2.8
Basal + 20% MC	0	4	19.5	144	2.8
Basal + 20% irradiated beef	6000	8	19.5	181	..
Experiment B (plus penicillin)[4]					
Basal	0	4	8.6	70	..
Basal	200	4	11.4	107	..
Basal	500	4	13.1	134	..
Basal	750	4	14.7	145	..
Basal + 5% irradiated beef	0	4	10.7	91	2.1
Basal + 5% nonirradiated beef	0	4	11.9	98	2.7
Basal + 10% irradiated beef	0	4	15.0	122	2.7
Basal + 10% nonirradiated beef	0	4	14.1	129	3.7
Basal + 20% irradiated beef	0	4	17.4	145	3.0
Basal + 20% nonirradiated beef	0	4	18.4	158	..

[1] Weanling rats (av. wt. 53 gm) were depleted of pyridoxine for three weeks prior to being placed on experiment.

[2] Irradiated in June, 1955 (3.2 megarep).

[3] Master control meat, which was not shipped to Arco, Idaho.

[4] Procaine penicillin G; 100 mg per kilogram of diet.

Results from 5 microbiological assays for pyridoxine (table 2) agreed very favorably with the results obtained by the rat biological assay. These data indicate that irradiation (approximately 3.0 megarep) destroyed about 26% of pyridoxine in beef, with a range of 20 to 29% destruction as indicated by the individual assays.

It is generally accepted that biological assay methods may not necessarily measure the total pyridoxine content of a substance because of the unequal activity of the naturally occurring forms of pyridoxine in promoting growth of higher animals. Linkswiler et al. ('51) reported that equal biological activity for synthetic pyridoxol, pyridoxal, and pyridoxamine was obtained by including aureomycin in the diet fed to rats. To determine whether higher assay values might be obtained by including an antibiotic in the diet, a rat-growth assay was conducted with penicillin (100 mg/kg) added to all the diets. Data from this assay (table 3, experiment B) are inconsistent. Because of the upward drift in assay values, no conclusions were drawn, although at the lower test levels, values obtained were comparable to those noted when penicillin was omitted from the diet. However, since the biological assay values obtained without penicillin supplementation (table 3, experiment A) were actually slightly higher than those obtained from microbiological assays (meat processed in June, table 2), it appears that all forms of pyridoxine in the beef were equally active in promoting rat growth in this assay. This may be related to the fact that much of the pyridoxine in beef is present in the bound form and may not be subject to bacterial interference when fed in this state. Also, the diet has been noted to influence the activity of the various forms of vitamin B_6 (Waibel et al., '52).

Linkswiler et al. ('51) pointed out the possibility of erroneous biological assay values in the assay of pyridoxine if the sample contained an antibiotic and the standard curve diets did not, or vice versa. The data obtained in the present study illustrate this again (table 3). In contrast to the findings of the above group, who reported only slight beneficial

effects from penicillin supplementation with no added synthetic vitamin B_6, a marked growth response was obtained in this study from penicillin supplementation even at the zero level of pyridoxine supplementation. Sauberlich ('52) reported an even greater sparing effect of penicillin on the pyridoxine requirement of the rat, when the diet contained dextrin as the source of carbohydrate.

In attempts to elucidate the growth-promoting action of antibiotics, several workers have measured the blood and tissue levels of certain vitamins and minerals in animals fed antibiotics and in control animals. Common et al. ('50), working with chicks, found that blood levels of riboflavin and calcium were increased when aureomycin was included in the diet. Lih and Baumann ('51) reported that aureomycin, streptomycin and penicillin administration increased the total amount of riboflavin in the blood and liver of rats, but the concentrations of this vitamin in plasma and liver were not increased. Monson et al. ('54) found that chicks receiving insufficient amounts of folic acid were benefited by antibiotic supplementation as evidenced by an increased rate of growth, and the folic acid content of the liver was increased. The sparing effect of antibiotics has been shown to include some of the fat-soluble vitamins as evidenced by increased liver storage of vitamin A per gram of liver and increased blood carotenoids when penicillin was fed to chicks (Burgess et al., '51 and others).

Evidence presented in table 4 reveals that supplementing diets containing suboptimum amounts of pyridoxine with penicillin resulted in an increase in the amount of pyridoxine stored in the liver. With graded levels of pyridoxine in the diets as used in bio-assays, the addition of penicillin to the diets increased the concentration of pyridoxine stored in the liver at all levels employed. Beaton and McHenry ('53) reported that subcutaneous injections of graded levels of pyridoxine resulted in a progressively increased liver storage of pyridoxine. Results summarized in table 4 substantiate this, with or without penicillin supplementation.

TABLE 4

Influence of dietary level of pyridoxine and penicillin on rat liver storage of this vitamin [1]

DIET	VITAMIN B_6 ADDED TO DIET	VITAMIN B_6 CONTENT OF RAT LIVER (wet basis)	
		No penicillin in diet	With penicillin in diet
	μg/kg	*μg/gm*	*μg/gm*
Basal	0	3.5	4.5
Basal	100	3.6	..
Basal	200	4.4	6.1
Basal	500	4.9	6.3
Basal	750	5.2	7.3
Basal	6000	9.8	..
Basal + 5% irradiated beef	0	3.7	5.5
Basal + 5% nonirradiated beef	0	3.8	5.6
Basal + 10% irradiated beef	0	4.5	5.9
Basal + 10% nonirradiated beef	0	4.6	6.2
Basal + 20% irradiated beef	0	5.8	6.9
Basal + 20% nonirradiated beef	0	6.8	8.5
Basal + 20% nonirradiated beef	6000	9.8	..

[1] When the growth bioassay for pyridoxine in beef was terminated, the rat livers from each group (table 3) were pooled and homogenized for determination of the pyridoxine content.

TABLE 5

Liver storage compared with growth as the criterion for the biological assay of pyridoxine in beef

ADDITIONS TO BASAL DIET	VITAMIN B_6 CONTENT OF BEEF (wet basis)			
	No penicillin in diet		With penicillin in diet	
	Method		Method	
	Liver storage [1]	Growth [2]	Liver storage [1]	Growth [2]
	μg/gm	*μg/gm*	*μg/gm*	*μg/gm*
5% Irradiated beef	2.2	2.2	2.0	2.1
5% Nonirradiated beef	2.8	2.8	2.3	2.7
10% Irradiated beef	2.3	2.2	2.2	2.7
10% Nonirradiated beef	2.5	3.0	2.6	3.7

[1] Calculated from the data presented in table 4.

[2] Taken from table 3.

Data presented in table 5 suggest that liver storage of pyridoxine might be used as the criterion for the biological assay of pyridoxine. With low levels of pyridoxine in the diets, the results obtained using liver storage of pyridoxine compared very favorably with the results obtained from the growth assays.

Niacin. Results obtained from microbiological assays for niacin in irradiated and nonirradiated beef (table 2) indicate that niacin is very resistant to the effects of gamma radiation. These data indicate that no destruction of niacin in beef occurred as a result of dosages of gamma radiation of approximately 3.0 megarep. Limited data obtained in this laboratory, which are not given in table 2, indicate that very little, if any, of the niacin in beef was destroyed by an irradiation dosage of 4.0 megarep. These results are in agreement with the findings of Proctor and Goldblith ('49) who found niacin to be relatively radio-insensitive when the medium was water.

Because of the nutritional interrelationship between niacin and tryptophan, the effect of irradiation on this amino acid was of special interest. Results of microbiological assays revealed that the tryptophan content of the beef was not reduced by the irradiation dosages employed in this study. Thus, according to these results, one would not need to be concerned with the destruction of niacin and tryptophan when beef is sterilized by means of ionizing radiation, since the 4.0 megarep level exceeds the dosage level usually considered necessary for sterilization.

Inositol. An irradiation dose of approximately 3.0 megarep did not appear to destroy any of the inositol present in the beef, according to the results of a microbiological assay (table 2). These results are in accord with the theory that secondary reactions are chiefly responsible for nutrient destruction. Substances that are relatively stable chemically have generally been found to be relatively radio-insensitive. Inositol is known to be very stable chemically.

SUMMARY

1. Gamma radiation of raw ground beef (approximately 3.0 megarep) resulted in the destruction of about 10% of the riboflavin and 25% of the pyridoxine, as indicated by results obtained from microbiological and biological (rat-growth) assays. Very little, if any, of inositol, niacin or tryptophan in beef was destroyed by 3.0 megarep doses of irradiation, according to data obtained from microbiological assays.

2. The supplementation of diets containing suboptimum amounts of pyridoxine with penicillin exerted a sparing effect on the pyridoxine requirement of rats, as was evidenced by an increased growth rate and an increased pyridoxine content of the liver. Rat liver storage of pyridoxine increased on a curvilinear basis as the level of pyridoxine was increased in the diet, with or without penicillin supplementation.

3. Comparable assay values for pyridoxine in beef were obtained by the microbiological and rat growth assays (without penicillin supplementation). This suggests that all forms of pyridoxine in beef were equally active for the rat in these studies.

LITERATURE CITED

ATKIN, L., A. S. SCHULTZ, W. L. WILLIAMS AND C. N. FREY 1943 Yeast microbiological methods for determination of vitamins. Ind. Eng. Chem. Anal. Ed., *15:* 141.

BEATON, G. H., AND E. W. MCHENRY 1953 Vitamin B_6 levels in rat tissues. Brit. J. Nutrition, *7:* 357.

BURGESS, R. C., M. GLUCK AND D. H. LAUGHLAND 1951 Effect of dietary penicillin on liver vitamin A and serum carotenoids in the chick. Arch. Biochem., *33:* 339.

COMMON, R. H., T. J. KEEFE, R. BURGESS AND W. A. MAW 1950 Modification of the biochemical responses of the immature pullet to estrogen by means of dietary aureomycin. Nature, *166:* 992.

HUBER, W., A. BRASCH AND A. ABSTRACK, JR. 1950 Radiation resisting vitamins. Chem. and Eng. News, *28:* 1380.

KUNG, H. C., E. L. GADEN AND C. G. KING 1953 Vitamins and enzymes in milk, effect of gamma-radiation on activity. J. Agr. Food Chem., *1:* 142.

LIH, H., AND C. A. BAUMANN 1951 Effects of certain antibiotics on the growth of rats fed diets limiting in thiamine, riboflavin, or pantothenic acid. J. Nutrition, *45:* 143.

LINKSWILER, H., C. A. BAUMANN AND E. E. SNELL 1951 Effect of aureomycin on the response of rats to various forms of vitamin B_6. Ibid., *43:* 565.

MARKAKIS, P. C., S. A. GOLDBLITH AND B. E. PROCTOR 1951 Effect of ionizing radiations on vitamin B_{12}. Nucleonics, *9:* 71.

MONSON, W. J., A. E. HARPER, M. E. WINJE, C. A. ELVEHJEM, R. A. RHODES AND W. B. SARLES 1954 Mechanisms of the vitamin-sparing effect of antibiotics. J. Nutrition, *52:* 627.

PROCTOR, B. E., AND J. P. O'MEARA 1951 Effect of high-voltage cathode rays on ascorbic acid. Ind. Eng. Chem., *43:* 718.

PROCTOR, B. E., AND S. A. GOLDBLITH 1949 The effect of soft x-rays on vitamins. Nucleonics, *5:* 56.

REPORT on the microbiological assay of riboflavin and nicotinic acid 1946. Analyst, *71:* 397.

SAUBERLICH, H. E., AND C. A. BAUMANN 1946 The effect of dietary protein upon amino acid excretion by rats and mice. J. Biol. Chem., *166:* 417.

SAUBERLICH, H. E. 1952 Effect of aureomycin and penicillin upon the vitamin requirements of the rat. J. Nutrition, *46:* 99.

STEELE, B. F., H. E. SAUBERLICH, M. S. REYNOLDS AND C. A. BAUMANN 1949 Media for *Leuconostoc mesenteroides* P-60 and *Leuconostoc citrovorum* 8081. J. Biol. Chem., *177:* 533.

WAIBEL, P. E., W. W. CRAVENS AND E. E. SNELL 1952 The effect of diet on the comparative activities of pyridoxal, pyridoxamine and pyridoxine for the chick. J. Nutrition, *48:* 531.

INFLUENCE OF SHADING UPON CHANGES IN THE ASCORBIC ACID AND CAROTENE CONTENT OF TURNIP GREENS AS COMPARED WITH CHANGES IN FRESH WEIGHT, DRY WEIGHT AND NITROGEN FRACTIONS

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It has been demonstrated a number of times that increased illumination resulted in a higher ascorbic acid content of plants (Åberg, '45; Asselbergs, '55; Babber, '50; Brown, '55; Crane and Zilva, '49; Hassan and McCollum, '54; Somers, Kelly and Hamner, '48; Somers, Hamner and Kelly, '50; see also Richardson, '54; Mapson, '55; Somers and Beeson, '48). However, there have been relatively few observations concerning the rate and reversibility of this response, although there has been the tacit assumption that such a reversibility occurs rather readily. A reversal of the effect of artificial illumination on turnip greens was observed by Hamner and Parks ('44) on a very limited scale. Relatively little is known concerning the physiology of ascorbic acid within the plant and the relationship of this vitamin to other plant constituents. This has hampered the interpretation of field experiments, since the apparent ascorbic acid response may depend upon the basis of expression. On a fresh weight basis

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the apparent response may be somewhat different than that on a dry weight, or per plant basis. Reder and Odell, '54, observed a high correlation between dry matter and ascorbic acid in turnip-green plants.

While it is well established that light influences the ascorbic content of plants, there is less evidence of such a relationship for carotene (see Somers and Beeson, '48; Fernández, '54; Goodwin, '55). Bandurski ('49) studied the influence of light upon the carotene content of isolated bean leaves and cited the results of earlier workers. He found that low light intensities resulted in the accumulation of less carotene. Moster and Quackenbush, '52; McCollum, '54; and Nettles, Hall and Dennison, '55, found light to have an influence on the carotenoid content of plants.

In the following experiments, changes in ascorbic acid and carotene content of turnip greens in response to changes in illumination were followed and were associated with other changes in the plant in an attempt to clarify some of the points mentioned above.

MATERIALS AND METHODS

Experiments were carried out during the summers of 1949 and 1950. The basic design of all of the experiments was the same, although they differed in a number of details (see table 1). The plants were grown outdoors on the lawn in pots of sand. A few weeks after planting about half of them were placed under a shade house covered with muslin which reduced the average illumination to about 25 to 30% of that in full sunshine. Care was taken to minimize, particularly in 1950, the differences in humidity and temperature between the shade and sun treatments. A week or more after the first plants were moved into the shade house the experiments were started. At the start of each experiment additional plants were moved from the sun into the shade and some of the plants in the shade house were moved back into the sun. Other plants remained in the sun or shade. Samples

TABLE 1

General conditions for the study of the effect of shade on the development of ascorbic acid and carotene in turnip greens

	1949	*1950*
Variety	Seven top	Shogoin
Planting details	Seed sown directly in 2-gal. pots of sand, May 31	Seed sown directly in 2-gal. pots of sand, May 19
Nutrient solution	Hoagland's no. 2 + micro elements (Hoagland and Arnon, '38) applied in excess on Mondays, Wednesdays and Fridays; with distilled water as required at other times.	Same as 1949
Thinning details	Thinned to 4 plants per pot in mid-June and to 2 plants per pot July 5.	Thinned to 5 plants per pot June 5 and to 3 plants per pot June 21.
Sampling procedure	6/29 to 7/11, inclusive, 2 samples of 10 plants each from 10 pots at random; 7/13 to 7/29, inclusive, 2 samples of 5 plants each from 5 pots at random.	Three samples of 5 plants each from 5 pots at random.
First plants shaded	June 23	June 26
First sampling date	June 29	Jnne 22
Other sampling dates	See figure 1	See figure 1
First experiment started	June 30	July 3
Second experiment started	July 11	None
Third experiment started	July 22	None

were taken at regular intervals for 7 to 11 days to follow the changes in composition of plants.

An outline of the experiments follows:

1949 experiments.

Experiment 1 (Started 30 days after planting; Seven Top variety used) Treatments were as follows:

A. Sun–Sun: Full sunshine continuously.

B. Sun–Shade: In full sunshine until the beginning of the experiment and then moved to shade.

C. Shade–Sun: Shaded when 23 days old; moved into full sunlight again at the beginning of the experiment.

D. Shade–Shade: Shaded from 23 days on.

Experiment 2

Same as Experiment 1, except that it was started 41 days after planting and before being analyzed the leaves of each plant were divided into two groups called "young" and "old" depending upon whether or not they appeared to be fully mature in size.

Experiment 3 (Started 52 days after planting).

A and D, same as Experiment 1.

B. (Sun–Shade)–Sun: In full sunshine until 41 days after planting, shaded from then until 52 days after planting and then moved into full sunshine again.

Leaves divided into "young" and "old" as in Experiment no. 2.

1950 Experiments.

Experiment 1 (Started 45 days after planting; Shogoin variety used) Treatments were as follows:

A. Sun–Sun: Full sunshine continuously.

B. Sun–Shade: In full sunshine until the beginning of the experiment and then moved to shade.

C. Shade–Sun: Shaded when 38 days old; moved into full sunlight at the beginning of the experiment.

D. Shade–Shade: Shaded from 38 days on.

The binomials such as Sun–Sun, Sun–Shade, etc., briefly describe the illumination treatments in each case. The first word refers to the illumination previous to the start of the experiment and the last word to the illumination during the experiment. In the case of (Sun–Shade)–Sun the first *two* words refer to the previous illumination.

Samples for analysis were taken at two- to three-day intervals. In 1949 the sampling period started the day

prior to start of experiment 1 and was used to establish the composition of the plants in the sun or in the shade before the experiment was started. In 1950 the sampling was started 4 days before any of the plants were shaded and was continued throughout the experimental period to give a more detailed description of the plants prior to the start of the experiment. These sampling dates are indicated in figures 1 and 2. The pots from which samples were to be taken were selected at random at the outset. Care was taken that all of the pots were watered thoroughly late the afternoon before samples were to be taken. All sampling was done between 8 and 9 A.M. The samples were individually wrapped in moist cloth and transferred immediately to the laboratory where they were stored at about 5°C. until they were further prepared for analysis. The harvesting, preparation and extraction of the samples for analysis were completed in less than 4 hours in all cases and the samples were prepared for analysis at random. In 1949 the samples were prepared for analysis by cutting off the leaves near the base of the petiole and removing the midrib. The leaf blades were cut into pieces about 1 cm square, or smaller, with sharp stainless steel knives. These pieces were mixed, samples were removed for extraction for ascorbic acid and carotene, and the remainder were dried in paper bags at 70°C. in a forced-draft oven. The preparation of the samples in 1950 was essentially similar except that the midribs and hypocotyls were also cut into pieces and analyzed.

Ascorbic acid was determined by the method of Nelson and Somers ('45) *i.e.*, the samples, usually 5 gm each, were ground in 100 ml of 3% metaphosphoric acid (or mixture of metaphosphoric acid and sodium metaphosphate acidified with an amount of hydrochloric acid equivalent to the sodium metaphosphate) in a Waring Blendor and were filtered. An aliquot of the filtrate was buffered at pH 3.9 and an excess of 2,6-dichlorobenzenoneindophenol solution was added. The excess indophenol was extracted with xylene and was determined colorimetrically.

Carotene was determined by the method of Ellis (see Hunter, Kelly and Somers, '50) which involved blanching the sample in boiling water, extraction with a Skellysolve B-ethanol mixture, and transfer of the carotene to Skellysolve B. This solution was dried with anhydrous sodium sulfate and an aliquot was chromatographed through a starch-Supercel mixture. The carotene content of the effluent solution was determined colorimetrically.

Total nitrogen was determined by a micro-Kjeldahl procedure. Aliquot portions (40 mg) of the dried and ground samples were digested with a sulfuric acid-selenium oxychloride mixture (Pepkowitz, Prince and Bear, '42) until light brown in color. After they had cooled somewhat about 1 ml of 30% hydrogen peroxide was added and the digestion continued until complete. More H_2O_2 was added followed by heating, if required, to give a colorless digest. Various tests showed that the digestion procedure used was such that a longer digestion time or more hydrogen peroxide did not give any higher values for total nitrogen. Following digestion the samples were diluted with water, an excess of concentrated NaOH solution was added, and the ammonia was aerated at about 70°C. into dilute H_2SO_4 using a Folin aeartion apparatus and Dow-Corning DC Antifoam A to permit vigorous aeration. The ammonia was determined by use of the Folin and Wu Nessler Reagent (Hawk, Oser and Summerson, '47). Ammonia added to samples at the outset of the digestion could be recovered completely (99.5%). Part of the nitrate nitrogen is included by this procedure, but analyses for nitrates indicated that only relatively small amounts were present.

Insoluble or "protein" nitrogen was determined by extracting a 50-mg sample of dried, ground tissue with 10 ml of boiling 70% ethanol for 10 minutes. The ethanol was discarded after centrifuging and the sample was washed with about 10 ml of distilled water at room temperature by centrifuging. The sample was then digested in the centrifuge

tubes. Soluble nitrogen was determined by the difference between total and insoluble nitrogen.

All of the results were reduced statistically using analysis of variance techniques for the data obtained in each experiment. No attempt was made to assign levels of significance at odds greater than 99 to 1 ($P < 0.01$). In expressing the data on a per plant basis no distinction was made between young and old leaves even when such a separation had been made prior to analysis.

RESULTS

Growth. Three measures of the growth of the plants as influenced by illumination were obtained. These were the fresh weight, dry weight and number of leaves. Each was expressed on a per plant basis and the results for three of the experiments are summarized in table 2. In this table each datum represents the mean of 8 samples of 10 plants each for 1949 experiment 1, of 8 samples of 5 plants each for 1949 experiment 2 and of 9 samples of 5 plants each for the 1950 experiment. These samples were collected over a period of several days (see table 1 and figs. 1 and 2). The third experiment of 1949 is not included in table 2, but insofar as it provided pertinent data the results essentially confirmed those of the other experiments. Illumination influenced growth as measured by any of these criteria, but the response depended upon the criterion in question. Thus, fresh weight was influenced by the illumination previous to the experiment in two cases, but did not respond significantly to differences in illumination during the experimental period. On the other hand, the dry weight of the leaf blades per plant was influenced markedly by the illumination during the experiment, but the previous illumination either had no effect or had a much smaller effect. The number of leaves per plant was similar to the fresh weight of the leaf blades in being influenced by the illumination treatment prior to the experiment. The fresh weight and number of leaves are

TABLE 2

Growth of turnip plants as influenced by the illumination previous to and during the experiment

	1949 EXPERIMENT 1						1949 EXPERIMENT 2						1950 EXPERIMENT					
	Previous			During exp.			Previous			During exp.			Previous			During exp.		
ILLUMINATION	Sun	Shade		Sun	Shade		Sun	Shade		Sun	Shade		Sun	Shade		Sun	Shade	
Fresh wt., leaf blades, gm/plant	19.7	17.8	*	19.1	18.4	NS	24.3	22.3	NS	23.2	23.5	NS	30.4	26.6	**	28.8	28.2	NS
Dry wt., leaf blades, gm/plant	2.46	2.16	*	2.68	1.95	**	3.05	2.83	NS	3.19	2.69	**	3.55	3.06	*	3.72	2.90	**
Leaves/plant	9.3	8.2	**	8.8	8.7	NS	12.8	11.1	**	12.2	12.2	NS	...	...		...	...	

NS = Not significant, * = significant ($P < 0.05$), ** = highly significant ($P < 0.001$).

These levels of significance refer to the comparison between plants in the sun and in the shade in each case.

obviously related since more leaves would give a greater fresh weight, unless the leaf blades were smaller on those plants with a greater number. That the leaves did differ in size is indicated particularly by the results of the second experi-

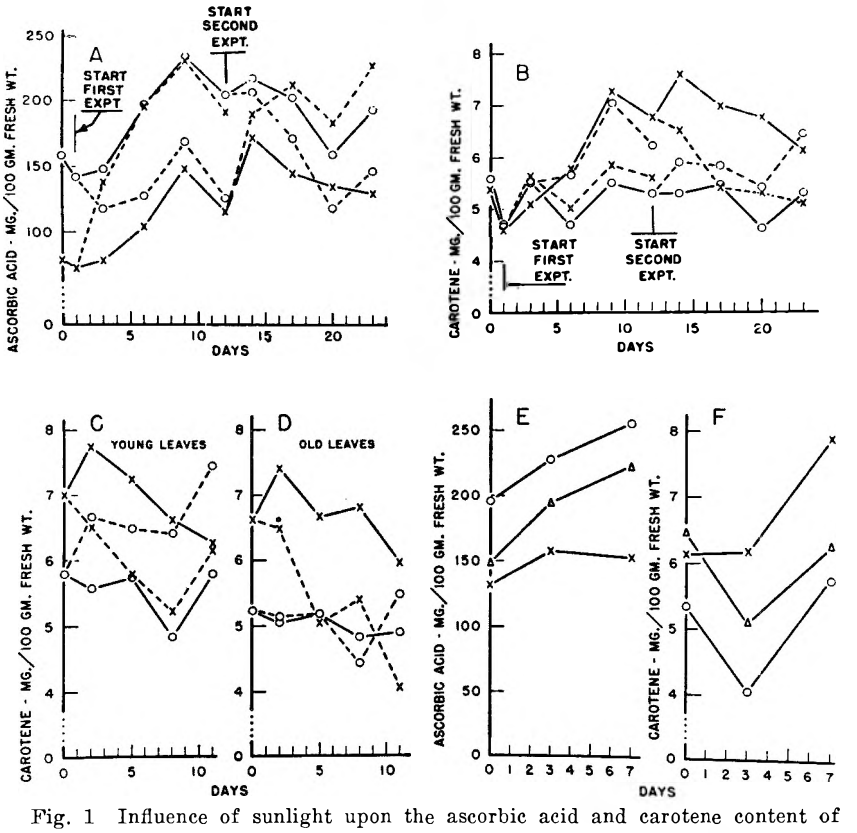

Fig. 1 Influence of sunlight upon the ascorbic acid and carotene content of turnip greens. 1949 experiment.

Plants grown continuously in full sunlight, ———O———. Plants moved from full sunlight into shade at times indicated in A and B, and at "O" days in C and D, – – – O – – –. Plants grown continuously in a shadehouse covered with muslin, ———X———. Plants moved from the shade into full sunlight at times indicated in A and B, and at "O" days in C and D, – – – X – – –.

