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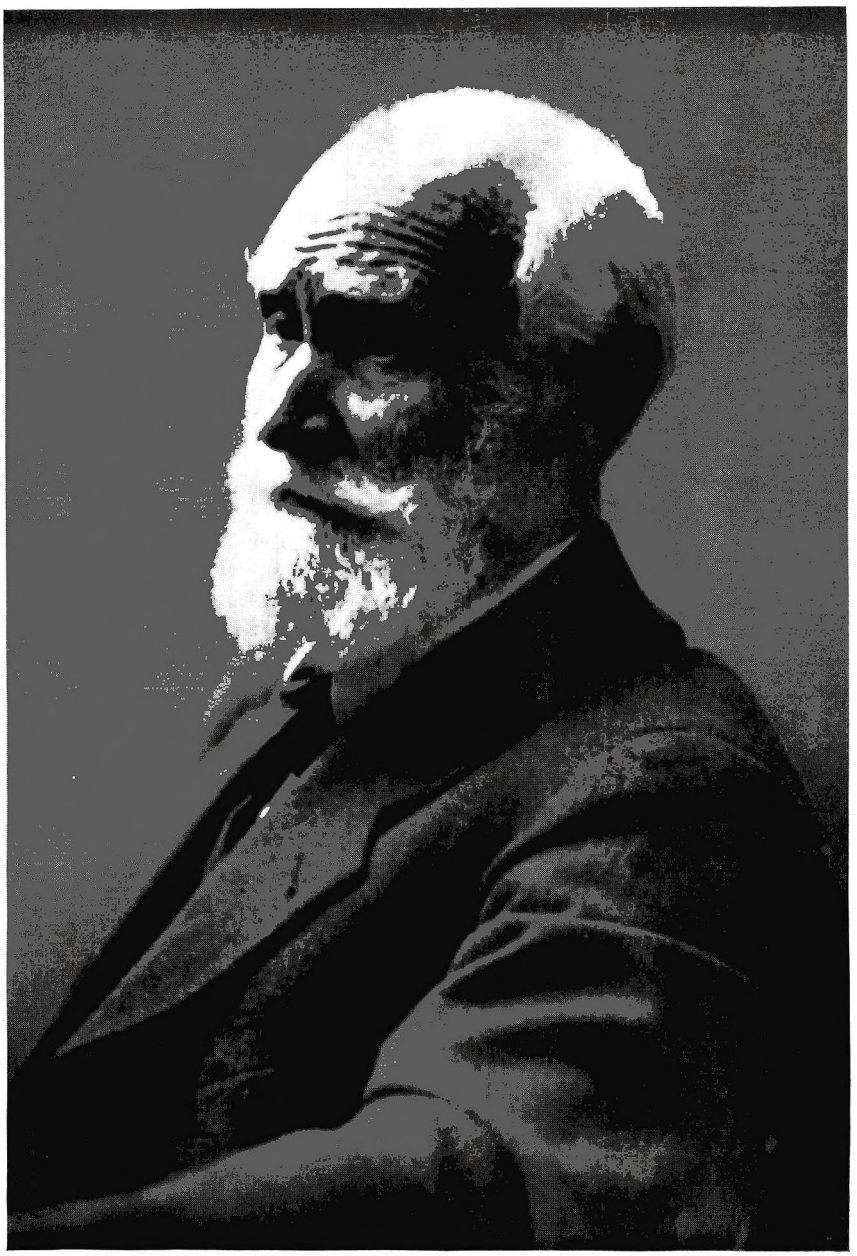

Ivan Petrovich Pavlov

Ivan Petrovich Pavlov

— A Biographical Sketch

(September 27, 1849 — February 27, 1936)

Ivan Petrovich Pavlov was the oldest son of a priest in the poor parish of Nikola Dolgoteli in Ryazan, Russia, who later became dean in one of the best parishes of Ryazan. Ivan's mother, Varvara Ivanova was the daughter of a priest. Quite naturally Ivan received a clerical education at the Ryazan Ecclesiastical High School, but he was not destined for the priesthood. The philosophy he studied at that time, however, influenced his later work and attitude toward helping his fellowmen. There were ten children in the family, six of whom died young. Of the three younger brothers who lived to adulthood, one became a priest and Dmitri, a year younger than Ivan and his closest associate, became professor of chemistry at the University of Petrograd.

From high school Ivan entered the University of Petrograd where he studied chemistry and physiology and graduated in 1875. While there he decided to become a physiologist. After the University he spent four years at the Military Medical Academy and was awarded a gold medal when he graduated in 1879. He was granted the degree of Doctor of Medicine in May, 1883, following four years of further study and research. While a student he lived with his brother Dmitri who practically nursed and cared for his every need. Ivan disregarded everything not directly connected with pursuing knowledge, so it was reported, but he did meet other young people, one of whom was to become his wife.

Seraphima Vasilievna Karchevskaya was the daughter of a navy doctor in Roslov-on-the-Don where she was born in 1855. She came to study at the Pedagogical Institute and met Pavlov while he was a student at the Medical Academy. They were married May 1, 1881. Because they were practically penniless they continued to share an apartment with his brother Dmitri until Ivan completed his education.

Seraphima was a pretty, vivacious and clever young woman, who became a true friend and companion, a devoted wife and model mother to their four children. Pavlov, being so little aware of everyday problems, in many respects depended upon her completely. She understood and appreciated her genius of a husband and made the best of their early years of poverty. It was not until 1890 when Pavlov received the chair of Pharmacology in the Military Institute that financial cares were relieved and life became easier for them.

After his marriage but while still a student, Pavlov spent a summer in Breslau, Germany, working in Rudolf Heidenham's laboratory and later spent two years in Germany studying under Heidenham and under Carl Ludwig at Leipzig. His field of work and techniques were greatly broadened by this experience outside his own country and strongly influenced by these two eminent scholars and teachers. His first publication in 1879 described his original method for establishing a pancreatic fistula, keeping the nerve supply intact — an improvement over the then accepted method of Heidenham, his teacher.

The next ten years Pavlov spent as an assistant in the physiology laboratory of the Military Medical Academy during which time he published a number of papers. During the 1880's, his investigations were on the heart and circulation, particularly the innervation of the heart and other abdominal organs. While studying the influence of the vagus nerves upon the process of secretion of the gastric glands, he developed his method for obtaining gastric juice from dogs — the famous "sham-feeding" technique. The results of

these experiments were so interesting and promising that he turned his entire attention to nerve physiology and the digestive organs.

From 1891 until his death in 1936, Pavlov was Professor at the Military Medical Academy and Director of the Institute for Experimental Medicine in Leningrad. By his 150 or more advanced students he is remembered as a great teacher who started many of them on famous careers. By the rest of the scientific world he is known for his contributions to the physiology and nervous control of digestion and for his later work on "conditioned reflexes."

Pavlov's esophageal fistula in dogs became standard technique not only in his laboratory but in those of his students and others around the world working along this line. The dog's esophagus was severed in such a way that swallowed food might be discharged at the upper fistula and unswallowed food might be introduced into the stomach through the lower opening. Thus the dog might be allowed merely to see and smell the food — "psychological feeding"; or the dog might be allowed to chew and swallow the food which would be discharged through the upper fistula — "sham feeding"; or a meal might be supplied by introducing food through the lower opening without the dog seeing, smelling or tasting the food — "true feeding." He thus demonstrated that sight, smell, taste and swallowing of food stimulated a copious and continuous flow of gastric juices and that this flow was stimulated by the vagus nerves, but was not affected when the splanchnic nerves were cut. He further demonstrated that the secretory fibers of the pancreas, as well as of the gastric glands, were in the vagi.

Pavlov and his pupils made many additional discoveries, among which were: that mechanical stimulation of the stomach by the introduction of food through the lower fistula while the dog was asleep did not necessarily stimulate secretion, contrary to popular opinion; that when psychical secretion is shut off the amount of secretion varies with the type of food given, being positive for meats and negative for other foods; that the degree of chemical stimulation varied with different types of foods; that the acid from the gastric juice stimulated the flow of pancreatic juice in the intestines; and that the proteolytic action of the pancreatic juice was due to "enterokinase" secreted by the duodenal membrane. Even to this day the activating enzyme in the intestines bears the name that Pavlov gave to it in 1899.

Pavlov's work on "conditioned reflexes," published in 1912, was instigated by his earlier observations on the importance of the relation of the brain and nervous system to the digestive functions. His name is best known in modern physiology texts for this work on conditioned reflexes. He studied in great detail the influence of appetite and hunger on the work of the digestive glands, noting responses that might have escaped a less keen observer. The showing of food to a dog constitutes an unconditioned stimulus; the musical note or bell which the dog associates with food becomes the conditioned stimulus. Failure to follow the conditioned by the unconditioned stimulus leads to weakening and eventually to the loss or extinction of the reflex. Repetition plays an important part in conditioned reflexes. It is not only necessary for the formation of the reflex but is essential for its maintenance, otherwise the reflex tends to decay. It is readily reinforced by repetition of the procedure of following up the conditioned by the unconditioned stimulus.

Pavlov went on to study the effect of the specific sensory and psychic stimuli or reflex actions showing that a musical tone, a bright color, a strong odor or a skin stimulus, if associated with the sight of food, causes salivation. The specificity of this reaction is illustrated by the fact that the flow of saliva which responds to the sound of a given note will cease if the note is raised or lowered by even a half-tone.

Thus Pavlov's contributions were significant in determining the neural mechanisms involved in the learning process. He emphasized the fact that learned behavior is built upon inherited behavior and that the learning process consists, in large part, of substituting new conditioned stimuli for the normal and inborn unconditioned ones. He maintained that rate of learning is largely determined by the rate of establishment of the conditioned reflex — that repetition "fixes" the response.

Learning by the establishment of conditioned reflexes becomes more complicated as education proceeds. One conditioned reflex may serve as the basis for another. Ultimately a complicated many-layered series of conditioned reflexes is acquired in this way. It is thus but a step from the conditioned reflex just described to the phenomenon of acquiring habits or habitual actions. Pavlov explained sleep as an active inhibition either internal or external such as protracted mild stimulation of a monotonous nature.

One of his students, W. N. Boldyreff, recalled that Pavlov's lectures always included demonstrations to show his students his method of operating and how the results were obtained. No lecture was given without experiments that had been patiently tried over and over again with meticulous attention to every detail before they were demonstrated to students. Failures or mistakes in lecture demonstrations were absolutely forbidden. Pavlov was also an eloquent speaker, but in his lectures to students much more stress was based upon action. He was one of the most popular professors in all Russia and many of his former students have become well-known scientists in various parts of the world.

Another former student, Peter Karpovich, who knew Pavlov only in his later years said of him: "Some teachers at his age lecture just by inertia, coming to life only when recollecting some incidents from their youth, or referring to the chicken farm on which they plan to retire. Pavlov was different. Always full of energy, he lectured in a manner that resembled mental moving pictures. Sometimes in stating a problem he would ask us to suggest a method of solution. He would wait a short time, then get impatient, and would start lashing us with not very complimentary statements as to the kind of brains in our possession. Although sometimes we were annoyed at this, nevertheless all was forgiven when someone in the class would succeed in making a proper suggestion and Pavlov would flatter him, saying 'I think you have brains'."

Pavlov formulated three rules which he maintained were a necessary foundation for physiological studies: 1) that it is necessary to carry on experimental work on normal, healthy animals, not merely on those poisoned or dying by vivisection; 2) that it is prerequisite to proper investigation of any organ to previously perform an operation which will render it easily accessible; and 3) that normal functioning of an organ must be observed after the animal has fully recovered from the operation. In all his experimental work he planned the minutest detail and, if possible, did the actual work himself at least until the procedure was standardized.

For his study of physiology Pavlov invented a series of operations on different organs: fistula of the pancreas, the isolated stomach, and a combination of esophagotomy with a gastric fistula. Then he combined these separate operations into a perfect and complete physiological surgical procedure. Then by means of his system of control experiments on healthy animals Pavlov created the most complete picture at that time of the physiology of digestion, and described the fundamental laws govering the activity of the salivary glands, the stomach, the pancreas, the liver and the intestinal glands.

He did not, however, enjoy writing up his results and published only about fifty articles under his own name, and most of them short. But his students wrote under his guidance something like 200 articles, and Pavlov himself was responsible for the greater part of the work reported. Jealousy of the success of others was unknown to him. When comment was made about his originating the famous experiments on conditioned reflexes, he would point out that Thorndike in America, independently of him, conducted similar experiments. When he lectured on the physiology of digestion, he always pointed to Heidenham as the source of his inspiration. He not only inspired his own students but encouraged qualified youth to enter the field of science for their life work.

As a student, Pavlov read literature as well as science continuously, and he had a vivid imagination. He was greatly influenced by his godfather, the Abbot of St. Trinity's Monastery near Ryazan.

As a teacher Pavlov used simple, clear language, illustrated his lectures with ex-

periments and demonstrations and always encouraged his students to ask questions.

As a research worker he was original in his thinking and nothing could change his mind once he had studied a problem thoroughly and was convinced that he was right. He was skilled as a surgical operator and handled animal tissues with the delicacy and speed necessary for his amazing successes. He was an honest and independent worker, an incorruptible scientific investigator.

Pavlov always enjoyed a joke, even when it was played on himself. When he was in Cambridge, England, in 1915, to receive an honorary degree a trick was played on him by the students in physiology and Professor A. V. Hill tells the story: "They thought they would have to do something to improve the occasion of the degree-giving. They went to a toy shop and bought a large and life-like dog, which they proceeded to decorate with rubber stoppers, glass tubes, pieces of rubber tubing and any other physical, chemical or physiological apparatus they could think of. They took it to the Senate House and suspended it from gallery to gallery by a long string. As Pavlov walked away, having received his degree, they let it down to him on the string. He was highly delighted, took the dog from the string and carried it under his arm. For many years he kept that dog in his study in Leningrad."

Pavlov's first honorary title was given to him by a Mexican scientific society in 1898. Previous to 1904 he had been elected an honorary member of many German and Russian medical societies. In 1904, he received the Nobel Prize for his discoveries in the physiology of digestion. Then followed many other honors: in 1907, Fellow of the London Royal Society and of the Petrograd Academy of Science; in 1912, the honorary doctor's degree from Cambridge University; in 1915, the Copely Medal from the London Royal Society; in 1923, an honorary doctor's degree from Edinburgh University. He was also a member of the Academy of Science of Paris, of Rome, Bologna and Denmark, and of the Irish, Belgian and American Medical Associations.

Just before he died he wrote the following bequest to the academic youth of Russia:

"What can I wish for the youth of my country who devote themselves to science?

"Firstly, GRADUALNESS: About this most important condition of fruitful scientific work I can never speak without emotion. Learn the ABC of science before you try to ascend to its summit. Never begin the subsequent without mastering the preceding. Never attempt to screen an insufficiency of knowledge even by the most audacious surmise and hypothesis. Howsoever the soap-bubble may rejoice your eyes by its play, it inevitably will burst and you will have nothing except shame. School yourselves to demureness and patience. Learn to accept drudgery in science. Learn, compare, collect the facts. Perfect as is the wing of a bird, it never could raise the bird without resting on air. Facts are the air of a scientist. Without them, you never can fly. Without them, your theories are vain efforts.

"Secondly, MODESTY: Never think that you already know all. However highly you are praised, always have the courage to say of yourself — I am ignorant.

"Thirdly, PASSION: Remember that science demands from a man all his life. If you had two lives that would not be enough. Be passionate in your work and your searchings."

It is impossible to estimate the extent of Pavlov's influence on the work of younger physiologists and nutritionists of his time and since. William Beaumont's earlier observations on factors affecting the flow of digestive juices were confirmed and explained by Pavlov and his school. Textbooks today continue to carry references to both of these pioneers and especially to Pavlov's experimental proof of the nervous control of the secretions.

The dynamic and imposing personality of "Academician Pavlov," who at eighty-seven presided at the 15th International Physiological Congress in Russia in 1935, impressed all who saw and heard him. He died the following winter. The first opportunity that many of us in this country had to meet this world-famous scientist whose name had long been familiar to students of physiology, had occurred twelve years ear-

lier in 1923, when he visited the United States for the first time. He was here again in 1929 to attend the 13th International Physiological Congress at Harvard as an honored guest.

His former student, Peter Karpovich of Springfield College, commented at that time: "Since the days of Tolstoy, hardly any other living Russian, not connected with politics, has attracted so much attention as has Pavlov. Different as they were, they had much in common. They were as two flaming torches placed at a distance from each other, lighting new ways into the darkness of the unknown. Tolstoy was an expression of the mystic search for truth, Pavlov was an exponent of the realistic, scientific search."

Dr. A. V. Hill wrote a glowing tribute to Pavlov at the time of his death in 1936, shortly after the International Physiological Congress:

"It was Pavlov's immense prestige and the deep affection which the physiologists the world over had for him which made the acceptance of an invitation to the Soviet Union possible. It was Pavlov's prestige and that affection together with the mixture of playfulness, sternness, impatience, devotion, and simplicity which formed his character, that made the Congress so successful, and opened up what one hopes is an era of friendly relations between physiologists in Russia and the rest of the world.

"Whenever Pavlov appeared in public — whether in Leningrad, London, Boston or elsewhere — his romantic and almost legendary figure, and the engaging simplicity and boyish humor of his bearing were apt to evoke prolonged and enthusiastic applause. He was sometimes rather impatient of his popularity.

"Pavlov was an old man in years, but he did not seem old in mind or in strength, and one of the memorable pictures of the Congress was of Pavlov giving his arm to a colleague ten years older than himself who came on the platform to address us. Partly by his age, partly by his repute, partly by his character, he was without peer among the scientists of his country, and he could be as tyrannical at one moment as he could be simple and boyish at another; but he was loved far more than he was feared. His single-hearted devotion to science and the cause of science was that of a religious man — as he was. I remarked to him that many great Englishmen were the sons of country parsons. He proudly replied that he was the son and the grandson of a priest. My obvious comment that he himself was a high priest drew chuckles of boyish pleasure.

"One of the charming things about Pavlov was his family relationships. In his later years, whenever he went abroad, he was always accompanied by one of his sons. A lawyer son had in recent years devoted himself, I believe exclusively, to acting as his father's secretary and agent. Pavlov himself did not easily speak any language but his own, though he was able to converse, not very readily in German. This son, however, was an extremely accomplished linguist and accompanied his father to such meetings as that of the Permanent International Committee of the Physiological Congresses, where conversation might be carried on in at least three languages and translated for him. I have the most vivid and charming memories of the old man and his son at these meetings, the latter taking part in the conversation in any language and rapidly giving his father in Russian the gist of all that was going on, the old man nodding and smiling and expressing his opinion with his hands and with smiles and nods all the while."

In conclusion I quote from a Leningrad newspaper of August 8, 1935:

"A monument to the Unknown Dog was unveiled today at the Institute of Experimental Medicine here. The inscription on the monument, bearing a stone image of a dog, reads: 'In memory of all dogs which have given their lives for physiological experiment for the purpose of prolonging life and improving human health'."

"Someone called Pavlov a modern saint. The main characteristics of a saint are: sincerity, simplicity, and a limitless devotion to high ideals. In Pavlov's person we have this combination and more. He was not only a saint but a prophet.

"Born in an obscure place that can hardly be found on the map, he grew in spite of obstacles into a giant, whose greatness was too big for any national borders

and reached every part of the civilized world."

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[2] Dr. Mitchell first met Pavlov when he visited in this country in 1923 and came to see his former student, W. N. Boldyreff, at Battle Creek College, where Dr. Mitchell was Professor of Nutrition. She met him again in 1935 when she attended the 15th International Physiological Congress in Moscow, of which he was the Honorary Chairman.

Composition of Aortic Tissue from Copper-deficient Chicks [1]

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ABSTRACT The effect of copper deficiency upon the composition of connective tissue in the vascular system of the chick was investigated. The aortas of 4-week-old chicks, which had been fed copper-deficient and control diets, were processed so as to separate soluble collagen, insoluble or mature collagen and elastin. The fractions were analyzed for nitrogen, hydroxyproline, and hexosamine. Chicks fed the deficient diet weighed approximately 30% as much as the controls fed copper but, expressed as percentage of body weight, their aortas were twice as heavy as controls. Based on wet weight, the saline-soluble fraction from copper-deficient aortas contained nearly twice as much nitrogen, nearly 4 times as much hydroxyproline and significantly more hexosamine. The percentage of nitrogen removed by autoclaving and by sodium hydroxide extraction was appreciably greater in the deficient aortas but it was not collagen. On the basis of dry weight, the control aortas contained about 47% elastin and the deficient about 26% whether determined by sodium hydroxide or formic acid purification. The copper-deficient aortas accumulated a large fraction of non-elastin, non-collagen protein and had an increased concentration of soluble collagen. The elastin isolated by sodium hydroxide treatment contained a greatly elevated concentration of lysine and significantly less desmosine and isodesmosine.

Copper deficiency results in connective tissue disorders as shown by recent observations made on vascular systems of the newborn rat (1), the chick (2) and the pig (3). Earlier work showed a deleterious effect upon bone (4, 5). Analysis of copper-deficient pig aortas has shown a decrease in elastin, an increase in hexosamine but no change in collagen content (6). In the chick the concentration of elastin in the aorta is decreased and it was suggested that the deficiency had an effect upon elastin biosynthesis (7). Elastin is an inert protein characterized largely by its insolubility and resistance to chemical reaction. For this reason methods for its determination are not highly specific and usually are based on the amount of residue remaining after dissolving the other tissue components in sodium hydroxide (8) or formic acid (9).

The present communication deals with the analysis of deficient and control chick aortas for the components soluble in cold saline, in the aqueous phase after autoclaving, and in hot sodium hydroxide. The amino acid composition of the elastin residue is presented and a comparison has been made of the elastin content of aortic tissue as determined by the above procedure and by the formic acid method.

EXPERIMENTAL

The composition of the basal diet,[2] shown in table 1, is similar to that used by Starcher et al. (7) and that previously used in this laboratory (2), differing chiefly in its higher zinc content. Control chicks received the basal diet supplemented with 50 ppm of copper as anhydrous $CuSO_4$. Diets and distilled water were supplied ad libitum.

Male Vantress × White Rock chicks were reared to 4 weeks of age in stainless steel battery brooders. At the end of the trial a portion of the thoracic aorta was removed, freed of adventitia, opened longitudinally, washed free of blood, blotted on filter paper and weighed. The tissue was stored frozen until analyzed.

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[2] The authors gratefully acknowledge gifts of vitamins from Merck and Company, Rahway, New Jersey and Hoffmann-LaRoche, Nutley, New Jersey. Arginine was donated by General Mills, Inc., Minneapolis.

TABLE 1

Composition of basal diet [1]

	%
Non-fat milk solids	60.0
Sucrose	29.5
Soybean oil	5.0
DL-Methionine	0.5
Arginine·HCl	1.0
Glycine	1.5
Choline·Cl	0.2
$CaHPO_4$ (N.F. hydrous)	2.0
NaCl	0.2
$MnSO_4 \cdot H_2O$	0.03
Ferric citrate	0.06
$ZnCO_3$	0.04
KIO_3	0.004

[1] The diet contained the following supplements per 100 g: (in milligrams) thiamine·HCl, 1.0; riboflavin, 1.0; pyridoxine·HCl, 1.0; Ca pantothenate, 3.0; niacin, 5.0; folacin, 0.2; biotin, 0.04; cyanocobalamin, 0.003; α-tocopheryl acetate, 2.5; menadione, 2.5; ethoxyquin, 12.5; and vitamin A palmitate, 2000 IU; and vitamin D, 430 ICU. By analysis the diet contained about 0.9 ppm of copper.

The principal method of analysis was similar to the fractionation procedure described by Weissman et al. (6) and was based on several analytical techniques previously described including those used by Neuman and Logan (8) and Levine and Gross (10). A single aorta (200 to 300 mg) was minced with scissors then homogenized in 6 volumes of buffered one-molar saline solution. This suspension was shaken in the cold (2°) for 18 hours and centrifuged at 80,000 × *g* for one hour. The residue was resuspended in about 4 volumes of M saline and again shaken for 18 hours and centrifuged. The supernatant solutions were combined and made to volume. The residue was washed with water to remove salt and extracted twice with 5 ml of 2:1 chloroform-methanol. Both the soluble and insoluble portions were dried in a vacuum oven. The insoluble portion will be referred to hereafter as the dry, fat-free residue although a portion of the non-fat solids had been removed by the saline extraction. The residue was rehydrated with 5 ml of water and autoclaved for 3 hours at 120°. The supernatant solution was removed, the residue autoclaved again with 4 ml of water in the same manner, and finally washed with 1 ml of water. The extracts were combined and made to volume. The residue was treated with 5 ml of 0.1 N NaOH at 100° for one hour and after centrifuging this was repeated for 15 minutes. The residue was washed thoroughly with water to remove sodium hydroxide. The extracts and washings were combined and made to a volume of 25 ml. The dried residue was considered to be elastin (8).

Nitrogen was determined by the micro-Kjeldahl procedure (11) and hydroxyproline by the method of Prockop and Udenfriend (12) after hydrolysis with 4 N HCl for 4 hours at 120°. This condition of hydrolysis gave maximal values for both hydroxyproline and hexosamine. Hexosamine was determined by a modification of the methods of Rondle and Morgan (13) and Blix (14). The acetylacetone solution contained 1.5 ml of acetylacetone in 50 ml of 1.25 N Na_2CO_3 and was prepared immediately before use. Ehrlich's reagent was prepared by adding 0.8 g of dimethylaminobenzaldehyde to 30 ml of absolute alcohol followed by 30 ml of concentrated HCl. The hydrolyzate was carefully neutralized to phenolphthalein and made to a volume of 2 ml. The concentration of salt in the solution affected color development; therefore all solutions, including the standards, were saturated with sodium chloride before addition of the acetylacetone reagent.

Elastin determination by the formic acid method (9) allowed the determination of dry matter as well. The whole tissue sample was dried to constant weight at 70° in a vacuum oven and the percentage of dry weight calculated. The tissue was then rehydrated overnight with the calculated amount of water and 0.2 ml of 90% formic acid/mg of dry tissue was added. The tissue was held at 45° for 36 hours then washed repeatedly with distilled water by decantation until the wash remained neutral after standing for one hour. The dried residue was considered to be elastin and the analysis was justified by amino acid analysis.

RESULTS AND DISCUSSION

Chicks fed the basal diet developed the typical symptoms of copper deficiency described previously (2) and there was a high rate of mortality resulting from rupture of major blood vessels. As shown by the data in table 2, the survivors weighed one-third as much as the controls, but their aortas were larger in proportion to body weight. This was due in part to in-

tramural hemorrhage, but was mostly the result of a thicker aortic wall. The deficient aortas contained significantly higher percentages of water (table 3) and lipid. Although the latter difference was statistically significant, the nature of the analytical procedure does not justify emphasis of this observation.

Extraction of tissue with cold saline solution removes, among other proteins, the soluble collagen component (10). The percentage of nitrogen extracted by saline, based on the wet weight of tissue, was nearly twice as high in the deficient aortas as in the controls (table 2). The concentration of hydroxyproline, which makes up about 13% of collagen, was nearly 4 times as great in the deficient tissue. However, it is clear that only a relatively small portion, not more than 25% of the total nitrogen, arises from collagen since the hydroxyproline present was only 3.4% of the crude protein in the extract from deficient aortas. Although the concentration of hexosamine in the deficient tissue was about 30% higher than in controls, the amount of nitrogen contributed by this component is an insignificant portion of the total. Thus it appears that there is an increased concentration of unidentified soluble protein.

Autoclaving collagen in water converts it into gelatin and thus solubilizes the mature insoluble component. The nitrogen and hydroxyproline content of this fraction of aorta is shown in section B of table

TABLE 2

Comparative analysis of aortas from copper-deficient and control chicks [1]

Measurement	Basal diet (M)	Diet M + copper	Statistical significance [2]
			$P <$
Body wt, g	143 ± 10 [3]	437 ± 30	0.001
Aorta wet wt, mg	251 ± 80	302 ± 30	—
Aorta/body wt, %	0.133 ± 0.01	0.069 ± 0.01	0.001
Lipid, % dry	11.8 ± 0.56	8.3 ± 0.72	0.005
Aorta dry, fat-free wt, mg	40.1 ± 4.3	56.4 ± 5.2	—
A. Saline extract, based on wet weight			
N, %	0.61 ± 0.04	0.35 ± 0.01	0.001
Hydroxyproline, mg/g	1.27 ± 0.11	0.35 ± 0.03	0.001
Hydroxyproline/N × 6.25, %	3.37 ± 0.39	1.74 ± 0.17	0.025
Hexosamine, mg/g	1.00 ± 0.05	0.76 ± 0.04	0.001
B. Autoclaved extract, based on dry, fat-free weight			
N, %	3.51 ± 0.23	2.24 ± 0.20	0.005
Hydroxyproline, mg/g	19.3 ± 1.9	14.6 ± 0.77	0.05
Hydroxyproline/N × 6.25, %	8.72 ± 0.42	10.6 ± 0.40	0.01
C. 0.1 N NaOH extract, based on dry, fat-free weight			
N, %	5.06 ± 0.53	3.26 ± 0.08	0.01
Hydroxyproline, mg/g	2.22 ± 0.26	1.41 ± 0.06	0.025
Hydroxyproline/N × 6.25, %	0.70 ± 0.03	0.69 ± 0.03	—

[1] Six chicks in each group analyzed individually.
[2] P values as determined by the *t* test.
[3] Mean ± SE.

TABLE 3

Elastin content of chick aortas as determined by sodium hydroxide and formic acid purification

Diet	Dry matter	Formic acid residue		Sodium hydroxide residue (D)
	%	*% dry*	*mg/100 g body wt*	*% dry, fat-free*
Basal (M)	18.5 ± 0.7 [1]	19.5 ± 1.9	4.7 ± 0.53	28.9 ± 1.8
M + 50 ppm Cu	21.4 ± 1.0	47.5 ± 1.4	6.6 ± 1.4	56.9 ± 1.3
$P <$	0.025	0.001	0.025	0.001

[1] Mean ± SE.

2 and is expressed on the basis of the dry, fat-free residue. This fraction of the deficient tissue also contained more nitrogen and hydroxyproline although the latter difference was of low statistical significance. The protein extracted was largely collagen as shown by the calculation of percentage hydroxyproline in the crude protein. There was a slightly higher percentage of other protein as well as of collagen in the extract from deficient tissue.

After autoclaving with water there still remained non-elastin protein (15) which could be solubilized with hot 0.1 N sodium hydroxide. The sodium hydroxide extract (section C, table 2) of the deficient tissue contained about 50% more nitrogen and hydroxyproline than control tissue, but there was obviously little collagen in this extract because hydroxyproline made up only about 0.7% of the crude protein. It is possible that a small amount of hydroxyproline arose from elastin, which contains approximately 1.5% of this amino acid, since elastin is slowly solubilized under the conditions used to purify it. In any case the sodium hydroxide extract from copper-deficient aortas contained more non-collagen, non-elastin nitrogen than a similar extract from controls.

The residue which remained after sodium hydroxide treatment is classed as elastin and the percentages of this fraction (D) based on the dry, fat-free residue, are shown in table 3. The deficient tissue contained about one-half as much elastin by this assay as the control tissue. Similar results were obtained by the formic acid method. The results are not directly comparable because the former is based on tissue which had been extracted with both saline solution and lipid solvents and the latter upon the total dry matter. Using average values and neglecting mechanical losses, the percentages of sodium hydroxide residue, calculated on total dry matter, were 26.0 and 47.4, respectively, for deficient and control tissue. These values are in agreement with those obtained by use of formic acid. Although the concentration of elastin in the aorta was markedly decreased by copper deficiency, the amount of elastin per unit of body weight was not changed to the same extent. It appears that not only is the synthesis of normal elastin impaired but there is also a significant deposition of other protein in the aorta.

The elastin isolated from copper-deficient aortas differs from normal in both physical characteristics and amino acid composition. The wet elastin has less elasticity and tensile strength and it tends to have a more sticky or adhesive character than normal. As shown in table 4 the copper-deficient elastin contained about 5 times as much lysine as the control protein. This is in agreement with the observation of Starcher et al. (7). The other significant difference was in the desmosine and isodesmosine concentrations which averaged about 60% of the control. The desmosines are tetra-amino-tetracarboxylic acids which were isolated from elastin and are believed to serve as crosslinking agents in this elastomer (16). It has been shown that lysine is a precursor of the desmosines and the evidence from both animal (17) and organ culture (18) experiments suggests that 4 moles of lysine are required for the synthesis of one mole of desmosine. However, the excess lysine in the copper-deficient elastin is more than

TABLE 4

Amino acid composition of aortic elastin from copper-deficient and control chicks [1] *(based on dry, fat-free protein isolated by the sodium hydroxide procedure).*

Amino acid	Copper-deficient, N = 16.62%	Control, N = 16.29%
	%	%
Aspartic acid	0.67	0.49
Threonine	0.58	0.59
Serine	0.40	0.46
Glutamic acid	2.19	2.16
Proline	16.20	16.78
Glycine	26.00	27.61
Alanine	17.30	17.08
Valine	23.09	22.43
Isoleucine	2.60	2.75
Leucine	6.80	7.47
Tyrosine	2.53	2.42
Phenylalanine	3.19	3.55
Isodesmosine	0.39	0.64
Desmosine	0.47	0.79
Lysine	2.68	0.56
Arginine	0.90	0.84
Hydroxyproline	1.73	1.56

[1] The protein was hydrolyzed for 72 hours in boiling 6 N glass-distilled HCl under nitrogen gas. The amino acids were determined by use of a Technicon Auto-analyzer (17).

would result from the decrease in desmosines. This suggests that the metabolic defect lies on the pathway between lysine and another compound, possibly an intermediate, which has not been identified. If lysine is the sole precursor of desmosine, the epsilon carbon of 3 moles of lysine must be oxidized to the aldehyde or its equivalent. Such reactions are catalyzed by amine oxidases and these enzymes are known to have copper as an essential component and to be reduced in the plasma of copper-deficient pigs (19).

Copper deficiency in the chick results in a failure of formation of the desmosine crosslink and it is probable that there is a decreased activity of a copper-containing enzyme which is involved in the biosynthesis of the desmosines. Although the concentration of desmosine was only mildly depressed, the amount of elastin isolated was markedly affected. This was to be expected inasmuch as only highly crosslinked protein would withstand the purification procedure. It is possible that synthesis of a non-crosslinked precursor proceeds normally but it is removed during the isolation and purification of elastin.

Histologically, there appears to be less elastin in the deficient tissue, but the most striking difference is the separation of the elastic lamellae by material that does not take up the Weigert stain. That there is a higher than normal concentration of non-collagen, non-elastin components in the deficient aorta is substantiated by the results presented here. Although the results of Weissman et al. (6) and Linker et al. (20) as well as those presented here show a higher mucopolysaccharide concentration in deficient aortas, the total amount present would not appreciably affect the concentration of elastin expressed as a percentage of dry weight. Contrary to the results of Starcher et al. (7), these results show a significantly higher water content in the copper-deficient tissue. Although they did not make a point of the observation because of high variability in the results, Weissman et al. (6) observed a greatly elevated concentration of hydroxyproline in the saline extract. This observation, confirmed in this study, strongly suggests a higher concentration of soluble collagen in copper-deficient aortas, a condition similar to that observed in lathyrism (10). Thus, there is a possibility that copper deficiency affects crosslinking in collagen in a manner analogous to that in elastin. Considering the fact that more soluble protein was extracted from deficient tissue, it is clear that copper deficiency affects not only elastin formation but also results in deposition of excess non-elastin protein in the aorta. This might be in part an elastin precursor.

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Comparison of Copper Deficiency and Lathyrism in Turkey Poults [1]

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ABSTRACT The pathology of copper deficiency and lathyrism was studied in turkey poults. Broad Breasted Bronze turkey poults were fed a series of 4 diets — a practical diet, the practical diet supplemented with 0.1% β-aminopropionitrile (BAPN), a basal milk diet low in copper and the basal milk diet supplemented with 50 ppm copper. Gross pathology was recorded and at the end of 4 weeks aortas were removed from survivors and analyzed chemically and histologically. The BAPN-fed poults developed typical gross symptoms of lathyrism, including leg weakness, subcutaneous hemorrhage and aortic rupture. The histological sections showed fragmentation of elastic lamellae, dissecting aneurysms, and accumulation of non-elastin material between the lamellae. The histopathology of the aortas from the copper-deficient poults was hardly distinguishable from those fed BAPN. Grossly the copper-deficient poults did not exhibit aortic rupture, but there was a high incidence of subcutaneous hemorrhage, perosis and enlarged hocks. Chemically the aortas of both copper-deficient and BAPN-fed poults contained a lower content of elastin than their controls and more nitrogen was extracted by cold molar saline and by hot sodium hydroxide solutions. There was no difference in the concentration of hexosamines extracted by cold molar saline but almost twice as much hydroxyproline was present in this fraction indicating a higher concentration of soluble collagen in both copper deficiency and BAPN toxicity. All attempts to counteract BAPN toxicity by dietary copper failed.

Aortic rupture occurs frequently among commercially reared turkeys (1) and the pathology, including dissecting aneurysms, is similar to that produced experimentally by feeding β-aminopropionitrile (BAPN) (2–4). These observations, coupled with those of dissecting aneurysms and spontaneous rupture of major vessels in copper-deficient chicks (5), suggested the turkey poult as a useful species for further study of connective tissue in copper deficiency. It was deemed desirable to compare copper deficiency and lathyrism in the same species, and the results obtained from such a comparison in turkey poults are presented here.[2]

EXPERIMENTAL

Groups of 10 Broad Breasted Bronze turkey poults were reared from hatching to 4 weeks of age in electrically heated stainless steel battery brooders. Feed and distilled water were supplied ad libitum. Four diets were used. The basal low copper diet (M), based on non-fat milk solids, contained less than 1 ppm of copper and is described elsewhere(6).[3] The control diet was supplemented with copper sulfate to supply 50 ppm of copper. The practical diet (N) was composed of soybean meal, 36.0; corn, 60.06; $CaHPO_4 \cdot 2H_2O$, 2.0; $CaCO_3$, 1.3; iodized salt, 0.5; DL-methionine, 0.1; $ZnCO_3$, 0.01; $MnSO_4$ (Tech.), 0.025; with the following vitamins added per kg of diet: (in milligrams) riboflavin, 5; Ca D-pantothenate, 10; niacin, 25; cyanocobalamin, 0.015; choline chloride, 250; menadione 4; and vitamin A, 4500 IU; vitamin D, 900 ICU; α-tocopheryl acetate, 22 IU. When added as a supplement, β-aminopropionitrile fumarate (BAPN: fumarate-2:1) was supplied at a level of 0.1%.

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[2] A preliminary account of this work has been presented. Savage, J. E., D. A. Ross, G. Reynolds and B. L. O'Dell 1962 Copper deficiency and beta-aminopropionitrile toxicity in turkeys. Federation Proc., *21:* 311 (abstract).

[3] The authors wish to express appreciation for the gifts of vitamins from Merck and Company, Rahway, New Jersey; Hoffmann-LaRoche, Nutley, New Jersey; Distillation Products Industries, Rochester, New York and Dawes Laboratories, Chicago. They are also grate-for the β-aminopropionitrile supplied by Abbott Laboratories, Chicago.

At the termination of the 4-week trial survivors were killed and a portion of the thoracic aorta was removed. The aorta was freed of adhering tissue, washed free of blood, blotted on filter paper, weighed and stored frozen until analyzed. The 2 methods of analysis were the same as described for chick aorta by O'Dell et al. (6) and involved formic acid extraction or a series of extractions using cold molar saline, chloroform-methanol, autoclaving with water, and hot 0.1 N sodium hydroxide. The residues from both methods are considered to be elastin. Tissue saved for microscopic examination was fixed immediately in Baker's fixative. After fixation the aortas were processed according to standard paraffin technique and the sections were stained with Weigert's stain.

RESULTS AND DISCUSSION

A summary of the gross pathology observed in the poults is presented in table 1. The symptoms of BAPN toxicity were similar to those reported by Barnett et al. (2). Growth was depressed and two-thirds of the poults died within 4 weeks. The chief cause of death was aortic rupture or internal hemorrhage. About 30% of the poults showed evidence of subcutaneous hemorrhage, particularly at pressure points such as on the breast or hocks. About 7% exhibited enlarged hocks and one-fourth had perosis.

The gross pathology exhibited by the copper-deficient turkey poult was similar to that of BAPN toxicity, but differed in that no internal hemorrhage occurred during the 4-week experimental period. This is also in contrast with the high incidence of hemopericardium observed in copper-deficient chicks. Apparently the chick is more subject to copper deficiency than the poult because when chicks and poults were fed diet M (0.9 ppm Cu) at the same time, the chicks exhibited more severe deficiency symptoms. The feathers of the deficient poults were strikingly depigmented as shown in figures 1–4, and a high percentage was afflicted with perosis and enlarged

TABLE 1

Comparison of the gross pathology of copper deficiency and lathyrism in turkey poults

Diet	No. started	Mortality	Weight	Hemorrhage		Enlarged hocks	Perosis
				Internal	Subcutaneous		
		%	g	%	%	%	%
M (milk basal)	40	50	340 ± 16 [1]	0	68	27.5	58
M + 50 ppm Cu	39	8	500 ± 15	0	3	2.6	0
N (practical)	30	0	546 ± 14 [1]	0	0	3.3	0
N + 0.1% BAPN [2]	30	67	417 ± 24	60	30	6.7	23

[1] Mean ± SE.
[2] BAPN indicates β-aminopropionitrile.

TABLE 2

Moisture and lipid content of turkey aortas

Diet	Wet wt	Aorta wt / Body wt	Dry matter	Lipid-soluble	Dry fat-free residue
	mg	%	%	% dry	mg
M (basal)	293 ± 16 [1] (11) [2]	0.08 ± 0.004 (11)	24.0 ± 0.4 (6)	6.2 ± 0.4 (10)	50.2 ± 3.9 (11)
M + 50 ppm Cu	439 ± 30 (10)	0.09 ± 0.006 (10)	27.7 ± 0.5 (5)	5.0 ± 0.6 (9)	87.6 ± 10 (10)
P [3] <	—	0.1	0.001	0.2	—
N (practical)	392 ± 20 (13)	0.06 ± 0.004 (13)	27.8 ± 0.5 (8)	4.9 ± 0.2 (12)	88.1 ± 4.5 (13)
N + 0.1% BAPN [4]	334 ± 18 (10)	0.08 ± 0.003 (10)	25.4 ± 0.4 (4)	5.9 ± 0.4 (10)	61.4 ± 4.9 (10)
P <	—	0.2	0.05	0.05	—

[1] Mean ± SE.
[2] Numbers in parentheses indicate number analyzed.
[3] P values as determined by the *t* test.
[4] BAPN indicates β-aminopropionitrile.

hocks (fig. 3). Copper deficiency caused mild anemia, the average hematocrit being 26% and hemoglobin, 6.1 g/100 ml in the deficient compared with 31% and 8.8 g/100 ml in controls. Less severe symptoms have been observed in poults fed low copper basal diets differing somewhat in composition and containing slightly higher levels of copper than the basal diet used in this study (7, 8).

The fresh weight of aortas analyzed and their relationships to body weight are shown in table 2. In contrast with the chick (6), the degree of copper deficiency produced by the basal diet used did not increase aorta weight in relation to body weight, but it did significantly increase the moisture content. No significance is attached to differences in lipid content. Similar results were observed in the BAPN-fed poults.

Significantly more nitrogen was extracted by cold molar saline from the aortas of both copper-deficient and BAPN-fed poults than from their respective controls (table 3). The percentage of nitrogen solubilized by autoclaving was greater in these groups, but the physiological significance of the differences was of a lower order especially in the case of BAPN. Approximately 30% more nitrogen was solubilized by hot sodium hydroxide from the aortas of both copper-deficient and BAPN-fed poults. The sodium hydroxide residue, elastin, constituted about 30% less of the dry, fat-free residue in the case of the copper-deficient and about 15% less in the BAPN-fed poults. Statistically, these are highly significant differences and the values are in reasonably good agreement with those obtained by formic acid extraction. The formic acid values are based on dry weight instead of dry, fat-free weight.

The chemical nature of part of the nitrogen extracted from aortas is summarized in table 4. There was no significant effect on the concentration of hexosamines in the saline extracts, but both copper deficiency and BAPN feeding more than doubled the concentration of hydroxyproline extracted by cold molar saline solution. The latter observation is indicative of a higher proportion of soluble collagen in turkey poults on either of the 2 dietary regimens. Similar observations have been made in the chick (6, 9) and the rat (10). Nevertheless, collagen constitutes a small portion of the total protein extracted into this fraction. There were no differences among groups in the concentration of hydroxyproline in the autoclaved extract. The nitrogen in this fraction was no doubt largely collagen in view of the high ratio of hydroxyproline to crude protein. Similarly, there were no significant differences among the sodium hydroxide extracts.

The histopathology of aortas produced by copper deficiency and BAPN toxicity is strikingly similar as illustrated by the photomicrographs shown in figures 5–12, respectively. Aortas from poults on both regimens have somewhat thicker walls and

TABLE 3

Nitrogen content of the soluble fractions and weight of insoluble residues of aortas

Diet	N in saline extract	N in autoclaved extract	N in 0.1 N NaOH extract	NaOH-insoluble residue	Formic acid-insoluble residue
	% wet	*% dry, fat-free*	*% dry, fat-free*	*% dry, fat-free*	*% dry*
M (basal)	0.62 ± 0.03 [1] (11) [2]	3.06 ± 0.18 (10)	4.32 ± 0.33 (11)	42.1 ± 4.1 (11)	36.3 ± 3.3 (6)
M + 50 ppm Cu	0.47 ± 0.02 (9)	2.31 ± 0.12 (10)	3.14 ± 0.17 (9)	59.0 ± 1.3 (10)	51.2 ± 2.1 (5)
P [3] <	0.01	0.005	0.01	0.005	0.01
N (practical)	0.48 ± 0.02 (12)	2.52 ± 0.10 (13)	2.86 ± 0.13 (12)	58.6 ± 1.5 (13)	53.1 ± 0.55 (8)
N + 0.1% BAPN [4]	0.61 ± 0.03 (10)	2.93 ± 0.11 (10)	3.81 ± 0.30 (10)	49.3 ± 1.9 (10)	49.6 ± 0.7 (4)
P <	0.01	0.02	0.005	0.001	0.005

[1] Mean ± SE.
[2] Numbers in parentheses indicate number analyzed.
[3] Probability as determined by the t test.
[4] BAPN indicates β-aminopropionitrile.

TABLE 4

Nitrogenous compounds in the soluble fractions of aortas

Diet description	Cold saline extract			Autoclaved extract			NaOH extract	
	Hexosamine	Hydroxyproline	Hydroxyproline/ N × 6.25	Hydroxyproline	Hydroxyproline/ N × 6.25		Hydroxyproline	Hydroxyproline/ N × 6.25
	mg/g fresh	*mg/g fresh*	%	*mg/g dry*	%		*mg/g dry*	%
Milk basal (M)	0.95 ± 0.15 [1] (5) [2]	0.75 ± 0.11(6)	2.01 ± 0.24(6)	16.0 ± 1.4(5)	8.4 ± 0.5(5)		2.90 ± 0.34(9)	1.10 ± 0.08(9)
M + 50 ppm Cu	1.00 ± 0.09 (5)	0.35 ± 0.03(5)	1.23 ± 0.06(5)	13.5 ± 0.8(6)	9.9 ± 0.5(6)		1.97 ± 0.14(8)	1.02 ± 0.06(8)
P [3] <	—	0.02	0.02	0.2	0.1		0.05	0.5
Practical (N)	0.99 ± 0.11 (7)	0.27 ± 0.01(6)	1.07 ± 0.11(7)	16.0 ± 0.8(4)	10.3 ± 0.5(4)		1.80 ± 0.5 (9)	1.01 ± 0.05(8)
N + 0.1% BAPN [4]	1.04 ± 0.13 (6)	0.57 ± 0.06(6)	1.68 ± 0.19(6)	14.8 ± 0.8(6)	8.3 ± 0.4(6)		2.09 ± 0.24(8)	0.90 ± 0.07(8)
P <	—	0.005	0.025	0.4	0.025		0.4	0.4

[1] Mean ± SE.
[2] Numbers in parentheses indicate number analyzed.
[3] P values as determined by the *t* test.
[4] BAPN indicates β-aminopropionitrile.

the elastic lamellae are widely separated by material that does not stain by the Weigert technique. This might be expected to be chiefly muscle tissue because of its location and because the extractable nitrogen was increased while the amounts of insoluble collagen and mucopolysaccharide were relatively unchanged. It might also include possible precursors of normal elastin. More focal breaks are apparent in the elastic lamellae of both groups and in general the elastin stained somewhat less intensely. Few dissecting aneurysms were observed in the copper-deficient aortas in contrast with the rather high incidence among the BAPN-fed poults. Lalich et al. (3) reported fragmentation of elastic fibrils, and dissecting medial hemorrhage in the aortas of BAPN-fed poults.

Despite several structural and chemical similarities between the aortas of copper-deficient and BAPN-fed turkey poults, all attempts to prevent BAPN toxicity by feeding copper salts have failed. A summary of part of the trials is presented in table 5. The addition of 50 ppm of copper to the practical diet (N) containing 0.1% BAPN lowered the rate of mortality and incidence of hemorrhage slightly but not significantly. Perosis was decreased but the incidence of enlarged hocks was actually higher when the copper supplement was fed. Needless to say, the practical diet contained adequate copper for normal performance in the absence of BAPN. When 0.1% of BAPN was added to the basal low copper diet (M), the rate of mortality was about 50%, not appreciably greater than without BAPN. The incidence of internal hemorrhage was increased from zero to about 7% and subcutaneous hemorrhage was unchanged. Supplementation of this diet with copper supported a rate of growth and level of performance about equal to that without BAPN. Thus, it appears that poults fed the milk diet are more resistant to BAPN toxicity than those fed a practical-type diet. Similar results have been obtained in other trials in which lower levels of BAPN were fed. It appears that excess dietary copper does not prevent BAPN toxicity nor does a deficiency appreciably accentuate it.