A and B summarize the changes in ascorbic acid and carotene content, respectively, of all the leaf blades. C and D summarize the changes in carotene content of the leaf blades of young and old leaves, respectively, during the second experiment. E and F summarize the changes in ascorbic acid and carotene content, respectively, during the third experiment, which started on the 23rd day.

ment of 1949. Here the number of leaves per plant differed as a result of the previous treatment, but neither the fresh weight nor the dry weight of the leaf blades varied significantly as a result of this same treatment. This is some measure of the differences in growth characteristics which

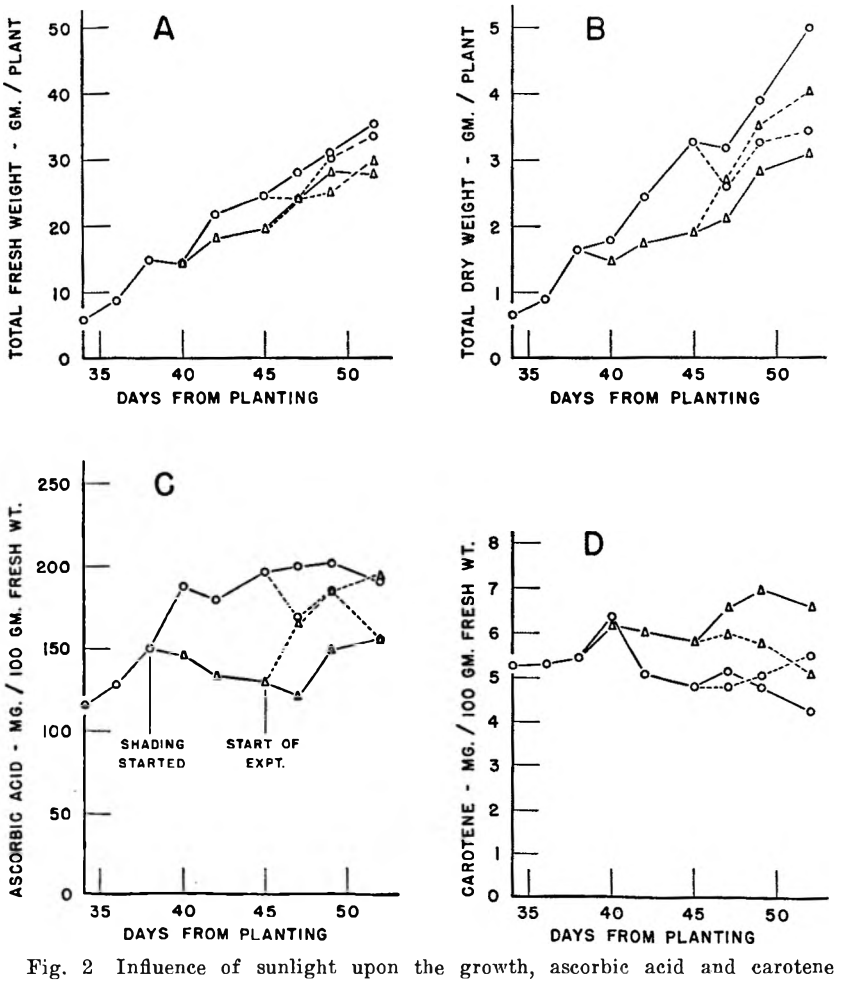

Fig. 2 Influence of sunlight upon the growth, ascorbic acid and carotene content of turnship greens. 1950 experiment.

Plants grown continuously in full sunsine, ———O———. Plants moved from full sunlight into the shade at the start of the experiment, – – – O – – –. Plants grown continuously in the shade from the date indicated, ———△———. Plants moved from the shade into full sunlight at the start of the experiment, – – – △ – – –.

resulted from shading. Thus it is clear that illumination influenced the growth of the plants in these experiments.

Composition of leaf blades; nitrogen fractions. Probably the most reliable criterion for assessing the influence of illumination upon the nitrogen fractions is the plant basis, *i.e.*, the average amount of each constituent in the total leaf blades of one plant. On this basis it was found (table 3) that the total nitrogen was not influenced significantly by illumination. The total nitrogen per plant increased as the plants grew, but this appeared to be independent of the illumination treatments. On a dry weight basis the total nitrogen content was influenced significantly by the illumination treatments. The shaded plants contained more nitrogen on this basis than those in the sun.

The insoluble or "protein" nitrogen content of the leaf blades was influenced significantly by the illumination treatment in two or more experiments on any basis of comparison. Where significant differences were observed on a per plant basis those plants in the sun contained more "protein" N than those in the shade.

The values for soluble N are subject to greater error than those for either of the other nitrogen fractions since they were obtained by subtracting the values from insoluble N from those for total N. Nevertheless, some significant responses of soluble N to the illumination treatments were observed. On a per plant basis the observed differences were small though significant in some cases. On a dry weight basis, full sunshine during the experiment resulted in a lower soluble N content. On a fresh weight basis previous illumination was without effect, but exposure to shade during the experiments resulted in a higher soluble nitrogen content.

Ascorbic acid. The amount of this vitamin in the leaf blades is summarized in table 3 (see also figs. 1 and 2). Those bases of comparison used with the nitrogen fractions are used also for ascorbic acid. In addition, this vitamin is expressed in terms of the total N content, since the amount of this

TABLE 3

The average composition of the leaf blades harvested during the experiment as influenced by the illumination previous to and during the experiment

	1949 EXPERIMENT 1						1949 EXPERIMENT 2						1950 EXPERIMENT					
	Previous			During exp.			Previous			During exp.			Previous			During exp.		
ILLUMINATION	Sun	Shade		Sun	Shade		Sun	Shade		Sun	Shade		Sun	Shade		Sun	Shade	
Soluble N																		
mg/100 gm fresh wt.	157	151	NS	144	164	NS	153	144	NS	141	156	*	107	97	NS	95	110	*
% dry wt.	1.32	1.34	NS	1.08	1.57	**	1.19	1.11	NS	0.99	1.32	**	0.94	0.89	NS	0.75	1.09	**
mg per plant	30	26	NS	27	29	NS	37	31	*	30	33	*	33	26	**	27	31	NS
Insoluble N																		
mg/100 gm fresh wt.	425	451	NS	469	406	**	467	490	NS	501	456	**	314	358	**	342	330	NS
% dry wt.	3.53	3.86	*	3.49	3.90	**	3.52	3.76	**	3.51	3.77	**	2.75	3.20	**	2.71	3.24	**
mg per plant	85	83	NS	92	76	**	100	97	NS	104	93	**	96	95	NS	98	93	NS
Total N																		
mg/100 gm fresh wt.	582	602	NS	614	570	NS	620	634	NS	642	612	NS	422	456	**	438	440	NS
% dry wt.	4.84	5.20	**	4.57	5.48	**	4.70	4.86	*	4.49	5.08	**	3.69	4.09	**	3.46	4.32	**
mg per plant	115	109	NS	119	105	NS	137	129	NS	134	131	NS	129	121	NS	126	124	NS
Ascorbic acid																		
mg/100 gm fresh wt.	167	152	**	195	125	**	182	177	NS	204	156	**	184	162	**	190	156	**
% dry wt.	1.36	1.27	**	1.44	1.20	**	1.36	1.34	NS	1.42	1.28	**	1.60	1.42	**	150	152	NS
mg per plant	33.8	28.5	**	38.5	23.8	**	78.4	74.9	NS	88.8	64.5	**	56.0	43.5	**	55.3	44.2	**
mg/gm total N	288	252	**	318	221	**	293	282	NS	320	254	**	432	356	**	438	351	**
Carotene																		
mg/100 gm fresh wt.	5.7	5.9	NS	5.4	6.2	**	5.6	6.2	**	5.4	6.4	**	4.9	6.2	**	5.2	5.9	**
mg/100 gm dry wt.	48	51	*	40	59	**	43	49	**	38	53	**	44	56	**	41	59	**
mg per plant	1.13	1.07	NS	1.03	1.17	*	1.27	1.32	NS	1.18	1.41	**	1.51	1.62	NS	1.45	1.68	*
mg/gm total N	9.9	9.8	NS	8.8	10.9	**	9.1	10.0	**	8.6	10.6	**	11.7	13.6	**	11.8	13.4	**

NS = Not significant, * = significant ($P < 0.05$), ** = highly significant ($P < 0.01$).
These levels of significance refer to the comparison between plants in the sun and in the shade in each case.

constituent on a per plant basis was found to be independent of the illumination treatments.

With only one exception in 12 comparisons, those plants which were in the sunshine during the experiment contained more ascorbic acid than those in the shade at the same time, regardless of the basis used for comparison. The dry weight basis of comparison showed smaller differences between the illumination treatments than any of the other bases. In fact, in 1950 no significant effect of the illumination treatment during the experiment was obtained with this basis of comparison.

In two experiments (1949 experiment 1 and 1950 experiment), those plants which had been in the sunshine prior to the start of the experiment contained more ascorbic acid when harvested during the experiment than those which had been in the shade. Thus, the illumination treatments given during these two experiments did not obliterate the effects of differences in illumination which the plant had received earlier.

In general, then, it seems rather clear that a greater ascorbic acid content of turnip leaf blades, except possibly on a dry weight basis, could be expected from plants growing in the sun than from comparable plants growing in the shade. The magnitude of the difference, however, may be determined not only by the illumination differences during any one period of time, but also by the previous illumination.

Carotene. The influence of the illumination during the experiment upon the carotene content of the leaf blades is summarized also in table 3 (see also figs. 1 and 2). Here it will be observed that, regardless of the basis of comparison, the leaf blades of those plants which were in the shade during the experiment had a higher carotene content than those in the sun. The illumination which the plants received prior to the start of the experiment influenced the carotene content of their leaf blades significantly in two experiments, except on a per plant basis.

The details of the analyses of variance used to interpret the above data are not given. However, in general it can be stated that there were few significant interactions observed between the illumination treatments given previous to the experiments and those given during the experiments. No significant interactions between these variables were obtained in the cases of fresh weigh per plant, dry weight per plant, or number of leaves per plant. In the case of ascorbic acid content, three significant interactions of this kind were observed. None were observed when the milligrams per plant basis of comparison was used. However, on a fresh weight basis, in 1949 experiment 2, the leaf blades of plants moved from the shade into the sun rapidly attained the ascorbic acid content of plants growing continuously in the sun, but plants moved from the sun into the shade contained on an average more ascorbic acid than those grown continuously in the shade. Similar differences in response were observed with ascorbic acid on a dry weight basis in 1949 experiment 1 and on a total N basis in 1949 experiment 2. The first of these interactions was significant ($P < 0.05$) and the latter two were highly significant ($P < 0.01$).

In the case of the carotene content of the leaf blades only two significant interactions were observed between the illumination treatments. Both of these were in the 1950 experiment and were highly significant ($P < 0.01$). On both a fresh and dry weight basis in this experiment plants moved from the sun into the shade increased in carotene content relatively less than those moved from the shade into the sun decreased in carotene content.

In a number of cases highly significant harvest-illumination interactions were observed in the composition data. These resulted from the fact that the changes in composition following a change in environment were not instantaneous, but required several days to reach the level characteristic of the new environment (see figs. 1 and 2).

Influence of age on leaves. On a fresh weight and dry weight basis the young leaves contained significantly more

of both vitamins (see table 4). On a total N basis the observed differences were so small that only in one case were they significant.

Composition of midribs and hypocotyls. On both a fresh weight basis and on a per plant basis midribs from plants in the sunshine contained more ascorbic acid than those from shaded plants (see table 5). The midribs were about as responsive to the illumination treatments as the leaf blades,

TABLE 4

Influence of age upon the ascorbic acid and carotene content of turnip leaf blades
Summer of 1949

BASES OF COMPARISON	ASCORBIC ACID					
	Experiment 2			Experiment 3		
	Young	Old		Young	Old	
mg/100 gm fresh weight	197	161	**	213	162	**
% dry weight	1.44	1.25	**	1.54	1.30	**
mg/gm total N	283	291	NS	316	326	NS

BASES OF COMPARISON	CAROTENE					
	Experiment 2			Experiment 3		
	Young	Old		Young	Old	
mg/100 gm fresh weight	6.4	5.5	**	6.6	5.2	**
mg/100 gm dry weight	47	44	*	49	44	**
mg/gm total N	9.0	10.1	**	9.8	10.4	NS

NS = not significant, * = significant ($P < 0.05$), ** = highly significant ($P < 0.01$).

on a fresh weight basis, but on the other bases they were less responsive. In any case the midribs contain much less ascorbic acid than the leaf blades of the same plants. The hypocotyls essentially did not respond to the illumination treatments insofar as the ascorbic acid content per unit of weight was concerned. The hypocotyls from plants in the sun were larger than those from plants in the shade. The mean dry weights of the hypocotyls from the various treatments were: sun–sun, 8.6; sun–shade, 5.1; shade–sun, 5.3; shade–shade, 3.8 gm per plant. These differences are re-

flected in the ascorbic acid content per plant. In any case the ascorbic acid content of the hypocotyls of these plants was small in comparison to that of the leaf blades.

Correlations. The analyses of variance discussed above omit any analyses which were made during the period when the plants were being subjected to treatments prior to the start of the experiment. In 1950, particularly, a large amount of data was not included in the analyses of variance. It seemed desirable to include all observations in the inter-

TABLE 5

The ascorbic acid content of midribs and hypocotyls of turnip plants for the 1950 experiment as influenced by the illumination previous to and during the experiment

	PREVIOUS			DURING EXPERIMENT		
ILLUMINATION	Sun	Shade		Sun	Shade	
Midribs						
mg/100 gm fresh weight	62	54	**	65	51	**
% dry weight	0.87	0.88	NS	0.87	0.89	NS
mg per plant	26.9	23.7	*	27.1	23.5	*
Hypocotyls						
mg/100 gm fresh weight	42	40	NS	43	39	*
% dry weight	0.51	0.50	NS	0.51	0.51	NS
mg per plant	13.8	9.2	**	13.9	9.1	**

NS = not significant, * = significant ($P < 0.05$), ** = highly significant ($P < 0.01$).

pretation of the results and to bring together all of the data for all experiments of one year. For this purpose all of the data available were expressed on a per plant basis and partial and multiple correlations were calculated among the amounts of the various constituents and the fresh weight, dry weight, and age of the plants. The results are summarized in table 6.

These data show that carotene responded in the same manner both years. As the fresh weight per plant or the age increased, the carotene content increased ($r_{12.34}$, $r_{14.23}$) but carotene was negatively correlated with the dry weight per plant ($r_{13.24}$). Ascorbic acid was not consistent in the as-

TABLE 6

Partial and multiple correlations between various constituents of turnip plants and fresh weight, dry weight, and age of the plants or plant parts

PLANT PART	VARIABLES IN CORRELATION [1]	$r_{12\cdot34}$		$r_{13\cdot24}$		$r_{14\cdot23}$		$r_{1\cdot234}$		n
1950 Samples										
Leaf blades	Ascorbic acid	0.47	**	0.77	**	—0.28	*	0.98	**	63
	Carotene	0.74	**	—0.63	**	0.38	**	0.97	**	63
	Total N	0.39	**	—0.83	**	—0.26	*	0.92	**	63
	Soluble N	0.47	**	—0.62	**	—0.17	NS	0.65	**	63
	Insoluble N	0.40	**	—0.73	**	—0.24	NS	0.90	**	63
Midribs	Ascorbic acid	0.13	NS	0.79	**	0.01	NS	0.97	**	63
Hypocotyls	Ascorbic acid	0.30	*	0.80	**	0.41	**	0.99	**	57
Whole plant	Ascorbic acid	0.14	NS	0.80	**	0.40	**	0.98	**	57
1949 Samples										
Leaf blades	Ascorbic acid	—0.53	**	0.92	**	0.78	**	0.99	**	84
	Carotene	0.68	**	—0.45	**	0.55	**	0.96	**	84
	Total N	0.78	**	0.40	**	0.48	**	0.99	**	84
	Soluble N	0.72	**	—0.48	**	0.17	NS	0.94	**	84
	Insoluble N	0.39	**	0.65	**	0.49	**	0.99	**	84

[1] The variables used in the correlations were: (1) the constituent in question, i.e., ascorbic acid, etc., (2) fresh weight per plant, (3) dry weight per plant, and (4) age of plant. These numbers are used to identify the various correlation coefficients.

NS = not significant, ($P > 0.05$), * = significant, ($P < 0.05$), ** = highly significant, ($P < 0.01$).

sociation with the fresh weight per plant or the age of the plant. However, it was consistently and highly significantly correlated with the dry weight of the plant. Both years this partial correlation coefficient ($r_{13.24}$) was large and positive. The differences in $r_{12.34}$ for ascorbic acid for the two years arise from differences between $r_{12.4}$ and $r_{13.4}$, *i.e.*, the relationship of this vitamin to fresh weight and to dry weight, since $r_{23.4}$, the relationship between fresh weight, dry weight and age, is nearly the same for the two years (1949 = 0.789; 1950 = 0.705).

The total nitrogen and insoluble nitrogen contents per plant behave in a very similar fashion, although only in the partial correlation with fresh weight is there any consistency from year to year. They were both positively correlated with fresh weight per plant both years.

From these partial correlations it can be concluded that those factors which had a tendency to produce a high dry weight per plant relative to the fresh weight and age also had a tendency to produce a high ascorbic acid content per plant. On the other hand, those factors which had a tendency to produce a large fresh weight per plant relative to dry weight and age had a tendency to produce a high carotene and a high total, soluble and insoluble nitrogen content per plant.

DISCUSSION

The results of these experiments confirm and extend the observations made in previous experiments. It can be concluded that for turnip greens, grown in sand culture, the amount of illumination is an important factor in determining their ascorbic acid and carotene content. Since these experiments were conducted out of doors, it follows that similar differences in illumination probably would produce similar responses under field conditions. However, the validity of this supposition and its practical importance can be adequately assessed only under the field conditions normally used in the production of this crop.

The reversibility of the responses of the plants to variations in illumination, insofar as their carotene and ascorbic acid content is concerned, suggests that by harvesting at the appropriate time the relative amounts of these vitamins could be varied somewhat. However, by simply timing the harvest to correspond to periods of favorable illumination both vitamins cannot be increased in amount simultaneously. Illumination conditions which favored a high ascorbic acid content would automatically give a low carotene content and vice versa.

The high degree of correlation between ascorbic acid content and dry matter per plant extends to the whole plant those observations which had previously been made with leaf discs (Somers and Kelly, '51) and confirms the observations of Reder and Odell ('54). It lends credence to the belief that observations made with leaf discs have significance for the whole plant.

It appears that various *d*-hexoses probably are precursors in the biological synthesis of ascorbic acid (Mapson, '55). The correlation between ascorbic acid content and dry matter may be the expression of such a functional relationship.

SUMMARY

1. Turnip plants were grown in sand culture outdoors two summers. The variety Shogoin was grown one year and Seven Top the other. When the plants were a few weeks old some of them were moved into a shade house which reduced the illumination to about 25 to 30% of full sunlight. Subsequently some plants were moved from shade into full sunshine once more and at the same time additional plants were moved into the shade. Frequent samples were analyzed over periods of 7 to 11 days for ascorbic acid (reduced), carotene, total N, soluble N and insoluble N.

2. The results show that the fresh weight of the plants and the number of leaves per plant were greater for those plants

which had been grown in full sunshine previous to the sampling period. Illumination during the sampling period had no effect on these two variables. On the other hand, the dry weight of the plants was influenced principally by the illumination *during* the sampling period. Plants in full sunshine contained more dry matter than those in the shade.

3. While the amount of soluble or insoluble N in the leaf blades on a per plant basis was influenced by the illumination treatments, the total N per plant was not influenced by these treatments.

4. On all bases used for comparison (fresh weight, dry weight, per plant and per gram total N) plants in full sunshine contained more ascorbic acid than those in the shade, although on a dry weight basis significant differences between treatment were not observed in all experiments. Illumination treatments both during and previous to the sampling period evoked significant responses.

5. On all bases used for comparison plants in full sunshine contained less carotene than those in the shade.

6. The responses of both carotene and ascorbic acid to changes in illumination were reversible.

7. Partial correlations between the composition of the plants, their fresh weight, their dry weight and their age showed a consistent positive correlation between ascorbic acid and dry weight. Carotene showed a consistent negative correlation with dry weight and a consistent positive correlation with fresh weight. The nitrogen fractions also showed a consistent positive correlation with fresh weight.

LITERATURE CITED

ÅBERG, B. 1945 Effects of light and temperature on the ascorbic acid content of green plants. Ann. Royal Agr. College, Sweden, *13:* 239–273.

ASSELBERGS, E. A. M. 1955 Studies on ascorbic acid synthesis in apple leaves. Diss. Abstr., *15:* 195–196.

BABBER, I. J. 1950 Significance of dehydro-ascorbic acid in plants. Indian J. Med. Research, *38:* 263–268.

BANDURSKI, R. S. 1949 Synthesis of carotenoid pigments in detached bean leaves. Bot. Gaz., *111:* 95–109.

BROWN, G. B. 1955 The ascorbic acid content of tomatoes as related to illumination. Proc. Amer. Soc. Hort. Sci., *65:* 342–348.

CRANE, M. B., AND S. S. ZILVA 1949 The influence of some genetic and environmental factors on the concentration of L-ascorbic acid in the tomato fruit. J. Hort. Sci., *25:* 36–49.

FERNANDEZ, M. DEL C. C. 1954 Effect of environment on the carotene content of plants. South. Coop. Series Bull., *36:* 24–42.

GOODWIN, T. W. 1955 Carotenoids. Ann. Rev. Biochem., *24:* 497–522.

HAMNER, K. C., AND R. Q. PARKS 1944 Effect of light intensity on ascorbic acid content of turnip greens. J. Am. Soc. Agron., *36:* 269–273.

HASSAN, H. H., AND J. P. MCCOLLUM 1954 Factors affecting the ascorbic acid content in tomatoes. Illinois Agr. Expt. Sta. Bull., *573:* 24 pp.

HAWK, P. B., B. L. OSER AND W. H. SUMMERSON 1947 Practical physiological chemistry, The Blakiston Co., Philadelphia, pp. 1229–1230.

HOAGLAND, D. R., AND D. I. ARNON 1938 The water-culture method for growing plants without soil. University of Calif. Col. Agr. Expt. Sta., Berkeley, Bull. 347.

HUNTER, A. S., W. C. KELLY AND G. F. SOMERS 1950 Effects of variations in soil moisture tension upon the ascorbic acid and carotene content of turnip greens. Agron. J., *42:* 96–99.

MAPSON, L. W. 1955 The biosynthesis of ascorbic acid. Vitamins and Hormones, *13:* 71–100.

MCCOLLUM, J. P. 1954 Effects of light on the formation of carotenoids in tomato fruits. Food Research, *19:* 182–189.

MOSTER, J. B., AND F. W. QUACKENBUSH 1952 The effects of temperature and light on the carotenoids of seedlings grown from three corn hybrids. Arch. Biochem. Biophys., *38:* 297–303.

NELSON, W. L., AND G. F. SOMERS 1945 Determination of ascorbic acid. Application of the indophenol-xylene extraction method of determination in large numbers of tomato and tomato juice samples. Ind. Eng. Chem., Anal. Ed., *17:* 754–756.

NETTLES, V. F., C. B. HALL AND R. A. DENNISON 1955 Influence of light on color development of tomato fruits. Proc. Amer. Soc. Hort. Sci., *65:* 349–352.

PEPKOWITZ, L. P., A. L. PRINCE AND F. E. BEAR 1942 A rapid Kjeldahl digestion method using perchloric acid. Ind. Eng. Chem., Anal. Ed., *14:* 856–857.

REDER, R., AND G. V. ODEL 1954 Vitamin content of turnip-green plants in relation to growth. Plant Physiol., *29:* 131–135.

RICHARDSON, L. R. 1954 Effect of environment on the ascorbic acid content of plants. Southern Coop. Series Bull., *36:* 6–23.

SOMERS, G. F., AND K. C. BEESON 1948 The influence of climate and fertilizer practices upon the vitamin and mineral content of vegetables. Advances Food Research, *1:* 291–324.

SOMERS, G. F., K. C. HAMNER AND W. C. KELLY 1950 Further studies on the relationship between illumination and the ascorbic acid content of tomato fruits. J. Nutrition, *40:* 133–143.

SOMERS, G. F., AND W. C. KELLY 1951 Ascorbic acid and dry matter accumulation in turnip and broccoli leaf discs after infiltration with inorganic salts, organic acids, and some enzyme inhibitors. Plant Physiol., *26:* 90–109.

SOMERS, G. F., W. C. KELLY AND K. C. HAMNER 1948 Changes in ascorbic acid content of turnip-leaf discs as influenced by light, temperature, and carbon dioxide concentration. Archives Biochem., *18:* 59–67.

DIETARY BULK AND AMINO ACID REQUIREMENTS [1]

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By measuring nutrient requirements of chicks as a percentage of the diet, a factor which translates these requirements into absolute amounts, namely food intake, is disregarded.

It is common knowledge that conditions making extra demands on the energy metabolism, such as exercise or low environmental temperatures, will trigger a mechanism causing increased food intake. Also, an animal will voluntarily consume more of a bulky diet, having a low caloric density, than of one having a greater caloric content. It is generally agreed that decreases in digestible energy per unit of feed are compensated for at least in part by increased feed intake (Brobeck, '48; Harte et al., '49; Sibbald et al., '56). Chicks fed a diet low in energy can still attain maximum growth by increasing their feed consumption, and their carcasses will have a low fat and high water content (Hill and Dansky, '54; Williams and Grau, '56a). By adding fibrous material to a diet sub-optimal in protein, Hill and Dansky ('50) obtained growth, due to increased intake, which compared well with that obtained on a diet containing sufficient protein. Probably the only work investigating the effects of decreases

[1] The experimental data in this paper are taken from a thesis submitted by the senior author to the Graduate School of the University of Illinois in partial fulfillment of the requirements of the Ph.D. degree in Animal Nutrition.

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in digestible energy per gram of feed on the intake of a diet moderately deficient in an amino acid was that of Williams and Grau ('56b), who worked with lysine-deficient semipurified diets.

The experiments reported in this paper were devised to study the growth response of young chicks to diets of varied caloric density and sub-optimal in either tryptophan (experiment 1), or arginine (experiment 2).