These observations do not preclude the possibility that BAPN acts at the same site

TABLE 5

Failure of copper supplementation to prevent gross symptoms of β-aminopropionitrile (BAPN) toxicity in turkey poults

Diet	No. started	Mortality	Weight	Hemorrhage		Enlarged hocks	Perosis
				Internal	Subcutaneous		
		%	g	%	%	%	%
M+0.1% BAPN	30	53	331±27 [1]	6.7	70	36.7	57
M+0.1% BAPN +50 ppm Cu	30	17	477±15	3.3	13	3.4	0
N+0.1% BAPN	30	67	417±24	60.0	30	6.7	23
N+0.1% BAPN +50 ppm Cu	30	47	394±20	43.3	20	36.7	0

[1] Mean ± SE.

as copper, that is, inhibits a copper enzyme. If such is the mechanism of its action, excess copper would not necessarily counteract the effect. It is also possible that copper and BAPN are concerned with different reactions along the pathway to elastin synthesis. In any case, it is unlikely that feeding excess copper affects either the plasma or tissue levels to a great extent because of the effective mechanisms that prevent excess absorption.

Dasler et al. (11) have suggested that all known lathyrogens possess the proper spacing of functional groups to form metallic complexes. Nevertheless, Levene (12) has observed that not all chelating agents are lathyrogens and he suggested that the complexation theory is ruled out. Preliminary results in this laboratory show that BAPN does complex with copper. Titration of BAPN with cupric ion (13) indicated that 2 moles of BAPN are complexed with one gram-atom of copper with a stability constant of approximately 10.6. This is not a highly stable complex and it appears more likely that BAPN interferes directly with the formation of the cross-linking compounds, desmosine and isodesmosine, rather than by decreasing the utilization of copper per se.

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PLATE 1

EXPLANATION OF FIGURES

1 A typical copper-deficient turkey poult fed the basal milk diet. Note depigmentation of the feathers. Age 4 weeks.

2 Control poult fed a diet similar to that fed to the poult above except 50 ppm of copper added.

3 Enlarged hocks typical of copper deficiency. Note also hemorrhagic appearance of hock and slight perosis in right leg.

4 Control, same as figure 2.

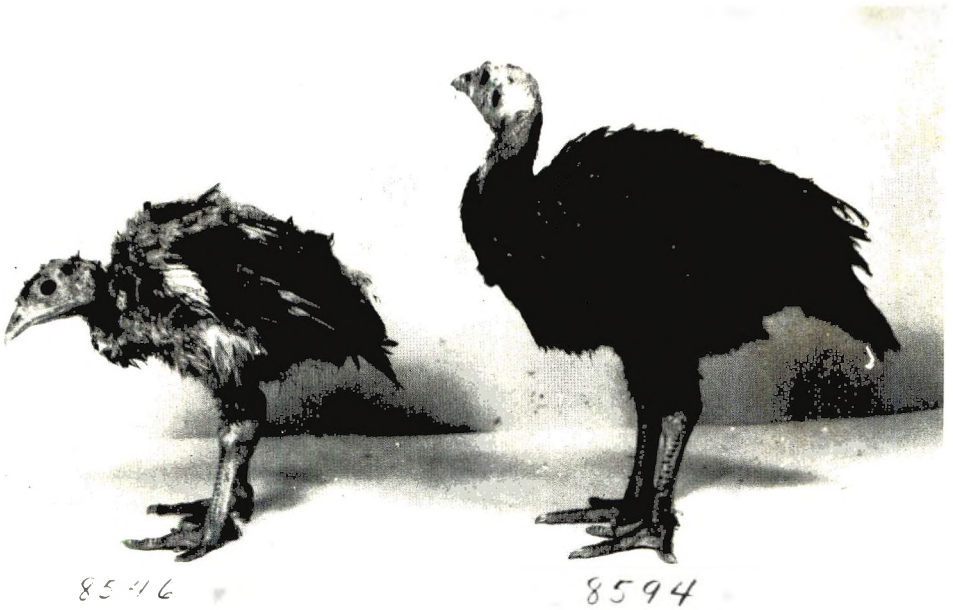

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PLATE 2

EXPLANATION OF FIGURES

Copper deficiency

5 Cross-section through arch of the aorta from control turkey poult fed the milk diet supplemented with 50 ppm of copper. Weigert's stain. × 24.

6 Cross-section through arch of aorta from copper-deficient poult. Weigert's stain. × 24. Note separation of elastic lamellae.

7 Control aorta as in figure 5. × 120.

8 Copper-deficient aorta as in figure 6. × 120. Note thicker wall, accumulation of non-elastin material and focal breaks.

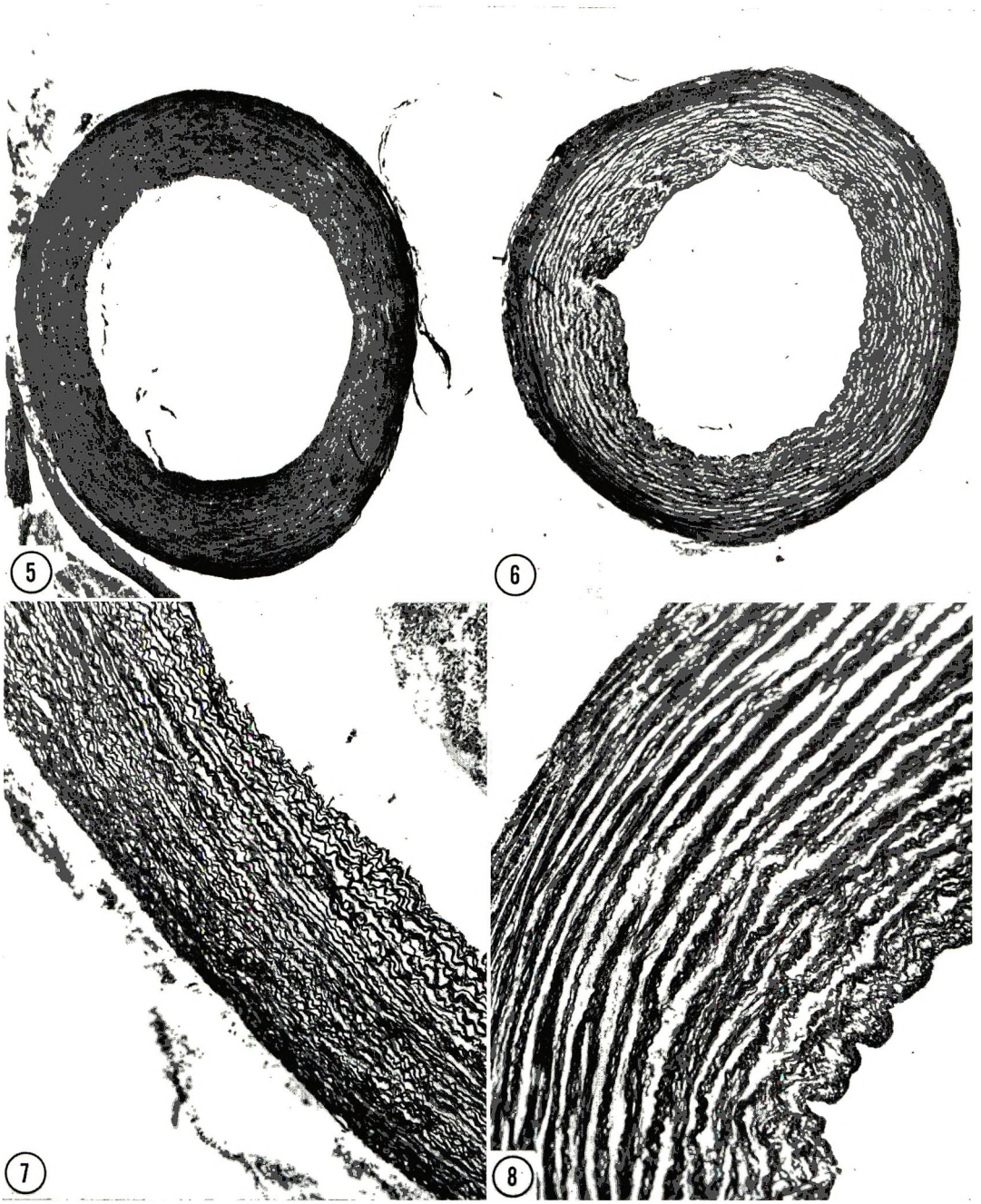
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PLATE 3

EXPLANATION OF FIGURES

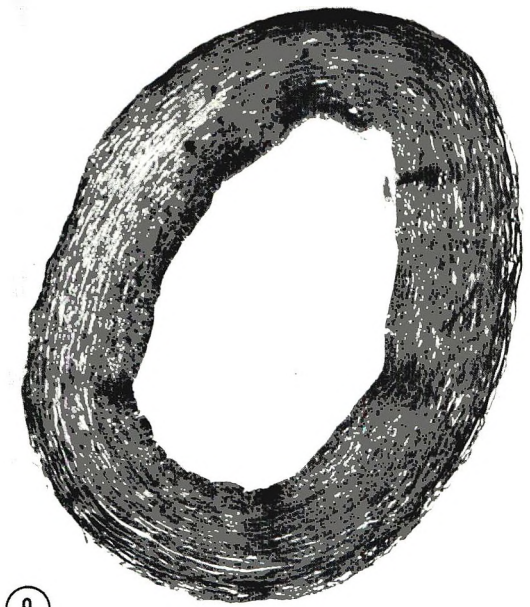
β-Aminopropionitrile (BAPN) toxicity

9 Cross-section through arch of aorta from turkey poult fed the practical control diet. Weigert's stain. × 24.

10 Cross-section through arch of aorta from poult fed practical diet supplemented with 0.1% BAPN. Weigert's stain. × 24. Note thickened wall and smaller size of lumen.

11 Control aorta as in figure 9. × 120.

12 Aorta from BAPN-fed poult as figure 10. × 120. Note separation of lamellae and disorganization of structure.



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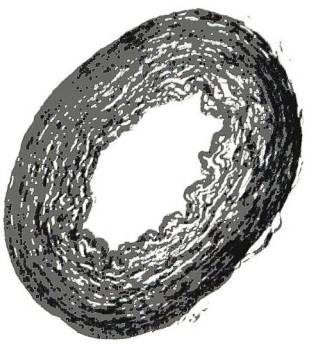

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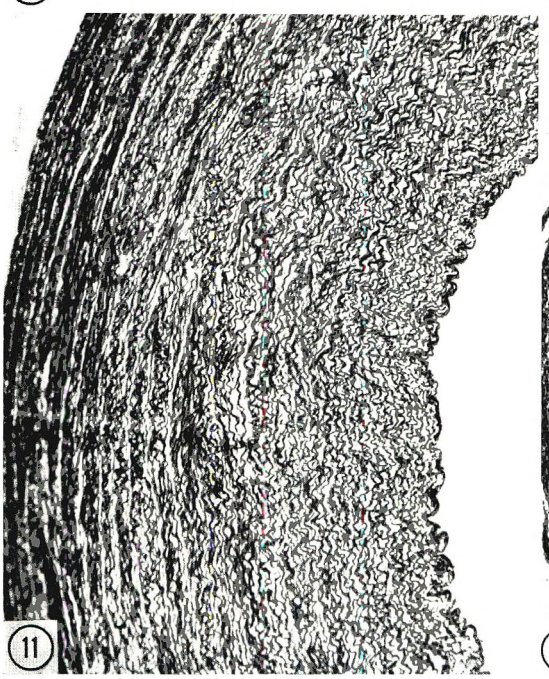

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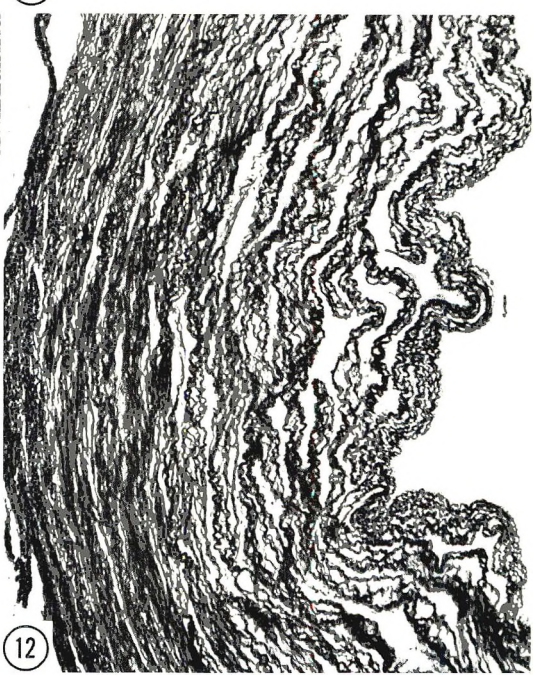

12

Oleic and Linoleic Acid Interaction in Polyunsaturated Fatty Acid Metabolism in the Rat [1,2]

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ABSTRACT Rats were raised with rations containing varying proportions of oleic and linoleic acids to study the interaction of these two acids as it influences the polyunsaturated fatty acid composition of liver and heart tissue. The ratio of 5,8,11-eicosatrienoic acid to arachidonic acid, an index of essential fatty acid nutrition, increased with an increase in the heart and liver oleate-to-linoleate ratio. In both heart and liver tissue, it was shown that linoleate inhibits the transformation of oleic acid to 5,8,11-eicosatrienoic acid. An inhibitory effect of oleic acid on linoleate metabolism was observed in liver tissue. Differences in fatty acid composition consistent with the lower susceptibility of female rats to essential fatty acid stress were also observed.

The polyunsaturated fatty acid composition of mammalian tissue is usually quite complex, being composed of a mixture of acids which are derived either from oleic, linoleic or linolenic acids. Oleic acid can be of either dietary or endogenous origin, whereas linoleic and linolenic acids are essentially of dietary origin only. Recent studies in Holman's laboratory (1–3) have demonstrated that linoleic acid can inhibit the transformation of linolenic acid into its derivative acids and vice versa. Linolenic acid was found to be the more effective since a given level of linolenic acid produces a greater effect on linoleic acid metabolism than does a comparable level of linoleic acid on linolenic acid metabolism.

An antagonistic effect of oleic acid on essential fatty acid metabolism has been reported by Dhopeshwarkar and Mead (4) who suggested that oleic acid might act competitively in inhibiting the transformation of linoleic acid to arachidonic acid. In our laboratory, analysis of the fatty acid composition of liver lipids from rats raised with rations containing high levels of oleic acid also provided some confirmatory evidence for this suggested competitive interaction (5).

The studies reported in the present paper were designed to extend the investigations of the interaction between oleic acid and linoleic acid in the synthesis of polyunsaturated fatty acids. Rats were raised with rations containing varying levels of these 2 acids and the fatty acid composition of liver and heart tissue were obtained.

Dhopeshwarkar and Mead (4) also reported on the effect of high dietary levels of oleate on the lipid composition of liver, serum and adipose tissue. An increase in total serum lipid was observed. This study extends these observations to include heart tissue.

EXPERIMENTAL

Ration composition and experimental design. The composition of the ration used is shown in table 1. The lipid was comprised of varying mixtures of coconut oil [3] and a fatty acid fraction derived from olive oil [4] thus giving varying proportions of oleic and linoleic acids. The level of linoleic acid in the olive oil fatty acids was reduced by urea adduct formation, and the fatty acid composition of the coconut oil and olive oil fatty acids is shown in table 2. The lipid composition of the 4 rations fed is summarized in table 3. The combinations used resulted in a large in-

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[1] Technical Paper no. 1933, Oregon Agricultural Experiment Station.

[2] This paper reports research undertaken in cooperation with the Office of the Surgeon General, Department of the Army, under contract DA-MD-49-193-62-G42. The opinions expressed are those of the authors and are not necessarily endorsed by the Department of the Army.

[3] Provided by Durkee Famous Foods, Division of the Glidden Company, Berkeley, California.

[4] Provided by the Wyandotte Olive Sales Company, Oroville, California.

TABLE 1
Ration composition

	g
Lactalbumin	225
Starch	459
Lipid	150
Salts [1]	40
Inositol	2
Choline chloride	3
Vitamin premix [2]	1
Liver powder	5
Guar gum	40
Cellulose [3]	75
	1000

[1] Jones, J. H., and C. Foster 1942 A salt mixture for use with basal diets either low or high in phosphorus. J. Nutrition, 24: 245.
[2] The vitamin mix contained the following: (in grams): thiamine, 4; riboflavin, 8; pyridoxine, 4; niacin, 4; Ca pantothenate, 20; *p*-aminobenzoic acid, 240; menadione, 4; potato starch to make 700 g. Fat-soluble vitamins fed twice a week, each dose providing 50 USP units of vitamin A, 5 USP units of vitamin D and 9.9 IU *dl*-*a*-tocopherol.
[3] Cellu Flour, Chicago Dietetic Supply House, Chicago.

crease in the level of oleic acid and only a slight decrease in the level of linoleic acid.

Five littermate groups (4 ♂ and 4 ♀) of weanling rats from our colony of Wistar strain rats were raised with these rations for 8 weeks. After this feeding period, the animals were killed and lipid analyses conducted.

Lipid analysis. Two-gram samples of liver tissue or the complete heart were extracted by homogenizing with chloroform: methanol using the procedure of Bligh and Dyer (6). Fatty acid esters were obtained directly from the liver lipids using 5% hydrogen chloride in anhydrous methanol. The lipid obtained from heart tissue was partitioned by thin-layer chromatography on Silica Gel G[5] using 15% ether in Skelly F. The required fractions were scraped from the plate and eluted with chloroform. Triglycerides were determined by glycerol analysis (7) after the methyl esters had been prepared using methanolic potassium hydroxide. Cholesterol and cholesterol esters were determined using the procedure of Chiamori and Henry (8). The phospholipid content was obtained by difference and the methyl esters of this fraction were prepared using methanolic HCl.

TABLE 3
Lipid composition of rations

Coconut oil/ olive oil acids	Ration 1	2	3	4
	100/0	90/10	75/25	50/50
18:1, %	7.7	15.1	26.3	44.8
18:2, %	1.9	1.8	1.5	1.2
Calories from 18:1, %	2.3	4.8	8.4	14.0
Calories from 18:2, %	0.6	0.6	0.5	0.4

The fatty acid methyl esters were chromatographed using a Beckman GC_2 instrument fitted with a hydrogen flame detector and a modified injection system (9). Separations were effected with a 6-foot column of 15% ethylene glycol succinate on Chromosorb P at 190°, the helium flow rate being 50–60 cm^3/minute.

To distinguish isomeric forms of different fatty acids as well as to denote metabolic relationships, the scheme proposed by Holman (1) was used. Fatty acids were classified by the number of carbon atoms after the terminal double bond in the molecule. Thus linoleic acid gives rise to an "ω6" series of acids and oleic acid, an "ω9" series.

RESULTS AND DISCUSSION

The rations fed provided reasonable growth rates, there being no differences observed among rations. No symptoms of essential fatty acid deficiency were observed and the high levels of dietary oleate did not result in a depression in growth rate. Guinea pigs have shown a depression in growth rate with similar rations (4), suggesting that this species may be more susceptible than the rat to essential fatty acid stress. Increasing levels of dietary oleate did not influence the composition of

[5] Research Specialities, Richmond, California.

TABLE 2
Fatty acid composition of coconut oil and olive oil acids

Fatty acid	8:0	10:0	12:0	14:0	16:0	18:0	18:1	18:2
	%	%	%	%	%	%	%	%
Coconut oil	3.2	5.0	49.9	19.1	10.4	2.9	7.7	1.9
Olive oil acids					14.6	2.8	82.1	0.5

TABLE 4

Dietary oleate and the composition of heart lipid

Ration	Total lipid	Phospholipid	Triglyceride	Cholesterol ester	Cholesterol
	% wet wt	*% total lipid*	*% total lipid*	*% total lipid*	*% total lipid*
1 ♂	2.86 ± 0.31 [1]	88.0 ± 2.2	9.5 ± 2.3	0.10 ± 0.02	2.43 ± 0.07
♀	2.87 ± 0.21	78.5 ± 4.6	17.6 ± 4.6	0.14 ± 0.05	3.67 ± 0.14
2 ♂	2.89 ± 0.39	85.3 ± 3.8	11.8 ± 3.1	0.15 ± 0.09	3.27 ± 0.04
♀	2.98 ± 0.17	81.2 ± 6.6	15.1 ± 5.8	0.22 ± 0.18	3.41 ± 0.14
3 ♂	2.93 ± 0.55	84.8 ± 1.2	11.2 ± 1.0	0.17 ± 0.07	3.52 ± 0.24
♀	3.15 ± 0.31	81.6 ± 4.3	13.9 ± 4.5	0.19 ± 0.04	3.41 ± 0.33
4 ♂	2.95 ± 0.15	86.2 ± 2.3	10.6 ± 1.1	0.16 ± 0.09	2.99 ± 0.73
♀	3.01 ± 0.14	82.7 ± 4.1	15.6 ± 4.6	0.15 ± 0.05	3.40 ± 0.20

[1] Mean ± SD.

heart lipids (table 4), although female animals showed consistently higher levels of triglyceride than males.

The fatty acid composition of liver lipids, heart phospholipids and heart triglycerides are summarized in table 5. Mean values are shown together with standard deviations for acids detected in all samples and present at levels $> 1\%$. The fatty acid composition of heart triglycerides parallels that of the dietary lipid. In liver tissue, the changes in fatty acid composition pertinent to this study are a decrease in the level of 18:2, ω6 and 20:4, ω6 with an increase in 20:3, ω9. Heart phospholipids show a smaller decrease in the level of 18:2, ω6, virtually no change in 20:4, ω6 and an increase in 20:3, ω9.

One criterion of essential fatty acid stress is an increase in the ratio of 20:3, ω9 to 20:4, ω6. This ratio increases in both heart and liver tissue with an increase in the ratio of 18:1, ω9 to 18:2, ω6 (fig. 1) which ratio is in turn determined by the dietary lipid. Those animals with the highest ratio thus appear to be bordering on an essential fatty acid-deficient state. Higher levels of oleate or lower levels of linoleate, or both, would be required to achieve a condition where gross symptoms are observed.

It has been suggested that competitive interactions exist in the synthesis of polyunsaturated fatty acids (1). If this is the case, it might be expected than an increase in the level of inhibitor would result in a decrease in the product-to-precursor ratio. In this system, oleic acid might be expected to inhibit the transformation of linoleic acid and vice versa. Using mean values for each ration and plotting the

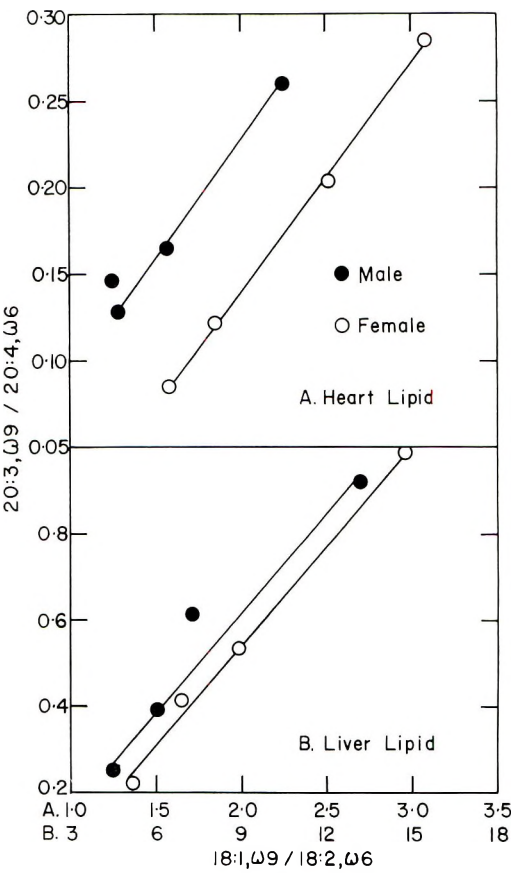

Fig. 1 Dependence of the 5,8,11-eicosatrienoic acid-to-arachidonic acid ratio on the tissue oleate-to-linoleate ratio.