EXPERIMENTAL

Male chicks originating from a mating of New Hampshire males and Columbian females were used in both trials. For each trial 240 chicks were selected at 7 days of age from larger populations by excluding the very small and the very large individuals; the chicks were then assigned to their

TABLE 1

Composition of tryptophan-deficient basal diet

INGREDIENT	AMOUNT
	%
Dextrin	55.908
Casein [1]	9.000
Casein, acid-hydrolyzed [2]	12.000
Gelatin	12.240
Salt mixture [3]	5.340
Alfalfa meal, sun-cured	2.000
Corn oil, refined	3.000
Choline Cl	0.200
DL-Methionine	0.300
L-Tryptophan	0.012
Total [4]	100.000

[1] Labco, vitamin-free.

[2] HY-CASE, a salt-free product of Sheffield Chemical Company, Inc., Norwich, N. Y.

[3] For composition of salt mixture see Fisher et al. ('54).

[4] Plus the following vitamins (milligrams per kilogram diet): thiamine-HCl 100.0; riboflavin 16.0; niacin 100.0; calcium pantothenate 20.0; pyridoxine 6.0; folic acid 4.0; biotin 0.6; cyanocobalamine 0.02; inositol 100.0; para-aminobenzoic acid 2.0; ascorbic acid 250.0; Menadione 5.0; alpha-tocopherol acetate 20.0; 10,000 I. U. vitamin A acetate and 600 I. C. U. vitamin D_3. Procaine penicillin G was added at the level of 15 mg/kg.

respective lots by weight with the help of random numbers within weight-blocks in such a way that each group was similar in weight range and average weight. The chicks were raised in electrically heated, thermostatically controlled batteries, and feed and water were kept before them at all times.

TABLE 2

Chick growth and feed consumption in relation to dietary levels of tryptophan and cellulose [1]

LOT [2]	FIBER [3,4]	TOTAL L-TRYPTOPHAN	AV. GAIN	FEED CONSUMPTION	
				Total	Nutrients
	%	%	*gm*	*gm*	*gm* [5]
1	. .	0.165	146	251	251
2	. .	0.149	131	242	242
3	. .	0.133	102	221	221
4	. .	0.117	68	178	178
5	10	0.165	159	271	244
6	10	0.149	141	265	239
7	10	0.133	125	252	227
8	10	0.117	77	206	185
9	20	0.165	153	287	230
10	20	0.149	152	288	230
11	20	0.133	138	275	220
12	20	0.117	98	238	190

[1] Gains and feed consumption during 12-day experimental period.

[2] Two pens of 10 chicks each per lot.

[3] Added at the expense of dextrin.

[4] Solka Floc, a product of the Brown Company, Chicago 3, Illinois, consisting of 99.5% pure cellulose.

[5] Fiber consumed disregarded in this column.

In the first experiment, 24 groups of 10 chicks each, averaging 94 gm, were fed a pretest diet, similar to the experimental diet, for three days after allocation. The basal experimental diet is given in table 1. This basal diet was modified into 12 different diets by the inclusion of 10 and 20% of non-digestible fiber,[3] at the expense of dextrin, and several levels of supplemental L-tryptophan, as shown in table 2. Each diet was fed to two groups of chicks selected

[3] Solka Floc, The Brown Company, Chicago, Ill.

at random. When fully supplemented (lots 1, 5, and 9), the ration contained, according to previous experiments (Griminger et al., '56), enough tryptophan to support optimum growth. Calculations of the tryptophan content of the rations were based on microbiological analyses of the dietary ingredients, using barium hydroxide hydrolysis and *S. faecalis*, according to a modification of the method of Miller and Ruttinger ('50). The chicks were kept on their experimental diets from the 10th to the 24th day.

In the second experiment the chicks were started on the experimental diets at 7 days of age. The average weight in each of the 30 lots of 8 chicks each was 88 to 89 gm. The

TABLE 3

Chick growth and feed consumption in relation to dietary levels of arginine and cellulose [1]

LOT [2]	DIET	FIBER [3]	TOTAL L-ARGININE [4]	AV. GAIN	FEED CONSUMPTION	
					Total	Nutrients
		%	%	gm	gm	gm [5]
1	A	..	1.70	201	370	370
2	B	..	1.53	197	366	366
3	C	..	1.36	185	340	340
4	A	10	1.70	219	405	364
5	B	10	1.53	198	386	347
6	C	10	1.36	181	376	338
7	A	20	1.70	207	425	340
8	B	20	1.53	210	440	352
9	C	20	1.36	190	405	324
10	A	9.1	1.55	199	389	354
11	B	9.1	1.39	179	385	350
12	C	9.1	1.24	173	385	350
13	A	18.2	1.39	184	405	331
14	B	18.2	1.25	174	412	337
15	C	18.2	1.11	160	381	312

[1] Gains and feed consumption during 15-day experimental period.

[2] Two pens of eight chicks per lot.

[3] Solka Floc, a product of the Brown Company, Chicago, Illinois; lots 4–9, added at the expense of cerelose; lots 10–15, added at the expense of the whole diet.

[4] After addition of Solka Floc.

[5] Fiber consumed disregarded in this column.

arginine-deficient basal diet contained 22% crude casein, 3% refined corn oil, 0.2% choline chloride, 1% glycine, 0.3% DL-methionine, 0.69% L-arginine-HCl, 67.47% cerelose, and the same vitamin and mineral supplements as the diet used in the first experiment. The 15 modifications of this basal diet are indicated in table 3. This basal diet was calculated to contain approximately 20% protein and 1.36% arginine (diet C, table 3). Diets B and A were supplemented with L-arginine-HCl to contain 1.53 and 1.70% L-arginine, respectively. All three diets were fed without and with the inclusion of non-nutritive fiber, replacing equal amounts of cerelose (lots 1 to 9), and with fiber included at the expense of the whole diet (lots 10 to 15). Each diet was fed to duplicate groups of chicks, selected at random.

Both experiments were carried out in an air-conditioned room, kept at 78°F. and 40 to 50% relative humidity.

RESULTS AND DISCUSSION

As expected, the reduction of the tryptophan levels of the diets reduced gains in all cases, though somewhat less in the diets containing the higher level of fiber. If gains in weight at equivalent percentage levels of tryptophan are compared at the 0 and 10% levels of fiber and at the 0 and 20% levels of this supplement, the average percentage increase resulting from the inclusion of fiber was 8.9, 7.6, 22.5, 13.2, and 4.8, 16.0, 35.3, and 44.1% respectively, as the tryptophan level decreases. These results indicate that diets low in the amino acid benefitted the greatest from supplemental fiber. Since lots 9 and 10 grew at identical rates, the tryptophan requirement of the chick does not appear to be greater than 0.149% when the diet contains 20% non-nutritive fiber but exceeds this amount in the absence of dietary bulk.

When the average gains of all 24 groups were correlated with the average tryptophan intake, a correlation coefficient of 0.974 was obtained. Figure 1 shows the regression between the absolute intake of tryptophan and the average gain in

body weight of the duplicate lots ($r = 0.979$). It is indeed noteworthy that the interaction of tryptophan level, dietary bulk and feed intake would influence growth sufficiently to yield such a high correlation.

The data supplied by lots 1 to 9 of the arginine experiment are similar in nature to those obtained from the tryptophan trial, but do not offer quite as clear a picture. When average gains of all groups were correlated with average arginine intakes, a coefficient of 0.808 was obtained.

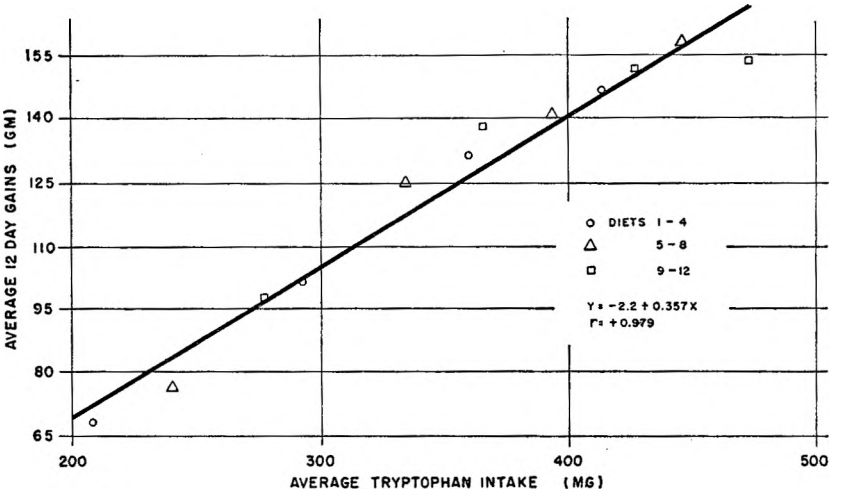

Fig. 1 Regression of average gains on average tryptophan intakes during 12-day experimental period. Each symbol represents the average of two pens of 10 chicks each.

In lots 10 to 15 non-nutritive fiber was included at the expense of the whole diet. While the chicks of lots 4 to 9 obtained, with increased feed intake, more of all nutrients except energy when compared with the control lots (1 to 3), the chicks in lots 10 to 12 would have had to eat 10% more, and those in lots 13 to 15, 20% more of their respective diets in order to obtain the same nutrients as those on the control diets. As the additional feed intake was not large enough to compensate for the lower nutrient concentration caused by the inclusion of fiber, there was a relative decreased nutri-

ent intake from the diets in which fiber replaced the total diet, while there was an increase in nutrient intake except energy when the fibrous material replaced cerelose.

It is not surprising that the tryptophan data are more conclusive than those obtained from the arginine experiment. Although both of these amino acids are dietary essentials for the growth of young chicks, there seems to be appreciable individual variability in the response to arginine-deficient diets (Fisher et al., '56). A number of previous experiments conducted under essentially the same dietary and environmental conditions (Griminger, '55) permitted a rather exact estimation of the tryptophan requirement. Although the data in table 3 indicate that diets B and C were deficient in arginine, it is less clear whether diet A represents the exact requirement under these circumstances.

An increase in the protein level increases the requirement for dietary tryptophan (Griminger et al., '56). This increase, however, is not in proportion to that in protein level, since less tryptophan, expressed as precentage of the protein, is required at higher protein levels. The addition of fiber, replacing dextrin, increased the consumption of protein and tryptophan and their percentage in relation to available energy, but did not change the ratio between protein and tryptophan. The high correlation between gains and tryptophan intake shows that additional tryptophan must have been utilized in the chicks getting more protein. It follows that better growth could be obtained when, accompanying the inclusion of bulk, a greater absolute amount of tryptophan was consumed, even when this was associated with the consumption of equally greater absolute amounts of protein.

It appears from the data in tables 2 and 3 that fiber in these basal diets stimulated chick growth. Davis and Briggs ('47) observed increased growth when graded levels of cellulose were added to their purified diet; Peterson et al. ('54) have expressed the opinion that such growth increases could be explained on the basis of increased food consumption at a marginal level of protein.

Fiber either stimulates feed consumption *per se*, as Fisher and Weiss ('56) have concluded from their experiments, or it increases feed consumption due to lowering the caloric density of the diet, or both. The fact that these purified diets have a rather high level of energy makes it seem unlikely that all increased food consumption and growth can be explained on the basis of the lower caloric density of the fiber-containing diets, which is still rather high when compared with practical-type rations. However, it is possible that the levels of protein employed in these experiments were not sufficiently high for optimum growth at the level of intake concomitant with these energy levels; thus the slight increase in growth at equal tryptophan levels when feed consumption was increased through the inclusion of fiber. This interpretation would agree with the conclusions of Rand et al. ('56) that the beneficial effect of fiber can be fully explained by the increased consumption of nutrients. Thus a purified diet without fiber and of a high caloric density might result in a sub-optimal intake of all nutrients except energy. Nutrients which are calculated to be just adequate when expressed as a percentage of the diet may prove to be deficient in terms of absolute intake. This situation would obviously be alleviated either through the replacement of some of the carbohydrate with fiber or through a higher level of those nutrients which appear to be marginal.

SUMMARY

The replacement of carbohydrate in purified or near-purified diets by non-nutritive fiber increased the voluntary food intake of chicks consuming such a diet. It is indicated that when such diets are deficient in an amino acid, increased food consumption might increase growth by supplying more of the limiting amino acid. A high correlation was shown to exist between gains and absolute intake of tryptophan and arginine respectively, when either of these was the limiting amino acid. This was observed whether the absolute intake was varied by providing more of the limiting amino acid in the

diet, expressed as percentage of the diet, or by inducing the birds to eat more of the deficient diet through the inclusion of bulk.

LITERATURE CITED

BROBECK, J. R. 1948 Regulation of energy exchange. Ann. Rev. Physiol., *10:* 315.

DAVIS, F., AND G. M. BRIGGS 1947 The growth-promoting action of cellulose in purified diets for chicks. J. Nutrition, *34:* 295.

FISHER, H., R. C. SALANDER AND M. W. TAYLOR 1956 Growth and creatine biosynthesis in the chick as affected by the amino acid deficiencies of casein. Ibid., *58:* 459.

FISHER, H., H. M. SCOTT AND R. G. HANSEN 1954 Further studies on the alfalfa factor and its relation to the liver and whey factors. Ibid., *52:* 13.

FISHER, H., AND H. S. WEISS 1956 Feed consumption in relation to dietary bulk and energy level: the effect of surgical removal of the crop. Poultry Sci., *35:* 418.

GRIMINGER, P. 1955 Effects of certain factors on the amino acid requirements of the chick. Doctoral thesis, University of Illinois, Urbana.

GRIMINGER, P., H. M. SCOTT AND R. M. FORBES 1956 The effect of protein level on the tryptophan requirement of the growing chick. J. Nutrition, *59:* 67.

HARTE, R. A., J. J. TRAVERS AND P. SARICH 1948 Voluntary caloric intake of the growing rat. Ibid., *36:* 667.

HILL, F. W., AND L. M. DANSKY 1950 Studies of the protein requirements of chicks and its relation to dietary energy level. Poultry Sci., *29:* 763.

———— 1954 Studies of the energy requirements of chicks. I. The effect of dietary energy level on growth and feed consumption. Ibid., *33:* 112.

MILLER, S., AND V. RUTTINGER 1950 An improved method of hydrolysis for use in the microbiological determination of tryptophan in human milk. Arch. Biochem., *27:* 185.

PETERSON, D. W., C. R. GRAU AND N. F. PEEK 1954 Growth and food consumption in relation to dietary levels of protein and fibrous bulk. J. Nutrition, *52:* 241.

RAND, N. T., H. M. SCOTT AND F. A. KUMMEROW 1956 The relationship of protein, fiber and fat in the diet of the growing chick. Poultry Sci., *35:* 1166.

SIBBALD, I. R., R. T. BERG AND J. P. BOWLAND 1956 Digestible energy in relation to food intake and nitrogen retention in the weanling rat. J. Nutrition, *59:* 385.

WILLIAMS, M. A., AND C. R. GRAU 1956a Energy intake and body composition of the chick in relation to the dietary concentrations of digestible carbohydrate and digestible food energy. Ibid., *59:* 255.

———— 1956b Food intake and utilization of lysine-deficient protein by the chick in relation to the digestible energy concentration of the diet. Ibid., *59:* 243.

AMINO ACID REQUIREMENTS OF MEN AND WOMEN

I. LYSINE [1]

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Fulfillment of the protein requirements of the world's population is a continuing problem of major nutritional significance. Although attention is being directed toward the production and consumption of dietary protein, the amino acids are the ultimate nutritional essentials. Information concerning the amino acid composition of foods in relation to amino acid requirements of human subjects is therefore of paramount importance. The first major contribution came from the laboratory of W. C. Rose whose classic work culminated in 1949 in a statement of the quantities of the 8 essential amino acids that are necessary for maintenance of nitrogen equilibrium in men. More recently Leverton ('54), Jones, Baumann and Reynolds ('56) and Swendseid and Dunn ('56) have estimated the quantitative requirements of women for several essential amino acids.

These investigators provided almost all of the dietary nitrogen as purified amino acids supplemented with urea or diammonium citrate. The customary dietary pattern of man is simulated more closely, however, by providing a reason-

[1] Contribution no. 15, Subproject 1, of the North Central Regional Cooperative Project NC-5, Nutritional Status and Dietary Needs of Population Groups. Journal paper 1030, Purdue Agricultural Experiment Station, Departments of Home Economics and of Biochemistry, Lafayette, Indiana. A preliminary report appeared in Fed. Proc., *15:* 546. 1956.

able proportion of amino acids as common foods and the remainder in purified form. The quantitative requirements of men and women for lysine therefore have been estimated when the diet contained cereals as well as purified amino acids.

PROCEDURE

Subjects

Five men and 5 women between 23 and 29 years of age cooperated in experiments that varied from 30 to 50 days in length. All were college graduates except one senior student, and several were doing advanced work in biochemistry or nutrition. The men varied in weight from 62 to 85 kg and the women from 45 to 80 kg. Data for the 10 individuals represent 755 person days, 4 subjects having served at least 120 days each. Two men and two women participated simultaneously in each test.

Sources of amino acids

Approximately half of the daily quota of 9.0 gm of nitrogen was supplied by wheat flour, corn meal and a few foods low in nitrogen, and the remainder by mixtures of purified amino acids and diammonium citrate. Wheat flour and corn meal were selected as sources of amino acids because they are dietary staples and also because the total lysine intake could be increased readily by supplementation with L-lysine monohydrochloride while other components of the diet remained constant. Each subject consumed 159 gm of flour [2] and 21 gm of corn meal [3] daily as baking powder biscuits and corn bread. The quantities of essential amino acids in the cereals were determined microbiologically and adjusted by apparent digestibility factors of 89% for flour and 76% for corn meal (Merrill and Watt, '55). The total amounts and sources of the essential amino acids consumed daily by each subject in

[2] Enriched white wheat flour, generously contributed by General Mills, Inc., Minneapolis, Minn.

[3] Purchased locally.

a typical experiment are stated in table 1. The total daily allotments of the essential amino acids, except lysine, and of cystine and tyrosine approximated those in 20 gm of egg protein (Mitchell and Block, '46). They exceeded the minimum requirements reported for women (Leverton et al., '56b; Swendseid and Dunn, '56) and for men (Rose, '49).

The L-isomers of the amino acids were incorporated in the supplementary mixtures, except for DL-isoleucine which was supplied in twice the amount indicated in table 1. The optical

TABLE 1

Quantities and sources of utilizable amino acids consumed daily

AMINO ACID	TOTAL [1]	FLOUR	CORN MEAL	OTHER FOODS [2]	PURIFIED AMINO ACIDS [3]
	mg	*mg*	*mg*	*mg*	*mg*
Arginine	1280	453	36		791
Histidine	420	359	30		31
Isoleucine	1600	638	79	27	856
Leucine	1840	1216	157	53	414
Methionine	820	279	30	158	353
Phenylalanine	1260	763	52	25	420
Threonine	980	468	39	43	430
Tryptophan	300	158	6	7	129
Valine	1460	717	52	61	630
Cystine	480	204	11		265
Tyrosine	900	502	36		362

[1] Equivalent to quantities in 20 gm of whole egg protein.

[2] As peaches, applesauce, orange and lemon juice, and a Litrison capsule that contained 150 mg of DL-methionine.

[3] L-isomers, except DL-isoleucine, of which twice the indicated quantity was used.

rotations of all amino acids were in good agreement with published values for the purified L-isomers. Arginine and histidine were included, almost all of the latter being derived from cereals. Although Rose and associates ('51) had demonstrated conclusively that neither arginine nor histidine was required for nitrogen equilibrium, clear-cut evidence that their omission did not induce other untoward effects became available only after this experiment was initiated (Rose et al., '55b).

The flour, corn meal and purified essential amino acids contributed daily 3.56, 0.28 and 0.75 gm of nitrogen, respectively. Fruits and other foods provided 0.38 gm. A mixture of glycine, glutamic acid and diammonium citrate supplied approximately 4.03 gm of nitrogen, one third from each source. The amount of this mixture was adjusted to maintain the total daily intake of nitrogen at or near 9.0 gm. The subjects ingested 36.5% of the total nitrogen at 7 a.m., 27.0% at noon, and 36.5% at 6 p.m. The difference in distribution resulted from the smaller amount of nitrogen supplied by corn bread, which was served at noon, than by biscuits.

One third of the daily quota of all supplementary amino acids was consumed at each meal. L-Lysine monohydrochloride, when used, was weighed separately and added to the others. Cystine and tyrosine were blended with butter oil. All others were dissolved in hot water, to which sugar and a solution of minerals in lemon juice then were added.

Foods and supplements

The amounts of wheat flour, corn meal and other ingredients that were incorporated in baking powder biscuits and corn bread are shown in table 2. Cornstarch pudding, wafers and butterfat [4] supplied additional calories, and 100 gm portions of applesauce, peaches and orange juice were served. One gram of decaffeinated coffee was allowed at each meal and mucilose flakes [5] were available.

Preliminary analysis of biscuits and corn bread indicated that some lysine was destroyed during baking. The preparation of individual portions of these products therefore was standardized. The servings of corn bread were mixed and baked at 425°F. for 20 minutes before lunch. The 8 biscuits, $1\frac{7}{8}$ inches in diameter, that were to be eaten by each subject in one day were frozen immediately after they were mixed

[4] Dry milk fat, Carnation Co., Oconomowoc, Wis.

[5] Psyllium seed. Winthrop Stearns Co., New York, N. Y.

and cut. Four frozen biscuits were baked at 450°F. for 20 minutes before breakfast, and 4 before dinner.

The daily diet of each subject included 7.8 gm of phosphate baking powder and 3.6 gm of a complete mineral mixture [6] (Leverton et al., '56a). One Litrison capsule [7] supplied in milligrams: DL-methionine 150, choline citrate 180, thiamine. HCl 3, riboflavin 3, panthenol 4.5, pyridoxine. HCl 3, niacinamide 9, folic acid 0.6, biotin 0.15, vitamin B_{12} 0.001, alpha tocopherol acetate 4.5, and 4500 U.S.P. units of vitamin A.

TABLE 2

Components of the basal diet

INGREDIENT	BISCUITS	CORN BREAD	WAFERS [1]	PUDDING [1]
	gm	*gm*	*gm*	*gm*
Flour, white wheat	138.0	21.0		
Corn meal		21.0		
Sucrose		14.0	20.0	30.0
Baking powder	5.0	1.8	1.0	
Sodium chloride	3.0	0.5	1.0	2.0
Mineral mix		0.7	1.1	
Butter oil		10.0	10.0	20.0
Vegetable fat	29.0		7.5	
Cornstarch			50.0	16.0
Mucilose flakes			2.0	
Water	85.0	43.0	30.0	200.0

[1] Spices or flavoring were added.

Quantities of lysine in the diet

The quantities of lysine in representative samples of biscuits and corn bread were determined microbiologically by the method of Steele et al. ('49), using *Leuconostoc mesenteroides* P-60. The mean concentration of lysine in biscuits prepared in one experiment was 125.0 ± 6.8 mg/gm of nitrogen, and in corn bread 127.0 ± 12.8 mg/gm of nitrogen. Products prepared from different lots of flour varied slightly in lysine content. Biscuits, corn bread and other foods supplied 75, 18 and 7%, respectively, of the amount of lysine,

[6] Prepared by Nutritional Biochemicals, Cleveland, Ohio.

[7] Generously supplied by Hoffman LaRoche, Nutley, N. J.

470 to 510 mg, present in the unsupplemented basal diet. The total intakes of lysine in different phases of the experiment ranged from that in the basal diet alone to 1500 mg which represented 20 gm of egg protein. Subjects were treated individually in regard to the quantity of lysine tested in a given period.

Caloric value of the diets

That the energy value of a diet influences nitrogen retention by human subjects who consume purified amino acids has been demonstrated (Leverton, '54; Rose et al., '54). Caloric intakes therefore were considered carefully although the diet contained cereal proteins as well as purified amino acids. In general, 40 to 45 Cal./kg of body weight were provided in the first experimental period for women and 42 to 47 Cal./kg for men. Maintenance of initial body weight then served as a criterion of caloric adequacy. All subjects carried on normal activities associated with classes, research and the home.

Biscuits, corn bread, wafers and fruit together contributed 1670 calories. The amounts of butterfat, jelly, sugar, cornstarch pudding, candy and carbonated beverage were modified as necessary. Breakfast, lunch and dinner supplied 25, 35 and 40%, respectively, of the total calories in a typical diet.

Experimental intervals and analyses

The subjects ate a diet of common foods that contributed the same amount of nitrogen as the test ration for two days preceding each experiment. Each subsequent dietary period continued for 6 days. Nitrogen balance data for the first 6-day period in each experiment, which are excluded from this report, indicated that satisfactory adjustment usually occurred in that interval.

Urine was collected in 1000-ml bottles containing 10 ml of 20% hydrochloric acid. Nitrogen was determined daily in triplicate by a macro-Kjeldahl method with potassium sulfate

and copper sulfate as catalysts.[8] The mean daily urinary nitrogen value in a given period agreed closely with that obtained from a composite representing the entire period. Creatinine was determined daily by a modification of the Folin-Wu procedure. Urine was examined twice weekly for reducing sugars and protein; all results were negative.

Feces were marked with carmine and held in frozen storage until the termination of the experiment. Material pertaining to each period was heated for 4 hours on a hot plate at moderate temperature with 25 ml of concentrated hydrochloric acid per 100 gm of feces, mixed in a blender and made to a known volume.

RESULTS AND DISCUSSION

Minimum lysine intakes for equilibrium

The minimum quantities of lysine that permitted equilibrium in 10 young men and women are summarized in table 3. An individual was judged to be in equilibrium when nitrogen balance was at or near zero. Although the errors inherent in balance tests involving human subjects were recognized, it seemed appropriate to examine the results of these experiments in relation to equilibrium rather than a higher level that allows for adult growth or a broad zone, since information was sought concerning not only the requirement for an amino acid but also the influence of various factors on it. Data for individuals are arranged in table 3 in order of increasing need for lysine. The number of periods during which each subject consumed the amount of lysine that permitted equilibrium is indicated.

Quantities of lysine between 500 and 900 mg permitted equilibrium when the subjects consumed approximately half of the total nitrogen intake as cereals and half as mixtures of amino acids and diammonium citrate. Certain individuals might have remained in equilibrium when ingesting slightly smaller amounts of lysine, but they were not always available for rechecking after fecal analyses were completed. In sum-

[8] Kelpak Powder no. 2, Laboratory Products Co., Kansas City, Mo.

mary, 500 mg of lysine were adequate for two subjects, 600 for two, 700 for three, and 800, 850 and 900 for others. Cereals supplied from 100 to 55% of the necessary amount of lysine. No striking difference was observed in the quantities of lysine required by men and women for nitrogen equilibrium.

The lysine requirements observed in this laboratory were approximately 100 mg higher than the range of 400 to 800 mg reported by Rose et al. ('55a) who employed mixtures of purified amino acids alone. They also exceed the 400 to 500 mg

TABLE 3

Minimum daily amounts of lysine that maintained nitrogen equilibrium in men and women [1]

SUBJECT				PERIODS	INTAKE OF		NITROGEN			CREATININE COEFF.
Initials	Sex	Body wt.	Surface area		Lysine	Calories	In urine	In feces	Balance	
		kg	*m²*		*mg*		*gm*	*gm*	*gm*	*mg/kg*
EG	F	45.2	1.38	1	500	2280	7.98	0.87	+ 0.22	20.7
EO	M	62.6	1.81	3	500	3000	8.10	0.93	— 0.03	26.6
VS	F	64.8	1.71	2	570	2320	8.07	0.95	+ 0.04	18.1
DB	F	53.6	1.52	3	620	2310	8.16	0.70	+ 0.15	23.0
GM	F	64.8	1.70	4	670	2380	8.07	0.92	+ 0.05	18.7
AP	M	68.2	1.80	2	670	3700	7.92	0.79	+ 0.30	22.6
RS	F	79.8	1.91	1	700	2550	8.31	0.41	+ 0.30	18.5
WC	M	85.5	2.12	1	770	3840	7.83	0.97	+ 0.26	21.0
LM	M	79.6	2.00	2	850	3700	8.05	0.83	+ 0.14	22.9
GN	M	71.7	1.95	1	900	4200	7.75	1.02	+ 0.23	24.8

[1] Data pertaining to intake and excretion represent mean daily values for the number of 6-day periods indicated. Subjects consumed approximately 9.0 gm of nitrogen daily.

which Jones, Baumann and Reynolds ('56) estimated to be sufficient for most women. The deviation in requirements reported from various laboratories may be attributable to differences in experimental procedure. For example, our subjects never received less than 470 mg of lysine whereas those tested by other investigators consumed a diet almost devoid of lysine in at least one period. The total nitrogen intake of our subjects was approximately 1.0 gm less than that of others. Also, the weights of 4 persons who ate the cereal-containing diets exceeded 70 kg. Statistical treatment of the

data in table 3 indicated that the relationship of lysine need to body weight, surface area and creatinine excretion was significant ($P < 0.05$), and to metabolic body size highly significant ($P < 0.01$). These findings differed from those of Rose et al. ('55a) who found no correlation. The minimum daily lysine requirement varied from 8.0 to 11.9 mg/kg, from 276 to 462 mg/m^2 of body surface, and from 22.6 to 35.0 mg/kg$^{\frac{3}{4}}$ of body weight. That the requirement for this amino acid should be influenced by muscular mass seems reasonable, since lysine functions principally as an essential structural unit present in high concentration in mammalian tissue.

The amounts of lysine indicated in table 3 are considered as minimum intakes compatible with nitrogen equilibrium under the conditions of this experiment rather than as a final statement of requirements. The true requirement of an individual for an amino acid may be influenced by many factors such as the total intake of nitrogen and of calories, interrelationships with other essential amino acids, distribution of the amino acid among the meals, and dietary history of the subject. To what extent a man or woman benefits nutritionally from consuming a larger quantity of an amino acid than necessary for equilibrium has not been established. Although a linear relationship existed between nitrogen balances and lysine intakes near the minimum requirement, this was not always true with larger quantities of the amino acid.

The effectiveness of amounts of lysine between 500 and 900 mg in fulfilling the needs of normal adults is pertinent to the suggestion of certain groups that bread should be enriched with lysine. The 159 gm of flour and 21 gm of corn meal in the basal diet were almost identical with the per capita consumption of these products in the United States (Handbook 62, '53). They supplied almost 500 mg of lysine, an amount that was adequate for two subjects and nearly so for two others. In the complete absence of animal protein, the highest lysine requirement recorded to date would be exceeded by doubling the amounts of cereals consumed by these subjects. A diet that includes even small amounts of lysine-rich

animal proteins such as meat, eggs or milk in addition to the per capita intake of cereals therefore should provide enough lysine for the normal man or woman. Moreover, bread that is enriched with 3 or 4% of non-fat dry milk solids already carries additional lysine.

The self-selected diets of one group of women in the North Central region contained from 1.3 to 7.3 gm of lysine (Mertz et al., '52); and of another group from 1.70 to 8.61 gm (Reynolds et al., '53). Diets that supplied 9.5 gm of nitrogen from a variety of foods contributed 3.56 gm of lysine on the average (Wharton et al., '53). Thus, the possibility of a general inadequacy of lysine in diets consumed by the adult population in this country seems remote.

Intakes of essential amino acids except lysine

Twice the quantities of all essential amino acids listed in table 1 were provided in one experiment, while the amount of lysine was varied. Nitrogen balances of subjects receiving amino acids equivalent to 20 or 40 gm of whole egg protein could not be distinguished over the range of lysine intakes tested. Large amounts of other essential amino acids therefore seem unnecessary in estimating minimum lysine requirements and are disadvantageous in terms of palatability, cost and time of preparation. Nevertheless, the question of balance among the essential amino acids merits further investigation in relation to the lysine requirement.

Caloric value of the diets

Caloric intakes were modified as necessary to maintain weights of the subjects at or near the point recorded at the beginning of each experiment. The 5 men maintained weight when consuming individually 45, 47, 47, 48 and 58 Cal./kg and the women with 29, 36, 38, 44 and 50 Cal./kg. Most of these intakes do not seem excessive when compared with the National Research Council Allowances ('53) which may be expressed as 49.2 and 41.8 Cal./kg, respectively, for the

reference man and woman who consume only natural foods. The subject with the highest lysine requirement (GN) had the greatest caloric need, but the reverse was not true. The relationship between caloric intake and lysine requirement is being investigated further.

SUMMARY

The quantities of lysine required for nitrogen equilibrium by 10 young men and women were estimated. A diet containing 159 gm of flour and 21 gm of corn meal was supplemented with purified essential amino acids so that the total amounts of the latter simulated 20 gm of whole egg protein, and also with a mixture of glycine, glutamic acid and diammonium citrate so that the subjects received 9.0 gm of nitrogen daily from all sources. Approximately half of the nitrogen was derived from white wheat flour, corn meal and a few foods low in nitrogen, and half from mixtures of purified amino acids and diammonium citrate. The quantities of flour and corn meal in the basal diet approximated the per capita consumption in this country and supplied nearly 500 mg of lysine.

The subjects attained equilibrium when consuming quantities of lysine between 500 and 900 mg. Only three subjects needed more than 700 mg per day. No striking difference in lysine needs of men and women was apparent. Nitrogen balances at a given lysine intake were not improved by doubling the amounts of all other essential amino acids.

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LITERATURE CITED

JONES, E. M., C. A. BAUMANN AND M. S. REYNOLDS 1956 Nitrogen balances of women maintained on various levels of lysine. J. Nutrition, *60:* 549–562.

LEVERTON, R. M. 1954 The amino acid requirements of man. Symposium on Protein Metabolism, National Vitamin Foundation, No. *8:* 55–74.

LEVERTON, R. M., M. R. GRAM, M. CHALOUPKA, E. BRODOVSKY AND A. MITCHELL 1956a The quantitative amino acid requirements of young women. I. Threonine. J. Nutrition, *58:* 59–82.

LEVERTON, R. M., J. ELLISON, N. JOHNSON, J. PAZUR, F. SCHMIDT AND D. GESCHWENDER 1956b V. Leucine. Ibid., *58:* 355–365.

MERRILL, A. F., AND B. F. WATT 1955 Energy value of foods. Agriculture Handbook *74.* United States Dept. of Agriculture, Washington, D. C.

MERTZ, E. T., E. J. BAXTER, L. E. JACKSON, C. E. RODERUCK AND A. WEIS 1952 Essential amino acids in self-selected diets of older women. J. Nutrition, *46:* 313–322.

MITCHELL, H. H., AND R. J. BLOCK 1946 Some relationships between the amino acid contents of proteins and their nutritive values for the rat. J. Biol. Chem., *163:* 599–620.

NATIONAL RESEARCH COUNCIL 1953 Recommended Dietary Allowances. National Academy of Sciences, Publ. *302.* Washington, D. C.

REYNOLDS, M. S., M. F. FUTRELL AND C. A. BAUMANN 1953 Nitrogen balances and amino acid content of self-selected diets of women. J. Am. Dietet. Assoc., *29:* 359–364.

ROSE, W. C. 1949 Amino acid requirements of man. Fed. Proc., *8:* 546–552.

ROSE, W. C., W. J. HAINES, D. T. WARNER AND J. E. JOHNSON 1951 The amino acid requirements of man. II. The role of threonine and histidine. J. Biol. Chem., *188:* 49–58.

ROSE, W. C., M. J. COON AND G. F. LAMBERT 1954 The amino acid requirements of man. VI. The role of the caloric intake. Ibid., *210:* 331–342.

ROSE, W. C., A. BORMAN, M. J. COON AND G. F. LAMBERT 1955a The amino acid requirements of man. X. The lysine requirement. Ibid., *214:* 579–587.

ROSE, W. C., R. L. WIXOM, H. B. LOCKHART AND G. F. LAMBERT 1955b The amino acid requirements of man. XV. The valine requirement; summary and final observations. Ibid., *217:* 987–995.

STEELE, B. F., H. E. SAUBERLICH, M. S. REYNOLDS AND C. A. BAUMANN 1949 Media for *Leuconostoc mesenteroides* P-60 and *Leuconostoc citrovorum* 8081. Ibid., *177:* 533–544.

SWENDSEID, M. E., AND M. S. DUNN 1956 Amino acid requirements of young women based on nitrogen balance data. II. Studies on isoleucine and on minimum amounts of the eight essential amino acids fed simultaneously. J. Nutrition, *58:* 507–517.

WHARTON, M. A., AND M. B. PATTON 1953 Amino acid excretion on different protein intakes. J. Am. Dietet. Assoc., *29:* 762–764.

U. S. DEPT. OF AGRICULTURE 1953 Consumption of food in the United States. Agriculture Handbook *62,* Washington, D. C.

EXUDATIVE DIATHESIS AND VITAMIN E DEFICIENCY IN TURKEY POULTS

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Most of the work on the role of vitamin E in nutrition has been concerned primarily with the determination of various deficiency symptoms in the several species. A variety of conditions has been attributed to the absence of vitamin E from the diet of the fowl. Pappenheimer and Gottsch ('31) reported the development of nutritional encephalomalacia. Dam and Glavind ('38, '39) obtained exudative diathesis as the primary symptom. Scott ('52) reported hock disorders and Pappenheimer and Goettsch ('34) observed muscular dystrophy. However, in most of these studies, the diets contained small amounts of vitamin E which necessitated the addition of an oxidizing fat such as cod liver oil or treatment with ferric chloride to destroy the vitamin E in the diet. These procedures served to complicate the picture and yielded a wide variety of results which make interpretation difficult.

Recently, Scott et al. ('55a), in an attempt to eliminate the complications resulting from the addition of oxidizing fats or ferric chloride to the diet, reported the development of a diet which appeared to be suitable for use in the study of uncomplicated vitamin E deficiency in the chick. The diet was a modification of that used for rats by Schwarz ('51). These workers used Torula yeast as the source of protein and demonstrated that the occurrence of exudative diathesis symptoms was not influenced to any measurable extent by the

presence or absence of prooxidants or antioxidants. Under these conditions, the primary symptom of vitamin E deficiency was exudative diathesis, a type of edema accompanied by hemorrhage, and a definite microcytic anemia with a low reticulocyte count. The symptoms could be completely alleviated by supplementation with alpha-tocopheryl acetate at a level of 5 mg per pound or by 10% of dried brewers' yeast which was reported by Scott ('53) to contain a factor that would spare vitamin E in its function of preventing enlarged hock disorders in turkeys. The occurrence of the edema suggested to these workers that vitamin E might in some way be involved in the maintenance of proper osmotic relationships between the intra-vascular and extra-vascular fluids within the body of the fowl.

The role of serum proteins as osmotic regulatory components of the blood has long been known. It would seem logical to assume that an alteration in the blood proteins could conceivably result in an edematous condition as seen in vitamin E deficiency. Some support for this assumption may be derived from the work of Schwarz ('51) in which it was demonstrated with rats that dietary necrotic liver degeneration occurs in animals fed a vitamin E-deficient diet containing Torula yeast as the protein source. Serum protein changes would, therefore, be possible in view of the fact that the liver is generally thought to be the site of formation of most of the blood albumin and globulin. Although Scott et al. ('55a) reported no liver degeneration in chicks with a Torula yeast diet, the possibility exists that some biochemical change takes place within the liver cells in vitamin E deficiency which would limit the formation of blood proteins.

In a later study, Scott et al. ('55b) placed the vitamin E requirement of the chick between 5 and 10 mg of alpha-tocopheryl acetate when the birds were maintained on the Torula yeast diet. The antioxidant, diphenyl-*p*-phenylenediamine, appeared to protect alpha-tocopherol, but was not required to protect alpha-tocopheryl acetate when these materials were used as sources of vitamin E. The vitamin E-sparing factor

in dried brewers' yeast seemed to be synthesized to a greater extent by yeasts grown under anaerobic conditions than by yeasts grown aerobically.

The purpose of the present experiments was to determine if exudative diathesis could be produced in turkey poults using a diet similar to that of Scott et al. ('55a) with Torula yeast as the protein source and to determine any electrophoretic changes in serum protein. The effectiveness of alpha-tocopheryl acetate and dried brewers' yeast for prevention of this disorder when these supplements were present in the maternal diets was also determined. In addition, serum from day-old poults hatched from hens fed both practical and synthetic diets supplemented with vitamin E and sources of unidentified factors was studied electrophoretically to ascertain the relationship between the serum proteins and the supplements to the diets of the dams.

EXPERIMENTAL

The vitamin E-deficient diet used for the production of exudative diathesis was similar to the chick diet of Scott et al. ('55a). It was calculated to contain 24% protein and consisted of: 76% Torula yeast, 5.41% cerelose, 5.00% lard, 3.00% wood pulp, 9.57% minerals, and 1.02% vitamins. The vitamin and mineral mixtures supplied the following per kilogram of diet: 16.03 gm $CaCO_3$, 60.10 gm $CaHPO_4 \cdot 2H_2O$, 5.0 gm NaCl, 1.14 gm $MnSO_4 \cdot 7H_2O$, 1.6 gm $FeC_6H_5O_7 \cdot 5H_2O$, 20.0 mg $CuSO_4 \cdot 5H_2O$, 14.0 mg $ZnCl_2$, 40.0 mg KI, 0.5 mg $CoCl_2 \cdot 6H_2O$, 6.0 gm KCl, 5.76 gm $MgSO_4 \cdot 7H_2O$, 8.0 mg riboflavin, 16.0 mg calcium pantothenate, 8.0 mg pyridoxine HCl, 120.0 mg niacin, 5.0 mg thiamine HCl, 2.0 mg folic acid, 20.0 mg para-aminobenzoic acid, 1.0 gm inositol, 0.2 mg biotin, 2.0 gm choline chloride, 0.5 mg vitamin K, 50.0 μg vitamin B_{12}, 4.0 gm methionine, 4.0 gm arginine, 10,000 I.U. vitamin A, 2,000 I.C.U. vitamin D_3. Beltsville Small White poults were obtained from dams fed 4 different practical type diets (table 1). The poults from each maternal source were divided

TABLE 1

Effect of maternal diet and vitamin E on growth, red blood cell count, hemoglobin, mean cell volume and albumin-globulin ratio in Beltsville Small White turkey poults at 4 weeks of age

POULT DIET [1]	EXUDATIVE DIATHESIS		WEIGHT AT 4 WEEKS		RED BLOOD CELLS		HEMOGLOBIN		HEMATOCRIT		M. E. V.		A/G RATIO	
	−E	+E	−E	+E	−E	+E	−E	+E	−E	+E	−E	+E	−E	+E
			gm	*gm*	*millions*		*gm %*	*gm %*	*%*	*%*	μ^3	μ^3		
Maternal diet: [2]														
Basal	++(8)	—	182	194	1.53	2.08 [3]	7.52	8.66	31.6	32.8	214	159	0.77	0.71
Vitamin E, 20 mg/lb	+(3)	—	187	211	1.68	2.25 [3]	7.21	9.64	30.8	35.0	183	156	0.67	0.84
Dried brewers' yeast, 5%	—	—	163	206	2.27 [4]	2.23	8.40	9.50	35.4	35.0	156	160	0.60	0.68
Vitamin E, 20 mg/lb + dried brewers' yeast, 5%	—	—	178	241	1.89 [5]	2.29 [6]	8.16	8.72	32.5	35.0	177	166	0.70	1.05
Average			178	214 [6]	1.84	2.21 [6]	7.82	9.14 [6]	32.6	34.5	182	162 [6]	0.69	0.82

[1] Synthetic Torula yeast diet, with and without vitamin E.

[2] Practical basal diet.

[3] Supplementation of maintenance diet with E significant at the 0.01 level of probability.

[4] Carry-over significant at the 0.01 level of probability.

[5] Carry-over significant at the 0.05 level of probability.

[6] Supplementation of maintenance diet with E significant at the 0.05 level of probability.

into two equal groups of 15 birds each; one group was fed the Torula yeast diet listed above and the other was fed the Torula yeast diet supplemented with 20 mg of *d*-alpha-tocopheryl acetate per pound. The composition of the maternal basal diet was as follows: 33.5% ground yellow corn, 34.0% ground milo, 25.0% soybean oil meal, 3.5% $CaHPO_4 \cdot 2H_2O$, 3.5% oyster-shell flour and 0.5% sodium chloride. In addition, the following were added per pound of diet: 160 mg $MnSO_4 \cdot 7H_2O$, 2 mg 2-methylnaphthoquinone, 2 mg riboflavin, 12.5 mg calcium pantothenate, 20 mg niacin, 400 mg choline chloride, 5 mg penicillin, 20 mg chlortetracycline, 6 μg vitamin B_{12}, 4,500 I.U. vitamin A, and 1,200 I.C.U. vitamin D_3.

The poults were reared in electrically heated batteries with raised wire floors. Feed and water were supplied ad libitum. Each group was weighed weekly and observed daily for appearance of exudative diathesis. Five birds were selected at random from each group upon the first appearance of deficiency symptoms and blood taken by heart puncture for hematological and electrophoretic analyses. Hematological studies consisted of erythrocyte count, hematocrit, and hemoglobin determinations. Mean erythrocyte volume was calculated according to Wintrobe ('42).

After removal of sufficient blood for the hematological studies, the remainder was allowed to coagulate at room temperature for one hour and the serum separated by centrifugation at 5,000 RPM for 15 minutes. The serum obtained was used for electrophoretic analysis in a Spinco Model R paper electrophoresis apparatus. The procedure employed was that recommended for serum by the manufacturer [1] of the apparatus employing a barbiturate buffer at pH 8.3, ionic strength 0.075, for a running time of 6 hours at a current of 15 milliamperes. Quantitative estimation of the percentages of each fraction present was accomplished by scanning with a Spinco Analytrol, and calculation of the area under the curve of each fraction.

[1] Spinco Division, Beckman Instruments, Inc., Belmont, California.

In addition to determination of serum protein changes upon appearance of exudative diathesis other experiments were carried out to determine the effect of maternal diet upon serum proteins of day-old Beltsville Small White poults. Poults were obtained from two sources for these experiments. The first source was identical to that for the exudative diathesis studies, namely, from dams fed the 4 practical maternal diets listed in table 1. Ten birds were selected representing each maternal diet and bled by severing the jugular vein and allowing the blood to collect in a test tube. The second source of birds was from dams fed a synthetic diet supplemented as shown in table 3. In this instance, 5 birds were selected representing each maternal diet, and bled by severing the jugular vein. The composition of the basal synthetic maternal diet was as follows: 24% soybean protein,[2] 29.0% cerelose, 30.0% starch, 3.0% soybean oil, 3.0% wood pulp, and 11.0% minerals. The mineral mix supplied the following per kilogram of feed: 35.62 gm $CaCO_3$, 46.72 gm $CaHPO_4$, 8.94 gm K_2HPO_4, 9.50 gm NaCl, 1.14 gm $MnSO_4$, 39.24 mg KI, 5.75 gm $MgSO_4 \cdot 7H_2O$, 1.56 gm $FeC_6H_5O_7 \cdot 6H_2O$, 16.75 mg $CuSO_4 \cdot 5H_2O$, 13.75 mg $ZnCl_2$, and 0.50 mg $CoCl_2 \cdot 6H_2O$. In addition, the following were included per kilogram of diet: 20.0 gm choline chloride, 1.0 gm inositol, 20.0 mg thiamine, 6.0 mg riboflavin, 15.0 mg calcium pantothenate, 100.0 mg niacin, 4.0 mg pyridoxine, 20.0 mg para-aminobenzoic acid, 2.0 mg biotin, 44.0 mg *d*-alpha-tocopheryl acetate, 50.0 μg menadione, 200.0 μg folic acid, 22.0 μg vitamin B_{12}, 2,640 I.C.U. vitamin D_3, 9,900 I.U. vitamin A, 7.5 gm methionine, 4.0 gm glycine and 22.0 mg penicillin.

Serum samples for studies on day-old poults were prepared, and electrophoretic analysis performed as indicated previously.

Wherever possible the data were treated statistically by analysis of variance according to Snedecor ('56).

[2] Drackett Assay C-1 Protein. The Dracket Products Company, Cincinnati, Ohio.

RESULTS AND DISCUSSION

Exudative diathesis studies. Appearance of exudative diathesis was observed when the poults were 4 weeks of age. Examination of the birds which died at that time revealed the characteristic hemorrhaging on the inner sides of the thighs and lower portion of the breast muscle. There was no evidence of the gross edema which has been reported in chicks, but the muscle and inner side of the skin exhibited an abnormal watery appearance which indicated that a slight edema had occurred. Only two groups were noted to contain any of these symptoms. These were the groups which were maintained on the vitamin E-deficient Torula yeast diet and had been hatched from dams receiving a basal diet, and that diet supplemented with vitamin E. The poults representing the basal maternal diet were affected to a greater degree than those representing the basal diet supplemented with vitamin E (table 1). Dried brewer's yeast was effective in the prevention of exudative diathesis when included in the maternal diet. There appeared to be no effect on weight which could be traced to the maternal diet, but supplementation of the Torula yeast maintenance diet with 20 mg of vitamin E per pound significantly increased the weight of the poults at 4 weeks (table 1).

The effect of the maternal diet on erythrocyte counts when the birds were maintained on a deficient diet was quite striking (table 1). There appeared to be a slight carry-over effect due to vitamin E from the hen through the egg to the poult, but the small increase was not found to be significant. However, a highly significant carry-over was evidenced by dried brewers' yeast as seen by the markedly increased erythrocyte count. Carry-over from the maternal diet supplemented with a combination of vitamin E and dried brewers' yeast was also significant, but this increase in cell count may have been due to the brewers' yeast effect alone. When the basal poult diet was supplemented with vitamin E, significant increases were noted in all instances except where

the maternal diet was supplemented with brewer's yeast alone which had already evidenced a high count due to carry-over. Although the hemoglobin responses were not as striking as the red cell counts, these still followed the same general pattern (table 1). A significant increase was noted upon supplementation of the basal maintenance diet with vitamin E.

TABLE 2

Effect of supplementation of maternal diet with vitamin E and dried brewers' yeast on serum A/G ratios of day-old Beltsville Small White poults

(Practical maternal diet)

SUPPLEMENT TO MATERNAL DIET	POULT A/G RATIO [1]
None	0.544
Vitamin E, 20 mg/lb	0.934 [2]
Dried brewers' yeast, 5%	0.712 [3]
Vitamin E, 20 mg/lb + dried brewers' yeast, 5%	0.812 [2]

[1] Represents 10 replicate analyses.

[2] Statistically significant at the 0.01 level of probability.

[3] Statistically significant at the 0.05 level of probability.

TABLE 3

Effect of supplementation of maternal diet with dried brewers' yeast and condensed fish solubles on serum A/G ratios of day-old Beltsville Small White poults in presence of vitamin E

(Synthetic maternal diet)

SUPPLEMENT TO MATERNAL DIET	POULT A/G RATIO [1]
None	0.732
Condensed fish solubles, 5%	0.682
Dried brewers' yeast, 5%	0.910 [2]
Condensed fish solubles, 5% + dried brewers' yeast, 5%	0.908 [2]

[1] Represents 5 replicate analyses.

[2] Statistically significant at the 0.01 level of probability.

Calculation of the mean erythrocyte volume indicated that the anemia present was that of a macrocytic type (table 1). When the maternal diets contained vitamin E or dried brewers' yeast or both, smaller mean cell volumes were obtained when the birds were maintained on the basal deficient diet. Addition of vitamin E to the basal poult diet significantly

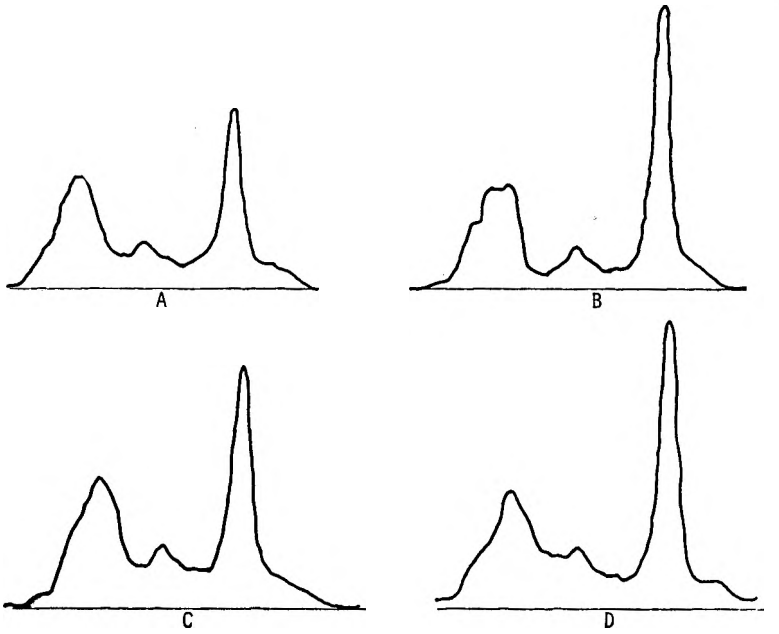

Fig. 1 A. Basal diet + vitamin E; B. Basal + vitamin E + dried brewers' yeast; C. Basal + vitamin E + condensed fish solubles; D. Basal + vitamin E + condensed fish solubles + dried brewers' yeast.

decreased the mean erythrocyte volume regardless of the maternal source of the poults. The occurrence of a macrocytic anemia in poults is not in agreement with the work of Scott et al. ('55a) with chicks in which a microcytic type of anemia was reported. However, if the anemia is due to hemorrhaging, a macrocytic anemia would be expected due to the release of reticulocytes into the blood stream.