TABLE 5

Fatty acid composition of liver lipid, heart triglyceride and heart phospholipid

Ration	% of total methyl esters											
	12:0	14:0	16:0	16:1	18:0	18:1, ω9	18:2, ω6	20:3, ω9	20:3, ω6	20:4, ω6	22:5, ω6	22:6, ω6
Heart tissue — phosphopilid												
1 ♂	1.25	0.28	11.2 ±2.1 [1]	0.47	26.7 ±3.1	15.0± 3.7	13.7 ±3.4	2.84±1.72	1.35	19.4±5.0	2.47±0.94	2.22±1.15
♀	1.24	0.13	12.3 ±2.8	0.58	28.7 ±5.2	13.2± 2.9	11.8 ±1.3	1.55±0.66	1.23	18.3±5.6	3.30±1.34	2.43±1.01
2 ♂	0.97	0.69	9.73±1.05	0.34	25.0 ±2.6	14.4± 2.2	14.2 ±0.9	2.73±0.44	1.28	21.3±1.3	3.00±0.92	3.11±1.33
♀	1.26	0.12	12.0 ±2.0	0.65	29.0 ±5.1	16.8± 1.2	12.0 ±1.2	2.15±0.74	0.89	17.6±3.7	2.60±0.63	2.11±0.69
3 ♂	0.96	0.09	9.61±0.86	0.32	24.9 ±1.8	15.1± 1.3	12.6 ±0.7	3.59±0.77	1.27	21.7±1.8	2.60±1.07	3.15±0.66
♀	0.95	0.10	11.4 ±1.6	0.37	28.9 ±2.3	18.0± 2.1	9.80±0.58	3.76±1.27	1.01	18.4±3.3	2.52±0.80	2.13±1.09
4 ♂	0.59	—	9.64±0.61	0.23	24.6 ±2.0	16.9± 1.0	10.2 ±0.9	5.67±1.22	1.42	21.8±1.7	2.29±0.29	3.74±0.61
♀	0.62	0.08	11.0 ±1.6	0.33	28.9 ±4.8	21.7± 2.7	9.45±1.23	4.62±0.55	1.12	16.2±5.6	2.09±0.71	1.78±0.66
Heart tissue — triglyceride												
1 ♂	25.2 ±2.7	15.2 ±4.7	23.6 ±4.4	2.33±0.41	6.21±0.93	22.2± 2.7	3.45±0.79	—	—	—	—	—
♀	24.8 ±3.2	16.4 ±0.7	19.1 ±1.1	2.62±0.25	4.63±0.25	28.9± 3.2	2.97±0.39	—	—	—	—	—
2 ♂	19.2 ±4.4	14.5 ±1.8	23.5 ±3.5	1.78±0.40	6.27±1.48	31.0± 6.3	2.97±0.47	—	—	—	—	—
♀	22.5 ±2.3	13.0 ±2.9	19.5 ±1.7	2.10±0.19	5.14±0.45	34.5± 2.9	3.12±0.52	—	—	—	—	—
3 ♂	18.5 ±5.8	12.7 ±3.5	18.7 ±2.1	1.71±0.33	5.63±1.76	39.2±11.4	2.71±1.38	—	—	—	—	—
♀	16.7 ±5.6	8.89±1.60	17.7 ±1.7	1.75±0.64	4.91±0.85	46.7± 5.7	3.06±0.53	—	—	—	—	—
4 ♂	12.5 ±3.9	7.64±2.18	15.6 ±1.5	1.59±0.38	4.66±0.45	54.1± 8.7	2.48±1.46	—	—	—	—	—
♀	12.0 ±4.8	8.11±1.84	16.7 ±1.0	1.48±0.19	4.71±0.25	53.3± 5.5	2.58±0.50	—	—	—	—	—
Liver tissue — total lipid												
1 ♂	0.67	3.04±0.51	20.1 ±3.0	1.40±0.19	19.3 ±2.0	21.3± 1.4	4.77±0.65	3.66±0.68	1.55	14.6±2.7	2.66±0.50	4.17±3.48
♀	0.79	2.22±0.54	16.1 ±0.5	1.47±0.28	23.7 ±1.7	20.1± 2.6	3.84±0.31	4.17±0.56	0.54	17.6±2.2	3.29±0.34	3.38±0.96
2 ♂	0.87	3.04±0.74	20.2 ±2.2	1.26±0.29	19.7 ±2.9	26.9± 2.5	4.42±0.51	4.74±0.55	1.87	12.3±2.0	2.32±0.43	2.27±0.59
♀	0.67	2.28±0.42	17.2 ±1.2	1.35±0.07	23.1 ±1.5	25.0± 2.0	3.65±0.34	5.90±1.18	0.81	14.0±0.9	2.77±0.50	2.26±0.57
3 ♂	0.43	2.03±0.70	18.4 ±3.3	0.92±0.09	18.5 ±1.2	27.7± 3.1	3.80±0.62	7.18±1.80	1.50	11.4±1.8	2.19±0.46	2.34±0.59
♀	0.36	1.58±0.81	14.1 ±2.5	0.93±0.17	23.5 ±3.6	28.1± 5.1	3.16±0.32	8.34±2.5	0.31	14.8±3.1	2.55±0.75	2.48±0.73
4 ♂	0.25	1.01±0.25	16.2 ±2.0	0.61±0.11	19.1 ±2.7	34.0± 6.5	2.57±0.50	10.1 ±1.6	0.32	10.8±3.8	1.53±0.52	2.07±0.60
♀	0.28	1.08±0.34	12.9 ±1.1	0.88±0.17	21.5 ±2.9	33.5± 3.7	2.26±0.22	11.0 ±2.3	0.28	11.0±1.1	1.78±0.41	2.24±0.80

[1] Mean ± SD.

20:3, ω9-to-18:1, ω9 ratio as a function of tissue linoleate, the inhibitory effect of linoleate on the 18:1,ω9 to 20:3,ω9 transition is observed in both heart and liver tissue (fig. 2). However, a similar treatment of the data does not show an inhibitory effect of oleate on the transition of 18:2, ω6 to 20:4, ω6. If the data are analyzed by plotting scatter diagrams of product-to-precursor ratios as a function of inhibitor level for individual animals, the inhibitory effect of both linoleate and oleate are observed together with the involvement of precursor level (figs. 3 and 4). As a first approximation, linear regressions can be fitted to these series of points. The relations demonstrated by these figures compare favorably with those predicted from a mathematical model of a 2-component interacting system.[6] These relations are observed in liver tissue. Analyses of data from heart tissue suggest similar relations but an adequate range of values is not obtained for a given precursor level to give a significant regression.

A number of sex differences are shown in the data presented in this paper (table 5). The differences most pertinent to this study are those illustrated in figures 1 and 2 for heart tissue. For a given ratio of 18:1, ω9 to 18:2, ω6 in the tissue, females show a lower 20:3, ω9-to-20:4, ω6 ratio. This is consistent with the observation that females are less susceptible to essential fatty acid stress than males (10). Females also show a lower 20:3, ω9-to-18:1, ω9 ratio than males for a given level of tissue linoleate. Apparently the 18:1, ω9 to 20:3, ω9 transition is more efficient in male than female rats.

These data, then, suggest that oleic acid inhibits the transformation of linoleic acid to arachidonic acid in liver tissue. The effect of oleic acid in these transformations is minimal and the ratio of dietary oleate to linoleate has to be appreciable to demonstrate an inhibitory effect. Under the conditions of this experiment, a comparable response was not observed in heart tissue probably because this tissue maintains a higher level of linoleic acid than liver and high tissue ratios of oleate to linoleate are not attained. Nevertheless, linoleic acid shows a marked effect on the

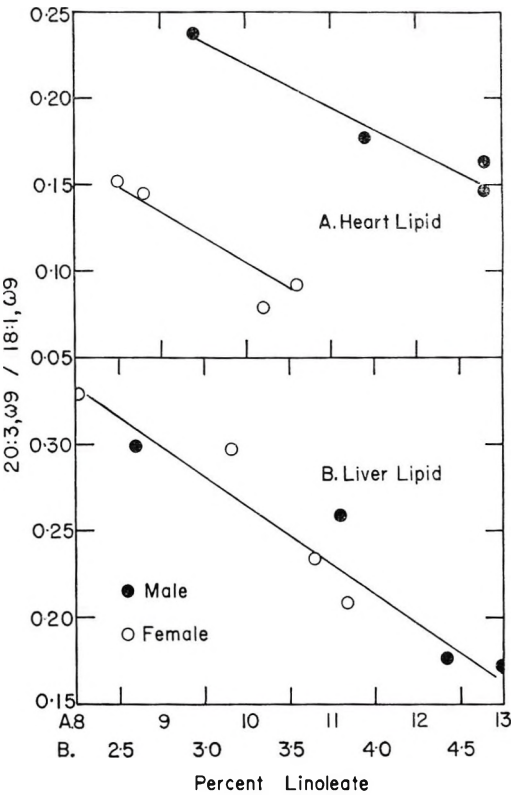

Fig. 2 Influence of tissue linoleate level on the transformation of oleic acid to its derivative eicosatrienoic acid.

transformation of oleic acid to its derivative acids. This response is observed in both heart and liver tissue and indicates that if linoleic and oleic acid react in the same system, the tendency for linoleate to be converted to its higher homologues is far greater than that for oleic acid.

The ratio of 20:3, ω9 to 20:4, ω6 increases as the level of dietary oleate increases. In the heart tissue, this change is due to the increase in the level of the trienoic acid since under the conditions of these experiments, no decrease in the level of arachidonate was observed. The increase of this ratio in liver tissue results from an increase in the level of the trienoic acid and a decrease in the level of arachidonic acid. The interaction of oleic acid

[6] Lindstrom, T., and I. J. Tinsley 1965 Interactions in polyunsaturated fatty acid metabolism — a mathematical model. Federation Proc., 24: 497 (abstract).

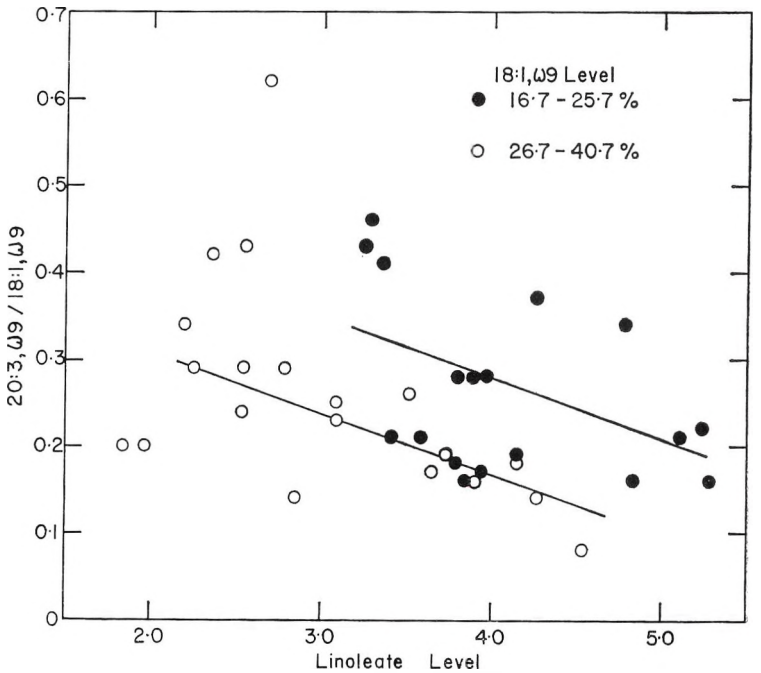

Fig. 3 Scatter diagram illustrating the interaction of linoleic acid in the transformation of oleic acid to eicosatrienoic acid.

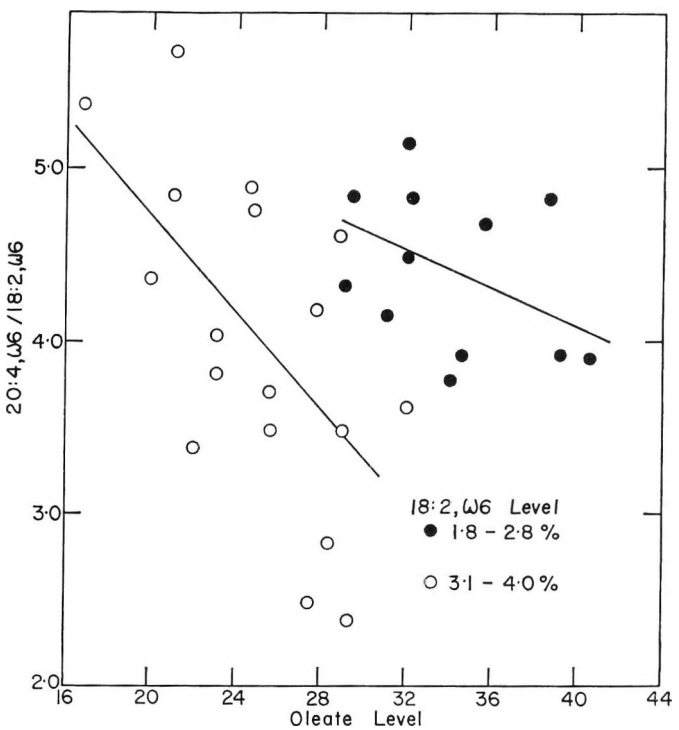

Fig. 4 Scatter diagram illustrating the interaction of oleic acid in the transformation of linoleic acid to arachidonic acid.

in the transformation of linoleic acid to arachidonic acid (fig. 3) suggests that the decrease in the level of aracihdonate is not simply a dilution effect. Oleic acid could become a factor in essential fatty acid nutrition if linoleic acid levels were marginal and oleic acid was present in appreciable quantities.

These studies serve to define some of the intractions involved in the synthesis of polyunsaturated fatty acids. A better understanding of these processes will result from a study of the actual synthetic systems involved in these interactions.

ACKNOWLEDGMENTS

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Metabolic Fate of ^{60}Co-labeled Cyanocobalamin in Tryptophan-deficient Rats

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ABSTRACT Absorpton, excretion, and tissue distribution of ^{60}Co-labeled cyanocobalamin were studied in rats maintained with a tryptophan-deficient diet. Absorption of orally administered ^{60}Co-cyanocobalamin was normal, but after either oral or parenteral administration the kidney uptake of the labeled vitamin B_{12} was increased above normal by tryptophan deficiency. The abnormal renal concentration of labeled vitamin B_{12} could be corrected for by subsequent dietary supplementation with the missing amino acid. In comparison, kidney concentrations of ^{60}Co were not increased after inorganic radiocobalt ($^{60}CoCl_2$) was injected into tryptophan-deficient rats. These observations suggest that tryptophan has a role in cyanocobalamin metabolism.

Our earlier observations in chicks have indicated that the administration of vitamin B_{12} increases plasma protein levels (1), and enhances hemoglobin regeneration when anemia had been induced by phenylhydrazine injection (2). Other reports have shown that vitamin B_{12} or coenzyme B_{12} also may affect the intermediate metabolism of various amino acids (3–5).

In the present report, data are presented to illustrate an apparent role of tryptophan in the tissue distribution of ^{60}Co-labeled cyanocobalamin in rats.

EXPERIMENTAL

Animals and diets. Male and female rats of the McCollum strain from 6 to 8 weeks old were used for this study. Most of the experimental groups consisted of 5 or 6 animals that were maintained with a tryptophan-deficient diet.

The percentage composition of the tryptophan-deficient diet was: sucrose, 66.17; corn oil, 5.0; non-nutritive bulk,[2] 2.0; gelatin, 20.0; salt mixture,[3] 4.0; amino acid mixture, 2.83, and all known vitamins.[4] The ingredients of salt mixture and vitamins added to the tryptophan-deficient diet have been indicated elsewhere (6).

The amino acid mixture was made up of L-cystine, 1.0; L-tyrosine, 2.4; DL-phenylalanine, 4.2; DL-isoleucine, 3.2; DL-histidine, 2.3; DL-methionine, 6.0; DL-valine, 7.0; and DL-threonine, 2.2 g/kg of deficient diet.

The control groups consisted of 6 rats each, matched with the experimental groups for age, sex, and weight. They received the same diet as the animals fed the deficient diet, with the exception that it was supplemented with 2.0 g of DL-tryptophan/kg. All of the animals were housed in individual screen-bottom cages and received their diets and water ad libitum.

Measurements of absorption, excretion and tissue distribution of ^{60}Co-labeled cyanocobalamin and cobalt chloride. Following a feeding period of 4 to 6 weeks, the absorption, tissue distribution, and excretion of ^{60}Co, administered as labeled vitamin B_{12} or as $^{60}CoCl_2$, were measured and compared between the tryptophan-deficient and supplemented groups. To accomplish this, 50 or 150 mμg of ^{60}Co-labeled cyano-

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[1] From the Radioisotope Service, VA Hospital, Fort Howard, Maryland.

[2] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

[3] Salt mixture had the following percentage composition: Ca carbonate, 29.315; Ca diphosphate, 7.328; Cu sulphate, 0.029; ferric citrate, 2.685; Mn sulfate, 2.687; Mg sulfate, 9.967; KI, 0.078; potassium phosphate (dibasic), 31.513; NaCl, 16.373; and Zn chloride, 0.024.

[4] Vitamin supplement consisted of the following per 100 g ration: vitamin A, 1553 IU; vitamin D, 220 USP units; (and in milligrams) α-tocopherol, 2.80; 2-methylnaphthoquinone, 0.26; thiamine, 0.22; riboflavin, 0.22; Ca pantothenate, 0.88; pyridoxine·HCl, 0.56; niacin, 0.88; biotin, 0.04; p-aminobenzoic acid, 0.88; folic acid, 0.04; choline chloride, 200.0; and inositol, 200.0.

cobalamin in 1.0 ml of distilled water were given by oral or parenteral administration to each rat. The specific activity of the tracer vitamin was 1 μc/ug. After 3 or 5 days following the administration of radioisotope, the test animals were anesthetized with sodium pentobarbital and killed by cardiac puncture. The feces, urine, and various tissue samples were separated and liquefied as described elsewhere (6), and concentrations of radioactivity in the solutions so prepared were measured by means of a crystal scintillation counter.

To compare the retention of inorganic radiocobalt with that of the ^{60}Co-labeled vitamin B_{12}, a single dose of 5.0 mμg of ^{60}Co-labeled cobalt chloride ($^{60}CoCl_2$) was injected intraperitoneally into a second series of tryptophan-deficient and supplemented rats. Following these injections urine and feces samples were collected until the animals were killed 72 hours later. The amounts of radioactivity contained in these specimens, and retained in liver, kidneys, and gastrointestinal tract were assayed as outlined above.

RESULTS

The growth of the animals that were fed the tryptophan-deficient diet was markedly depressed, but this was prevented by the addition of DL-tryptophan to the diet, as table 1 indicates. In addition, rats fed the tryptophan-deficient diet excreted pale, gray stools, whereas the stools from rats fed the tryptophan-supplemented diet appeared normal.

The growth effects observed were associated with wide differences in the plasma concentrations of vitamin B_{12}, which are summarized in table 2. The vitamin B_{12} assay method used to obtain these values was based on a procedure in which *Lactobacillus leichmannii* 4797 was used as a test organism (7). The technique used for the preparation of liver and kidney homogenates in this test has been described previously (8).

The results of the radioisotopic assay for ^{60}Co in liver, kidney, gastrointestinal tract, and feces of animals that received orally administered radiocyanocobalamin are compiled in table 3. In tryptophan-deficient rats the renal concentrations of ^{60}Co were consistently about 60% greater than in the animals that had received a tryptophan supplement. The difference was statistically significant ($P < 0.05$). A very similar trend at the same level of statisti-

TABLE 1

Body weights of tryptophan-deficient and normal rats

Dietary supplement	Sex	No. of animals	Body weight	
			Initial	Final, (4 weeks)
			g	*g*
No tryptophan	M	6	184 ± 5 [1]	148 ± 2
	F	5	197 ± 6	167 ± 6
Tryptophan	M	6	168 ± 2	220 ± 4
	F	5	160 ± 5	192 ± 4

[1] SE of mean.

TABLE 2

Vitamin B_{12} activity in plasma, liver, and kidney of tryptophan-deficient and normal female rats

Dietary supplement	No. of animals	Vitamin B_{12} activity		
		Plasma	Liver	Kidney
		μμg/ml	*mμg/g*	*mμg/g*
No tryptophan	6	105 ± 30 [1]	83 ± 12	61 ± 7
Tryptophan	5	239 ± 16	71 ± 16	56 ± 7

[1] SE of mean.

TABLE 3

Distribution of radioactivity after oral administration of ^{60}Co-labeled cyanocobalamin to tryptophan-deficient rats [1]

Dietary supplement	Sex	No. of animals	Dose	Radioactivity			
				Kidney	Liver	GI tract	Feces
			mμg	*% of oral dose*		*% of oral dose*	
No tryptophan	M	6	50	8.4 ± 1.3 [2]	9.1 ± 0.8	12.6 ± 1.2	40.6 ± 1.6
Tryptophan	M	6	50	5.1 ± 0.6	8.3 ± 1.3	10.9 ± 0.8	43.2 ± 2.5
No tryptophan	F	5	150	13.0 ± 1.6	8.0 ± 0.6	10.8 ± 2.0	39.4 ± 3.5
Tryptophan	F	5	150	8.1 ± 0.8	7.1 ± 0.6	8.8 ± 0.7	40.7 ± 2.9

[1] Animals were killed 5 days following oral administration of ^{60}Co-labeled cyanocobalamin.
[2] Mean % of oral dose ± SE of mean.

cal significance was also observed in kidney when the oral dose of labeled vitamin B_{12} was increased from 50 to 150 mμg. Here, the kidney concentrations appeared to be increased in some proportion to the greater dosage used, suggesting in part that gastrointestinal absorption was unimpaired. However, it could not be ascertained whether these increases were also related to the fact that only female animals received the higher dosage. In the liver, gastrointestinal tract, and in feces the amounts of radioactivity did not differ significantly between the groups when they were compared on a basis of the use of dietary supplement, dose level of radiocyanocobalamin, or the sex of the animals.

To establish that these results probably represented uptake of labeled vitamin B_{12} instead of only some nonspecific incorporation of ^{60}Co by kidney, the concentration differences were compared with the distribution and excretion of injected $^{60}CoCl_2$. In addition, because the kidney concentration differences could possibly have been induced by some deficiency of vitamin B_{12}, a nonradioactive supplement of this vitamin also was administered to tryptophan-deficient animals to correct for this possible defect. For this purpose animals maintained with the tryptophan-deficient diet that had been injected with nonradioactive vitamin B_{12} twice weekly for 3 weeks were also injected with the ^{60}Co cyanocobalamin. In table 4, the results of the assay of ^{60}Co in kidney and other tissues 72 hours after the labeled vitamin was administered to these animals are compared with the distribution of inorganic ^{60}Co.

The distribution and excretion of ^{60}Co differs grossly between the groups that received the labeled vitamin as compared with those that received the inorganic radiocobalt. More specifically, results shown in this table indicate that tryptophan deficiency had no effect on kidney concentration of inorganic ^{60}Co. Furthermore, the data show that the nonradioactive vitamin B_{12} supplementation was not a relevant factor to affect renal concentration of the labeled vitamin induced by dietary tryptophan deficiency. As observed originally, after injection of labeled vitamin B_{12}, dietary supplementation with tryptophan was sufficient to pre-

TABLE 4

Distribution and excretion of ^{60}Co following intramuscular injection of labeled cyanocobalamin or cobalt chloride [1]

Dietary supplement	No. of female animals	Radioactivity					
		Kidney	Liver	Spleen	Lung	GI tract	Feces	Urine
					% of injected doses			
				^{60}Co-labeled vitamin B_{12} injected				
No tryptophan	6	13.9 ± 1.8 [2]	4.2 ± 0.1	0.10 ± 0.08	0.29 ± 0.01	9.8 ± 0.2	3.3 ± 0.6	4.3 ± 0.8
No tryptophan [3]	5	16.7 ± 1.1	3.8 ± 0.4	0.14 ± 0.04	0.18 ± 0.04	9.5 ± 0.7		
Tryptophan	5	6.7 ± 1.8	4.7 ± 0.6	0.22 ± 0.08	0.24 ± 0.03	10.6 ± 0.7	5.2 ± 0.5	4.1 ± 0.1
				^{60}Co-labeled cobalt chloride injected				
No tryptophan	5	1.9 ± 0.2	2.9 ± 0.3			2.4 ± 0.3	13.5 ± 1.4	71.5 ± 2.3
Tryptophan	5	1.9 ± 0.2	4.9 ± 0.7			6.2 ± 0.5	13.2 ± 0.5	60.7 ± 6.2

[1] Rats were killed 72 hours after injection of 50 mμg of ^{60}Co-labeled cyanocobalamin or of 5 mμg of $^{60}CoCl_2$.
[2] Mean % of intramuscular dose ± SE of mean.
[3] One microgram nonradioactive vitamin B_{12} injected intraperitoneally twice/week for 3 weeks.

TABLE 5

Distribution of intramuscularly injected ^{60}Co-labeled cyanocobalamin in pair-fed rats

Dietary supplement	Experimental period	Days after isotope injection	Radioactivity					
			Kidney		Liver		Urine	GI tract and feces
			Total	Per g	Total	Per g		
	days		*% of injected dose*		*% of injected dose*			*% of injected dose*
No tryptophan	21	3	18.8 ± 1.9 [1]	17.9 ± 1.9	11.9 ± 0.4	3.3 ± 0.2	4.3 ± 0.8	26.7 ± 1.2
Tryptophan	21	3	9.7 ± 0.3	7.5 ± 0.1	12.0 ± 0.5	2.3 ± 0.1	4.1 ± 0.1	30.5 ± 1.3
No tryptophan	28	10	14.5 ± 0.8	14.4 ± 0.9	17.8 ± 0.2	4.6 ± 0.2	10.7 ± 0.9	30.2 ± 1.7
Tryptophan	28	10	9.8 ± 0.3	7.0 ± 0.5	17.4 ± 0.1	2.6 ± 0.1	8.4 ± 0.8	30.3 ± 1.8

[1] Mean % of injected dose ± SE of mean. Each group contained 6 female animals.

vent above normal ^{60}Co concentrations in kidney.