Electrophoretic analysis of the serum from all groups of poults fed the Torula yeast diet for a period of 4 weeks indicated no significant changes in the ratio of albumin to globulins. No changes in total serum protein concentration were found as determined by biuret reaction. This fact was not too surprising in view of the fact that only a mild edema occurred in the deficient poults.

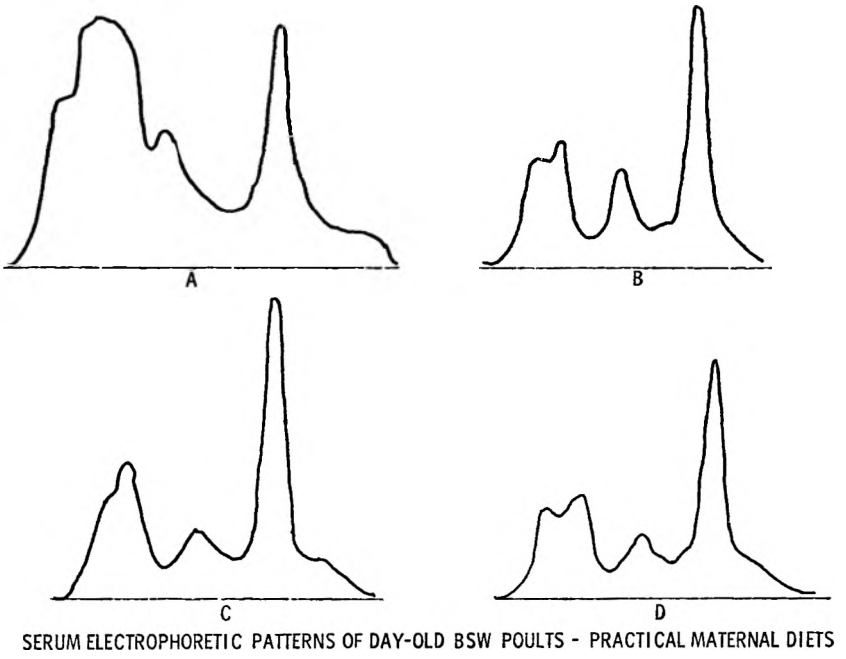

Fig. 2 A. Basal diet; B. Basal + dried brewers' yeast; C. Basal + vitamin E; D. Basal + vitamin E + dried brewers' yeast.

When subjected to gross examination, the livers from poults which died of exudative diathesis appeared healthy and normal with no indication that degeneration had taken place.

Electrophoretic studies on day-old poults. Results of these studies indicate that vitamin E and a similar factor present in dried brewers' yeast are in some way involved in the metabolism of serum proteins. Day-old poults hatched from dams which were fed a practical diet without added vitamin

E had grossly reduced A/G ratios (table 2). Supplementation of that maternal diet with 20 mg per pound of vitamin E significantly increased the albumin-globulin ratio. Identical results were obtained when the diet was supplemented with 10% of dried brewers' yeast or a combination of vitamin E and brewers' yeast. In a similar study using poults from dams fed synthetic diets containing 44 mg of vitamin E per kilogram supplementation of the basal diet with 5% of condensed fish solubles had no effect on the A/G ratios of poults from this source. However, addition of 5% dried brewers' yeast to the maternal diet produced significant increases in the A/G ratios when fed alone and in combination with condensed fish solubles. The response obtained from the combination supplemented group was the same as that for brewers' yeast alone (table 3). Typical electrophoretic patterns from poults obtained from synthetic maternal diet sources are seen in figure 1. Patterns from poults obtained from practical diets are represented by figure 2. In the cases where the A/G ratios are reduced, it appears to be due to reductions in the amount of albumin present. However, further studies are required to substantiate this observation.

SUMMARY

Exudative diathesis was produced in 4 weeks with Beltsville Small White turkey poults by feeding a diet using Torula yeast as the source of protein. The symptoms were similar to those described in chicks except that the anemia was found to be macrocytic. The condition was also characterized by hemorrhaging on the inner sides of the thighs and lower portion of the breast muscle and by a mild edema. All symptoms of the condition were prevented by addition of alpha-tocopheryl acetate at a level of 20 mg per pound of feed. The factor in dried brewers' yeast which spares vitamin E was found to be transmitted to a high degree from the dam through the egg to the poult and was effective in the prevention of exudative diathesis. No changes were noted in the protein components of blood serum as determined electrophoretically.

Studies carried out on the serum of day-old poults from dams fed practical and synthetic diets indicated that poults from practical maternal diet sources had reduced A/G ratios which could be significantly increased by supplementation of the basal diet with alpha-tocopheryl acetate or dried brewers' yeast or both. Birds from synthetic maternal diet sources exhibited increased A/G ratios when the basal diet was supplemented with dried brewers' yeast even in the presence of vitamin E. Supplementation of this maternal diet with condensed fish solubles had no effect on the A/G ratio of poults from that source.

ACKNOWLEDGMENTS

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LITERATURE CITED

DAM, H., AND J. GLAVIND 1938 Alimentary exudative diathesis. Nature, *142:* 1077–1078.

———— 1939 Alimentary exudative diathesis, a consequence of vitamin E deficiency. Ibid., *143:* 810–811.

PAPPENHEIMER, A. M., AND M. GOETTSCH 1931 A cerebellar disorder in chicks, apparently of nutritional origin. J. Exp. Med., *53:* 11–26.

———— 1934 Nutritional myopathy in ducklings. Ibid., *59:* 35–42.

SCHWARZ, K. 1951 Production of dietary necrotic liver degeneration using American Torula yeast. Proc. Soc. Exp. Biol. Med. *77:* 818–823.

SCOTT, M. L. 1952 Prevention of enlarged hock disease in turkeys and ducks. Proc. of 1952 Cornell Nut. Conf. for Feed Mfrs., pp. 86–91.

———— 1953 Prevention of the enlarged hock disorder in turkeys with niacin and vitamin E. Poultry Sci., *32:* 670–677.

SCOTT, M. L., F. W. HILL, L. C. NORRIS, D. C. DOBSON AND T. S. NELSON 1955a Studies on vitamin E in poultry nutrition. J. Nutrition, *56:* 387–402.

SCOTT, M. L., L. C. NORRIS, G. F. HEUSER AND T. S. NELSON 1955b Further chick studies on vitamin E and a vitamin E-like factor in dried brewers' yeast. Poultry Sci., *34:* 1220.

SNEDECOR, G. W. 1956 Statistical Methods. The Iowa State College Press, Ames, Iowa.

WINTROBE, M. M. 1942 Clinical Hematology. Lea and Febiger, Philadelphia, Pennsylvania.

CASEIN AS A SOURCE OF PROTEIN FOR THE CHICK[1]

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We recently reported (Hogan, Wietlake, O'Dell and Kempster, '53; Wietlake, Hogan, O'Dell and Kempster, '54) that as a source of protein for the chick, the casein-gelatin combination commonly used in experimental diets is partially inadequate. It was also reported that the arginine requirement of the newly hatched chick is higher than previous estimates, and that creatine could replace part of the arginine. Griminger, Fisher and Scott ('55) and Snyder, Morrison and Scott ('56) confirmed our report. When sucrose was the carbohydrate Monson, Harper, Benton, Winje and Elvehjem ('55) observed more rapid gains in weight when casein was supplemented with arginine and glycine than when it was supplemented with gelatin. Fisher, Salander and Taylor ('56a) gave chicks a basal diet that contained 20.4% of protein in the form of casein. It was necessary to add enough arginine to raise the total quantity to 9% of the protein in order to obtain the maximum rate of gain.

Fisher, Scott and Johnson ('55) and Fisher, Salander and Taylor ('56b) reported an acceleration in the growth rate when 2 to 5% of glycine is added to a diet that contains casein and 1% of arginine. The basal diet of Wixom, Pipkin and Day

[1] Supported in part by a grant from Merck and Company, Rahway, New Jersey. Contribution from the Missouri Agricultural Experiment Station, Journal Series 1688.

('55) in their experiment XII contained casein and arginine and the average gain in two weeks was 133 gm. When 1.5% of glycine was added to this combination the average gain was 149 gm. At this rate the shortage of glycine would not seem to be critical.

The objectives of the investigation to be described in the following pages were to determine: (1) what amino acid is the limiting factor in the casein-gelatin mixture; (2) how many amino acids are a serious limiting factor when casein is the only source of protein; (3) whether the biological value of casein, after suitable supplementation with arginine, glycine and creatine is inferior to that of other high grade proteins.

EXPERIMENTAL AND RESULTS

The chicks were Single Comb White Leghorns. They were housed in electrically heated batteries, on raised screen floors, and were allowed food and water ad libitum. The basal diet, described by Wietlake et al. ('54) contained the following constituents in per cent: casein 35, glucose [2] 46.5, cellulose 3, soybean oil 10, salts 5 and DL-methionine [3] 0.5. The original publication should be consulted for a description of the vitamin [4] supplements, and for other details. When gelatin was included in the basal diet, no. 3237, it replaced an equal weight of casein. When amino acids or creatine were added to the diet they replaced an equal weight of cerelose. In order to conserve space only the variable constituents, the nutritional factors under investigation, are shown in the following tables.

Ration 3319 has consistently supported rapid gains in weight, and has been used as a standard of excellence. It does not contain casein and it contains soybean oil meal (solvent-extracted) 55%, and cerelose 26.4%. In all other respects it is the same as ration 3237.

[2] Cerelose

[3] Courtesy of the Dow Chemical Co., Midland, Michigan.

[4] Folic acid was supplied by the Lederle Laboratories, Pearl River, N. Y. All other vitamins except A and D were supplied by Merck and Co., Rahway, N. J.

As the investigation developed and the combinations of amino acids were changed, it became impossible to repeat all the old rations whenever a new one was tried. It was observed that as a rule the variability within groups was larger than it was between groups. The question arose then as to whether all chicks that received the same ration should be included in the tabulations, even though some of those within a comparison were not under observation at the same time. As a rule all of our data are shown in the tables, but in a few cases a comparison of chicks observed at the same time changed markedly the mathematical significance of the difference of the means. The disparity in the ratios of males to females in the various groups could interfere with the statistical treatment. In an effort to avoid that complication the weights of the chicks were converted to "comparative final weights", as described by Wietlake et al. ('54).

Casein-gelatin mixture as a source of nitrogen. As has been mentioned, the combination of casein and gelatin, ration 3251, is partially inadequate as a source of nitrogen for the chick. An attempt was made to determine what amino acid was deficient in this combination, and the results are shown in table 1.

Ration 3251 contains 25% of casein and 10% of gelatin, and it supported only a moderate rate of growth. Our analyses indicated that the casein [5] was normal in composition. It contained 13.8% of nitrogen. When calculated to a base of 16% nitrogen it contained 3.8% of arginine, 0.64% of cystine and 3.10% of methionine. One other observation deserves some emphasis, the enormous variability on this diet. Some of the chicks were small and barely survived. In contrast to these, some were unaffected, and were as heavy and thrifty as those on our best diet. When 1.5% of glycine was included in the diet, as in ration 3490, the average weights were slightly higher than those shown in the first line, but the difference was a little under the 5% level of significance. However, our

[5] We are indebted to Dr. C. W. Gehrke and Dr. Laura M. Flynn for these analyses.

TABLE 1

Arginine, glycine, and creatine as supplements to the casein-gelatin mixture

RATION NUMBER	SUPPLEMENT [1]			WEIGHT RECORDS AT 4 WEEKS		COMPARATIVE FINAL WEIGHT
	C	A	G	Males	Females	
	%	%	%	*gm*	*gm*	%
3251				294 (130)[2]	288 (122)	84
				118–472 [3]	108–414	S.D.[4] 25.1
3490			1.5	301 (45)	305 (67)	89
				168–464	146–446	S.D. 21.7
3489		0.83				
3654		1.25		362 (49)	334 (65)	101
				230–458	218–398	S.D. 13.2
3491		0.83	1.5	393 (21)	344 (28)	106
				316–476	174–394	S.D. 12.4
3758	1.5			385 (12)	345 (16)	106
				300–456	272–428	S.D. 12.6

The "t" value for significance of difference.

Rations compared	Found	Required	
		0.05	0.01
R3490 > R3251	1.868	1.968	
R3489, R3654 > R3490	5.011		2.601

[1] C = Creatine hydrate, A = Arginine, G = Glycine.

[2] Figures within parentheses are numbers of chicks.

[3] Range in weights.

[4] Standard deviation.

observations on ration 3251 extended over a long period of time, and included chicks under observation when none were receiving ration 3490. When these were excluded and comparison limited to chicks under observation on the same dates, the difference of the means was larger and was far above the 1% level of significance. It seems certain that the rate of gain is accelerated somewhat when glycine is included in the diet, but in our hands the effect was somewhat inconsistent.

Ration 3489 contained 0.83% of added arginine [6] and ration 3654 contained 1.25%, but the rates of gain were practically

[6] Supplied as L-arginine HCl, but the amounts quoted represent the free base.

identical and the data from the two rations were combined. The rates of gain were excellent by most standards and the increase in the rate of gain over that observed on ration 3490 is highly significant. Ration 3491 contained additions of both arginine and glycine and the average weights were definitely higher than on ration 3489. However, when the comparison included only chicks observed at the same time the difference was small and of no statistical significance. If glycine does improve a ration such as no. 3489, which contains an adequate supply of arginine, a considerable increase in numbers would be necessary before the small differences observed had mathematical validity.

The chicks on diet 3758 received a supplement of creatine, and the weights, which were exceptionally high, were practically the same as those on diet 3491. The deficiencies of the casein-gelatin mixture were remedied completely by creatine. However, the weights on diet 3758 were no higher than those on diet 3489, when the comparison is limited to chicks under observation on the same dates.

One may conclude that a combination of 25% casein and 10% gelatin is an unsatisfactory source of nitrogen, because it does not supply sufficient arginine. It may be that either creatine alone, or a combination of arginine and glycine, is superior to arginine alone, but if so the number of chicks was too small to demonstrate the fact.

Casein alone as a source of nitrogen. The next phase of the investigation was an attempt to determine whether chicks would gain as rapidly on a diet which contained 35% of casein supplemented with amino acids as they do on a casein-gelatin diet supplemented with amino acids. Some of the rations were described in an earlier publication (Wietlake et al., '54) but most were rerun, in order to have larger numbers for the comparisons. As was shown previously, 0.66% of added arginine is insufficient. A few chicks, omitted from the table, received a diet that contained 0.83% of added arginine, and the comparative final weight of the females was a trifle over

100. However, the comparative final weight of the three males was only 75, and this trial was not repeated. The other data are shown in more detail in table 2.

Reference to lot I shows what when casein was supplemented with creatine, or with creatine and glycine, the rate of gain was moderate and the variability was excessive. Each ration in lot II contained at least 0.5% each of added arginine and creatine, as in ration 3309, or 1.25% arginine, as in rations 3310 and 3529. Creatine can replace only a small part of the arginine that is required. The average rates of gain on all the rations in this lot were about the same, though as will be brought out later it may be that some of the differences among them are significant. The average weight for the entire lot is considerably higher than in lot I, and is about as high as we expect to get over a long period of time on any ration. The rations in lot III contained 1.5% of creatine and 1.25% of added arginine. The mean weight is significantly higher than in lot II. However, when the comparison is limited to chicks under observation on the same dates, the difference of the means did not reach statistical significance. Diet 3702, lot IV, contains a total of 3.8% of arginine, but the decrease in weight indicates that this quantity is excessive.

Diet 3319, lot V, has consistently supported a maximum rate of gain, and we have used it as a standard. The weights shown in lot III are higher than those in lot V, but the difference of the means was far short of statistical significance. When the comparison was limited to chicks under observation on the same dates, the difference disappeared. It seems certain that when they are properly supplemented with amino acids the three sources of protein, casein, casein plus gelatin, and soybean oil meal, all support about the same rate of growth.

The data on the importance of glycine in chick nutrition are inconsistent. Thus diet 3529 contains a total of 2.5% of arginine, and there was some acceleration in the rate of gain when glycine was added to the diet, as in no. 3310. However,

TABLE 2

Arginine, glycine and creatine as supplements to casein

LOT	RATION NUMBER	SUPPLEMENT [1]			WEIGHT RECORDS AT 4 WEEKS		COMPARATIVE FINAL WEIGHT
		C	A	G	Males	Females	
		%	%	%	*gm.*	*gm.*	%
I	3233	1.5			305 (11)[2]	249 (20)	79
					146–424 [3]	96–382	S.D.[4] 29.9
	3234	1.5		1.5	333 (16)	260 (15)	86
					168–426	110–388	S.D. 30.0
II	3309	0.5	0.5	1.5	339 (6)	333 (10)	99
	3308	1.0	0.5	1.5	317 (8)	347 (8)	97
	3236	1.5	0.5		346 (18)	317 (13)	95
	3252	1.5	0.5	1.5	349 (51)	326 (58)	93
	3529		1.25		352 (24)	313 (38)	95
	3310		1.25	1.5	368 (26)	339 (34)	103
	Sum				351 (133)	326 (161)	98
					126–534	132–480	S.D. 19.3
III	3389	1.5	1.25	1.5	381 (6)	369 (3)	107
	3492	1.5	1.25		384 (12)	342 (8)	105
	Sum				383 (18)	349 (11)	106
					336–444	302–388	S.D. 8.6
IV	3702		2.5		321 (44)	289 (35)	88.5
					238–424	170–370	S.D. 15.3
V	3319 [5]				364 (120)	326 (145)	100
					200–490	176–472	S.D. 16.5

The "t" value for significance of difference.

Rations	Found	Required	
		0.05	0.01
Lot 3 > Lot 5	1.090	1.965	
R3234 > R3233	0.868	2.000	
R3310 > R3529	2.577 [6]	1.984	2.626
R3252 > R3236	0.536	1.979	

[1] C = Creatine hydrate, A = Arginine, G = Glycine.

[2] Figures within parentheses are numbers of chicks.

[3] Range in weights.

[4] Standard deviation.

[5] Soybean oil meal ration.

[6] When chicks on Ration 3310 at odd times were included, the difference of the means was not significant.

the data on ration 3310 include only chicks that were under observation at the same time as those on no. 3529. There were 30 more on diet 3310, run at a different time. When these 30 chicks are included in the calculations the equivalent final weight is 99.1, and the difference of the means is not significant. The "t" value required for significance of the difference of the means at the 5% level, was 1.976 and 1.091 was found. Diets 3233, 3236 and 3492 do not contain glycine, and all three contain creatine. When glycine was added to these diets, as in nos. 3234, 3252 and 3389, there was a slight increase in the rate of gain, which did not reach statistical significance.

The reports of other laboratories leave no room to doubt that glycine has some unique property in the nutrition of the chick. It may be of some significance that although the effect of glycine in our investigations was small, there was an almost uniform increase in the rate of gain when glycine was supplied. Presumably, the increase would have been significant, if the number of chicks had been sufficiently large. This would seem to show though that glycine is not of critical importance, and that it is not an essential amino acid in the same way that lysine or tryptophan is essential. It could be of direct importance as a structural unit, or of indirect importance as a precursor of creatine. No attempt was made to determine whether or not the effect of glycine was specific for that amino acid.

It has been known for some time that creatine has unique dietary properties for the chick. One would suppose that it is necessary for the chick to synthesize a large amount of this compound and that its amino acid precursors are essential dietary constituents. If creatine is supplied preformed it has a sparing effect on the requirement for both arginine and glycine. The sparing effect on arginine is more marked, presumably, because the requirement for this amino acid is more critical. Apparently the chick can synthesize little if any arginine, but can synthesize glycine at a rate insufficient to

support the maximum rate of gain. For the chick the deficiency of arginine is of major importance. The deficiency of any other amino acid in casein is much less important.

No attempt was made to determine the minimum amounts of arginine and glycine that would support the optimum rate of gain, but our data are of interest in that connection. The amounts of the pertinent nitrogenous constituents, according to Block and Bolling ('51), are shown in table 3.

TABLE 3

Amounts of arginine and glycine in the two basal diets

RATION NUMBER	PROTEIN SOURCE	PROTEIN	ARGININE	GLYCINE
	%	%	%	%
3251	Casein 25	21.5	0.9	0.45
	Gelatin 10	10.0	0.8	2.35
	Total 35	31.5	1.7	2.8
3237	Casein 35	30.0	1.3	0.6

Ration 3251, table 1, contains 1.7% of arginine, and diet 3235 (Wietlake et al., '54) contained 1.8% or practically the same amount, and this was not enough. Diet 3310 contained 2.5%, and that is sufficient. As to the amount of glycine required, diet 3529 contained 0.6% and there is some doubt that this is sufficient for the maximum rate of gain. At present Almquist's ('52) estimate of 1% is preferable.

SUMMARY

When a synthetic diet which contained 25% of casein and 10% of gelatin was supplemented with 1.5% of glycine, there was an acceleration in the rate of gain, of uncertain significance. When supplemented with 1% of arginine, or with 1.5% of creatine, the chicks gained at the maximum rate for this laboratory.

Casein alone as a source of protein for the chick is grossly deficient in arginine. The deficiency is completely, or almost completely, remedied by adding 1.2% of arginine to the diet.

The optimum amount of total arginine in this diet is over 1.8% and probably less than 2.5%.

When 1.5% of glycine was added along with 1.25% of arginine, the chicks gained at our maximum rate, but the increase in rate of gain due to glycine was small. The data give some support to Almquist's estimate that the diet of chicks should contain at least 1% of glycine.

When suitable amino acid supplements were added to casein, to the casein-gelatin mixture, or to soybean oil meal, the rates of gain were unusally rapid and approximately equal.

LITERATURE CITED

ALMQUIST, H. J. 1952 Amino acid requirements of chickens and turkeys — A review. Poultry Sci., *31:* 966–981.

BLOCK, R. L., AND D. BOLLING 1951 The amino acid composition of proteins and foods. Second Edition. Charles C. Thomas, Publisher, Springfield, Illinois.

FISHER, H., R. C. SALANDER AND M. W. TAYLOR 1956a The influence of creatine biosynthesis on the arginine requirement of the chick. J. Nutrition, *59:* 491–499.

———— 1956b Growth and creatine biosynthesis in the chick as affected by the amino acid deficiencies of casein. Ibid., *58:* 459–470.

FISHER, H., H. M. SCOTT AND B. C. JOHNSON 1955 The role of glycine in chick nutrition. Ibid., *55:* 415–430.

GRIMINGER, P., H. FISHER AND H. M. SCOTT 1955 On the arginine requirement of the growing chick. Poultry Sci., *34:* 1247–1249.

HOGAN, A. G., A. W. WIETLAKE, B. L. O'DELL AND H. L. KEMPSTER 1953 Amino acid deficiencies of casein. Abstracts of Communications, XIX, International Physiological Congress, p. 473.

MONSON, W. J., A. E. HARPER, D. A. BENTON, M. WINJE AND C. A. ELVEHJEM 1955 Effect of arginine and glycine on the growth of chicks receiving complete, purified diets. Poultry Sci., *34:* 186–190.

SNYDER, J. M., W. D. MORRISON AND H. M. SCOTT 1956 The arginine requirement of chicks fed purified and corn-soya diets. Ibid., *35:* 852–855.

WIETLAKE, A. W., A. G. HOGAN, B. L. O'DELL AND H. L. KEMPSTER 1954 Amino acid deficiencies of casein as a source of protein for the chick. J. Nutrition, *52:* 311–323.

WIXOM, R. L., G. E. PIPKIN AND P. L. DAY 1955 Interrelationship of serine and glycine for chick growth. Ibid., *56:* 409–422.

THE BIOASSAY OF THIAMINE IN BEEF EXPOSED TO GAMMA RADIATION [1]

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The military and industrial importance of "cold sterilization" by means of ionizing radiations has received increasing attention in both scientific literature and the lay press. Because of this, the effect that radiation sterilization has on the nutritive quality of the product is of special interest to the nutritionist and biochemist.

Results obtained in this laboratory (Day et al., '57) indicate that little, if any, of the niacin, inositol, and tryptophan was destroyed by gamma radiation doses of 3.0 megarep (one million rep) [2]; only about 10% of the riboflavin and 25% of the pyridoxine were destroyed by the same radiation level. In contrast to this, other investigators have shown certain vitamins (vitamin A, ascorbic acid, tocopherols, carotenes, vitamin B_{12} and riboflavin) in raw milk (Markakis et al., '51; Kung et al., '53) and the ascorbic acid in orange juice (Huber, '50; Proctor and O'Meara, '51) to be very radiosensitive.

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[2] Rep = roentgen equivalent physical.

Results are presented in this paper indicating considerable destruction of thiamine in beef when exposed to sterilization doses of gamma radiation (approximately 3.0 megarep). Also included are supplementary experiments concerned with possible interfering substances in the biological and microbiological assays for thiamine.

EXPERIMENTAL

The methods employed in handling the beef samples, experimental diets and animals (Sprague-Dawley rats) were essentially the same as those previously described (Day et al., '57). Ground beef was sealed in no. 2 metal cans and frozen prior to shipment to the reactor. Three different shipments of raw ground beef received gamma radiation dosages of 3.0, 3.0 and 3.2 megarep, respectively. Unirradiated control samples received the same treatment, except for the irradiation in all cases. An additional control, "master control," received the same treatment as the unirradiated control sample of beef, except that it was stored at — 8°C. in the laboratory at Auburn whereas the other beef was sent in dry ice to Arco, Idaho, where one-half of the sample was irradiated each time.

The basal diet used in the rat-growth assays for thiamine had the following percentage composition: sucrose 67, extracted casein 20, lard 6, salts 4,[3] glycerol 2, and cod liver oil 1. The following vitamins, in milligrams, were added per kilogram of diet: riboflavin, 6; pyridoxine, 6; niacin, 25; calcium pantothenate, 30; inositol, 1,000; 2-methyl-1,4-naphthoquinone, 5; biotin, 0.5; alpha-tocopherol, 50; alpha-tocopherol acetate, 50; choline chloride, 2,000; vitamin B_{12}, 0.03; and folacin, 2. Thiamine was omitted from the diets, except as noted in the text and tables. This same basal diet was used in the supplementary experiments, except that in some cases, the sucrose was replaced with dextrin and the glycerol omitted. Unless otherwise indicated, fat and protein were kept constant in the rat-growth assay diets by decreasing the levels of casein and lard as beef was added.