In an effort to establish a definite difference between the accumulation of ^{60}Co cyanocobalamin in kidneys induced by tryptophan deficiency and a similar effect that was reported by Rosenthal (9) to accompany starvation, appropriate experiments with the tryptophan-deficient diets were carried out, using a paired feeding technique. Each rat was fed 12 g/day of its respective experimental diet during total feeding trial periods of 3 and 4 weeks. On the eighteenth day of the trial each animal was injected intramuscularly with 50 mμg of ^{60}Co-labeled cyanocobalamin. One-half of the total number of animals was killed 3 days later, and the other half was killed 10 days after the radioisotopic administration. Concentrations of ^{60}Co in their tissues and excreta were assayed. The data obtained are shown in table 5. Exclusive of any effect that starvation might have in animals, the data show again that feeding of a tryptophan-deficient diet was closely associated with enhanced renal accumulation of ^{60}Co. These data indicate very clearly the specificity of this deficiency on radiocyanocobalamin.

In addition to this observation, these data show that another trend prevailed during the interval between the third and tenth postinjection day. As compared with the tryptophan-supplemented group, kidney concentrations of ^{60}Co decreased considerably in the deficient group, whereas in both groups of animals the liver concentrations increased by about 40%. Here, the fact that the kidney retention in the normal groups was unchanged raises a question as to whether vitamin B_{12} in tryptophan-deficient animals is transferred from kidney to liver as some investigators have suggested (9–11).[5] Although these differences observed do not entirely coincide with this possibility, at the same time they appear to be partial evidence that the loss of radiocobalt from kidney in the deficient group might represent transfer, but to other, unmeasured tissue locations.

Repletion of deficient animals with tryptophan should correct for the increased

[5] Okuda, K., and B. F. Chow 1960 Absorption and tissue distribution of radioactive 5,6-dimethylbenzimidazole-B_{12} coenzyme. Federation Proc., *19*: 417. (abstract).

TABLE 6

Kidney and liver retention of ^{60}Co-labeled cyanocobalamin after tryptophan repletion

Dietary supplement	Experimental period	No. of animals	Radioactivity	
			Kidney	Liver
	weeks		*% of injected dose*	
No tryptophan	4	6	16.5±0.7 [1]	8.0±0.1
Tryptophan	4	5	8.2±0.7	9.4±0.2
Tryptophan repletion [2]	6	5	7.5±0.5	9.6±0.2
Tryptophan	6	5	7.3±0.8	11.9±0.6

[1] Mean % of injected dose ± SE of mean.
[2] Animals that were initially fed a tryptophan-deficient diet for 4 weeks subsequently received a tryptophan-supplemented diet for the next following 2 weeks.

kidney concentration of labeled vitamin B_{12} that accompanies tryptophan deficiency, and demonstrate whether this manifestation is a specifically reversible defect.

To resolve this possibility, eleven 6-week-old male rats were fed a tryptophan-deficient diet and compared with 10 control rats maintained with the same diet, but supplemented with the missing amino acid. After 4 weeks on these regimens, 6 rats from the deficient group and 5 rats from the supplemented group were injected intraperitoneally with 25 mμg of ^{60}Co-labeled cyanocobalamin, and finally killed 72 hours later. Concentrations of the ^{60}Co were measured in kidney and liver.

At the completion of 4 weeks of feeding the deficient diet, 5 rats that remained in the deficient group were started and maintained for an additional 2 weeks with the tryptophan-supplemented diet that the remaining control animals were receiving. Then, at the end of the 6-week period these animals were injected with the labeled vitamin, killed 72 hours later, and the ^{60}Co concentrations measured in kidney and liver.

The results of these assays (table 6), support the conclusion that dietary tryptophan deficiency induces an increased, but reversible renal uptake of injected ^{60}Co-labeled vitamin B_{12}, which can be corrected for fully by reincorporation of the missing amino acid into the diet.

DISCUSSION

This study has shown that feeding a diet low in the amino acid tryptophan to rats results in an increased uptake by the kidney of orally or parenterally administered ^{60}Co-labeled cyanocobalamin. This increase can be readily normalized by specifically supplementing the inadequate diet with tryptophan. That administration of inorganic ^{60}Co was unaffected by tryptophan deficiency indicates that the effect of this amino acid may reflect only that cyanocobalamin molecule is different from inorganic cobalt, even though this ion is a component of the vitamin. Perhaps, it is not unexpected that storage of a vitamin would differ from that of one of its elemental constituents. However, the evidence obtained from this study suggests that the differences of ^{60}Co activity in kidney uptake associated with tryptophan deficiency were much more related to storage of radioactive vitamin than to elemental ^{60}Co.

Although direct evidence to support the concept that kidney is the major site of vitamin B_{12} storage in rats is lacking, Harte et al. (10) have demonstrated that the uptake of ^{60}Co-labeled vitamin B_{12} in the rat kidney was higher than in the liver. The present studies confirm this observation. However, the belief that vitamin B_{12} is transferred from the kidneys to the liver, or other sites (11) cannot be completely reassessed from the data obtained in this present study because it is based on a relatively short period of observation. The work of Okuda and Chow [6] has shown that cyanocobalamin has a strong affinity for deposition in the kidney, but that when it is converted to its coenzyme, dimethylbenzimidazolylcobamide, it shows comparatively a greater affinity for liver. Whether the accumulation of ^{60}Co-cyanocobalamin in the kidney of tryptophan-deficient rats indicates some diminished ability to con-

[6] See footnote 5.

vert the vitamin to the coenzyme is not known.

Another possibility that could help to explain the retention of cyanocobalamin by kidney might be that tryptophan deficiency induces deterioration of renal function. If, as a recent study (12) indicates, the vitamin B_{12} binding capacity of α-globulin and the rate of glomerular filtration are closely related, then an elevated plasma level of vitamin B_{12} might be expected to be associated with impaired excretion. Our observation that vitamin B_{12} activity is reduced in tryptophan-deficient rats is contrary to this eventuality. This result will require additional correlation with the renal clearance of cyanocobalamin, which is not clearly defined at the present time.

Evidence from another source (13) also fails to explain the increased kidney concentration of ^{60}Co-labeled cyanocobalamin observed in tryptophan deficiency. These workers reported some renal hypertrophy in tryptophan-deficient rats, but no other pathological changes were observed. This evidence for renal damage would be sufficient to account for the radioisotopic accumulations reported here, except that this study demonstrates clearly that tissue concentration of radiocobalamin in kidney expressed as percentage of injected dose per gram of wet weight was more than doubled in the tryptophan-deficient rats (table 5). From these observations tryptophan appears to have a capacity to affect the metabolism of cyanocobalamin, but no detailed explanation for this can be given. Additional work on this problem could bring some insight into the mechanisms of action associated with tryptophan deficiency.

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Availability of Niacin in Wheat for Swine [1,2]

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ABSTRACT An experiment was conducted with 24 barrows to determine the availability of niacin in wheat for swine. Urinary excretion of N′-methylnicotinamide (NMN), N′-methyl-2-pyridone-5-carboxamide (2PY) blood levels of diphosphopyridine nucleotide (DPN), rate of gain, feed conversion and digestibility and retention of nitrogen were used as criteria for determining availability. Average daily gains and feed intake were higher ($P < 0.05$) for the pigs fed the 80% wheat ration (no supplemental niacin) than those fed 40%, or 40% (high niacin) wheat rations. No significant differences were noted in feed required per kilogram of gain. Urinary NMN and 2PY excretion increased in a linear manner ($P < 0.005$) for the pigs fed the 80, 40 and 40% (high niacin) rations, respectively. Blood DPN levels were lower ($P < 0.05$) for the pigs fed the 80% wheat ration than for those fed the 40% wheat rations. Nitrogen digestibility was lower ($P < 0.01$) for the pigs fed the 80% wheat ration than for those fed the 40% wheat rations. No significant differences were noted in nitrogen retention. The results from the excretion patterns of NMN and 2PY and the blood DPN levels observed indicate that the niacin of wheat is not appreciably available to swine.

Little information has been reported on the biological availability of niacin in cereal grains for swine. Some research indicates that the niacin of white corn is largely unavailable to swine (1, 2) and recently a similar observation was reported for both yellow corn and milo.[3] No information could be found in the literature concerning the biological availability of niacin in wheat for swine. However, considerable evidence has been reported indicating that the niacin of wheat is in a bound form and is largely unavailable to rats and microorganisms (3–6).

The present work was conducted to determine the availability of niacin in hard red winter wheat for swine.

EXPERIMENTAL PROCEDURE

Twenty-four Hampshire-Yorkshire crossbred pigs which averaged 27.3 kg live weight were allotted at random to 3 ration treatments, 8 pigs/treatment. The experiment was conducted over a period of 42 days. The pigs were housed and group-fed in concrete-floor pens which were equipped with self-feeders and waterers. The experimental unit was completely enclosed and was equipped with a thermostatically controlled heating system. Temperature in this unit was maintained at approximately 20°.

Composition of the experimental rations is presented in table 1. The rations fed were an 80% wheat ration supplying 42.2 mg of niacin/kg of feed exclusively from wheat and 2 rations with 40% wheat which contained 21.1 mg of niacin/kg of feed from wheat.

One of the rations with 40% wheat was supplemented with 21.1 mg of crystalline niacin/kg of feed, whereas the other, designated a 40% wheat (high niacin) ration, was supplemented with 41.3 mg of crystalline niacin/kg of feed. Cornstarch was used as the additional energy source in the 40% wheat ration. Vitamin-free casein and gelatin were used as protein supplements to maintain all rations at approximately 16.0% protein. Tryptophan (calculated, 0.19%) was held constant in all rations.

Six pigs, two from each ration treatment, were chosen at random on day 21, 28 and 35 of the experiment and placed in circular metabolism cages constructed from expanded metal material. The cages

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[2] Presented in part at the meeting of the American Society of Animal Science, East Lansing, Michigan. August, 1965. J. Animal Sci., in press (abstract).

[3] Luce, W. G. 1965 The availability of niacin in certain cereal grains for swine. Ph.D. Thesis. University of Nebraska, Lincoln, Nebraska.

TABLE 1

Composition of experimental rations

	Ration A	Rations B, C [1]
	80% wheat	40% wheat
Ingredients, %		
Ground wheat	80.0	40.0
Casein	4.0	10.0
Gelatin	2.5	2.5
Sucrose	4.0	4.0
Cornstarch	2.1	35.5
Lard	2.5	2.5
Monosodium phosphate	1.5	2.1
Ground limestone	1.8	1.8
Salt (iodized)	0.5	0.5
Trace mineral mix [2]	0.1	0.1
Amino acid and vitamin premixes	1.0 [3]	1.0 [4]
Protein (chemical analysis), %	16.88	16.38
Tryptophan (calculated), %	0.19	0.19
Niacin (microbiological assay), mg/kg	42.29	21.15
Calcium (calculated), %	0.72	0.70
Phosphorus (calculated), %	0.70	0.70

[1] Supplemented with 21.15 and 42.29 mg of niacin/kg of feed for rations B and C, respectively.
[2] Percentage composition: Mn, 10.0; Fe, 10.0; Cu, 1.0; Co, 0.10; I, 0.30; Zn, 10.0; and Ca, 9.1; obtained from the Calcium Carbonate Company, Quincy, Illinois.
[3] Amino acid and vitamin premixes contributed the following (per kg of feed): vitamin A, 4409.0 IU; vitamin D_2, 396.8 IU; vitamin B_{12}, 22.0 μg; and (in milligrams) pantothenic acid, 8.8; choline chloride, 264.5; riboflavin, 4.8; DL-methionine, 599.6; L-lysine (monohydrochloride), 3300.3; and DL-tryptophan, 39.7.
[4] Vitamin premix contributed the following (per kg of feed): vitamin A, 4409.0 IU; vitamin D_2, 396.8 IU; vitamin B_{12}, 22.0 μg; and (in milligrams) pantothenic acid, 13.2; choline chloride, 572.2; and riboflavin, 5.1.

were equipped with self-feeders and waterers and the animals were fed ad libitum.

Total collections of urine and feces were made for 3 days after a 3-day adjustment period. The feces were collected twice daily and immediately frozen in tightly sealed plastic bags until they could be processed. The urine was collected daily in gallon glass jugs containing 20.0 ml of concentrated hydrochloric acid, and an 8.0% aliquot was immediately refrigerated. At the end of each trial, 10 ml of blood were collected from the brachial vein in a glass syringe equipped with a 5-cm, 18-gauge needle. The blood was immediately transferred to a 50 ml glass bottle which contained 20 mg of ammonium oxalate to prevent coagulation.

The total feces collected from each pig for 3 days were thawed and thoroughly mixed with a home-type twin beater food mixer. The aliquots of urine collected for 3 days from each pig were thoroughly mixed by rapid inversion and were then frozen until chemical analysis could be made.

Nitrogen analyses were determined by the method of Kjeldahl as outlined by AOAC (7). Analyses of the urine samples for N′-methylnicotinamide (NMN) were made by the fluorometric procedure of Huff and Perlzweig (8). Niacin content of the wheat was determined by the microbiological method as outlined by Methods of Vitamin Assay (9). Analysis for diphosphopyridine nucleotide (DPN) in blood samples was made by the method of Levitas et al. (10). Hematocrits were made on 1.0 ml of blood by the technique of Wintrobe (11).

Determinations of N′-methyl-2-pyridone-5-carboxamide (2PY) were made on urine samples by the column chromatography method of Price (12) with the following modifications: The columns were 1.6 cm in diameter and similar to those described by Walters et al. (13). Dowex 1 (10% cross linkage) 200–400 mesh and Dowex 50 (4% cross linkage) 200–400 mesh were used as the ion exchange resins. Twenty milliliters of urine were washed through each column. Distilled water effluents col-

lected while washing the columns before running the urine samples through were used as the blank determinations as suggested by Vivian et al. (14).

Student's t test as outlined by Steel and Torrie (15) was used to determine differences between treatment means. A probability level of $P < 0.05$ was accepted as being significant.

RESULTS

The results of the growth study are presented in table 2. The greatest average daily gains (0.96 kg) were made by the pigs fed the 80% wheat ration which contained no supplemental niacin. These gains were significantly greater ($P < 0.05$) than the 0.82 kg observed for the pigs fed the 40% wheat rations. The average daily feed intake of the pigs fed the 80% wheat ration was greater ($P < 0.05$) than that of those fed the 40% wheat or 40% wheat (high niacin) rations. No significant differences were noted among treatments in feed required per kilogram of gain.

NMN and 2PY excretion patterns for the pigs fed the different ration treatments are shown in figure 1. Average daily urinary

TABLE 2

Summary of average daily gain, average daily feed intake and feed required per kilogram of gain [1,2]

	Ration A [3]	Ration B [3]	Ration C [3]
	80% wheat	40% wheat	40% wheat, high niacin
Niacin, mg/kg	42.2 (all from wheat)	42.2 (1/2 crystalline)	63.4 (2/3 crystalline)
Avg initial wt, kg	27.1	27.4	27.4
Avg final wt, kg	47.2	44.6	44.6
Avg daily gain, kg	0.96 [4]	0.82 [4]	0.82 [4]
Avg daily feed intake, kg	2.10 [5]	1.79 [5]	1.81 [5]
Feed/kg gain, kg	2.18	2.20	2.22

[1] Duration of experiment, 42 days.
[2] Eight pigs/treatment.
[3] See table 1 for exact compositions.
[4] Ration A higher ($P < 0.05$) than B or C.
[5] Ration A higher ($P < 0.02$) and ($P < 0.05$) than B and C, respectively.

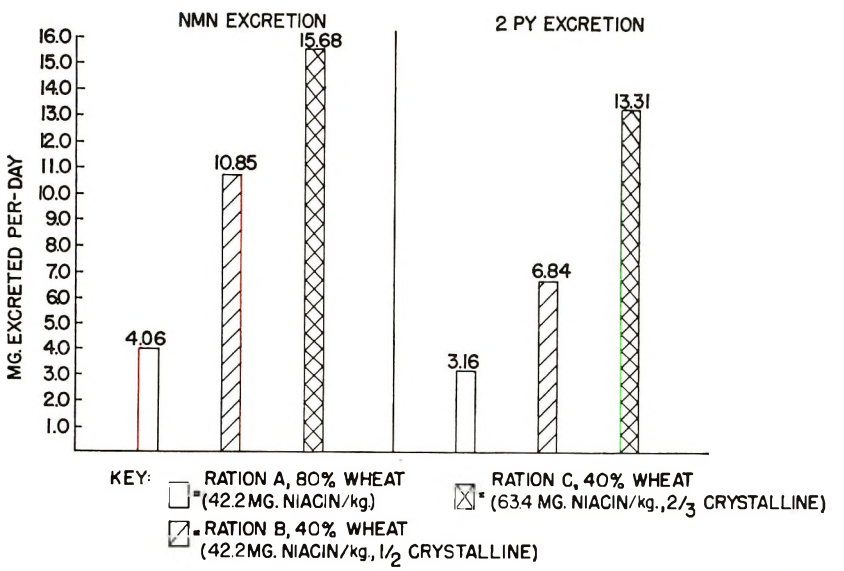

Fig. 1 Effect of ration treatment on urinary excretion of N'-methylnicotinamide (NMN) and N'-methyl-2-pyridone-5-carboxamide (2PY) by swine.

excretion of NMN was 4.06, 10.85 and 15.68 mg for the 80, 40 and 40% (high niacin) wheat rations, respectively. A significant ($P < 0.005$) linear increase in NMN excretion was observed with increases of supplemental niacin. The average daily urinary excretion of 2PY was 3.16, 6.84 and 13.31 mg for pigs fed the 80, 40 and 40% (high niacin) wheat rations, respectively. The linear effect of supplemental crystalline niacin on 2PY excretion was significant ($P < 0.005$).

Blood DPN levels for the pigs fed the different ration treatments are shown in figure 2. DPN levels were 23.0, 29.4 and 28.7 μg/ml of erythrocytes for the pigs fed the 80, 40 and 40% (high niacin) wheat rations, respectively. Blood DPN values were significantly lower ($P < 0.05$) for the pigs fed the 80% wheat ration than for those fed the 40% wheat and the 40% wheat (high niacin) rations.

Nitrogen digestibility and retention data are shown in table 3. The pigs fed the 80% wheat ration showed a lower ($P < 0.01$) nitrogen digestibility than the pigs fed either of the 40% wheat rations.

No significant differences were noted in nitrogen retention.

DISCUSSION

The excellent gains made by the pigs fed the 80% wheat ration without supplemental niacin indicates that a wheat ration with a moderate level of tryptophan (0.19%) is adequate for growing pigs

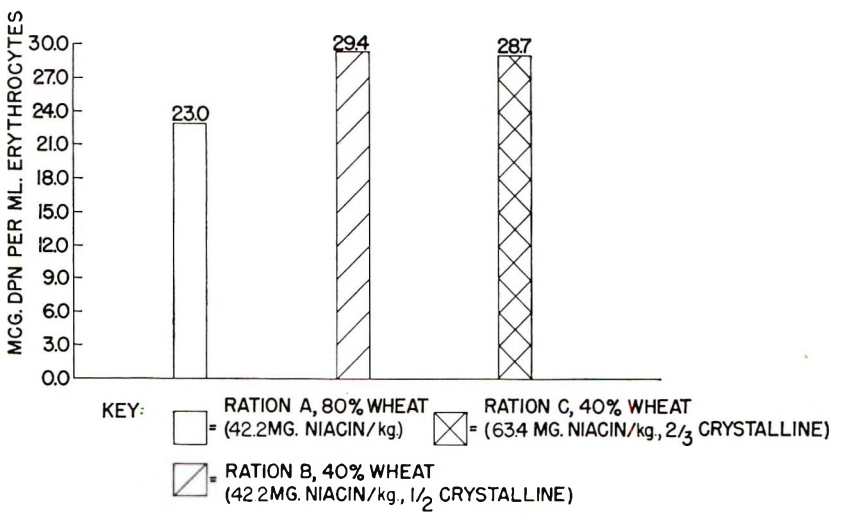

Fig. 2 Effect of ration treatment on levels of blood diphosphopyridine nucleotide (DPN).

TABLE 3

Summary of nitrogen digestibility and retention

	Ration A [1]	Ration B [1]	Ration C [1]
	80% wheat	40% wheat	40% wheat, high niacin
Niacin, mg/kg	42.2 (all from wheat)	42.2 (1/2 crystalline)	63.4 (2/3 crystalline)
Avg daily nitrogen intake, mg	61.60	52.89	49.06
Avg daily nitrogen digestibility, %	90.0 [2]	95.1 [2]	95.2 [2]
Avg daily nitrogen retention, % of intake	51.4	48.5	51.8

[1] See table 1 for exact composition.
[2] Ration A lower ($P < 0.01$) than ration B or C for nitrogen digestibility.

without added niacin. The gains made by the pigs fed both 40% wheat rations, although lower than those made by the pigs fed the 80% wheat ration, were also considered to be excellent. The lowered gains were probably a result of the lowered feed intake since the 40% wheat rations were semi-purified which appears to affect feed consumption. Niacin intake did not appear to be involved since those fed the 40% wheat rations consumed amounts of niacin similar to those of the pigs fed the 80% wheat ration. An addition of 21.1 mg of crystalline niacin/kg of feed appeared to be adequate for the pigs fed the 40% wheat ration since no increase in gains was noted with the higher level of crystalline niacin.

The highly significant linear increase in NMN and 2PY excretions of the pigs fed the 80, 40 and 40% (high niacin) wheat rations was attributed to the increase of crystalline niacin since the 80% wheat and the 40% wheat ration were equal in total niacin content. The pigs fed the 40% wheat ration excreted approximately 2.7 and 2.2 times more NMN and 2PY, respectively, than the pigs fed the 80% wheat ration, yet their total niacin intake was the same. The niacin in the 80% wheat ration originated exclusively from wheat, whereas one-half of the niacin in the 40% wheat ration was from a crystalline source.

The niacin in wheat was not as effective as crystalline niacin for blood pyridine nucleotide synthesis as shown by the significant decrease in blood DPN levels when the pigs received all their niacin from wheat as compared with the pigs fed the rations containing crystalline niacin. Failure of the pigs fed the 42.2 mg of crystalline niacin/kg of feed to show an increase in blood DPN levels as compared with those fed 21.1 mg of crystalline niacin/kg of feed for the 40% wheat rations does not agree with the work of Morrison et al. (16) who reported that either niacin or tryptophan added in excess of dietary needs further increased blood DPN levels in rats. Perhaps the blood DPN levels of the pigs fed the ration containing 21.1 mg of crystalline niacin had reached their physiological limits upon which additional niacin could have no further effect.

The differences observed in nitrogen digestibility were thought not to be a reflection of niacin levels but of differences in sources of protein. When the wheat levels were changed from 80 to 40% in the experimental rations, casein was used to equalize protein content in all rations. The replacement of part of the wheat protein with casein undoubtedly was the cause for the increase in nitrogen digestibility. Increased nitrogen digestibility did not result in increased nitrogen retention.

The results indicate that the niacin from wheat is largely unavailable to swine. This work is in agreement with that of several workers (3–6, 17 and 18) who have also observed the niacin of wheat to be largely unavailable to microorganisms, rats and ducks.

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Effect of Isoniazid on the Loss of Pyridoxal Phosphate from, and its Distribution in, the Body of the Rat [1]

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ABSTRACT The effect of isoniazid (INH) on the pyridoxal phosphate (PALP) content of tissues of rats was determined. INH induced an increase of PALP in liver and a decrease in muscle, regardless of the adequacy of the diet in vitamin B_6. When the diet was adequate in vitamin B_6, INH caused an exceedingly slight increase in leukocytes and a decrease in PALP in plasma; when the diet was deficient in the vitamin, the deficiency itself brought about such a severe depletion of PALP in leukocytes and plasma that INH caused no further change. Considering the 4 body components, leukocytes, plasma, liver and muscle, INH caused a net loss of PALP. When INH was withdrawn, the PALP content of the liver decreased; some PALP probably went to the muscles since the PALP content of metabolizing muscle did not decrease.

Before studies of the requirement of vitamin B_6 can be initiated the body stores of the vitamin must be reduced to the level required for normal functioning. The object of the present study was to determine whether, in rats, isoniazid (INH), a compound used widely in the treatment of tuberculosis and known to produce certain vitamin B_6-deficiency symptoms above certain dosages, hastens depletion. A presumption was made that the pyridoxal phosphate (PALP) content of leukocytes, the only representative metabolizing tissue readily accessible from human subjects, would serve as an indicator of the state of vitamin B_6-depletion of the entire body. Wachstein and Moore (1) had reported leukocytes to be a good indicator for rats fed a vitamin B_6-deficient diet. Therefore the PALP content of leukocytes was determined. Since plasma was obtained in the extraction of leukocytes, its PALP content was determined also. The PALP content of the livers of rats was also investigated as the liver is a storage organ and would presumably decrease in PALP content if the stores of the coenzyme were being reduced. The observation was made, however, that instead of causing a reduction of liver PALP, INH increased the stores; therefore, the investigation was broadened to include the PALP content of muscle in an attempt to determine whether a net reduction of body PALP occurred insofar as could be estimated from knowledge of the PALP content of leukocytes, plasma, liver and muscle. This led to an investigation of whether the PALP stored in the liver under the influence of INH is released for use in muscle tissue after cessation of INH treatment.