[3] Salmon, W. D., J. Nutrition, *33:* 155 (1947).

The microbiological method employed for thiamine determination was essentially that of Sarett and Cheldelin ('44). Basal medium IV (Sauberlich and Baumann, '46) and a commercial medium [4] were used, with the addition of D-xylose (10 gm/liter) in all cases. The thiochrome method as given by the Association of Official Agricultural Chemists ('50) was used for the chemical determination.

RESULTS AND DISCUSSION

Rat bio-assays. Preliminary experiments were conducted to determine the levels of thiamine that should be added to the experimental diets to permit construction of the response curve for the assay of thiamine. It was found that unless 2% of glycerol was added to the diets, as previously shown by Kandutsch and Baumann ('53) and Waibel et al. ('54), the rats failed to grow in spite of the addition of a fairly high level of thiamine to the diet.

In other preliminary experiments, it was noted that the growth of rats fed the basal diet (no meat) fully supplemented with thiamine (6 mg/kg) was somewhat inferior to that of rats fed the same diet supplemented with 30% of beef. However, this effect was eliminated when the protein content of the diet was kept constant. On the basis of these results, the protein level was kept constant in all diets and glycerol was added to each diet, unless otherwise indicated.

Data are summarized in table 1 on the rat-growth assay for thiamine in irradiated beef. The meat was processed in April, 1955 (3.0 megarep of gamma radiation). A base level of 750 μg of thiamine per kilogram of diet was used in this experiment as indicated in table 1. The results from this assay indicate that the gamma radiation reduced the thiamine content of the beef (wet basis) from 1.40 μg per gram to about 0.45 μg per gram or a destruction of approximately 67%. No apparent difference was noted in the thiamine content of the two control samples of non-irradiated meat.

[4] Difco.

Microbiological assays. Results obtained from microbiological assays of thiamine in irradiated and non-irradiated beef are summarized in table 2. The data from the assays on the three separate shipments of meat indicate that the irradiation destroyed from 61 to 67% of thiamine, with an

TABLE 1

Biological assay of thiamine in beef as measured by growth of weanling male rats [1]

DIET	THIAMINE ADDED TO DIET	NO. OF RATS	AV. DAILY FOOD INTAKE /RAT	AV. WT. GAIN /RAT (4-wk. period)	THIAMINE CONTENT OF BEEF (wet basis)
	μg/kg		*gm*	*gm*	*μg/gm*
Basal	750	8	3.0	4	...
Basal	900	8	4.3	27	...
Basal	1,100	8	7.0	75	...
Basal	1,300	8	10.4	133	...
Basal	1,500	8	14.2	178	...
Basal	6,000	8	14.7	179	...
Basal + 10% irradiated beef [2]	750	8	7.0	46	0.45
Basal + 10% non-irradiated beef	750	8	8.2	70	1.44
Basal + 10% MC [3]	750	4	8.4	71	1.39
Basal + 20% irradiated beef [2]	750	8	11.0	92	0.47
Basal + 20% non-irradiated beef	750	8	13.4	127	1.33
Basal + 20% MC	750	4	15.3	146	1.31
Basal + 30% irradiated beef [2]	750	8	13.0	113	0.44
Basal + 30% non-irradiated beef	750	8	18.9	186	1.55
Basal + 30% MC	750	4	19.7	187	1.43
Basal + 30% irradiated beef [2]	6,000	8	20.4	196	...

[1] Weanling rats were depleted of thiamine for a 10-day period prior to being placed on experiment.

[2] Irradiated in April, 1955 (3.0 megarep).

[3] Master control meat. This was not shipped to Arco, Idaho.

average of 64% destruction. The results of the microbiological assays and rat-growth assay for thiamine in the processed beef were in reasonable agreement with each other as may be noted in table 2.

Various investigators have suggested that irradiation may result in greater destruction of nutrients that are in close

proximity to the metal container. Since thiamine was found to be very radiosensitive, it was chosen for testing this supposition. Samples of beef were obtained from the center and from within one-eighth of an inch of the metal of irradiated and non-irradiated containers. Results of microbiological

TABLE 2

Summary of biological, microbiological and chemical determinations of thiamine in irradiated and non-irradiated beef

SAMPLE	THIAMINE CONTENT OF BEEF (wet basis)		
	Chemical assay [1]	Rat growth assay [2]	Microbiological assay [3]
	μg/gm	*μg/gm*	*μg/gm*
Irradiated beef [4]	0.45	0.44	0.52
Non-irradiated beef [4]	1.13	1.14	1.31
Master control [4]	1.13	1.30	1.32
Irradiated beef [5]	0.43	0.46	0.43
Non-irradiated beef [5]	1.05	1.44	1.31
Master control [5]	1.12	1.38	1.35
Irradiated beef [6]	...	...	0.45
Non-irradiated beef [6]	...	...	1.13
Master control [6]	...	...	1.13
Destruction by irradiation, %	61	66	64

[1] Thiochrome method employed; three separate determinations were conducted.

[2] The experimental design for this assay was approximately the same as for the rat growth assay summarized in table 1.

[3] Turbidimetric assay employing *L. fermenti*-36 as the test organism. These values represent the averages from 7 assays.

[4] Processed in February, 1955. The irradiated sample received a dosage of 3.0 megarep.

[5] Processed in April, 1955 (3.0 megarep).

[6] Processed in June, 1955 (3.2 megarep).

assays revealed that there was very little, if any, difference in the thiamine content of beef with respect to its location within the metal container.

The essentiality of thioctic acid (lipoic acid) for certain bacteria (Stokstad et al., '50; Snell and Broquist, '49) and for a protozoan (Kidder and Dewey, '49) is well known. The possibility of thioctic acid in beef interfering in the micro-

biological assays for thiamine because of a growth-stimulating or thiamine-sparing effect was investigated. The results of these studies indicated no influence on the growth of the assay organism *Lactobacillus fermenti*-36 by the presence or absence of thioctic acid in the medium regardless of whether or not optimal or suboptimal levels of thiamine were employed.

Chemical determinations. The results obtained by the thiochrome analyses for thiamine indicate that about 61% of the thiamine in the beef was destroyed by the gamma radiation (table 2). These values were slightly lower than those obtained by microbiological or rat assays, which indicated 64 and 66% destruction of the thiamine, respectively.

Effect of certain supplements on thiamine deficiency. Because of the physiological relationship between thiamine and thioctic acid (Gunsalus, '54; Reed and DeBusk, '54) in the oxidation of alpha-keto acids in bacterial and animal tissue systems, it seemed possible that this substance might exert a sparing effect on the thiamine requirement of the rat even if no need for it could be demonstrated in the presence of adequate thiamine. DeBusk and Williams ('55) reported that thioctic acid improved the growth rate of rats and chicks. However, Stokstad et al. ('53) were unable to obtain evidence of a thioctic acid deficiency in either rats or chicks fed purified diets.

In view of the conflicting evidence and the possible interference of thioctic acid in the rat bio-assay for thiamine, a series of experiments was conducted. Results of these studies are summarized in tables 3 to 5. In experiments A and B (table 3), weanling rats were fed a thiamine-deficient basal diet for a depletion period of 10 days and then placed on the sucrose basal diet supplemented with 750 μg of thiamine per kilogram of diet. Glycerol was present in all diets at a level of 2%, except where noted. Results from experiment A (table 3) indicated that the supplementation of thioctic acid either by diet or by stomach tube exerted no sparing effect on rats fed suboptimal levels of thiamine. The presence of glycerol in the diet had no effect upon the thioctic acid supplements.

However, glycerol did have a protective effect upon the thiamine added to the diet. The addition of thioctic acid at a level of 10 mg per kilogram of diet also failed to improve growth of animals fed suboptimal amounts of thiamine (experiment B, table 3). In fact, the results indicated that the higher level of thioctic acid under certain conditions may have aggravated the thiamine deficiency (tables 3 and 5). The addition of thioctic acid (10 mg/kg) to diets fully supplemented with thiamine (6 mg/kg) also failed to produce significant improvement in growth. These results appear to be in agreement with results obtained by Stokstad et al. ('53).

TABLE 3

Effect of thioctic acid and of glycerol on the growth of rats fed a sucrose diet supplemented with suboptimal amounts of thiamine [1]

SUPPLEMENTS TO SUCROSE BASAL DIET [2]	SURVIVAL AND NO. OF RATS	AV. DAILY FOOD INTAKE	AVERAGE BODY WEIGHT AT		
			0 wk.	3 wk.	4 wk.
		gm	*gm*	*gm*	*gm*
Experiment A					
Glycerol, 2% + vitamin B_1 (750 μg)	6/6	4.7	59	76	...
Glycerol, 2% + vitamin B_1 (750 μg) + thioctic acid (1 mg)	6/6	4.3	57	70	...
Glycerol, 2% + vitamin B_1 (750 μg) + thioctic acid (stomach tube)[3]	6/6	4.1	59	74	...
No glycerol + vitamin B_1 (750 μg)	6/6	3.3	56	46	...
No glycerol + vitamin B_1 (750 μg) + thioctic acid (1 mg)	6/6	3.5	57	53	...
Experiment B					
Glycerol, 2% + vitamin B_1 (750 μg)	5/9	2.1	75	53	57
Glycerol, 2% + vitamin B_1 (750 μg) + thioctic acid (10 mg)	2/6	1.9	77	56	49
Glycerol, 2% + vitamin B_1 (6 mg)	6/6	13.3	74	201	241
Glycerol, 2% + vitamin B_1 (6 mg) + thioctic acid (10 mg)	6/6	14.3	78	209	250

[1] The weanling rats were fed a thiamine-deficient diet for 10 days prior to being placed on experiment. The average initial weight of the animals was 55 gm in experiment A and 45 gm in experiment B.

[2] Values within parentheses indicate amounts added per kilogram of basal diet.

[3] Amounts of thioctic acid equivalent to a level of 2 mg/kg of diet were given daily by means of a stomach tube.

Results from rats fed diets containing dextrin in place of sucrose are presented in tables 4 and 5. No thiamine was added to the diets except as noted. Glycerol (2%) was added to all diets, except in experiment C, table 4. The rats were placed on the respective diets without a preliminary thiamine-depletion period. As may be noted in table 4, the addition of penicillin to the thiamine-free diet permitted over 80%

TABLE 4

Effect of various supplements on growth rate of weanling rats fed a thiamine-deficient diet containing dextrin

SUPPLEMENTS TO DEXTRIN BASAL DIET [1]	SURVIVAL AND NO. OF RATS	AV. DAILY FOOD INTAKE	AVERAGE BODY WEIGHT AT [2]			
			0 wk.	2 wk.	3 wk.	4 wk.
		gm	*gm*	*gm*	*gm*	*gm*
Experiment C						
No glycerol	3/6	5.1	36	70	65	61
No glycerol + penicillin (100 mg)[3]	6/6	8.1	36	99	136	175
No glycerol + penicillin (100 mg) + thioctic acid (1 mg)	6/6	8.6	35	93	125	164
No glycerol + thioctic acid (1 mg)	4/6	5.5	35	73	65	53
No glycerol + thioctic acid [4]	3/6	5.4	35	75	61	46
No glycerol + ascorbic acid (5 gm)	3/6	5.8	35	73	71	84
No glycerol + vitamin B_1 (6 mg)	6/6	11.6	35	115	157	200
No glycerol + vitamin B_1 (6 mg) + thioctic acid (1 mg)	6/6	11.7	35	116	158	205
Experiment D						
Glycerol, 2%	3/6	6.1	35	72	67	66
Glycerol, 2% + penicillin (100 mg)[3]	6/6	9.1	36	92	126	170
Glycerol, 2% + penicillin (100 mg) + thioctic acid (1 mg)	6/6	10.0	35	96	138	171
Glycerol, 2% + thioctic acid (1 mg)	2/6	6.0	36	70	60	60
Glycerol, 2% + thioctic acid [4]	5/6	5.1	35	76	72	58
Glycerol, 2% + ascorbic acid (5 gm)	5/6	7.7	36	99	103	97
Glycerol, 2% + vitamin B_1 (6 mg)	6/6	10.4	36	112	148	194
Glycerol, 2% + vitamin B_1 (6 mg) + thioctic acid (1 mg)	6/6	11.5	35	117	167	212

[1] Values within parentheses indicate amounts added per kilogram of basal diet.

[2] Average weight of rats alive at end of period indicated.

[3] Procaine penicillin G.

[4] Amounts of thioctic acid equivalent to 2 mg/kg diet were administered daily by means of a stomach tube.

of the normal growth obtained when adequate thiamine was present. This effect has been previously noted by Lih and Baumann ('51) and by Sauberlich ('52). The presence of thioctic acid or glycerol in the diet had no apparent effect on the action of the penicillin (table 4). It may also be noted in contrast to findings presented in table 3 that glycerol had

TABLE 5

Effect of certain supplements on growth and liver storage of thiamine in weanling rats fed a thiamine-deficient diet containing dextrin

SUPPLEMENTS TO DEXTRIN BASAL DIET [1]	SURVIVAL AND NO. OF RATS	AV. DAILY FOOD INTAKES	AVERAGE BODY WEIGHT AT [2]			THIAMINE CONTENT OF LIVER (wet basis)
			0 wk.	2 wk.	4 wk.	
		gm	*gm*	*gm*	*gm*	*μg/gm*
Glycerol, 2%	5/6	4.1	41	78	43	0.60
Glycerol, 2% + penicillin (100 mg)[3]	6/6	11.3	42	119	201	2.41
Glycerol, 2% + L-penicillamine (25 mg)	6/6	4.4	43	82	56	0.42
Glycerol, 2% + D-penicillamine (25 mg)	6/6	5.1	45	87	66	1.17
Glycerol, 2% + ascorbic acid (5 gm)	6/6	5.9	41	85	90	2.07
Glycerol, 2% + thioctic acid (10 mg)	3/6	4.2	43	77	57	0.52
Glycerol, 2% + vitamin B_1 (6 mg)	6/6	11.9	43	120	206	15.2
Glycerol, 2% + vitamin B_1 (6 mg) + thioctic acid (10 mg)	6/6	12.8	44	126	210	16.6

[1] Values within parentheses indicate amounts added per kilogram of basal diet.
[2] Average weight of rats alive at end of period indicated.
[3] Procaine penicillin G.

no effect on the growth of the animals fed the thiamine-free dextrin diet. The growth-promoting action of glycerol was demonstrated only in the presence of suboptimal amounts of thiamine in the diet. This would indicate that the action of glycerol is one of protecting dietary thiamine.

The addition of relatively high amounts of ascorbic acid to the thiamine-free dextrin diet (5 gm/kg) gave partial protec-

tion (tables 4 and 5). This effect appeared to be enhanced somewhat by the presence in the diet of glycerol, which may have protected the ascorbic acid. The ability of ascorbic acid to exert a protective or sparing effect on certain other B vitamins required by animals has been noted by Daft ('51) and by McDaniel and Daft ('54).

In table 5, it may be noted that rats fed thiamine-free dextrin diets supplemented with either penicillin or ascorbic acid had an increased storage of thiamine in the liver as compared with rats fed an nnsupplemented thiamine-free dextrin diet (2.41 μg, 2.07 μg and 0.60 μg per gram of fresh liver, respectively). Although the penicillin-supplemented, thiamine-free diet permitted growth equal to that obtained when the diet was fully supplemented with thiamine (40 gm per week), the former diet permitted only about one-seventh of the liver storage of thiamine permitted by the latter. D-Penicillamine, the natural degradation product of penicillin, exhibited only a slight thiamine-sparing effect when fed at a molar level approximating that of penicillin. L-Penicillamine, however, appeared to have no thiamine-sparing effect. Thiotic acid was also without effect on growth and liver storage of thiamine.

Although certain substances may possibly interfere in the rat bio-assay for thiamine, the values obtained in the present study on raw ground beef with this method compared very favorably with values obtained by microbiological assay. However, the presence of penicillin in test samples would undoubtedly produce erroneous thiamine values with the rat bio-assay. Thiamine values obtained by the chemical method appeared to be somewhat lower than values obtained by the microbiological or rat bio-assay method.

SUMMARY

A study has been made on the stability of thiamine in beef when exposed to sterilization doses of gamma radiation. Supplementary studies were made with respect to possible

interfering substances in the biological and microbiological assays for thiamine. The following results were obtained:

1. Gamma radiation (approximately 3.0 megarep) destroyed a large portion of the thiamine in raw beef. Results obtained from biological, microbiological and chemical determinations indicated 66, 64 and 61% destruction, respectively. The thiamine assay values obtained by the different methods compared very favorably. The metal container appeared to have very little influence upon the destructive effects of irradiation in these studies.

2. Supplements of thioctic acid had no influence on the growth of rats fed various diets containing optimal or suboptimal levels of thiamine.

3. Glycerol had a protective effect when added to diets containing suboptimal amounts of thiamine but was without effect when added to diets completely deficient in thiamine.

4. The addition of high amounts of ascorbic acid to diets devoid of thiamine increased the growth rate and longevity of rats.

5. While dietary supplements of penicillin had a very marked influence on growth of rats fed thiamine-deficient diets, D-penicillamine had only slight effect and L-penicillamine was without effect.

6. The storage of thiamine in the liver of rats fed thiamine-deficient diets was increased about 4-fold by the addition of penicillin to the diet. A similar effect was also observed with D-penicillamine and ascorbic acid.

LITERATURE CITED

ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS 1950 Official methods of analysis. 7th Edition, Washington, D. C.

DAFT, F. S. 1951 Effect of vitamin C on pantothenic-deficient rats. Fed. Proc., *10:* 380.

DAY, E. J., H. D. ALEXANDER, H. E. SAUBERLICH AND W. D. SALMON 1956 Effects of gamma radiation on certain water soluble vitamins in raw ground beef. J. Nutrition, *62:* 27.

DEBUSK, B. G., AND R. J. WILLIAMS 1955 Effect of lipoic acid on the growth rate of young chicks and rats. Arch. Biochem. Biophys., *55:* 587.

GUNSALUS, I. C. 1954 Oxidative and transfer reactions of lipoic acid. Fed. Proc., *13:* 715.

HUBER, W., A. BRASCH AND A. ABSTRACK, JR. 1950 Radiation resisting vitamins. Chem. and Eng. News, *28:* 1380.

KANDUTSCH, A. A., AND C. A. BAUMANN 1953 Factors affecting the stability of thiamine in a typical laboratory diet. J. Nutrition, *49:* 209.

KIDDER, G. W., AND V. C. DEWEY 1949 Studies on the biochemistry of tetrahymena. XI. Components of factor II of known chemical nature. Arch. Biochem., *20:* 433.

KUNG, H. C., E. L. GADEN AND C. G. KING 1953 Vitamins and enzymes in milk. Effect of gamma-radiation on activity. J. Agr. Food Chem., *1:* 142.

LIH, H., AND C. A. BAUMANN 1951 Effects of certain antibiotics on the growth of rats fed diets limited in thiamine, riboflavin or pantothenic acid. J. Nutrition, *45:* 143.

MARKAKIS, P. C., S. A. GOLDBLITH AND B. E. PROCTOR 1951 Effect of ionizing radiations on vitamin B_{12}. Nucleonics, *9:* 71.

MCDANIEL, E. G., AND F. S. DAFT 1954 Effect of ascorbic acid in preventing or delaying multiple B-vitamin deficiencies in rats. Fed. Proc., *13:* 468.

PROCTOR, B. E., AND J. P. O'MEARA 1951 Effect of high-voltage cathode rays on ascorbic acid. Ind. Eng. Chem., *43:* 718.

REED, L. J., AND B. G. DEBUSK 1954 Enzymatic synthesis of a lipoic acid coenzyme. Fed. Proc., *13:* 723.

SARETT, H. P., AND V. H. CHELDELIN 1944 The use of *Lactobacillus fermentum* 36 for thiamine assay. J. Biol. Chem., *155:* 153.

SAUBERLICH, H. E. 1952 Effect of aureomycin and penicillin upon the vitamin requirements of the rat. J. Nutrition, *46:* 99.

SAUBERLICH, H. E., AND C. A. BAUMANN 1946 The effect of dietary protein upon amino acid excretion by rats and mice. J. Biol. Chem., *166:* 417.

SNELL, E. E., AND H. P. BROQUIST 1949 On the probable identity of several unidentified growth factors. Arch. Biochem., *23:* 326.

STOKSTAD, E. L. R., H. P. BROQUIST AND E. L. PATTERSON 1953 Role of protogen in animal nutrition. Fed. Proc., *12:* 430.

STOKSTAD, E. L. R., C. E. HOFFMANN AND M. BELT 1950 Role of protogen in the nutrition of an unidentified corynebacterium. Proc. Soc. Exp. Biol. Med., *74:* 571.

WAIBEL, P. E., H. R. BIRD AND C. A. BAUMANN 1954 Effect of salts on the instability of thiamine in purified chick diets. J. Nutrition, *52:* 273.

RELATION OF THYROID ACTIVITY TO INCREASED METABOLISM INDUCED BY FAT DEFICIENCY [1]

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One of the consistent departures from the normal in rats fed a fat-free diet is an increase in basal metabolic rate. As early as 1931, Wesson and Burr made reference to this manifestation of fat deficiency, and in 1937 Burr and Beber extended these observations. Recently, Panos and Finerty ('53, '54) and Panos, Finerty and Wall ('55) reopened the problem and pointed out that the increased metabolic rate as indicated by increase in basal oxygen consumption is one of the earliest manifestations of the fat-deficiency syndrome, being recorded within 7 to 14 days after the start of the experimental diet in immature rats.

Since such a disturbance in energy balance is of obvious fundamental importance, it becomes pertinent to attempt to determine the mechanism(s) involved. Several explanations have been proposed. That it is not due to hyperactivity of the animal was demonstrated by Burr and Beber ('37) using electrical recording devices. It is likely that at least one factor involved is the extra energy expenditure due to the increased conversion of carbohydrate to fat (Krogh and Lindhard, '20; Wesson, '27; Burr and Beber, '37). Increased

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water loss through the skin, with an attendant loss of energy, due to increased permeability of the skin, has been suggested as a possible major factor by Sinclair ('52).

The possibility that fat deficiency is associated with hyperthyroidism has received some attention in the past (Wesson and Burr, '31). Recent reports from this laboratory (Panos and Finerty, '53, '54; Panos, Wall and Finerty, '55) tend to contradict this possibility. Morphological studies in both male and female rats showed a decrease in relative thyroid weights in fat-deficient animals and histological observations revealed no difference from the normal. A preliminary study of the 4-hour thyroid I^{131} uptake by Panos, Wall and Finerty ('55) showed no significant difference from controls.

It was the purpose of the present study to investigate more critically the activity of the thyroid gland in rats fed a fat-deficient diet as compared with others fed a similar diet supplemented with fat. The following indices of thyroid activity were measured: (1) biological decay of I^{131} in vivo; (2) 24-hour thyroidal I^{131} uptake; (3) radio-iodine conversion ratio; (4) radio-iodine T/S ratio (activity per milligram thyroid/activity per milliliter serum; (5) thyroid weight; (6) thyroid histology. In addition, the pituitaries were assayed for thyrotrophic activity and oxygen consumption was determined.

PROCEDURE

Feeding methods. Forty-eight weanling rats of the Holtzman strain were separated into three groups and fed as follows: (1) fat free ad libitum — a synthetic diet containing all essential minerals and vitamins, carbohydrate and protein, but entirely lacking in fat. This diet is described in detail by Panos and Finerty ('53). (2) fat free plus fat, isocaloric — given the same food as above but with fat in the form of cottonseed oil [3] (about 50% linoleic acid), substituted isocalorically for carbohydrate. This diet contained 30% of its calories as fat. The amount fed daily was calculated by determining the

[3] Wesson oil.

previous day's consumption of the fat-free group and calculating from the relation; 1 gm fat-free food was equal calorically to 0.846 gm fat-free plus fat mixture. (3) fat free plus fat, equal weight; this series of rats was given the same food as the previous group but in an amount necessary to maintain the mean body weight the same as that of the fat-free group.

Animals were kept in identical cages with 4 or 5 animals in each cage and food consumption and body weights were determined daily. The air-conditioned animal room was kept

TABLE 1

Relation of body growth to caloric intake

DIET	NO. RATS	MEAN FINAL BODY WT.	MEAN WT. GAIN IN 91 DAYS	MEAN DAILY CALORIC INTAKE	TOTAL CALORIC INTAKE / TOTAL WEIGHT GAIN
		gm	*gm*		
Fat-free	8 [1]	292	227	60	23.6
Fat-free plus fat, iso-caloric	8	342	277	60	19.7
Fat-free plus fat, to equal wt.	8	303	238	55	21.0

[1] Body weights are those of rats in table 2; caloric intake calculated from 16 rats per group.

at a temperature of from 69 to 78°F. with a humidity of about 70%. The dietary regimen was continued from the day of weaning until sacrifice. Food consumption and body weight changes are shown in table 1.

Biological decay. After the animals had been on the experimental diet for 13 weeks, 8 rats from each group were given an intraperitoneal injection of 15 μc carrier-free iodine^{131}. After 6, 24, 48 and 72 hours, the radioactivity of the thyroids was measured by placing the animal over a scintillation detector employing a thallium-activated sodium iodide crystal.[4] The end of the tube was collimated to an area of about one half inch diameter and the rat thyroid

[4] Tracerlab P-20A detector mounted in a crystal well shield.

approximated over this area after anesthesia.[5] An equal-sized area over the epigastric region was counted as "background," which was subtracted from the thyroid count.

I^{131} uptake, PBI^{131}, serum I^{131}. Similarly, 8 rats from each group were injected with 15 μc carrier-free radio-iodine^{131} and autopsied 24 hours later, at which time blood was taken from the abdominal aorta and the thyroids were removed, weighed, and placed in test tubes containing 10% formalin. Activity of the thyroids was determined by placing the tube in a thallium-activated sodium iodide well scintillation detector,[6] after which histological sections were prepared. The activity of 1 ml of serum was similarly determined while another milliliter of serum was treated to separate the proteins. This was done by adding 3 ml of 10% trichloracetic acid (TCA), centrifuging, then washing the precipitate twice with 2 ml of 10% TCA. The remaining TCA was decanted and the precipitate was then counted in the original centrifuge tube. The conversion ratio was calculated according to the formula: PBI^{131}/total serum I^{131} (Clark et al., '49). The T/S ratio was calculated according to the formula: activity of 1 mg thyroid/activity of 1 ml serum.