EXPERIMENTAL

Experimental diets were fed to 60, 24, and 32 male rats of the Holtzman strain with mean weights of 144, 131 and 123 g, at 46, 42 and 39 days of age, respectively, in experiments designated as A, B and C.

In all 3 studies the basal diet contained: (in percent) "vitamin-free" casein,[3] 18; glycine, 1; methionine, 0.2; corn oil, 5; salt mix USP XIV, 4; agar, 2; vitamin mixture,[4] 2.2 (with pyridoxine·HCl omitted); and enough sucrose to make 100. Pyridoxine·HCl was added at levels of 4, 2 and 2 mg/kg of food in experiments A,

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[3] The casein contained 0.63 μg of vitamin B_6/g. The basal diet, therefore, contained 0.113 mg vitamin B_6/kg.

[4] The vitamin mixture supplied in each 45.5 kg (100 pounds) of diet: (in grams) vitamin A (200,000 units/g), 4.5; vitamin D conc (400,000 units/g), 0.25; α-tocopherol, 5.0; ascorbic acid, 45.0; inositol, 5.0; choline chloride, 75; riboflavin, 1.0; menadione, 2.25; *p*-aminobenzoic acid, 5.0; niacin, 4.5; thiamine·HCl, 1.0; Ca pantothenate, 3.0; and (in milligrams) biotin, 20; folic acid, 90; and vitamin B_{12}, 1.35. (Vitamin Diet Fortification Mixture in Dextrose, with pyridoxine·HCl omitted, obtained from Nutritional Biochemicals Corporation, Cleveland.)

B and C, respectively. In experiment A, 1.5 g of INH/kg of food were added and in experiments B and C, 1 g. In all experiments the rats were pair-fed. The mean daily food consumption per rat was 8.5, 9.8 and 12 g, respectively, in experiments A, B and C.

Experiment A. Sixty rats were distributed equally among 4 groups. One group was fed the basal vitamin B_6-deficient diet, the other 3 were fed the basal diet plus pyridoxine·HCl or INH or deoxypyridoxine (DOP). The observations with DOP will be reported in a later paper. The rats were arranged in 15 groups of 4 rats each, with one rat in each group fed each diet for the purpose of pair-feeding. DOP administration was discontinued after 21 days and INH after 28 days. One group of 4 was killed after one week, a second one after 3 weeks and thereafter 2 or 3 groups of four each week until all had been killed at the end of the seventh week.

Experiment B. Twenty-four rats were divided into 4 groups of 6 rats each. For one week all received the basal diet plus pyridoxine·HCl. Thereafter, 2 groups were fed the basal vitamin B_6-deficient diet; INH was added to the food of one. The other 2 groups both received pyridoxine·HCl; INH was added to the food of one. They were treated as 6 groups of four each for pair-feeding; within each group of four was one rat fed each diet. At the end of the fifth week INH was removed from the diet of the rats not receiving pyridoxine·HCl. After the 4 diets had been fed for 7 weeks, one group of four was killed each day.

Experiment C. Thirty-two rats were divided into 2 groups of 16 each. The diets of both groups contained pyridoxine·HCl; INH was added to the food of one group. After 3 weeks both groups were divided into 2 parts of 8 each: one part continued to be fed a modified diet, and one part was killed. The half of each group (8 rats) which continued to be fed experimental diets was given a diet containing neither pyridoxine·HCl nor INH for 2 more weeks, and then was killed.

Methods. All rats were etherized and an incision made into the thoracic cage. Blood was removed from the heart with a siliconized needle and syringe, transferred to a siliconized test tube and sodium EDTA was added and mixed with the blood to prevent coagulation. In experiments B and C the livers were removed, weighed and placed in a freezer within 5 minutes of the time of death of the animal. In study C, the gluteal muscle was removed from both hind legs.

White cells were removed from the blood, hydrolyzed with sodium hydroxide and neutralized according to the procedure of Boxer et al. (2). Homogenates made from the muscles and the left sections of the cystic lobes of the livers were also hydrolyzed with sodium hydroxide. Plasma was not treated with sodium hydroxide but was diluted 6 times according to the procedure of Wachstein et al. (3). The L-tyrosine decarboxylase method of Boxer et al. (2) was used for the determination of PALP.

To learn whether the L-tyrosine decarboxylase method determines PALP in the hydrazone form, as well as free PALP, the hydrazone was synthesized by the method of Gonnard (6). Equimolar amounts of the synthetic hydrazone and free PALP were heated with NaOH, neutralized with HCl according to the procedure used in the present study and the PALP was determined by the L-tyrosine decarboxylase method. Approximately three-fifths of the hydrazone was determined as PALP. The results were the same whether the reaction was carried out in a buffer or in buffer plus liver homogenate. When the PALP determination was not preceded by heating with NaOH (plasma was not so treated) only one-fifth of the PALP residue was determined as PALP. Probably, therefore, about three-fifths of the amount of hydrazone present in the leukocytes, livers and muscle was determined in the present study provided the sodium or potassium salts present in vivo behave in a manner similar to the acid form of the hydrazone used in the test. Thus our total PALP values are probably somewhat low for the periods when INH was fed.

RESULTS

Effect of INH on the PALP content of leukocytes. If INH has an effect on the PALP content of the leukocytes of rats fed a diet adequate in vitamin B_6, INH

causes an increase rather than a reduction of the coenzyme. After all the rats in experiment C had been fed a vitamin B_6-adequate diet for 3 weeks, the leukocytes of those rats fed INH contained more PALP than those of their counterparts not fed the drug (table 1). The difference was small and on the borderline of significance ($P < 0.05$) according to paired t tests.[5]

When the diet was devoid of vitamin B_6, INH caused no additional loss of PALP from leukocytes. The vitamin B_6-deficient rats fed INH (exp. A) lost no more PALP from their leukocytes than counterparts not fed the drug (table 1). Also the leukocytes of those rats from whose diets INH had been withdrawn after it had been administered to them for varying times, contained no less PALP than those of the control rats that never had received the drug (table 1).

Thus if INH causes depletion of vitamin B_6 in the body as a whole, the depletion cannot be assessed solely from observations on the PALP content of leukocytes.

Effect of INH on the PALP content of plasma. INH reduces plasma PALP when fed to rats supplied with diets adequate in vitamin B_6. In both experiments B and C, INH reduced plasma PALP more than one-half (table 1). In experiment B the difference was on the borderline of statistical significance ($P < 0.05$) because the value for one rat in the control group was very low but in experiment C the reduction of plasma PALP was highly significant ($P < 0.01$).

When the diet was deficient in vitamin B_6 (exps. A and B) INH caused no further reduction of plasma PALP than the drastic reduction induced by the vitamin B_6 deficiency (table 1). When rats had been deprived of vitamin B_6 for 2 weeks, after having had the vitamin for 3 weeks (exp. C), the PALP content of the plasma was greatly reduced but was not significantly lower among those animals that had been fed INH earlier than among those that had never received the drug (fig. 1).

Effect of INH on the PALP content of liver. INH caused an increase in PALP per gram and also in whole livers of rats fed diets both adequate and inadequate

[5] The levels of significance were calculated by paired t tests with animals paired for food intake and time of killing, except in a few cases where comparisons were made with group t tests because comparisons were made perpendicularly rather than horizontally on rats that had not been paired.

TABLE 1

Effect of isoniazid (INH) on the amount of pyridoxal phosphate (PALP) in leukocytes, plasma and liver in experiments A, B and first 3 weeks of experiment C

Exp.	Pyridoxal phosphate				No. of rats/ group	Time fed diets
	Diet with vitamin B_6		Diet without vitamin B_6			
	No INH	INH	No INH	INH		
						weeks
			Leukocytes, mμg/10^6			
A	1.05 ± 0.2 [1]		0.61 ± 0.1	0.69 ± 0.1	5	4 or less
A	1.32 ± 0.2		0.56 ± 0.1	0.59 [2] ± 0.1	7	4 to 7
C	0.57 ± 0.1	0.68 ± 0.1			7	3
			Plasma, mμg/ml			
A	177.9 ± 27.2		8.2 ± 1.0	8.5 ± 3.8	4	2, 5, 6
B	104.0 ± 17.3	47.5 ± 5.1	6.8 ± 0.9	7.9 [3] ± 0.9	6	7
C	52.2 ± 6.3	20.8 ± 2.3			7	3
			Liver, μg/whole			
B	48.4 ± 4.0	65.7 ± 4.1	16.1 ± 0.7	23.5 [3] ± 1.7	6	7
C	50.1 ± 2.6	78.2 ± 4.1			8	3
			Liver, μg/g			
B	8.1 ± 0.3	10.9 ± 0.5	2.2 ± 0.1	3.6 [3] ± 0.4	6	7
C	7.1 ± 0.3	10.8 ± 0.5			8	3

[1] SE of mean.
[2] INH fed for varying periods of time, then INH omitted.
[3] INH omitted at the end of the fifth week.

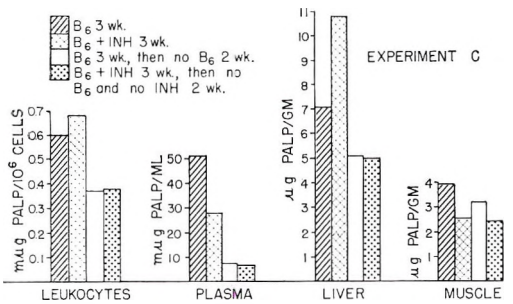

Fig. 1 The pyridoxal phosphate (PALP) content of plasma, leukocytes, liver and muscle of 2 groups of 8 rats after 3 weeks with pyridoxine hydrochloride (B_6) or vitamin B_6 plus isoniazid (INH) in the diet and 2 other groups of 8 rats treated in the same way and then vitamin B_6 and INH omitted from the diet for 2 additional weeks.

in vitamin B_6. In experiment B rats given INH for 7 weeks stored more PALP than their counterparts to which the drug was not administered when the diet was adequate and also when it was deficient in vitamin B_6 (table 1). The differences were significant ($P < 0.02$) on the basis of per gram of wet liver and even more highly significant ($P < 0.01$) on the basis of whole liver. In experiment C, in which all rats were fed diets adequate in vitamin B_6 during the first 3 weeks, those rats receiving INH stored significantly more PALP ($P < 0.01$) both per gram and in whole livers (table 1).

Experiment C was continued 2 weeks longer to determine whether the rats would be forced to use some of the PALP stored in their livers under the influence of INH when both INH and pyridoxine·HCl were withdrawn. On the basis of per gram of liver and of whole liver, rats that had been fed pyridoxine·HCl as well as those that had been fed pyridoxine·HCl plus INH, lost PALP from their livers (fig. 1). According to group *t* tests, the differences were equally significant ($P < 0.01$). Rats that had never been fed INH lost 28% per gram and 18% per whole liver, whereas rats fed INH for 3 weeks, and which consequently had a larger supply of PALP lost more, namely, 54% per gram and 48% per whole liver. At the end of 5 weeks, the livers of both groups contained about the same amount.

Effect of INH on the PALP content of muscle. In experiment C, following the first 3 weeks during which all rats were given adequate supplies of vitamin B_6, the PALP content of the muscle of the rats to which INH had been administered was significantly lower ($P < 0.01$) than that of partners not fed the drug (fig. 1). After 2 more weeks, during which pyridoxine·HCl was omitted from the diets to force the rats to use their stored vitamin B_6, the PALP content of the muscle of the control rats that had never received INH, decreased 18%, a significant amount ($P < 0.01$) according to group *t* tests; on the contrary, the rats that had received INH for the first 3 weeks but not the last two, showed no significant decrease in muscle PALP according to group *t* tests. Since PALP is needed for the metabolism of muscle tissue and the muscle content did not decrease, the needed PALP must have been drawn from body stores. Presumably the liver supplied at least some of this PALP because liver stores decreased during this time. Possibly the muscle PALP of the control rats decreased following the removal of pyridoxine·HCl because no withdrawal of PALP occurs from liver stores until the content of muscle has decreased to a certain point below normal and that point had not been reached. Another possible explanation is that with less PALP the liver relinquishes its store less readily.

DISCUSSION

In a survey of the literature, studies of the effect of INH on the PALP content of leukocytes, plasma and muscle were not found; a study involving liver was reported by Bain and Williams (5). In the present study the effect of INH on all 4 tissues in rats was investigated.

The PALP content of leukocytes of rats did not decrease under the influence of INH, thus leukocyte PALP, although a good criterion in a simple vitamin B_6-dietary deficiency, cannot be used to measure the vitamin B_6-depleting effect of INH.

INH induced an increase of PALP in the liver of rats when fed diets both adequate and deficient in vitamin B_6: a total of 40 rats was involved, one-half of which were controls.

Bain and Williams (5) observed that the livers of 5 mice injected with INH con-

tained less PALP than that of 2 control mice even when PALP in the hydrazone form was included. In a dog, however, the total free and combined PALP in the liver increased after treatment with INH.

The present study on rats supplies evidence that PALP stored in livers, when INH is administered, can be withdrawn and used when INH treatment ceases; when INH treatment was stopped, the PALP content of the livers decreased and that in muscle did not decrease despite the requirement of PALP in normal metabolic processes.

Probably some of the PALP in the livers of the rats fed INH existed as isonicotinyl PALP-hydrazone. Bain and Williams (5), who used column chromatography to separate 6 forms of vitamin B_6 and two of their hydrazones in livers from mice fed INH, observed about one-fifth as much PALP in the hydrazone as in free PALP. Therefore, the livers of the rats fed INH in the present study probably contained some of the hydrazone. If so, two questions arise.

One question is: does the L-tyrosine decarboxylase method determine the hydrazone? A second question is: does the formation of a hydrazone limit the use of PALP as a coenzyme? In answer to the first question evidence was obtained in the present study that the method used determines about three-fifths of the hydrazone. In answer to the second question, considerable evidence exists to show that although INH hastens the symptoms of vitamin B_6-deficiency, the reason is not the inability of the apoenzyme to use the hydrazone but rather some other action of INH. Recently several workers have synthesized the hydrazone and compared its activity with that of free PALP as a coenzyme for several apoenzymes. Gonnard and Fenard (7), Torchinsky (8), Makino et al. (9) and Bonavita and Scardi (10) have reported that it acts as a coenzyme. At this time investigators do not agree as to whether the activity is brought about by a freeing of the PALP from the hydrazone or by the intact hydrazone.

The original object of the present study was to learn whether INH could be used to hasten the loss of vitamin B_6 from the bodies of rats. A gain in liver PALP, no change in leukocyte PALP, and a loss in muscle and plasma PALP probably resulted in a small net loss. A rough estimate can be made from some of the data (exp. 3): after 3 weeks the muscle of rats fed INH contained 1.35 μg/g less than that of their counterparts not given INH. Assuming that 40% of the body weight was skeletal muscle, then the loss of PALP from muscle was 104.8 μg for rats averaging 194 g; the loss of PALP from plasma would add a little to this. In the same period of time the gain of PALP in the livers was 28.1 μg. The loss is so much greater than the gain that there undoubtedly was a net loss of PALP from the body caused by INH. This is not unexpected because animals given INH eventually display some vitamin B_6-deficiency symptoms. Moreover, Biehl and Vilter (4) working with human beings and Bain and Williams (5) in a study on a dog, reported some loss of vitamin B_6 in urine caused by INH. Thus although INH causes some depletion of vitamin B_6, INH is not highly efficient in this respect; it would not be expected to markedly accelerate the depletion of human subjects.

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Effect of Deoxypyridoxine on the Amount of Pyridoxal Phosphate in the Livers and Leukocytes of Rats and on Leukocyte Number, Size, and Type [1]

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ABSTRACT When deoxypridoxine (DOP) was administered orally to rats fed a vitamin B_6-deficient diet, the amount of pyridoxal phosphate (PALP) in the livers increased both for per gram of wet tissue and for the whole organ. DOP caused an increase in the PALP content of leukocytes per million cells and also in the amount of PALP contributed by leukocytes to 1 ml of whole blood. Thus, DOP cannot be used to increase the rate of loss of PALP from these 2 tissues and when it is administered the PALP content of leukocytes cannot be used as a criterion of depletion. DOP caused a decrease in the number of lymphocytes and an increase in the mean size of leukocytes.

The overall object of the investigation, of which this study is a part, was to find a way to hasten the depletion of the vitamin B_6 stores of subjects eating low vitaimn B_6 diets to prepare them for studies of requirement. Two compounds, isoniazid (INH) and deoxypyridoxine (DOP), known to inhibit vitamin B_6, were tried on rats to test the usefulness of the compounds for accelerating depletion. The inhibition of vitamin B_6 by DOP is caused by the competition of DOP-phosphate with pyridoxal phosphate (PALP) for the active site on PALP-dependent apoenzymes. The mechanism of inhibition by INH is not known although several theories have been proposed. If the inhibition of INH and DOP is brought about by different mechanisms one of them may be better than the other in hastening removal of the vitamin from the body. The work with INH has been reported (1); the work with DOP is the subject of the present paper.

The amount of PALP in the liver and in the leukocytes of DOP-treated rats was determined. When the PALP content of leukocytes per million cells was found to be high, an investigation was made to determine the reason and also to learn how the high content of PALP per million cells affected the amount of PALP contributed by leukocytes to a milliliter of whole blood.

EXPERIMENTAL METHODS

This report includes 2 experiments, A and D. Experiment A was described as experiment 1 in the paper reporting the work on INH (1). In experiment D, 16 male rats of the Holtzman strain, 47 days of age, and weighing an average of 151 g, were fed the basal vitamin B_6-deficient diet described previously (1); to the food of half the animals 1 g of DOP/kg was added. Because the rats were pair-fed the food consumption of the 2 groups was alike: the mean daily intake per rat was 9.8 g. The animals were killed at the rate of a pair a day beginning after they had been fed the experimental diets for 3 weeks.

The removal of the livers and blood and the separation of the leukocytes from the whole blood has been described previously (1). Whole blood taken from the heart was used for counting leukocytes. Blood from the tail was used for differential counts. To find the relative size of the leukocytes, the size of the button of packed leukocytes following centrifugation from the leukocyte-containing plasma drawn off the top of the packed red cells was estimated. From the volume of the leukocyte-containing plasma and the count of leuko-

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cytes in it, the number of cells per 0.1 ml of packed cells was estimated. This was a rough estimate because the volume of packed cells in the bottom of the graduated conical centrifuge tubes could be judged only approximately; the method was used, however, because it required simply adding the observed size of the cell button to measures required for other purposes. Also, the difference between the size of the cells of the control animals and those fed DOP appeared to be large enough to be measured by means which were not precise.

The PALP content of the liver and leukocytes was determined by the L-tyrosine decarboxylase method of Boxer et al. (2).

RESULTS

Effect of DOP on the PALP content of liver. In experiment D the PALP content of the livers of rats fed DOP was higher than that of their counterparts not fed the compound (table 1). The difference was statistically significant both per gram of wet tissue ($P < 0.02$) and per whole liver ($P < 0.01$), according to paired t tests.[3] This observation is in agreement with that of Stoerk (3) who reported that the livers of rats fed vitamin B_6-deficient diets and administered DOP contained more PALP per gram than those of control rats not administered DOP. He did not, however, report the content of whole livers. Since his rats fed DOP weighed less than his controls, they might have had smaller livers; thus the whole livers might not have contained more PALP. The results of the present study answer the question with respect to whole livers. The 2 studies show conclusively that DOP causes an increase in liver PALP. DOP, therefore, does not cause depletion of PALP from liver. In this respect DOP behaves similarly to INH which also induced liver storage of PALP (1).

Effect of DOP on the amount of PALP per million leukocytes and its cause. In experiment A, at the end of the first week the leukocytes of the one rat that had been administered DOP contained more PALP per million cells than any of the other 3 rats in the group, even more than the one rat fed the vitamin B_6-adequate diet. Since this observation had also been made during some preliminary work, the decision was made to discontinue the administration of DOP one week before the rats were killed, on the theory that the number of leukocytes would return to normal and only the depleting effect of DOP on PALP, if DOP has such an effect, would be measured. After 21 days the administration of DOP to all rats was discontinued, because they were in poor health.

[3] The levels of significance have all been calculated by paired t tests with animals paired for food intake and time of killing.

TABLE 1

Effect of deoxypyridoxine (DOP) on the amount of pyridoxal phosphate (PALP) in the leukocytes and livers of rats

Exp.	Pyridoxal phosphate			No. of rats/ group	Time fed diets
	Diet with vitamin B_6	Diet without vitamin B_6			
	No DOP	No DOP	With DOP		
					weeks
		Leukocytes, mμg/10^6			
A	1.19 ± 0.1 [1]	0.53 ± 0.1	0.67 [2] ± 0.1	14	2 to 7
D		0.42 ± 0.1	1.25 ± 0.2	7	3
		Leukocytes, mμg/ml [3]			
D		2.42 ± 0.3	4.08 ± 0.3	7	3
		Liver, μg/whole			
D		34.8 ± 1.0	42.5 ± 2.0	7	3
		Liver, μg/g			
D		5.33 ± 0.2	6.85 ± 0.4	7	3

[1] SE of mean.
[2] These rats were administered DOP but it was discontinued 3 to 20 days before they were killed.
[3] Millimicrograms of PALP in the leukocytes present in 1 ml of whole blood.

Whether DOP had been present in the diets for a long or a short time before it was discontinued made no difference in the PALP content; hence the values for the leukocytes for all rats treated with DOP, except for the one rat fed DOP up to the time of killing, were combined. The PALP content of the leukocytes of these rats was slightly higher than that of the controls which had not been administered DOP at any time (table 1) but the difference was of borderline significance ($P < 0.05$). Values for both groups of rats deprived of vitamin B_6 were about half that for rats fed diets adequate in vitamin B_6 (table 1). The differences were significant for both groups ($P < 0.01$). Thus, removal of DOP from the diet soon reduced the PALP to values normal for vitamin B_6-deprived animals but not below that norm; therefore DOP had had no depleting effect on the PALP content of leukocytes.

In experiment A, while DOP was in the diet and the diet did not contain vitamin B_6, DOP increased the PALP content per million cells in only one rat; therefore experiment D was set up to determine whether this observation could be verified. The leukocytes of the rats in experiment D fed DOP up to the day of killing, contained about 3 times more PALP per million cells than those of their partners not fed the compound (table 1). The difference was statistically significant ($P < 0.01$).

To determine whether the cause of the high PALP content per million cells was an increase in size of the leukocytes induced by DOP, experiment D was conducted. For every pair of control and treated rats, the number of cells contained in 0.1 ml of packed cells was smaller in the blood of rats treated with DOP; the mean count for the DOP-treated rats was about one-third of that for their partners not so treated (table 2). Since the mean volume of cells was increased 3 times and the PALP per million cells was also increased 3 times (table 1), the concentration of PALP within the cells remained the same. Thus, the cause of the high PALP content can be attributed to an increase in the mean size of the leukocytes.

Because the increase in cell size may have been the result of a change in the proportion of certain types of leukocytes, differential counts were made to learn whether a shift in the proportion of certain cell types had occurred. The number of lymphocytes in the blood of rats treated with DOP was about one-third of that in the blood of their partners (table 2). The difference was significant ($P < 0.01$). A similar decrease in lymphocyte count caused by DOP was reported by Hawkins and Evans (4); others have observed it in the monkey (5), dog (4, 5), mouse (6) and human (7). In contrast, the number of neutrophils in the blood of rats fed DOP, although slightly higher than that in the blood of their partners, was not significantly higher. Hawkins and Evans also reported no change in the number of neutrophils in rats fed vitamin B_6-deficient diets although they noted an increase when the diet was adequate in vitamin B_6. Mushett et al. (5) reported that DOP caused an increase in neutrophils in the blood of dogs.

Effect of DOP on the amount of PALP contributed by leukocytes to a milliliter of whole blood. Whether the high PALP content per million leukoctyes in the blood of DOP-treated rats affected the amount of PALP contributed by leukocytes to whole blood was a question. The blood of the rats in experiment D that were administered DOP contained about half as many leukocytes as that of the blood of their counterparts not treated with the compound (table 2). The difference was significant ($P < 0.01$). Even though the number of leukocytes for DOP-treated rats was

TABLE 2

Effect of deoxypyridoxine (DOP) on the number, type and size of the leukocytes of rats after oral administration of DOP for 3 weeks

	Rats fed DOP [1]	Control rats [1]
Leukocytes, 10^6/0.1 ml packed cells	9.6 ±0.9 [2]	28.9 ±6.1
Leukocytes, 1000/mm^3 blood	3.55±0.4	7.13±0.9
Lymphocytes, 1000/mm^3 blood	1.80±0.2	5.69±0.8
Neutrophils, 1000/mm^3 blood	1.72±0.2	1.37±0.2

[1] Both groups of rats contained 8 animals and both were fed the basal vitamin B_6-deficient diet.
[2] SE of mean.

smaller, their PALP content was so high that the amount of PALP contributed by them per milliliter of whole blood exceeded that of their untreated partners (table 1). The difference was significant ($P < 0.01$).