Oxygen consumption. Oxygen consumption of 8 rats in each series 13 weeks after the beginning of the special diets was measured in a closed circuit respirometer similar to that described by Holtkamp and coworkers ('55). Three separate determinations of 15 minutes each were made on each animal in the fasting (6 hours), resting state, after a 30 minute acclimitization period in the oxygen chamber. Oxygen consumption is expressed in terms of milliliters of oxygen consumed per 100 gm body weight at standard temperature and pressure.

Pituitary assay. Pituitaries removed at autopsy were weighed and placed in acetone. Two weeks later, they were removed, air dried, weighed and ground in physiological saline. Injections of these suspensions were made subcu-

[5] Nembutal, Abbott.

[6] Tracerlab P20AW mounted in a crystal well shield.

taneously into day-old White Leghorn cockerels such that each chick received 0.75 mg dry powder divided into 5 injections of 0.02 ml each. The first injection was given on the afternoon of receipt of the chicks, others were given on the morning and afternoon of the next two days and the birds were autopsied and thyroids weighed on the morning of the 4th day of age. The birds were watered but kept without feed during the entire period. From 10 to 18 chicks were used in each series, and a control series of 18 chicks was injected with physiological saline. Thyrotrophic activity is expressed as the increase in weight of the thyroids of the assay chicks over those of a control series receiving saline.

RESULTS

Growth rate and food consumption. Elimination of fat from the diet reduced the nutritional efficiency as expressed by growth rate. The fat-free rats gained less weight in the 13-week period than did a similar group fed the same number of calories containing 30% of the calories as fat (table 1). This difference is expressed in the last column of table 1, which shows the number of calories required for the rat to gain 1 gm of body weight. Furthermore it can be seen that the fat-deficient rats consumed more calories to reach a final weight of 292 gm than did other rats (final weight, 303 gm) which were fed a diet with fat supplement.

Oxygen consumption. The data in table 2 confirm repeated observations made earlier in this laboratory. Fat deficiency definitely resulted in a significant increase in oxygen consumption. The rate of oxygen uptake of the rats in the fat-free series was about 30% greater than in any groups receiving fat in the diet.

Thyroid weight and histology. The thyroids of the rats fed a fat-free diet were noticeably lighter in weight than any of the other series (table 2). This became particularly evident when calculated on a basis of milligrams per 100 gm body weight and again confirms previous observations.

Histologically the thyroids of all groups appeared very slightly hypoactive. More follicles with flattened acinar cells were evident although the presence of numerous areas of apparently normal follicles suggested that the glands were not markedly hypoactive. While cell height measurements

TABLE 2

The effect of fat deficiency on metabolic rate and thyroid activity

	FAT-FREE	FAT-FREE PLUS FAT ISO-CALORIC	FAT-FREE PLUS FAT TO EQUAL WT.
Number of rats	8	8	8
Body weight, gm	292 ± 16 [1]	342 ± 11 [2]	303 ± 25
Oxygen consumption, ml O_2/min./100 gm body wt.	168 ± 20	129 ± 5 [2]	129 ± 5 [2]
Thyroid weight, absolute	10.64 ± 1.04	14.81 ± 1.18 [2]	12.85 ± 0.74 [2]
Thyroid weight, mg/100 gm body wt.	3.66 ± 0.25	4.33 ± 0.35 [2]	4.24 ± 0.35 [2]
24-hr. I^{131} uptake, cpm/mg thyroid	488 ± 42	422 ± 150	479 ± 47
24-hr. PBI^{131}, cpm/1 ml serum	2565 ± 403	2194 ± 926	2111 ± 1042
24-hr. conversion ratio, PBI^{131}/total serum I^{131}	40 ± 10	22 ± 9 [2]	22 ± 4 [2]
24-hr. T/S ratio, cpm 1 gm thyroid/ cpm 1 ml serum	8.84 ± 1.66	4.89 ± 1.93 [2]	6.04 ± 1.58 [2]
TSH pituitary assay (chick assay), increase in thyroid wt., mg	2.21 ± 1.40	2.34 ± 1.05	2.36 ± 1.13

[1] Standard deviation.

[2] Difference from fat-free group, $P < 0.01$.

were not made, the impression was that the thyroids of the fat-free rats were slightly more active than those of the others on the experimental diets.

I^{131}uptake and biological decay. The ability of the thyroid to accumulate radioiodine over a 24-hour period was essentially the same in all three groups (table 2). Likewise, the decay curves shown in figure 1 for the three series do not

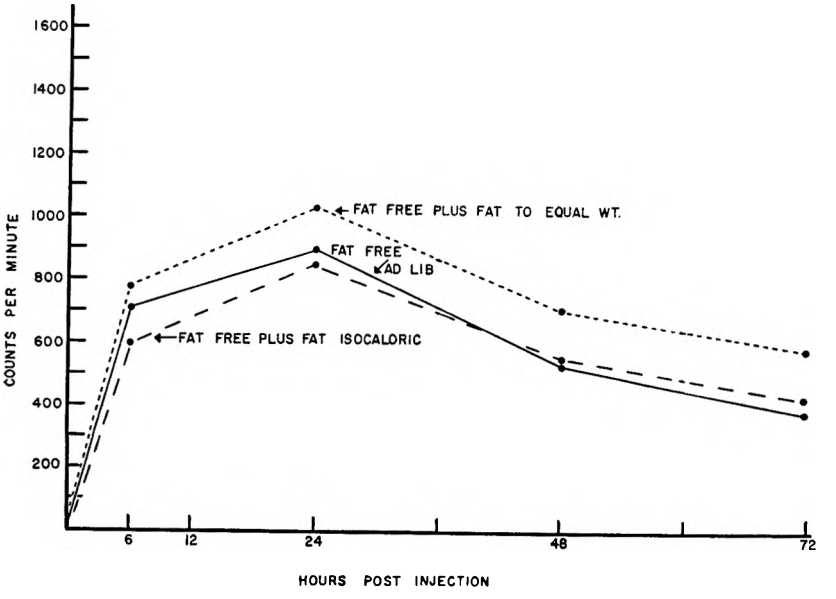

Fig. 1 Effect of fat deficiency on the radioiodine activity of the thyroid, in vitro, after the injection of I^{131}. Values are given per 100 gm body weight.

seem to differ significantly. Certainly, that for the fat-free group is not indicative of a hyperactive thyroid gland.

PBI^{131}. The ability of the thyroid to incorporate radioiodine into a protein which presumably represents the active thyroid hormone, was not affected by fat deficiency (table 2).

Conversion ratio and T/S ratio. Both of these ratios were significantly higher in the fat-free series than in either of the other two experimental groups. This is due to the lower value for total serum I^{131} in the fat-free group (table 2).

Pituitary assay. The various diets studied had no apparent effects upon the thyrotrophic activity of the pituitaries (table 2).

DISCUSSION

From the data obtained on caloric intake, weight gain and oxygen consumption, it is obvious that the rate of energy metabolism is increased by fat deficiency. Rats kept on a diet completely free of fat showed an increased rate of oxygen consumption, and made less efficient use of caloric intake as measured by body weight gain. This confirms previous reports from this laboratory (Panos and Finerty, '53, '54; Panos, Finerty and Wall, '55).

The reduced rate of growth seen in the rats fed a fat-free diet was not due to a reduced caloric intake since other rats fed isocalorically with a fat supplement showed a significantly greater growth rate. It is also pertinent that it required fewer calories to maintain a given body weight in rats fed a complete diet with fat than to maintain the same weight in rats on a fat-deficient mixture.

The hypothesis that this altered metabolic pattern is due to hyperthyroidism induced by the fat-deficient diet is untenable in view of the data presented here. Indeed, morphological studies point toward hypothyroidism in fat-deficient rats, inasmuch as a significant decrease in both absolute and relative thyroid weights was recorded. Histological examination of the thyroids suggests a mildy more active gland in the fat-deficient series as compared with those receiving fat in the diet although it is by no means marked and cannot account for the altered oxygen consumption seen in these animals.

A more accurate criterion of physiological activity of the thyroid is provided by the I^{131} studies. No significant difference in either the ability to accumulate radioiodine over a 24-hour period or to manufacture protein-bound iodine materials was induced by the fat deficiency. It is also seen (fig. 1) that the initial uptake of radioiodine (after 6 hours)

was not affected by fat deficiency. Furthermore, the rate of release of radioactive materials from the glands in the fat-free series was not appreciably different from that seen in the iso-caloric series with fat supplement.

The conversion ratios and the T/S ratios would seem to suggest that the fat-deficient animals had a greater capacity for accumulating radioiodine and converting it to thyroxine. However, in view of the data on PBI^{131} and thyroid I^{131} uptake, one must conclude that the difference seen in these ratios is due to a lower total serum I^{131} in the fat-deficient animals. This in turn suggests that in these animals there is a greater loss of the injected iodine by way of the alimentary canal, urinary tract, or other outlet.

It is concluded that the thyroid activity of rats fed a diet deficient in fats is not significantly different from that of the thyroid of control animals maintained on a similar diet supplemented with fat at an isocaloric level or at a level devised to maintain a similar rate of growth.

SUMMARY

A study was made of the growth rate, oxygen consumption and thyroid activity of rats maintained on a fat-free diet for 13 weeks. Similar observations were made on rats maintained isocalorically on a diet containing 30% of its calories in the form of cottonseed oil. These were compared with data from rats fed the complete diet in an amount necessary to maintain the same growth rate as that of animals in the fat-deficient series.

Data confirm previous observations that fat deficiency causes an increased basal oxygen consumption and an increased energy expenditure manifested by less weight gain per calorie. The fat-free diet did not cause an increase in thyroid activity as measured by thyroid weight and histology, 24-hour I^{131} uptake, 24-hour PBI^{131}, and biological I^{131} decay. An increase in rate of removal of inorganic I^{131} by some route other than the thyroid was suggested by the altered T/S

and conversion ratios observed in the rats on the fat-deficient diet. It is concluded that the recorded increase in oxygen consumption seen in the fat-deficient syndrome is not due to an altered thyroid function.

LITERATURE CITED

BURR, G. O., AND A. J. BEBER 1937 Metabolism studies with rats suffering from fat deficiency. J. Nutrition, *14:* 553.

CLARK, DWIGHT E., R. H. MOE AND E. E. ADAMS 1949 The rate of conversion of administered inorganic radioactive iodine into protein-bound iodine of plasma as an aid in the evaluation of thyroid function. Surgery, *26:* 331.

HOLTKAMP, D. E., S. OCHS, C. C. PFEIFFER AND A. E. HEMING 1955 Determination of the oxygen consumption of groups of rats. Endocrinology, *56:* 104.

KROGH, A., AND J. LINDHARD 1920 The relative value of fat and carbohydrates as sources of muscular energy. Biochem. J., *14:* 290.

PANOS, T. C., AND J. C. FINERTY 1953 Effects of a fat-free diet on growing female rats, with special reference to the endocrine system. J. nutrition, *49:* 397.

———— 1954 Effects of a fat-free diet on growing male rats with special reference to the endocrine system. Ibid., *54:* 315.

PANOS, T. C., J. C. FINERTY AND R. L. WALL 1955 Influence of fat in diet on oxygen consumption. Am. J. Dis. Child, *59:* 621.

PANOS, T. C., R. L. WALL AND J. C. FINERTY 1955 Oxygen consumption in relation to thyroid activity in fat-free rats. Federation Proc., *14:* 110.

SINCLAIR, H. M. 1952 Essential fatty acids and their relation to pyridoxine. Biochemical Society Symposia no. 9. Lipid metabolism. Cambridge University Press, London.

WESSON, L. G. 1927 Fat formation under abnormal conditions from carbohydrate by the rat, and its relationship to a possible new dietary factor. J. Biol. Chem., *73:* 507.

WESSON, L. G., AND G. O. BURR 1931 The metabolic rate and respiratory quotients of rats on a fat-deficient diet. Ibid., *91:* 525.

THE COMPARATIVE EFFECTS OF COTTONSEED OIL AND LARD ON CHOLESTEROL LEVELS IN THE TISSUES OF RATS [1,2,3]

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INTRODUCTION

Although many investigators (Wilkinson et al., '50; Keys et al., '55; Gofman et al., '50) believe that serum cholesterol levels are controlled by fat intake regardless of the source of the fat, there exists evidence that the origin of the fat may be vitally concerned with the regulation of these cholesterol levels. Schettler ('48, '49, '49a) fed to mice diets in which various animal and vegetable fats were included and found significant increases in plasma and liver cholesterol levels in the mice fed the animal fat diets as compared with those fed the diets containing vegetable fat. Tsai et al. ('54) reported that dogs had lower serum cholesterol levels on a vegetable fat diet than when fed a commercial diet containing variable amounts of animal fat and cholesterol. When Kinsell and coworkers (Kinsell et al., '52, '53, '53a; Cochrane et al., '53) substituted vegetable fat for animal fat in the diet of

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[4] Deceased, April 17, 1956.

diabetic and non-diabetic patients with vascular diseases, a rapid and maintained decrease in serum cholesterol and phospholipids resulted. When animal and vegetable fats were alternated, a decrease in phospholipids and serum cholesterol obtained on the vegetable fat diet which rose to the previous higher levels when animal fat was fed. A statistical study by Hardinge and Stare ('54) demonstrated that strict vegetarians have lower cholesterol levels than those vegetarians who eat dairy products, and that both groups have lower cholesterol levels than the general population. Ahrens et al. ('54) reported that 6 subjects with obesity of exogenous origin showed approximately 20% reductions in serum cholesterol and phospholipids when plant fats were substituted isocalorically for animal fats.

The following investigation was undertaken in an attempt to clarify the role of dietary animal and vegetable fat in cholesterol metabolism.

EXPERIMENTAL

Male and female rats of the University of Southern California strain were placed at weaning on the diets listed in table 1. The two test fats employed in this study were lard [5] and cottonseed salad oil.[6] Groups of rats given the L (lard) and CsO (cottonseed oil) regimens were sacrificed after 6, 12 and 24 weeks on diet; groups of rats fed the diets supplemented with cholesterol were sacrificed after 6 and 18 weeks, the latter animals, however, receiving the cholesterol diet for the last 6 weeks only. In all cases, the rats were sacrificed after anesthesia,[7] and withdrawal of blood from the heart. The liver was quickly extirpated and blotted to remove excess blood and body fluid. The remaining carcasses were ground and the lipids were extracted with an ethanol-ethyl ether mixture (3:2) in a Soxhlet apparatus. Lipids in plasma

[5] Obtained from Swift and Co., and containing no added tocopherol as antioxidant.

[6] Furnished by The Best Foods, Inc.

[7] Nembutal, Abbott.

and liver were extracted by the procedure of Thompson et al. ('49) and the extracts were analyzed for free and total cholesterol by a modification of the Schonheimer-Sperry procedure (Nieft and Deuel, '49). Total lipids were determined gravimetrically on the petroleum ether extracts of the original livers.

In experiments to determine cholesterol absorption, feces were collected for a two-week period from male and female rats fed the different experimental diets. Food consumption was measured concomitantly. Fecal fat analyses were performed after Soxhlet extractions.

TABLE 1

Composition of diets

COMPONENT	LARD AND CHOLESTEROL	LARD	COTTONSEED OIL AND CHOLESTEROL	COTTONSEED OIL
	%	%	%	%
Lard	15.00	15.00	....	....
Cottonseed oil	....	....	15.00	15.00
Cholesterol [1]	1.00	....	1.00	....
Bile salts [2]	0.25	0.25	0.25	0.25
Celluflour [3]	4.00	4.00	4.00	4.00
Salt mixture [4]	4.00	4.00	4.00	4.00
Casein, commercial [5]	24.00	24.00	24.00	24.00
Sucrose	51.30	52.30	51.30	52.30
Choline	0.24	0.24	0.24	0.24
Vitamin mixture [6]	0.19	0.19	0.19	0.19
Vitamin A and D oil [7]	0.012	0.012	0.012	0.012
Alpha-tocopherol [8]	0.012	0.012	0.012	0.012

[1] U.S.P.; Merck Co.

[2] Difco Laboratories, Inc., Detroit, Mich.

[3] Solka-floc, Brown Co., San Francisco, Cal.

[4] Osborne and Mendel [Science, *75:* 339 (1932)] Wesson Modification; Nutritional Biochemicals Corp., Cleveland, Ohio.

[5] Lactic casein, Challenge Dairy Co., Los Angeles, Cal.

[6] The vitamin mixture consisted of 38.57% *p*-amino-benzoic acid, 31.88% inositol, 12.75% ascorbic acid, 4.59% thiamine hydrochloride, 3.82% niacin, 3.82% Ca-pantothenate, 1.72% riboflavin, 1.72% pyridoxine, 0.64% folic acid, 0.32% menadione, 0.16% biotin, and 0.00004% vitamin B_{12}; Merck Co. and Nutritional Biochemicals Corp.

[7] The Nopsol solution contains 100,000 I. U. of vitamin A per gram and 20,000 I. U. of vitamin D per gram. Nopco Chemical Co., Harrison, N. J.

[8] Nutritional Biochemicals Corp., Cleveland, Ohio.

RESULTS

The plasma cholesterol levels in male and female rats maintained for 6, 12 and 24 weeks on diets containing either 15% lard or 15% cottonseed oil are shown in table 2. It can be seen that although no significant differences are apparent

TABLE 2

The plasma cholesterol levels in male and female rats fed a diet containing either 15% lard or 15% cottonseed oil for 6, 12 and 24 weeks

WEEKS	SEX AND NO.	LARD (L)		
		Plasma cholesterol		
		Total [1]	Free [1]	% Free
		mg %	*mg %*	
6	M (10)[2]	80.5 ± 3.2	23.8 ± 1.8	29.4
12	M (6)	75.4 ± 4.7	23.6 ± 1.2	31.6
	F (9)	88.4 ± 4.8	28.3 ± 1.4	32.0
24	M (10)	77.9 ± 3.5	24.0 ± 1.4	30.8
	F (9)	78.0 ± 4.8	21.3 ± 1.4	27.3

WEEKS	SEX AND NO.	COTTONSEED OIL (CsO)		
		Plasma cholesterol		
		Total [1]	Free [1]	% Free
		mg %	*mg %*	
6	M (10)[2]	81.4 ± 4.2	20.9 ± 1.5	24.6
12	M (6)	79.6 ± 5.5	22.9 ± 1.2	28.9
	F (9)	81.4 ± 2.8	22.1 ± 1.8	26.9
24	M (10)	61.9 ± 3.9	22.4 ± 1.7	36.1
	F (9)	69.4 ± 4.5	22.9 ± 1.4	32.9

[1] Including standard error of the mean.

[2] Numbers in parentheses are numbers of animals in each group.

after 6 or 12 weeks on the respective diets, at 24 weeks, the plasma cholesterol levels of the rats fed the diet containing cottonseed oil are significantly lower than those of rats receiving the lard diet. This decrease also obtains when total liver lipid and total liver cholesterol values are compared (table 3), although here the differences are already apparent at the end of 12 weeks on the diets; i.e. 3.25 mg cholesterol

TABLE 3

The average liver lipid and cholesterol levels in male and female rats fed a diet containing either 15% lard or 15% cottonseed oil for 6, 12 and 24 weeks

CATEGORY	SEX	LARD (L)			COTTONSEED OIL (CsO)		
		6 wks.	12 wks.	24 wks.	6 wks.	12 wks.	24 wks.
No. rats	M	10	6	10	10	6	10
	F	...	9	9	...	9	10
Liver wt., gm	M	9.8	10.4	12.5	9.8	10.7	12.2
	F	...	6.9	8.8	...	7.1	7.5
Lipids, mg/gm liver [1]	M	49.1 ± 2.0	58.4 ± 3.6	58.4 ± 3.6	48.6 ± 2.8	53.2 ± 4.2	38.9 ± 2.0
	F	...	52.7 ± 2.0	50.1 ± 2.0	...	49.6 ± 1.7	46.0 ± 2.0
Cholesterol, mg/gm liver [1]							
Total	M	2.77 ± 0.39	3.25 ± 0.14	3.18 ± 0.17	2.76 ± 0.26	2.66 ± 0.14	2.48 ± 0.12
	F	...	2.58 ± 0.03	2.65 ± 0.05	...	2.40 ± 0.06	2.37 ± 0.09
Free	M	2.22 ± 0.28	2.42 ± 0.09	2.43 ± 0.10	2.15 ± 0.20	2.36 ± 0.12	2.15 ± 0.04
	F	...	2.27 ± 0.04	2.13 ± 0.05	...	2.22 ± 0.06	2.07 ± 0.07
%	M	80.5	75.0	76.4	78.0	88.7	86.6
	F	...	88.0	80.3	...	92.8	87.3

[1] Including standard error of the mean.

TABLE 4

The effect of age and cholesterol feeding on male and female rats fed a diet containing either 15% lard or 15% cottonseed oil as reflected by plasma cholesterol levels

PLASMA CHOLESTEROL	SEX	DIETARY TREATMENT [1]			
		LC, 6 wks.	L, 12 wks. followed by LC, 6 wks.	CsOC, 6 wks.	CsO, 12 wks. followed by CsOC, 6 wks.
Total, mg % [2]	M	118.1 ± 9.1 (10)[3]	104.7 ± 11.7 (9)	85.6 ± 4.4 (9)	89.4 ± 6.8 (10)
	F		165.4 ± 19.3 (9)		141.6 ± 16.7 (10)
Free, mg % [2]	M	26.6 ± 2.0	37.1 ± 6.2	23.0 ± 1.4	26.5 ± 1.8
	F		57.1 ± 4.7		34.4 ± 5.0
%	M	23.3	35.4	26.7	29.6
	F		34.5		24.2

[1] L = lard; LC = lard + cholesterol; CsO = cottonseed oil; CsOC = cottonseed oil + cholesterol.

[2] Including standard error of mean.

[3] Numbers in parentheses are the numbers of animals in each group.

per gram of liver for male rats fed the lard diet as compared with 2.66 mg cholesterol per gram of liver for male rats fed the cottonseed oil diet.

When 1% of cholesterol is added to the diets, the differences are accentuated (table 4). After 6 weeks, the plasma choles-

TABLE 5

The effect of age and cholesterol feeding on male and female rats fed a diet containing either 15% lard or 15% cottonseed oil as reflected by liver lipid and liver cholesterol levels

CATEGORY	SEX	DIETARY TREATMENT [1]			
		LC, 6 wks.	L, 12 wks. followed by LC, 6 wks.	CsOC, 6 wks.	CsO, 12 wks. followed by CsOC, 6 wks.
No. rats	M	10	9	9	10
	F		9		10
Liver wt., gm	M	11.7	11.6	12.2	12.4
	F		7.9		7.8
Lipids, mg/gm liver [2]	M	117.4 ± 8.8	131.5 ± 8.1	100.7 ± 7.4	127.3 ± 9.4
	F		119.7 ± 8.1		97.1 ± 6.1
Cholesterol, mg/gm liver [2]					
Total	M	26.9 ± 2.9	39.8 ± 3.0	22.0 ± 1.9	32.1 ± 3.5
	F		31.6 ± 3.7		16.4 ± 1.7
Free	M	7.4 ± 0.8	20.1 ± 1.4	4.4 ± 0.4	5.7 ± 0.6
	F		7.3 ± 1.1		5.6 ± 0.8
%	M	26.2	51.8	21.4	18.7
	F		25.4		34.6

[1] L = lard; LC = lard + cholesterol; CsO = cottonseed oil; CsOC = cottonseed oil + cholesterol.

[2] Including standard error of mean.

terol concentration of the male rats on the lard and cholesterol diet is 118.1 mg % whereas the plasma cholesterol concentration of the male rats on the cottonseed oil diet supplemented with cholesterol is 85.6 mg %. This difference is also exhibited by the older animals pre-fed the diets without cholesterol for 12 weeks followed by the cholesterol-supplemented diet for 6 weeks, i.e. 104.7 mg % for the lard + cholesterol diet and

89.4 mg % for the cottonseed oil + cholesterol diet. A sex difference in plasma cholesterol levels is apparent here with the female rats in both groups exhibiting much higher plasma cholesterol levels than do the male rats.

In table 5, the total lipid, and free and total cholesterol concentrations in the liver of animals on the various diets are reported. In all cases, the values for the animals on the cottonseed oil + cholesterol diets are lower than those on the lard + cholesterol diets. The most striking change is exhibited in the female rats where the liver cholesterol value

TABLE 6

The absorption of cholesterol in male and female rats fed a diet containing either 15% lard or 15% cottonseed oil for two weeks [1]

DIET	SEX	LIPIDS			CHOLESTEROL		
		Ingested	Excreted	% Absorbed [2]	Ingested	Excreted	% Absorbed [2]
		gm	*gm*		*gm*	*gm*	
LC [3]	M	28.5	2.7	90.4 ± 0.7	1.9	1.0	48.2 ± 1.5
	F	24.9	1.7	92.8 ± 0.8	1.5	0.7	55.5 ± 2.6
CsOC	M	30.2	2.2	92.3 ± 0.1	1.9	1.0	47.0 ± 2.5
	F	21.2	1.3	93.7 ± 0.2	1.3	0.6	54.2 ± 2.2

[1] Five animals per group.

[2] Including standard error of mean.

[3] LC = lard + cholesterol; CsOC = cottonseed oil + cholesterol.

of the animals on the cottonseed oil + cholesterol diet is approximately half of that in the female rats on the lard + cholesterol diet (16.4 mg/gm liver as compared with 31.6 mg/gm liver). This value is also approximately one-half of that shown by the male rats on the same diet (16.4 mg/gm liver as compared with 32.1 mg/gm liver).

In an attempt to explain these differences, the absorption of cholesterol on these various diets was determined to discover whether or not there was an effect due to the vehicular fat. Since fats of vegetable origin contain phytosterols, a possible interference with the absorption of cholesterol might obtain. Also, a sex difference in the absorption of cholesterol (i.e. a decreased absorption) might explain the lower liver

cholesterol values which obtained in the female rats. The results are shown in table 6. Not only is the absorption of cholesterol in the male rat the same on both fat diets, but also the absorption of the cholesterol is higher in the female rat than in the male animal.

TABLE 7

Average cholesterol levels in carcasses, excluding liver, of rats raised on lard and cottonseed oil diets for 24 weeks [1]

GROUP	SEX	AVERAGE BODY WT.	CHOLESTEROL		
			Total per carcass	Free % of total	Total % of carcass [2]
		gm	*mg*		
L	M	358	6741	84.1	2.06 ± 0.09
L	F	244	4571	82.3	2.02 ± 0.03
CsO	M	375	5969	87.0	1.69 ± 0.06
CsO	F	230	3984	87.4	1.88 ± 0.09

[1] Five rats per group.