DISCUSSION

Since DOP caused an increase in PALP in the 2 tissues examined, a conclusion regarding depletion of the body as a whole cannot be made. Thus, when DOP is fed, neither liver- nor leukocyte-PALP can be used to measure depletion. DOP, when fed to rats in the absence of vitamin B_6, had the same effect as INH in causing liver storage, but DOP caused more retention of PALP per million leukocytes than INH (1). A decrease in the number of lymphocytes caused by DOP would make it less desirable than INH for use in bringing about depletion of PALP.

Why DOP increased the concentration of PALP in the liver, is not known. Possibly DOP-phosphate occupied some of the sites on the apoenzymes which PALP would otherwise have occupied and the unutilized PALP accumulated, or possibly PALP which could not be used elsewhere in the body was carried to the liver. The concentration in the liver poses the question as to whether the stored PALP can be utilized when DOP is discontinued in the food. If so, a temporary remission in vitamin B_6-deficiency symptoms would occur. Such a remission was observed in DOP-treated rats: 2 weeks after removal of DOP from their diets, the severe acrodynia which had developed in 3 weeks of DOP treatment had regressed considerably.

ACKNOWLEDGMENT

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Dietary Control of Selenium Volatilization in the Rat [1,2]

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ABSTRACT Rats injected with single subacute doses of selenite (2.0 mg Se/kg) volatilized selenium in amounts that depended upon the diet fed previously. Volatilization was minimal, about 10% of the dose, with a good purified basal diet, and with certain crude diets. Volatilization could be increased two- to three-fold by adding 5 ppm of selenium to the basal diet, by increasing the protein and methionine content of the basal diet, or by feeding appropriate crude materials. These latter included 2 commercial rat feeds and a combination of whole wheat bread, milk and lettuce. The volatilizing activity in the commercial feed did not appear to reside solely in its content of selenium, nor of protein and methionine. A large number of nutrients and food materials tested separately appeared to be devoid of volatilizing activity, including linseed meal, a substance that minimizes the effects of chronic selenium poisoning in rats.

In a previous study the elimination of volatile selenium compounds in the expired air by rats injected with labeled sodium selenite was shown to vary with the nature of the diet fed prior to the injection (1). Volatilization was relatively low with a purified diet that would be considered nutritionally adequate under most other circumstances, but it was increased greatly when a crude commercial diet was fed. In the present study an extensive survey has been made for well-defined diets favoring volatilization. Methyl donors were tested since the volatile product is thought to be dimethyl selenide (2, 3). The effect of previous exposure to selenium was studied, because such exposure alters both the urinary excretion and the retention of physiologically active amounts of selenite (4). Data on other nutrients are also presented.

METHODS

A series of 19 experiments was carried out over a period of 12 months with only minor variations from the following procedure. Weanling male rats of the Holtzman strain, weighing 40 to 60 g, were distributed into groups of four or five and housed in individual wire cages. The experimental diets and distilled water were fed ad libitum for a 2-week period. Assay of the animals for ability to volatilize a subacute dose of labeled sodium selenite began with the heaviest animals on the fourteenth day and extended through the fifteenth or sixteenth day. Four rats from each group were injected with 2 μc of $Na_2{}^{75}SeO_3$ (2.0 mg Se/kg of body weight) in isotonic saline and placed in glass metabolism chambers (1). Lower doses of selenite were given in the initial series. Volatile radioactive compounds exhaled during the subsequent 10-hour collection period were trapped in 8 N nitric acid and counted in a well-type scintillation counter, as described previously (1). Volatilization was expressed as the percentage of the dose exhaled within 10 hours.

A negative control group, included in most of the experiments, received the purified basal diet described previously (1) which consisted of sucrose, 71; corn oil, 5; salts, 4; casein, 20; and generous amounts of vitamins. All experiments included a positive control group fed Purina Laboratory Chow,[4] or the basal diet supplemented with either 50% of ground Chow or with an active fraction of Chow. Substances to be tested for activity were

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added to the basal diet at the expense of sucrose unless otherwise indicated.

RESULTS

Purified diet vs. Chow. Table 1 summarizes the data on the positive and negative control rats from the series given 2.0 mg of selenite Se/kg. The details of the separate experiments are recorded elsewhere.[5,6] The rats fed substantial amounts of the crude diet volatilized a large portion of the injected selenite, an average of 27.1% of the dose; those fed the purified basal diet volatilized much less, an average of only 9.7%. The average of the coefficients of variability (standard deviations expressed as percentage of sample means) was larger for the negative controls (42%) than for the positive controls (16%). The characteristic responses to these diets were observed whether the selenite was given intraperitoneally or subcutaneously. However, in later experiments the subcutaneous rout was preferred because it avoided the possibility of an intraintestinal injection, which sometimes occurred in individuals fed the crude diet.[7]

Various crude diets. Animals fed the basal diet supplemented with 24% of linseed meal (expeller process) volatilized only 8% of a 2-mg dose of selenite Se/kg, whereas their respective positive controls volatilized 33%. In a survey of other crude diets fed ad libitum for two or more weeks, selenium volatilization was increased by some diets, whereas others were no more active than the purified diet used as the negative control (table 2). A diet of whole wheat bread, milk, and lettuce was fully adequate for selenium volatilization, in agreement with the results of Schultz and Lewis (5). A second commercial laboratory diet (Rockland)[8] was also equal to the positive control diet in promoting selenium volatilization. In contrast, the Steenbock stock diet (6), which contains ground yellow corn and linseed meal as major ingredients, was devoid of activity. Another natural diet consisting mainly of ground wheat, and similar to that used by Moxon and associates (7) in studies of selenium toxicity, also failed to increase volatilization appreciably. Similarly, a diet of whole milk fortified with iron and cop-

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[6] Levander, O. A. 1963 The effect of diet on the volatilization of selenium in the rat. M. S. Thesis, University of Wisconsin.

[7] See footnotes 5 and 6.

[8] Rockland D-free Rat Diet, A. E. Staley Manufacturing Company, Decatur, Illinois; plus vitamins A and D orally as for basal diet.

TABLE 1

Volatilization of injected selenite by rats fed crude or purified control diets

Exp. no.	Dosage of Se and route	Positive control diet	% of dose volatilized [1]	
			Positive control (crude)	Negative control (purified)
	mg/kg			
18	2.0 sc	Purina Chow	33 ± 4.3	11 ± 4.8
19	2.0 sc	Purina Chow	33 ± 2.7	14 ± 11.6
13	2.0 sc	Purina Chow	29 ± 4.8	7 ± 2.5
16	2.0 sc	Purina Chow	27 ± 2.7	8 ± 3.7
14	2.0 sc	Acetone residue [2]	26 ± 3.2	8 ± 2.7
15	2.0 sc	Acetone residue [2]	25 ± 2.8	12 ± 6.8
12	2.0 ip	50% Purina Chow [2]	30	—
6	2.0 ip	Purina Chow	28	12 ± 6.7
8	2.0 ip	Purina Chow	27 ± 0.4	5 ± 1.2
7	2.0 ip	50% Purina Chow	26 ± 6.6	11 ± 3.2
10	2.0 ip	50% Purina Chow	24 ± 3.3	9 ± 1.5
9	2.0 ip	50% Purina Chow	23 ± 7.8	—
11	2.0 ip	Acetone residue	21 ± 5.2	—
			Avg 27.1	Avg 9.7

[1] Percentage of radioactivity exhaled within 10 hours after injection of 2.0 mg Se/kg as $Na_2{}^{75}SeO_3$; each value is an average of 2 to 4 animals ± SD. Mean weight of positive control groups 130 to 158 g, average 140; for negative control groups 123 to 135 g, average 130.

[2] The negative control diet supplemented with 50% of acetone-extracted Purina Chow or Purina Chow itself at the expense of sucrose.

TABLE 2

Volatilization of injected selenite by rats previously fed various crude diets ad libitum

Exp. no.	Diet	Avg wt	% of dose volatilized [1]
		g	
8	Whole wheat bread, milk, and lettuce	129	26 ± 2.4 [2]
7	Rockland [3]	125	30 ± 1.8
18	Steenbock (6) [4]	125	10 ± 4.4
18	Moxon (7) [5]	124	12 ± 5.5
12	Mineralized milk [6]	138	14 ± 4.5

[1] The average reference values for positive and negative controls are 27.1 and 9.7%, respectively. Control values for each specific series are given in table 1.

[2] Average ± SD.

[3] Rockland D-free Rat Diet, A. E. Staley Manufacturing Company, Decatur, Illinois; plus vitamins A and D orally as for basal diet.

[4] Ground yellow corn, 400; soybean meal, 100; linseed meal, 150; alfalfa leaf meal, 30; iodized NaCl, 10; $Ca_3(PO_4)_2$, 10; skim milk powder, 200; and butter fat, 100.

[5] Crude casein, 100; Wesson salts (8), 10; Crisco (Procter and Gamble Co., Cincinnati), 30; halibut liver oil:corn oil (1:10 w/w), 15; brewer's yeast, 10; and ground wheat to 990.

[6] Included 0.5 mg Fe and 0.05 mg Cu (as $FeCl_3$ and $CuSO_4$, respectively) per rat daily in milk.

per permitted a good rate of growth but did not increase selenium volatilization compared with that of the negative control.

A number of natural substances added individually to the basal diet produced no more than a moderate increase in the ability of rats to volatilize selenite. These supplements, and the percentages in the diet at which they were fed, included liver powder 5; dried skim milk, 10; alfalfa leaf meal, 25; wheat germ, 10; fish meal, 5; sardine solubles, 5; brewer's yeast, 18; and Torula yeast, 40. In addition, supplementation of the basal diet individually with bread, milk, or lettuce or with pairs of these substances demonstrated that the activity of the bread-milk-lettuce combination was not present exclusively in any one of the 3 components.[9]

Activity of fractions. The activity of the crude positive diet was moderately labile (table 3). Dry heating caused some loss in the activity of Purina Chow, as did autoclaving at pH 3, 7, or 9, although substantial activity remained in all cases. The dry ash of Chow was completely inactive. These results suggested that organic substances were associated with the effectiveness of the crude diet in promoting selenium volatilization. Preliminary fractionation experiments indicated that the volatilization-promoting factors in Purina Chow were not extracted by acetone. Extracts and residues of water or 50% aqueous ethanol fractionations had only partial activity; recombination of the aqueous ethanol extract and residue, however, appeared to restore full activity.[10]

Stimulatory nutrients. Supplementation of the negative control diet with large amounts of methionine or casein (table 4) increased the volatilization of selenium, but the values for individual animals were highly variable and the average never exceeded 71% of the appropriate positive control. A tenfold increase in the level of water-soluble vitamins (line 5) had little

[9] See footnotes 5 and 6.

[10] See footnote 6.

TABLE 3

Stability of the Se-volatilizing factor in an active crude diet

Exp. no.	Treatment	Chow equivalent [1]	Avg wt	% of dose volatilized [2]
			g	
9	Heated 48 hours at 110°	50	148	17 ± 3.4 [3]
12	Autoclaved [4] at pH 3	50	148	18 ± 4.4
12	Autoclaved at pH 7	50	147	18 ± 6.9
12	Autoclaved at pH 9	50	158	21 ± 4.2
4	Ashed 12 hours at 600°	100	150	9 ± 2.3 [5]

[1] Grams of Purina Laboratory Chow represented by fraction added, per 100 g of diet; all fractions were added to the negative control diet at the expense of sucrose.

[2] Specific positive and negative controls for these experiments are shown in table 1. They average 27.1 and 9.7%, respectively.

[3] Average ± SD.

[4] 750 g of ground Chow was autoclaved 1 hour at 120° in 3 volumes of distilled water; pH adjusted with HCl or NaOH.

[5] Dose of selenium was 1.5 mg Se/kg, instead of 2 mg/kg; percentage volatilization by appropriate controls averaged 20 ± 1.4 and 9 ± 5.2 for positive and negative controls, respectively.

effect on volatilization ability. The presence of 5% of cellulose in the diet or the replacement of sucrose with dextrin (lines 6 and 7) likewise failed to increase volatilization.

Active fortified diets. Attempts to produce additive effects with more elaborate combinations of nutrients (table 5) were only partially successful, and included a variant of the basal diet which contained an additional 0.5% of methionine, tenfold levels of the water- and fat-soluble vitamins, and 0.25% of ascorbic acid (line 1). But when the latter diet was supplemented with an additional 16% of casein (total 36%), a fully active diet was produced (line 2). The substitution of dextrin for sucrose was not a contributing factor in the active diets (table 5, line 9 vs. 2). The activity of the high casein-methionine combination was confirmed in the next experiment (line 3) and was also observed with a slightly modified diet in a third experiment.

The omission of individual components from these so-called "fortified basal diets" indicated that the extra vitamins were not essential for the volatilizing effect (lines 6–8), whereas the omission of either the methionine (line 4) or the "extra" 16% of casein (line 5) eliminated much of the response. Volatilization was improved somewhat by the substitution of sucrose for dextrin in the active combination (line 9 vs. line 8).

Effect of prefeeding selenium. Rats fed 5 ppm of selenium as sodium selenite in our negative control diet for 2 weeks

TABLE 4

Activity of certain nutrients in promoting selenium volatilization

Line no.	Exp. no.	Supplements to negative control diet [1]	Avg wt	% of dose volatilized [2]
			g	
1	18	DL-Methionine,0.5%; pyridoxine·HCl, 45 mg/kg	132	17 ± 4.9 [3]
2	11	DL-Methionine, 1% to 0.5%; [4] vitamin mix, 1.8%	136	14 ± 5.2
3	13	Casein, 16%	131	20 ± 7.7
4	18	Casein, 16%; pyridoxine·HCl, 45 mg/kg	135	23 ± 7.9
5	6	Vitamin mix, 1.8%	138	10 ± 7.5
6	15	Cellulose,[5] 5%	120	10 ± 3.7
7	13	Dextrin, 70.7%	133	14 ± 2.4

[1] All supplements added at the expense of sucrose.
[2] Reference values for positive and negative controls for these series are in table 1; they average 27.1 and 9.7%, respectively.
[3] Average ± SD.
[4] Level of added methionine was decreased from 1% to 0.5% on eighth day of feeding.
[5] Cellu Flour, Chicago Dietetic Supply House, Chicago.

TABLE 5

Volatilization of injected selenium by rats fed fortified casein diets

Line no.	Exp. no.	Supplements to negative control diet [1]						Avg wt	% of dose volatilized [3]
		Methionine	Casein	Vitamin mix	Fat-soluble vitamins [2]	Ascorbic acid	Dextrin		
		%	%	%		%	%	*g*	
1	13	0.5	—	1.8	+	0.25	—	136	20 ± 9.3 [4]
2	15	0.5	16	1.8	+	0.25	70.7	134	30 ± 3.3
3	16	0.5	16	1.8	+	0.25	70.7	132	26 ± 3.4
4	16	—	16	1.8	+	0.25	70.7	136	14 ± 1.8
5	16	0.5	—	1.8	+	0.25	70.7	132	20 ± 7.8
6	16	0.5	16	—	+	0.25	70.7	128	28 ± 9.6
7	16	0.5	16	1.8	—	0.25	70.7	129	32 ± 6.7
8	16	0.5	16	1.8	+	—	70.7	132	29 ± 4.7
9	16	0.5	16	1.8	+	0.25	—	126	39 ± 8.6

[1] All supplements were added at the expense of sucrose.
[2] Plus sign indicates that each animal received a total of 20 drops/week (10 × normal amount) of the fortified halibut liver oil (1).
[3] Reference values for positive and negative controls are in table 1; they average 27.1 and 9.7%, respectively.
[4] Average ± SD.

showed a marked increase in their ability to volatilize a subsequent dose of labeled selenite (table 6). Two such groups volatilized an average of 24 and 30% of the dose, corresponding to 96 and 100% of the volatilization by their respective positive controls. Thus it was apparent that volatilization could be increased by the simple expedient of feeding selenite in the negative control diet for a 2-week period, without the addition of the organic factors that enhanced volatilization when low selenium diets were fed (tables 4 and 5). Rather large amounts of selenite were necessary to produce this effect; an additional 0.5 ppm of this form of selenium had only a slight effect, whereas 3 ppm produced an intermediate response. Selenite was able to produce an additional response even when added to a fortified basal diet which was already fully as active as the positive control, resulting in one case in a volatilization of 48% of the dose (table 6, line 8) the highest observed in any of our experiments to date.

The prefeeding of 5 or 10 ppm of tellurium, another group VI element volatilized by animals (9), did not increase the volatilization of selenium. Molybdenum, which is not a component of the modified Wesson salt mixture used in the basal diet, did not increase selenium volatilization at the 5-ppm level. However, some effect was noted when the basal diet was supplemented with a combination of 1 ppm of selenium and 5 ppm of molybdenum.

Selenium content of diets. The effectiveness of dietary sodium selenite in stimulating the volatilization of a subsequent dose of ^{75}Se raised the question whether the enhanced volatilization obtained with crude or fortified basal diets might not have depended upon their content of selenium. Accordingly 3 to 6 samples of the negative control diet and of the fortified basal diets were analyzed by the micro-colorimetric method of Kelleher and Johnson (10) and by neutron activation analysis in the University of Wisconsin reactor;[11] Purina Chow was analyzed by a semi-micro modification of the Kelleher and Johnson procedure and also by activation analysis. An average value of approximately 0.05 ppm was obtained for the fortified basal diet (colorimetric method = 0.04 ± 0.01 ppm; activation analysis = 0.07, 0.07 ppm) and 0.5 ppm for Purina Chow (colorimetric method = 0.49 ± 0.02 ppm; activation analysis = 0.46, 0.56 ppm). The negative control diet contained only 0.03 ppm (colorimetry

[11] See footnote 6.

TABLE 6

Effect of prefeeding selenite and certain other minerals on the volatilization of injected selenite [1]

Exp. no.	Addition to negative control diet	Se, Te, or Mo	Avg wt	% of dose volatilized [2]
		ppm	*g*	
12	Na_2SeO_3	0.5	142	14 ± 4.2 [3]
18	Na_2SeO_3	0.5	131	13 ± 6.3
19	Na_2SeO_3	1.0	128	10 ± 4.4
13	Na_2SeO_3	3.0	129	19 ± 10.9
15	Na_2SeO_3	3.0	120	22 ± 8.0
12	Na_2SeO_3	5.0	110	30 ± 4.9
15	Na_2SeO_3	5.0	103	24 ± 3.5
16	Na_2SeO_3 (fortified diet) [4]	5.0	128	48 ± 4.2 [4]
15	K_2TeO_3	5.0	121	11 ± 3.1
19	K_2TeO_3	10.0	117	15 ± 8.6
19	Na_2MoO_4	5.0	135	10 ± 3.4
19	Na_2MoO_4 + Na_2SeO_3	5.0 + 1.0	130	18 ± 8.7

[1] Diets fed for 2 weeks; injected selenite = 2.0 ^{75}Se/kg.
[2] Positive and negative controls averaged 27.1 and 9.7%, respectively; see table 1 for values in the specific series.
[3] Average ± SD.
[4] The fortified diet contained 36% casein, 0.5% methionine, and extra vitamins (table 5, line 3); volatilization in the absence of pre-fed Se was 26%.

= 0.02 ± 0.01 ppm; activation analysis = 0.04, 0.05 ppm). The amounts of selenium in the active diets, crude or fortified, were therefore much less than the 3 ppm that produced a marginal response when added to the basal control diet (table 6, lines 4 and 5).

DISCUSSION

The present results demonstrate clearly the existence of at least 2 independent ways by which diet can enhance the ability of the rat to volatilize injected selenite. One is to pre-feed the animal with 3 to 5 ppm of selenium. Another is to feed a low selenium diet high in casein and methionine. That there may be a third way is suggested by the fact that rats volatilize selenite very well when fed certain crude diets which contain only moderate amounts of selenium, protein, and methionine — levels insufficient by themselves to enhance volatilization. Such diets lose activity under treatments too mild to cause any substantial destruction of these nutrients (table 3). It is possible, however, that some forms of dietary selenium are more active than others in inducing the metabolic changes that enable a rat to volatilize a subsequent challenging dose of selenite. Factor 3-selenium and certain organic forms of this mineral are about 3 times as active as selenite in the alleviation of deficiency symptoms (11). But if the activity of Purina Chow under our conditions were to be ascribed solely to its selenium content, then the selenium in the Chow would have to be at least 10 times (5.0 ppm selenite/0.5 ppm in Chow) as active as selenite-Se. The possibility of a decomposition of selenite in the diet as suggested by Shrift (12) likewise does not appear to be of sufficient magnitude to account for the discrepancy in the activities of the 2 diets.

It is known that appropriate diets may enhance the ability of animals to resist levels of selenium that cause symptoms of chronic toxicity. Higher levels of protein tend to increase resistance, and linseed meal or concentrates thereof are particularly effective (13). However, under our conditions, linseed meal failed to enhance the volatilization of single sublethal doses of selenite.

Since the conversion of selenite to volatile selenium takes place largely in the liver,[12] it appears that the overall dietary effects described in this study are essentially an expression of effects of diet upon liver enzymes.

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[12] See footnotes 2 and 5.

Distribution of Microgram Quantities of Selenium in the Tissues of the Rat, and Effects of Previous Selenium Intake [1,2]

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ABSTRACT Small doses of radioselenium were injected as selenite into rats previously fed one of 3 types of low selenium diets for 2 weeks, or into rats fed these diets supplemented with graded levels of selenite up to 5 ppm of selenium. The rats fed the low selenium diets retained over 50% of such doses in their carcasses after 24 hours, and they excreted only 10 to 20% of the dose in the urine. Urinary excretion of the labeled dose increased progressively with previous selenium intake to 47% by rats fed the most selenite, and retention of the labeled dose decreased correspondingly in the carcass. Neither the general character of the basal diet, nor the presence of vitamin E altered this inverse relationship. But in contrast with the rest of the carcass, the liver tended to retain a relatively constant percentage of the labeled single dose, despite previous selenium intake.

Other studies in this series deal with dietary factors which can alter the distribution of rather large doses of selenium in the rat (1–3). The question arose whether similar differences could be obtained when the very small amounts of selenium that produce desirable physiological responses (4–6) were used. Data on the distribution of subtoxic amounts of ^{75}Se in the tissues of rats and other animals are available (7–9), but relatively little is known regarding the metabolic fate of very small amounts of selenium. The present study concerns the effect of previous selenium intake on the distribution of microgram quantities of labeled selenium injected into rats.

PROCEDURE

Three diets were used in these studies: a semipurified Torula yeast diet deficient in selenium, a purified diet containing 20% casein (1), and a crude diet of commercial laboratory chow.[4] The Torula yeast diet contained the following ingredients: (in grams per kilogram) sucrose, 510; Torula yeast,[5] 400; stripped lard,[6] 50; $CaCO_3$, 23.5; Torula salts,[7] 16.5. All animals also received 2 drops of ADK solution [8] per week. The crude diet was ground to pass through a medium screen of the Wiley mill. Sodium selenite was premixed with sucrose and added to the diets at levels of 0.1, 1.0 and 5.0 ppm of selenium; additions to the purified diets were made at the expense of sucrose, additions to laboratory chow at the expense of chow. The highest level of selenium used is sufficient to cause a chronic selenosis in rats.

Male weanling rats of the Holtzman strain were used exclusively except for an experiment with "pre-depleted" weanlings which were obtained from pregnant rats fed the Torula yeast diet during gestation and lactation. The rats were housed individually in wire-bottom cages and were

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[2] Further details are recorded in a Ph.D. thesis entitled, "Contrasting nutritional responses to vitamin E and selenium." L. L. Hopkins, University of Wisconsin, 1962.

[3] Present address: Division of Nutrition, Food and Drug Administration, Washington, D. C.

[4] Purina Laboratory Chow, Ralston Purina Company, St. Louis.

[5] Food grade; Lake States Yeast Corporation, Rhinelander, Wisconsin.

[6] Distillation Products Industries, Rochester, New York.

[7] Salt mixture used for diets high in Torula yeast: NaCl, 67.05; KCl, 26.82; MgO, 2,68; ferrous phosphate, 2.95; KIO_3, 0.295; $MnSO_4 \cdot H_2O$, 0.174; and $CaCl_2 \cdot 6H_2O$, 0.027.

[8] ADK solution contained 4 mg of menadione/ml of halibut liver oil (Haliver oil, Abbott Laboratories, North Chicago, Illinois).

given their diets and distilled water ad libitum. Following a 2-week feeding period, the rats were injected intraperitoneally with a single dose of radioselenite [9] containing approximately 3 μc of ^{75}Se; appropriate additions of carrier selenite were made to the various dose solutions. Then the animals were placed in all-glass metabolism chambers which were equipped with sintered-glass columns filled with 8 N HNO_3 for trapping the volatile selenium compounds exhaled by the rats (1).