[2] Including standard error of mean.

TABLE 8

The average plasma and liver cholesterol levels and liver lipid levels of male rats fed a diet containing 15% lard with and without the addition of vitamin E and with and without the addition of 1% cholesterol

CATEGORY	− CHOLESTEROL		+ CHOLESTEROL	
	− Vit. E	+ Vit. E	− Vit. E	+ Vit. E
No. rats	10	10	10	9
Liver lipids, mg/gm [1]	48.2 ± 2.0	43.8 ± 2.0	118.4 ± 7.4	100.6 ± 6.7
Liver cholesterol, mg/gm [1]				
Total	2.76 ± 0.24	2.50 ± 0.02	37.3 ± 4.9	23.3 ± 3.3
Free	2.30 ± 0.19	2.22 ± 0.02	4.00 ± 0.02	3.50 ± 0.02
%	83.3	88.8	10.9	15.0
Plasma cholesterol, mg % [1]				
Total	69.5 ± 1.0	62.8 ± 3.4	93.9 ± 7.9	92.4 ± 2.1
Free	18.9 ± 1.5	17.4 ± 1.8	19.3 ± 1.8	22.4 ± 3.2
%	27.2	26.1	20.5	24.2

[1] Including standard error of mean.

To determine whether the small amount of cholesterol (0.08%) present in the lard used in these diets might account for the differences in cholesterol levels observed on the two diets, male rats were fed cottonseed oil, to which 0.08% of cholesterol had been added, for 6 weeks. Values for cholesterol in plasma and liver, and total lipids in liver are no different from those obtained when unsupplemented cottonseed oil was fed. The possibility existed that the differences in cholesterol concentration in the liver on the two fat diets might be caused by differences in distribution. Therefore, animal carcasses, excluding blood and liver, were analyzed for cholesterol content (table 7). The carcass cholesterol concentration is also significantly higher in the animals fed the lard diet than in those fed the cottonseed oil diet.

It was conceivable that some of the differences which obtained in liver cholesterol on the lard and cottonseed oil diets might be due to the fact that lard is quite low in vitamin E as compared with cottonseed oil. Therefore, the lard diets, with and without cholesterol, were supplemented with α-tocopherol (5 mg/day/rat). The results are shown in table 8. The effect of the excess vitamin E on endogenous cholesterol metabolism as judged by plasma and liver cholesterol levels and total liver lipids is negligible (in plasma, 62.8 mg % of cholesterol on the vitamin E supplemented diet compared with 69.5 mg %; in liver 2.50 mg cholesterol/gm compared with 2.76 mg cholesterol/gm, and 43.8 mg lipid/gm compared with 48.2 mg/gm). However, when vitamin E was fed simultaneously with 1% of cholesterol, more marked changes are apparent in the liver although the plasma cholesterol levels are still unaffected by the vitamin supplement. In the liver, the effect of the vitamin E is to decrease the total lipid from 118.4 mg/gm liver to 100.6 mg/gm and similarly to decrease the total cholesterol concentration from 37.3 mg/gm to 23.3 mg/gm. These results indicate that vitamin E is a factor in regulating proper cholesterol metabolism in the liver of animals fed a high cholesterol diet, since the total liver cholesterol

was significantly decreased by the addition of large amounts of vitamin E to the lard diet containing 1% of cholesterol.

DISCUSSION

The need for essential fatty acids for the normal transport and metabolism of cholesterol has previously been postulated by this laboratory (Alfin-Slater et al., '54). When these fatty acids are not present in adequate amounts in the diet, cholesterol cannot be readily transported and therefore tends to accumulate in certain organs, notably the liver. The results reported in this paper can be explained on the basis of the higher content of essential fatty acids found in cottonseed oil over that present in lard, 49.2 versus 8.6%. In addition, the sex differences observed here are probably due to the much smaller requirement of the female rat for essential fatty acids (Greenberg et al., '50). Therefore, more of the essential fatty acids ingested by the female rats are available to keep cholesterol in a mobile state than from the same quantity of essential fatty acid ingested by the male rats. The implication that lard is objectionable because of its high saturated fatty acid content is not tenable. Rather the criticism should be leveled at the low essential fatty acid content of lard since saturated fatty acids cannot duplicate the positive biochemical functions of the polyunsaturated essential fatty acids.

The influence of vitamin E in eliminating to a great extent the differences between lard and cottonseed oil in their effects on cholesterol metabolism is probably largely due to its action as an antioxidant *in vivo;* in this capacity it allows the smaller amounts of essential fatty acids present in the lard to be more effectively utilized. It is well established in the chick that with an increased intake of polyunsaturated fatty acids, there is an increased requirement for vitamin E. It may therefore follow that with a limited intake of labile polyunsaturated fatty acids in a diet low in vitamin E, supplementation with additional vitamin E is equivalent to

increasing the content of the polyunsaturated fatty acids in the diet thereby permitting greater participation of these acids in essential biological functions.

Kritchevsky and associates ('54) have shown that the severity of atherogenesis is markedly reduced in rabbits when corn oil, a potent source of essential fatty acids, is added to a cholesterol-containing diet. If the hypothesis herein presented is applicable to man, then fats containing adequate amounts of essential fatty acids not only would not cause the deposition of cholesterol in the aorta but would tend to combat its deposition by maintaining normal transport and metabolism of this sterol. Data have accumulated over the past years in our laboratory to support this hypothesis, and these findings will be presented in forthcoming publications.

SUMMARY AND CONCLUSIONS

Plasma and liver cholesterol levels and liver lipids of male and female rats maintained on diets containing 15% lard or 15% cottonseed oil, with and without cholesterol, were determined after 6, 12, 18 and 24 weeks. In all cases, higher cholesterol concentrations were found in the livers of animals on the lard diets. Differences in plasma cholesterol levels and total liver lipids were not apparent unless cholesterol was present in the diet, at which time animals receiving the cholesterol-supplemented lard diets exhibited higher cholesterol concentrations in these categories as well.

A sex difference in cholesterol metabolism has been observed; on both dietary regimens, female rats exhibited higher plasma and lower liver cholesterol levels than those found in the corresponding male rats.

Increased cholesterol concentrations were also noted in the carcasses (excluding liver) of animals of both sexes which had been fed the diet containing lard as compared with the cholesterol content of carcasses of animals on the cottonseed oil diet.

The absorption of cholesterol in male and female rats when fed with either lard or cottonseed oil was found to be the same regardless of the vehicle. However, a sex difference in lipid metabolism was again exhibited, since on both lipid diets, female rats absorbed more cholesterol than did the male rats.

The possibility that the small quantities of cholesterol present in lard might account for the increased liver cholesterol in the animals on this diet was eliminated, since, when the cholesterol content of cottonseed oil was equalized with that normally present in lard, no effect on liver and plasma cholesterol levels as compared with regular cottonseed oil was observed.

The effect of vitamin E, in which lard is practically deficient, was studied by adding a 5-fold excess of vitamin E to the lard diets with and without cholesterol. The increased quantities of vitamin E definitely decreased the liver cholesterol concentration of the rats fed cholesterol to a point where the differences in liver cholesterol content of animals on both the lard + cholesterol diet and the cottonseed oil + cholesterol diet were non-existent. Vitamin E, however, was without effect in the rats on the lard diet from which exogenous cholesterol was omitted.

It is hypothesized that the difference in action of the two fats is due to the greater quantity of essential fatty acids found in cottonseed oil over that which occurs in lard. These essential fatty acids are required for normal cholesterol transport and metabolism; an inadequate supply will tend to cause the accumulation of cholesterol in certain tissues in the animal body.

LITERATURE CITED

AHRENS, E. J., JR., D. H. BLANKENHORN AND T. T. TSALTAS 1954 Effect on human serum lipids of substituting plant for animal fat in the diet. Proc. Soc. Exp. Biol. Med., *86:* 872–878.

ALFIN-SLATER, R. B., L. AFTERGOOD, A. F. WELLS AND H. J. DEUEL, JR. 1954 The effect of essential fatty acid deficiency on the distribution of exogenous cholesterol in plasma and liver of the rat. Arch. Biochem. Biophys., *52:* 180–185.

COCHRANE, G. C., G. D. MICHAELS AND L. W. KINSELL 1953 Dietary modifications of plasma cholesterol and phospholipid levels in diabetic patients. The effects of mixed diets high in vegetable fat. J. Clin. Nutrition, *1:* 295–298.

GOFMAN, J. W., H. B. JONES, F. T. LINDGREN, T. I. LYON, H. A. ELLIOTT AND B. STRISOWER 1950 Blood lipids and human atherosclerosis. Circulation, *2:* 161–178.

GREENBERG, S. M., C. E. CALBERT, E. E. SAVAGE AND H. J. DEUEL, JR. 1950 The effect of fat level of the diet on general nutrition. VI. The interrelation of linoleate and linolenate in supplying the essential fatty acid requirement in the rat. J. Nutrition, *41:* 473–486.

HARDINGE, M. G., AND F. J. STARE 1954 Nutritional studies of vegetarians. 2. Dietary and serum levels of cholesterol. J. Clin Nutrition, *2:* 83–88.

KEYS, A., J. T. ANDERSON, F. FIDANZA, M. H. KEYS AND B. SWAHN 1955 Effects of diet on blood lipids in man. Clinical Chem., *1:* 34–52.

KINSELL, L. W., J. PARTRIDGE, L. BOLING, S. MARGEN AND G. MICHAELS 1952 Dietary modifications of serum cholesterol and phospholipid levels. J. Clin. Endocrine Metabolism, *12:* 909–913.

KINSELL, L. W., G. MICHAELS, L. DEWIND, J. PARTRIDGE AND L. BOLING 1953 Serum lipids in normal and abnormal subjects (observations on controlled experiments). Calif. Med., *78:* 5–10.

KINSELL, L. W., G. D. MICHAELS, J. W. PARTRIDGE, L. A. BOLING AND H. E. BALCH 1953a Effect upon serum cholesterol and phospholipids of diets containing large amounts of vegetable fat. J. Clin. Nutrition, *1:* 224–231.

KRITCHEVSKY, D., A. W. MOYER, W. C. TESAR, J. B. LOGAN, R. A. BROWN, M. C. DAVIES AND H. R. COX 1954 Effect of cholesterol vehicle on experimental atherosclerosis. Am. J. Physiol., *178:* 30–32.

NIEFT, M. L., AND H. J. DEUEL, JR. 1949 Studies on cholesterol esterase. I. Enzyme systems in rat tissues. J. Biol. Chem., *177:* 143–150.

SCHLETTLER, G. 1948 Blut und Organcholesterin der weissen Maus nach verfütterung pflanzlicher Öle und tierischer Fette mit Phytosterinzusatz. Klin. Woehenschr., *26:* 566–567.

———— 1949 Studien über den Cholesterinstoffwechsel der Maus. 1. Mitteilung Die Beeinflussung des Blut und Organcholesterins durch Verschiedene Fette und Öle ohne Cholesterinzusatz. Biochem. Z., *319:* 349–358.

———— 1949a Studien zum Cholesterinstoffwechsel der Maus. 4. Mitteilung Cholesterinmast bei verwendung verschiedener Öle und Fette. Biochem. Z., *319:* 444–452.

THOMPSON, S. Y., J. GANGULY AND S. K. KON 1949 The conversion of β-carotene to vitamin A in the intestine. Brit. J. Nutrition, *3:* 50–78.

TSAI, S. Y., E. D. FUTCH, M. KOBAYASHI, L. G. MAY AND R. L. GREGORY 1954 The effect of cholesterol-free diet on serum cholesterol in dogs. Texas Reports Biol. Med., *12:* 423–428.

WILKINSON, C. F. JR., E. BLECHA AND A. REIMER 1950 Is there a relation between diet and blood cholesterol? Arch. Intern. Med., *85:* 389–397.

EFFECT OF QUANTITY AND SOURCE OF DIETARY NITROGEN ON THE UTILIZATION OF THE HYDROXY ANALOGUES OF METHIONINE AND GLYCINE BY CHICKS [1]

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The net utilization of ammonium nitrogen by growing rats fed the 10 essential amino acids was demonstrated by Lardy and Feldott ('49, '50) and by Rose et al, ('49). Apparently ammonium compounds can serve as a source of nitrogen for the synthesis of the non-essential amino acids in the rat. No such effect of ammonium compounds or urea has previously been observed in chicks.

The commercial availability of methionine hydroxy analogue (2-hydroxy-4-methyl thio-butyric acid, calcium salt) for use in poultry feeds has raised some interesting questions regarding the possibility of supplying fragments of essential amino acids and letting the chicken finish the job of synthesis. F. H. Bird ('52) reported that chick growth obtained from methionine hydroxy analogue paralleled that obtained from DL-methionine when both were supplements at molecular equivalent levels to a 20% protein diet low in methionine. Gordon et al. ('54) reported that feed conversion by broiler chickens was significantly improved by the addition of methionine hydroxy analogue to corn-soybean oil meal diets containing 20% protein. Reid et al. ('54) found that chicken

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

[2] Wisconsin Alumni Research Foundation fellow.

growth and the reduced glutathione content of the liver were significantly increased through the addition of DL-methionine, or the hydroxy analogue, to a diet low in methionine. Gordon and Sizer ('55) reported that L-methionine, D-methionine, and the hydroxy analogue were equal on the molar basis as supplements to chick diets. These workers found DL-methionine to be less active than the other three methionine supplements.

Since all previous studies of methionine hydroxy analogue (MHA) utilization by the chicken were conducted with 20% protein diets it was of interest to study the effects of MHA in the presence of lower protein levels. Studies of chick utilization of sodium glycolate, the hydroxy analogue of glycine, were also conducted.

EXPERIMENTAL

Straight run chicks originating from the mating of New Hampshire males to Single Comb White Leghorn females were used in this study. One group of 10 birds was assigned to each treatment. The chicks were weighed, wing-banded, and placed on experiment in electrically heated battery brooders at one day of age; water and experimental diets were supplied ad libitum. The composition of the basal ration is given in table 1. The protein content of this diet was calculated to be 14% and eventually this value was lowered to 12% by decreasing the soybean protein [3] and increasing the sucrose. Amino acid levels of these low-protein diets were calculated to correspond to the reduced protein levels. Theoretical amino acid requirements were determined as follows: 14/20 X NRC values (Bird et al., '54) or 12/20 X NRC values if the basal diet contained 14% protein or 12% protein respectively. Both glycine and methionine were needed in these low-protein diets; this permitted the testing of hydroxy analogues of these two amino acids. [4]

[3] Drackett Assay C-1 Protein. The Drackett Products Company, Cincinnati, Ohio.

[4] Supplies of DL-methionine and MHA were furnished respectively by E. I. du Pont de Nemours & Company, Newark, Delaware, and the Monsanto Chemical Company, St. Louis 1, Missouri.

TABLE 1

Composition of basal diet (14% protein)

INGREDIENT	PER KILOGRAM DIET
	gm
Sucrose	714.70
Soybean protein [1]	156.00
Mineral mixture *	60.00
Soybean oil	45.00
DL-Methionine [2]	3.36
Glycine [3]	1.26
Choline chloride (70%)	2.00
Inositol	1.00
Vitamin premix in sucrose **	5.00
Penicillin [4]	0.70
Alpha tocopherol acetate	10.00 mg
Vitamin D_3 (1500 I.C.U./gm)	600 I.C.U.
Vitamin A	15,000 I.U.

MINERAL MIXTURE *		VITAMIN PREMIX **	
Ingredient	Per kilogram diet	Ingredient	Per kilogram diet
	gm		*mg*
$CaCO_3$	22.8192	Vitamin B_{12} in carrier [5]	20.00
K_2HPO_4	15.4800	*d*-Biotin	0.20
$CaHPO_4 \cdot 2H_2O$	7.1400	Menadione	0.50
NaCl	8.0400	Pyridoxine HCl	4.00
$MgSO_4 \cdot 7H_2O$	4.8960	Pteroylglutamic acid	4.00
Ferric citrate $\cdot 6H_2O$	1.3200	Riboflavin	6.00
$MnSO_4 \cdot H_2O$	0.2400	Ca pantothenate	20.00
KI	0.0384	Thiamine HCl	10.00
$CuSO_4 \cdot 5H_2O$	0.0144	Niacin	50.00
$ZnCl_2$	0.0120	*p*-Aminobenzoic acid	100.00
		Sucrose	4,785.30

[1] Drackett C-1 Assay Protein. The Drackett Products Company, Cincinnati, Ohio.

[2] Ommitted when methionine hydroxy analogue used.

[3] Omitted when sodium glycolate used.

[4] Each pound equivalent to the activity of 4 gm of procaine penicillin (equivalent to 2.4 gm of penicillin G master standard).

[5] Each gram equivalent to 1 mg of anhydrous cyanocobalamin.

RESULTS AND DISCUSSION

Growth response curves resulting from the addition of three graded levels of DL-methionine and three corresponding levels

of MHA to methionine-deficient 14% protein diets are shown in figure 1. Both supplements improved chick growth over that on the methionine-deficient basal diet. At each corresponding level of supplementation DL-methionine produced greater gains than did MHA. A repeat test using the same 14% basal ration gave almost identical results.

Figure 1 also shows chick growth response curves resulting from the addition of three graded levels each of DL-methionine and MHA to methionine-deficient 12% protein diets. Again

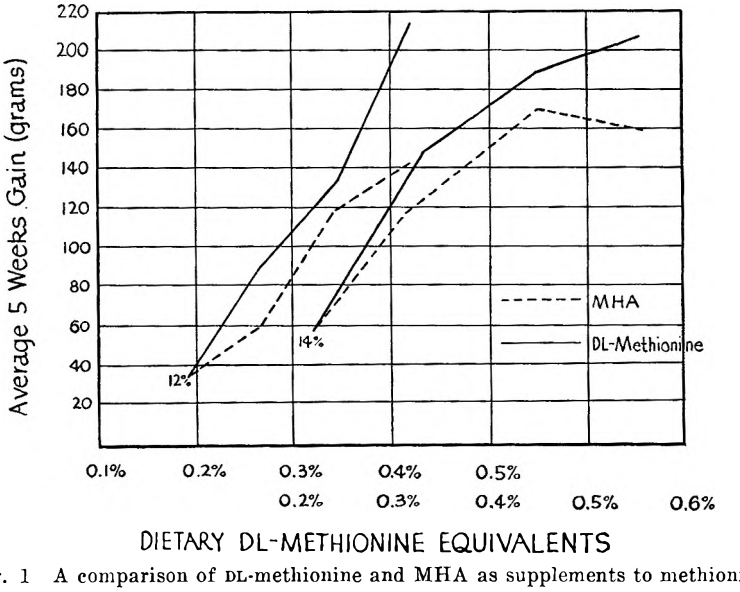

Fig. 1 A comparison of DL-methionine and MHA as supplements to methionine-deficient 12% and 14% protein diets for chicks.

both supplements improved chick growth response over the deficient basal diet. Each level of DL-methionine produced greater chick gains than did the corresponding levels of MHA. The greatest difference in growth response, 69 gm. occurred at the highest level of supplementation.

To improve the MHA diets, it appeared that some source of nitrogen must be made available to the chicken. Three nitrogen compounds were tested: urea, diammonium citrate and glycine. These compounds were added to the 12% protein

diet along with MHA or DL-methionine. Additions of nitrogen were made on an equal molar basis in which each mole of supplemental MHA or DL-methionine was combined with one mole of nitrogen from one of the nitrogen sources. Two tests were conducted to study the effect of adding these nitrogen compounds to MHA and DL-methionine supplemented chick diets as described above. Typical data from these tests are presented in table 2.

TABLE 2

Chick feed efficiency and weight gains as affected by the addition of urea and diammonium citrate to methionine hydroxy analogue (MHA) and DL-*methionine- supplemented 12% protein diets*

MODIFICATIONS OF BASAL RATION	AVERAGE GAIN IN 5 WEEKS		GRAMS FEED PER GRAM GAIN	
	Exp. 5	Exp. 6	Exp. 5	Exp. 6
	gm	*gm*		
DL-Methionine, 0.288%	127	118	2.89	3.62
DL-Methionine, 0.288% + urea, 0.06%	124	96	3.42	3.62
DL-Methionine, 0.288% + diammonium citrate, 0.226%	131	...	3.34	...
MHA, 0.347%	90	90	4.26	4.24
MHA, 0.347% + urea, 0.06%	128	111	2.88	3.52
MHA, 0.347% + diammonium citrate, 0.226%	125	118	3.07	3.67

Chicks receiving the DL-methionine-supplemented diets gained (table 2) at least 28 gm. more than those receiving the MHA-supplemented diets. Additions of either urea or diammonium citrate to the MHA diets increased chick gains to equal those of the DL-methionine group. These compounds added to the DL-methionine diets did not increase chick growth over methionine alone, and in one test, urea added to the DL-methionine-supplemented diet caused a decrease in chick gains to the extent of 22 gm. In both tests the chicks receiving the MHA-supplemented diets showed poorer feed efficiency (table 2) than those receiving the DL-methionine-supplemented diets. Additions of urea and diammonium citrate to the MHA

diets improved chick feed efficiency to equal that of the chicks receiving the DL-methionine diets. Additions of urea and diammonium citrate to DL-methionine diets may have decreased chick feed efficiency in one test.

Data from the addition of glycine to the MHA diets have been omitted; however, both growth response and feed efficiency were markedly improved when glycine was added to the MHA diets.

Studies of chick tolerance to increased levels of ammonium nitrogen were interesting; it appeared that chicks were more tolerant to dietary urea and diammonium citrate in the

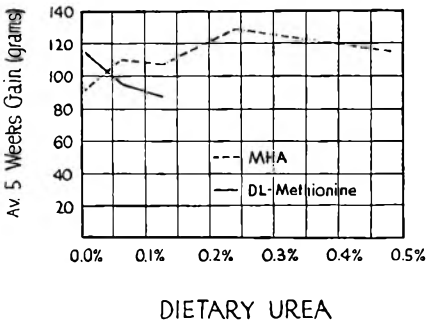

Fig. 2 Effects of increasing levels of urea in MHA and DL-methionine-supplemented 12% protein diets for chicks.

presence of MHA than in the presence of DL-methionine. The effect of increased levels of urea in the presence of these two methionine supplements is shown in figure 2. When urea was added to the DL-methionine diets, growth response appeared to decrease as the level of supplemental urea was increased. Considerable amounts of urea could be fed with MHA without any decrease in chick growth response. Increased levels of diammonium citrate had the same general effect as urea when combined with MHA and DL-methionine in low-protein chick diets. Essentially the same results were obtained in two other tests in which the dietary ammonium nitrogen was increased as described.

The hydroxy analogue of glycine, sodium glycolate, was tested in the 12% protein diet which has been described previously. This diet was deficient in glycine to the extent of 0.11%. Molecular equivalents of glycine, sodium glycolate, and sodium glycolate plus urea were added to this glycine-deficient diet. Urea was added to the sodium glycolate diets in a manner previously described in the MHA discussion. The data from these studies are presented in table 3.

TABLE 3

Effect of glycine, sodium glycolate, and sodium glycolate plus urea as supplements to a glycine-deficient 12% protein diet for chicks

MODIFICATIONS OF BASAL RATION	AVERAGE GAIN IN 5 WEEKS		GRAMS FED PER GRAM GAIN	
	Exp. 10	Exp. 12	Exp. 10	Exp. 12
	gm	*gm*		
Glycine, 0.11%	79	91	4.64	4.20
Sodium glycolate, 0.147%	41	85	6.32	4.59
Sodium glycolate, 0.147% + urea, 0.045%	74	127	5.41	3.96

Chicks receiving the glycine-supplemented diets, in both these tests, showed greater gains than those receiving the sodium glycolate-supplemented diets. The addition of urea to the sodium glycolate diets increased chick gains to equal or exceed those of the glycine group. Chicks receiving the sodium glycolate-supplemented diets showed poorer feed efficiency (table 3) than those receiving the glycine-supplemented diets. When urea was added to the sodium glycolate-supplemented diets, feed efficiency was improved.

SUMMARY

1. The hydroxy analogues of methionine and glycine as supplements in low-protein chick diets failed to support growth equivalent to the alpha amino compounds (DL-methionine and glycine).

2. Additions of nitrogen in the form of urea or diammonium citrate to the MHA diets caused a marked increase in chick growth response over MHA alone. Urea, added to the sodium glycolate-supplemented diets, increased chick gains over sodium glycolate alone.

3. Feed efficiency of chicks receiving MHA and sodium glycolate-supplemented diets was poorer than that of chicks receiving diets supplemented with DL-methionine and glycine. Additions of urea or diammonium citrate to MHA or sodium glycolate-supplemented diets improved feed efficiency.

4. Chicks appeared to be more tolerant of dietary urea and diammonium citrate in the presence of MHA than in the presence of DL-methionine.

5. The increased chick growth response to dietary urea or diammonium citrate is believed to be the first such observation in chick experiments.

LITERATURE CITED

BIRD, F. H. 1952 A comparison of methionine and two of its analogues in the nutrition of the chick. Poultry Sci., *31:* 1095–1096.

BIRD, H. R., H. J. ALMQUIST, W. W. CRAVENS, F. W. HILL AND J. MCGINNIS 1954 Nutrient requirements for poultry. Publication 301: National Research Council, Washington, D. C.

GORDON, R. S., K. H. MADDY AND S. KNIGHT 1954 Value of methionine hydroxy analogue supplementation of broiler diets. Poultry Sci., *33:* 424–425.

GORDON, R. S., AND I. W. SIZER 1955 The biological equivalence of methionine hydroxy analogue. Ibid., *34:* 1198.

LARDY, H. A., AND G. FELDOTT 1949 The net utilization of ammonium nitrogen by the growing rat. J. Biol. Chem., *178:* 509–510.

———— 1950 The net utilization of ammonium nitrogen by the growing rat. Ibid., *186:* 85–91.

REID, B. L., S. GABAY AND J. R. COUCH 1954 Effect of methionine hydroxy analogue and vitamin B_{12} on the glutathione level in the chick. Poultry Sci., *33:* 1090.

ROSE, W. C., L. C. SMITH, M. WOMACK AND M. SHANE 1949 The utilization of the nitrogen of ammonium salts, urea, and certain other compounds in the synthesis of non-essential amino acids in vivo. J. Biol. Chem., *181:* 307–316.

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