After a 24-hour collection period, the rats were stunned and decapitated and a 1-ml sample of blood was taken. The various organs were removed and placed in tubes or beakers. The contents of the gastrointestinal tract were flushed out by washing with 0.9% NaCl and stripping 3 times; the feces were added to the intestinal contents. All tissue fractions, including the carcass, were digested in 20% KOH (a few KOH pellets were added to the saline-gastrointestinal mixture). The samples were allowed to stand for 1 or 2 days, about 100 ml of 95% ethanol were added to the carcass, and the mixtures incubated at 50° until the solutions became clear. All samples were made to appropriate volumes, and 2-ml aliquots were taken for counting in a Baird-Atomic well-type scintillation counter. The data are expressed as percentages of the dose given.

RESULTS

Distribution of injected selenium. The distribution of various small doses of radioselenite in the tissues of depleted rats fed the Torula yeast [10] diet is summarized in table 1. Urinary excretion of selenium was greater by the rats receiving 1 μg of selenium than by the rats receiving 0.25 μg of selenium or less; 16% of the dose was excreted by the former vs. only 5% by the latter. Increased urinary output was accompanied by a moderate decrease in the percentage retention of selenium in the carcass. Substantial amounts of selenium were noted in the liver, kidneys, testes and blood, and the percentages of the doses retained tended to be rather constant over the entire range of doses administered.

Effect of previous intake of dietary selenium. Both the amounts of radioselenite retained in the carcass and the amounts excreted in the urine appeared to depend upon the level of selenium that had been added to the diets fed previously. Marked, progressive decreases in the percentage of the ^{75}Se retained in the carcass occurred as the level of previous selenium intake increased, and these decreases in carcass retention were accompanied by more or less corresponding increases in the urinary output of selenium (table 2). For example, the retention of ^{75}Se in the carcasses of rats fed the yeast diet unsupplemented with selenium was more than 50% of the dose and only about 15% was excreted in the urine. These figures were essentially reversed when

[9] Oak Ridge National Laboratory, Oak Ridge, Tennessee.

[10] The Torula yeast contained 0.04 ppm selenium, the basal diet, 0.05 ppm, and the crude diet 0.5 to 0.7 ppm (9).

TABLE 1

Percentages of small doses of $Na_2{}^{75}SeO_3$ in rats 24 hours after injection [1]

Radioselenium injected [2]	% of dose recovered			
	1.0 μg/100 g	0.25 μg/100 g	0.025 μg/100 g	1.0 g/100 g
	%	%	%	%
Urine	16.4 ± 0.68	5.3 ± 0.59	5.0 ± 0.80	14.8 ± 1.6
GI contents and feces	4.8 ± 0.32	3.4 ± 0.51	4.2 ± 0.74	7.7 ± 0.78
Carcass	60.7 ± 1.2	70 ± 0.34	68 ± 2.2	52.4 ± 2.3
Liver	7.0 ± 0.29	7.9 ± 0.24	6.5 ± 0.62	6.4 ± 0.16
Kidneys	6.3 ± 0.40	7.5 ± 0.40	6.6 ± 0.28	5.4 ± 0.20
Blood (1 ml)	1.6 ± 0.12	1.8 ± 0.11	1.6 ± 0.10	1.3 ± 0.11
Testes	3.7 ± 0.35	5.9 ± 0.33	6.3 ± 0.53	—
Wt gain in 2 weeks, g	40 ± 3.7	39 ± 3.4	40 ± 4.0	42 ± 6.8

[1] Percentages are means ± SE; rats were fed Torula yeast diet; 4 to 5 rats/group.

[2] Columns 1–3 represent experiments performed simultaneously; column 4, data from a later experiment.

TABLE 2

Effect of previous level of dietary selenium upon the distribution of a subsequent injected dose of 1 μg of ^{75}Se in rats

Dietary Se	Avg gain	% of injected dose					
		Carcass	Urine	Feces and GI contents	Liver	Kidney	Blood (1 ml)
ppm	*g/2 weeks*	%	%	%	%	%	%
		Series 1, Torula diet					
0	42 ± 6.8 [1]	52 ± 2.3	15 ± 1.6	7.7 ± 0.78	6.4 ± 0.16	5.4 ± 0.20	1.3 ± 0.11
0.1	44 ± 4.8	37 ± 1.5	22 ± 1.5	9.3 ± 1.1	7.9 ± 0.50	4.1 ± 0.21	1.1 ± 0.12
1.0	40 ± 5.2	27 ± 0.55	39 ± 1.0	8.0 ± 0.39	6.8 ± 0.15	3.0 ± 0.07	0.79 ± 0.07
5.0	26 ± 3.4	16 ± 0.66	49 ± 0.86	9.4 ± 0.71	5.5 ± 0.23	2.1 ± 0.11	0.42 ± 0.03
		Series 2, Torula diet, pre-depleted weanlings [2]					
0	18 ± 5.7	51 ± 2.0	8.3 ± 1.4	24 ± 2.7	9.1 ± 1.3	8.5 ± 0.42	2.3 ± 0.22
0 + E [3]	37 ± 6.0	52 ± 2.7	11 ± 1.9	16 ± 0.55	7.3 ± 0.75	6.8 ± 0.66	1.9 ± 0.22
0.1	35 ± 3.0	42 ± 1.0	24 ± 2.4	14 ± 0.65	9.9 ± 0.34	6.0 ± 0.28	2.1 ± 0.26
1.0	30 ± 2.0	27 ± 2.7	43 ± 1.3	12 ± 0.82	7.8 ± 0.32	4.0 ± 0.23	1.5 ± 0.08
5.0	27 ± 3.0	16 ± 0.93	53 ± 5.6	12 ± 2.4	8.2 ± 0.73	5.0 ± 1.5	0.79 ± 0.06
		Series 3, Casein diet					
0	81 ± 4.2	67 ± 1.3	15 ± 0.75	4.6 ± 0.29	8.8 ± 0.31	5.9 ± 0.18	1.2 ± 0.05
0.1	72 ± 6.0	49 ± 1.2	27 ± 0.87	7.0 ± 0.26	10 ± 0.37	4.9 ± 0.10	1.0 ± 0.06
1.0	75 ± 5.6	35 ± 0.53	43 ± 2.1	7.5 ± 0.93	8.8 ± 0.33	3.8 ± 0.17	0.63 ± 0.04
5.0	41 ± 1.4	20 ± 1.6	37 ± 2.8	13.8 ± 3.2	11 ± 2.8	11 ± 3.7	0.47 ± 0.03
		Series 4, Crude diet					
0	87 ± 5.2	33 ± 0.95	53 ± 1.0	8.2 ± 1.6	6.6 ± 0.27	3.1 ± 0.14	0.58 ± 0.02
0.1	82 ± 4.0	33 ± 0.76	52 ± 1.8	7.9 ± 0.96	6.1 ± 0.30	3.1 ± 0.15	0.58 ± 0.08
1.0	88 ± 5.6	27 ± 0.28	61 ± 0.97	6.6 ± 0.86	5.5 ± 0.27	2.7 ± 0.06	0.44 ± 0.02
5.0	81 ± 3.0	19 ± 0.20	64 ± 2.2	7.7 ± 0.77	6.3 ± 0.97	2.5 ± 0.41	0.28 ± 0.03

[1] Mean ± SE; 4 to 5 rats/group.
[2] Gastrointestinal tract included with contents and feces.
[3] Received 20 mg α-tocopherol/week/rat.

5 ppm of selenium as selenite were added to this diet and fed for a 2-week period: carcass retention decreased to 16% of the dose, whereas urinary excretion increased to over 50% of the injected selenium. Intermediate levels of dietary selenium intake resulted in intermediate effects on the retention and excretion of injected radioselenite. This ability of previously fed dietary selenite to alter the fate of a subsequent dose of $Na_2{}^{75}SeO_3$ was observed with all 3 diets (table 2).

The percentage retention of ^{75}Se in the blood and kidneys appeared to parallel that in the rest of the carcass, but was relatively constant in the liver in all series (table 2) and did not appear to depend on the previous dietary intake of selenium. The gastrointestinal excretion of selenium had no obvious dependence upon the previous level of selenium intake when the yeast (series 1 and 2) or the crude (series 4) diets were fed. With the casein diet (series 3), elimination into the intestinal contents increased from 4.6% for the group receiving no added selenium to 14% for the group receiving 5 ppm of selenium in the diet. Exhalation of volatile selenium compounds in the expired air generally was less than 1% and never exceeded 2% of the dose when these very low levels of selenium were injected; selenium volatilization was not increased under these conditions by feeding the crude diet, as it is when larger amounts of selenium are injected (1).

Effect of basal diet. Vitamin E supplementation had little effect upon the distribution of ^{75}Se although it permitted a good rate of growth and prevented the deficiency symptoms observed in the unsupplemented lot (table 2, series 2). The severely deficient lot lost weight during the latter part of the 2-week feeding period, and 2 animals died near the end of the collection period. Comparison of the pre-depleted weanling lots (series 2) and the commercial weanling lots (series

1) revealed a slightly greater retention of the ^{75}Se in the organs of the deficient animals. The largest carcass retentions of radioselenium were observed in the animals fed the casein diet.

Supplementation of the crude diet with 0.1 ppm of selenium failed to alter the distribution of the injected ^{75}Se, presumably because of the relatively high selenium content of the crude diet itself (0.5 ppm) as compared with 0.03 to 0.05 ppm in the Torula or casein diets (3). The retention of radioselenium in the carcasses of the rats fed the unsupplemented crude diet was similar to that observed in the animals fed the purified diets supplemented with 1 ppm of selenium. As in certain studies cited by Ganther and Baumann (1), added selenite appeared to be less toxic in the crude diet than in the purified diet; this was true even though the unselenized crude diet contained more selenium than the purified diets. The growth of animals fed the crude diet plus 5 ppm selenium was depressed only slightly, whereas a similar addition of selenium to the purified diets reduced growth by about 50%. The addition of 5 ppm of selenium in the casein diet also caused mottled livers in the animals fed this diet.

Recoveries and irregularities. The total recoveries of injected ^{75}Se in the various groups fed the purified diets were 82 to 90% of the dose in series 1, 95 to 102% in series 2, and 95 to 102% in series 3. Recoveries with the crude diet ranged from 102 to 105%. In the course of these studies, the amounts of ^{75}Se recovered in the gastrointestinal contents of certain individual rats were occasionally very high, from 2 to 10 times those for the remaining rats in their respective group. These high values appeared to be due to error in technique, some of the radioselenite having been injected into the intestinal lumen. Accordingly, these values were not included in the averages recorded here, although they are tabulated elsewhere.[11]

Arsenic, cadmium, zinc, thyroxine. Earlier work demonstrated that both arsenic and cadmium could have marked effects on the metabolism of subacute doses of selenium (1), but neither of these agents had any influence on the distribution of the microgram quantities of selenium used in the present studies. Arsenic and cadmium were injected intraperitoneally on each of 3 days prior to dosage with ^{75}Se; the amounts administered were 50 μg As/day as $NaAsO_2$ and from 63 to 250 μg Cd/day as $CdCl_2$. Three hours after the last injection of cadmium or arsenic, the rats were injected with from 0.025 to 1.0 μg Se/100 g as $Na_2{}^{75}SeO_3$. The average values for ^{75}Se in the testes from cadmium-treated animals were considerably lower than for untreated animals, probably as the result of injury to these organs; otherwise, very little difference was observed in the distribution of radioselenium as a result of arsenic or cadmium injection.[12] Also, similar administration of zinc chloride at a level of 500 μg Zn/day or the feeding of the Torula yeast diet containing 50 mg of thyroxine plus 0.1 mg vitamin B_{12} per kg of diet for 3 weeks did not cause any appreciable changes in the distribution of a subsequent dose of injected radioselenite.

DISCUSSION

These studies demonstrate that the retention of microgram doses of injected radioselenite can be decreased in the carcass of rats by the previous ingestion of dietary selenium, and that the decreases in carcass retention are largely the result of enhanced urinary excretion. But whereas the retention of selenium in the blood and kidneys showed a dependence on previous selenium intake similar to that of the rest of the carcass, the percentage retention of ^{75}Se in the liver was relatively constant for any given diet and did not appear to depend upon the level of selenium fed previously. This suggests a metabolic difference between the liver and the rest of the body as far as selenium is concerned. It may be significant that gross abnormalities of the liver develop both in selenium toxicity (hob-nailed cirrhosis) and in selenium-vitamin E deficiency (necrosis). The results are consistent with a relatively rapid turnover of selenium in the liver, a conclusion

[11] See footnote 2.
[12] See footnote 2.

TABLE 3

Percentages of injected selenium eliminated by the rat in various ways

Dose injected	Diet	Urine	Feces and GI contents	Air	Reference
mg/kg		%	%	%	
2.0	Purified	12	7	21	(1)
1.5	Purified	14	11	8	(1)
0.01	Purified	15	4.6	0.29	table 2
0.00025	Purified	5.0	4.2	—	table 1
2.0	Crude	14	7	39	(1)
1.5	Crude	16	10	17	(1)
0.01	Crude	53	8.2	0.65	table 2

reached by McConnell (8) and by Heinrich and Kelsey (9) from data on the rate of elimination of selenium from the liver.

In contrast with the marked dependence of urinary selenium upon the level of previous intake of this element, the proportion of the dose appearing in the urine did not appear to vary much with the size of the test dose. Table 3 summarizes data on the elimination of single doses of selenium via 3 pertinent pathways. The data represent negative controls not previously exposed to selenium but injected with selenite at 0.01 mg of Se/kg of body weight (table 2). Data for much higher levels of injection, 1.5 to 2.0 mg/kg are taken from a previous study (1). The proportion of the test dose appearing in the urine was remarkably constant over a 200-fold range in the size of the test dose. An apparent exception, 53% of the small test dose excreted in the urine of rats fed the crude diet, is ascribed to the selenium content of the crude diet, 0.5 to 0.7 ppm [13] which supplied about 5 times as much selenium daily as the single test dose. The proportion of the test dose excreted into the gastrointestinal tract likewise did not vary much over the 200-fold range of the test dose.

In agreement with the present results, Jensen et al. (11) have reported that vitamin E had no effect on the distribution of selenium in chicks, but that the feeding of selenium itself had a marked effect on the uptake and retention of radioselenium.

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[13] See footnote 10.

Determination of Optimal Dietary Protein Requirements of Young and Old Dogs [1]

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ABSTRACT The purpose of these investigations was to determine the optimal nitrogen intake that would produce maximal filling of the protein reserve of the animal. Data were obtained which indicate that the present method of calculating protein maintenance requirements as 2 mg of nitrogen/basal kcal is considered to be endogenous nitrogen and may not adequately fill the "protein reserves" of the animal. Both young dogs (one-year-old) and old dogs (12 to 13 years of age) can be placed in nitrogen equilibrium and maintained with 0.2 to 0.6 g casein nitrogen/day/kg of body weight. Liver and muscle protein to DNA ratios reached maximal values in young dogs that were fed 0.4 g of casein nitrogen/day/kg of body weight, whereas the older animals required 0.6 g of nitrogen to obtain optimal filling of the protein reserves. Under these conditions, the dogs cannot be placed in positive nitrogen balance by feeding more dietary protein. Liver and muscle RNA to DNA values are closely correlated with changes in protein to DNA levels. When all dogs reached nitrogen equilibrium when fed 0.5 g of casein nitrogen/day/kg, higher cellular RNA concentrations and rate of incorporation of radioactive leucine into tissue protein were obtained in the liver and muscle of young adults as compared with the older animals. A kinetic model was developed which can be used to explain the change in tissue protein metabolism that is associated with variations in the intake of dietary nitrogen.

Data have been obtained in our laboratories to demonstrate that feeding 0.6 g of casein nitrogen/day/kg of body weight (g/day/kg) will maximally fill the protein reserves of the adult dog (1, 2). Under these conditions the dog was most resistant to the toxic effects of such drugs as the phosphoramides (used in chemotherapy of cancer) and 2-aminofluorene (a carcinogen) (3, 4). This intake of casein is considered by many workers to be greater than is needed to maintain an adult dog in nitrogen equilibrium and in good health. It is customary to calculate the need for maintenance for both man and animals as equivalent to 2 mg of nitrogen/basal kcal (5, 6). Feeding this amount of nitrogen, which is equivalent in the dog to approximately 0.15 g of casein nitrogen/day/kg of body weight, produced animals that appeared clinically to be in good condition and that were, in fact, in nitrogen equilibrium (1). They were, however, much more susceptible to the toxicity of the drugs (3, 4). Although it is possible to maintain nitrogen equilibrium by feeding as little as 0.15 g of casein nitrogen/day/kg of body weight, in so doing the animal is being maintained in a depleted state with markedly reduced protein reserves. This suggests that the maintenance of nitrogen equilibrium is not an adequate criterion to measure the protein requirements of an individual. In fact, some excess nitrogen in the diet may be necessary to develop maximally cellular proteins in various tissues (7). Large excesses, however, may be harmful, particularly to animals with certain pathologies such as kidney damage (8, 9). There is, therefore, need to determine the optimal intake of dietary protein that is necessary to maintain adequate reserves and optimal health in adult dogs.

Recent data have indicated that the total essential amino acid nitrogen which is required for nitrogen balance is greater in men over 50 years of age than in young adults (10–13). Thus, in the present

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study, young dogs (one to two years of age) and older dogs (12 to 13 years) were used to evaluate the level of dietary protein which is necessary to maintain adequate protein reserves.

EXPERIMENTAL METHODS

Dietary regimens and care of young adult dogs. Nine- to twelve-month-old Beagles, that had been maintained with a commercial diet, were divided into 2 groups containing 6 littermates in each group. Biopsy samples of liver, muscle and serum were collected from each dog and stored for chemical analysis. All dogs were fed agar-gel diets (4) which supplied 70% of the calories as varying amounts of casein, sucrose, dextrose and dextrin (carbohydrate sources in a 1:3.75: 1.8 ratio, respectively); 30% of the calories as lard; Wesson salt mixture (14); agar, and a vitamin mixture. The vitamin mixture contained per kilogram of diet: (in milligrams) thiamine, 2.0; riboflavin, 1.6; niacin, 16.0; Ca pantothenate, 13.0; pyridoxine, 1.0; folic acid, 0.6; biotin, 0.6; α-tocopherol, 30; and (in micrograms) 2-methyl-naphthoquinone, 2.6; vitamin B_{12}, 28; and (in grams) choline chloride, 1.0; and vitamin A, 55,000 units and vitamin D, 11,000 units. All dogs were fed these diets at 80 kcal/kg of body weight/day. The dogs in one group (A) were first fed a diet which supplied 0.3 g of nitrogen/day/kg of body weight (9.4% of the calories from protein). When the dogs were initially fed this diet and at 4 weeks after feeding the diet, 4-day specimens of urine and feces were collected and analyzed for their nitrogen content. After 5 weeks of feeding this diet, biopsy samples of liver, muscle and serum were collected from all dogs and stored for chemical analysis. Similar experimental periods of urinary and fecal collections and biopsies were followed on all subsequent dietary regimens. One week after the biopsy samples were taken, the dogs were fed a diet which supplied 0.6 g N/day/kg (18.8% of the calories from protein). This regimen was then followed by an 8-week experimental period in which the animals were fed diets which supplied 0.5 g N/day/kg (15.6% of the calories from protein), 8 weeks of feeding 0.4 g N/day/kg (12.4% of the calories from protein), and 4-week feeding experiments with diets which supplied 0.2 g N/day/kg (6.2% of the calories from protein) and 1.2 g N/day/kg (37.6% of the calories from protein).

The second group of dogs (B) was initially fed a diet which supplied 0.4 g N/day/kg. After biopsy, the animals were fed a diet which supplied 0.5 g N/day/kg. This regimen was followed by 8-week experimental periods in which the dogs were first fed a diet which supplied 0.6 g N/day/kg and then 0.4 g N/day/kg, and 4-week experiments with diets which initially supplied 0.6 g N/day/kg and then 0.15 g N/day/kg (4.7% of the calories from protein).

Dietary regimens and care of older dogs. Six Beagle dogs, (group C), that were 12 to 13 years old, were placed on experimental dietary regimens which were characterized by feeding 80 kcals/kg of body weight/day of an agar-gel diet (4) which contained a varying amount of nitrogen from casein, by initial and 4-week urinary and fecal collection and by initial and terminal biopsy samples from liver, muscle, and serum. To determine what nitrogen intake of casein was necessary for optimal filling of the protein reserves, dogs were first given a diet which supplied 0.3 g N/day/kg. After biopsy, the animals were given a dietary intake of 0.4 g N/day/kg, and this regimen was followed for 8 weeks by a diet which supplied 0.6 g N/day/kg. This regimen was followed by experimental periods in which the dogs were fed diets that supplied 0.2 g N/day/kg, 0.4 g N/day/kg for 16 weeks, 0.6 g N/day/kg for 8 weeks and 0.5 g N/day/kg for 4 weeks.

For both experiments, weekly body weights were recorded for each dog. All animals were housed at a temperature of 22 $\pm$ 1°, with a relative humidity of 35%. The animal quarters were illuminated from 6 AM to 6 PM.

Biopsy procedure and analyses. Dogs were anesthetized with sodium thiamylal (25 mg/kg), 0.5- to 1.0-g, samples of liver were removed by means of an abdominal laparotomy, and similar samples of semitendinosus muscle were taken by means of a laparotomy of the hind leg. The tis-

sues from the liver and muscle of each dog were homogenized into a suspension with 4 ml of ice-cold distilled water. As described previously (15), this suspension was used to determine the concentration of total protein, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). All urinary and fecal samples were collected for 4 days and were analyzed for nitrogen by means of micro-Kjeldahl procedure.

When young and old adult dogs had obtained nitrogen equilibrium with 0.4 g of casein nitrogen/day/kg, each animal received, at 2 hours before biopsy, an intravenous injection of 0.05 mc/kg of DL-leucine-4,5-^{3}H (1.52 μg/kg). Radioactivity of tissue proteins was determined on the KOH extract as described previously (15). Samples of liver and muscle homogenates were used to measure tissue

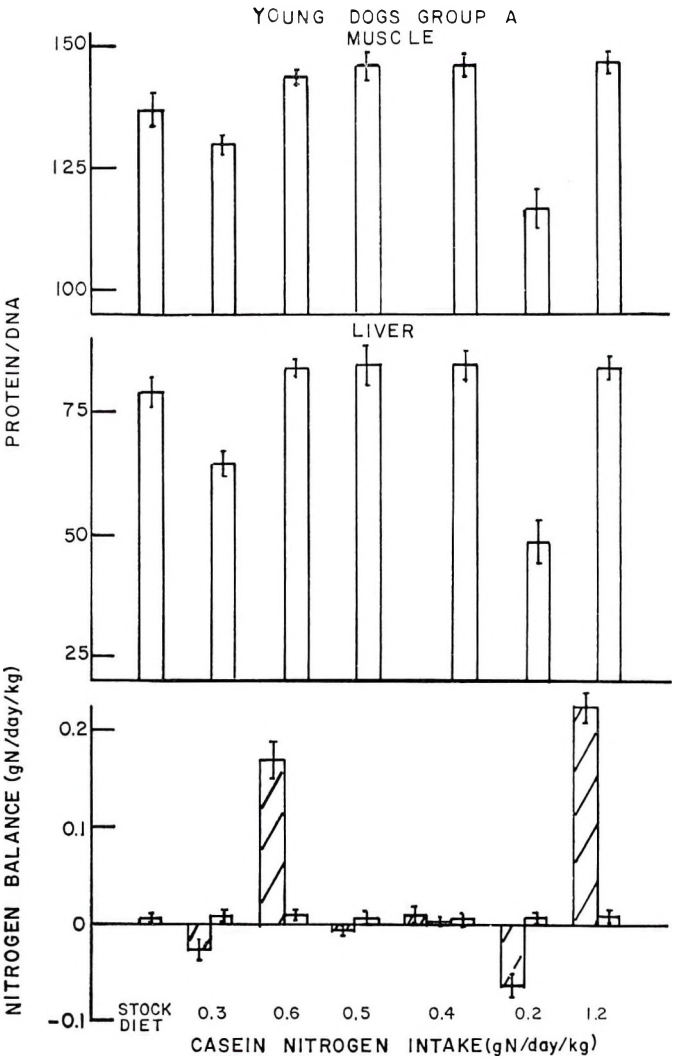

Fig. 1 Nitrogen balance (g N/day/kg) and protein-to-DNA ratios (mg/mg) of liver and muscle from a group of young dogs (A) fed at various levels of casein nitrogen (g N/day/kg). Bars with slanted lines are the nitrogen balance values when the dogs were initially changed to a new diet. Each value is the mean of 6 dogs and the line in the middle of each bar is the standard error of the mean.

free amino acid by ion exchange chromatography. The uptake of radioactive leucine into protein was then corrected for difference in free amino acid pool size (16).

Standard errors were determined for all values. If the probability value was less than 0.01, the difference between the mean was considered significant.

RESULTS AND DISCUSSION

Data which have been accumulated on young dogs indicate that these animals could be placed in nitrogen equilibrium and maintained with protein intakes which were varied from 0.15 to 1.2 g of casein nitrogen/day/kg of body weight (figs. 1 and 2). Liver and cellular protein were definitely lower in young adults fed

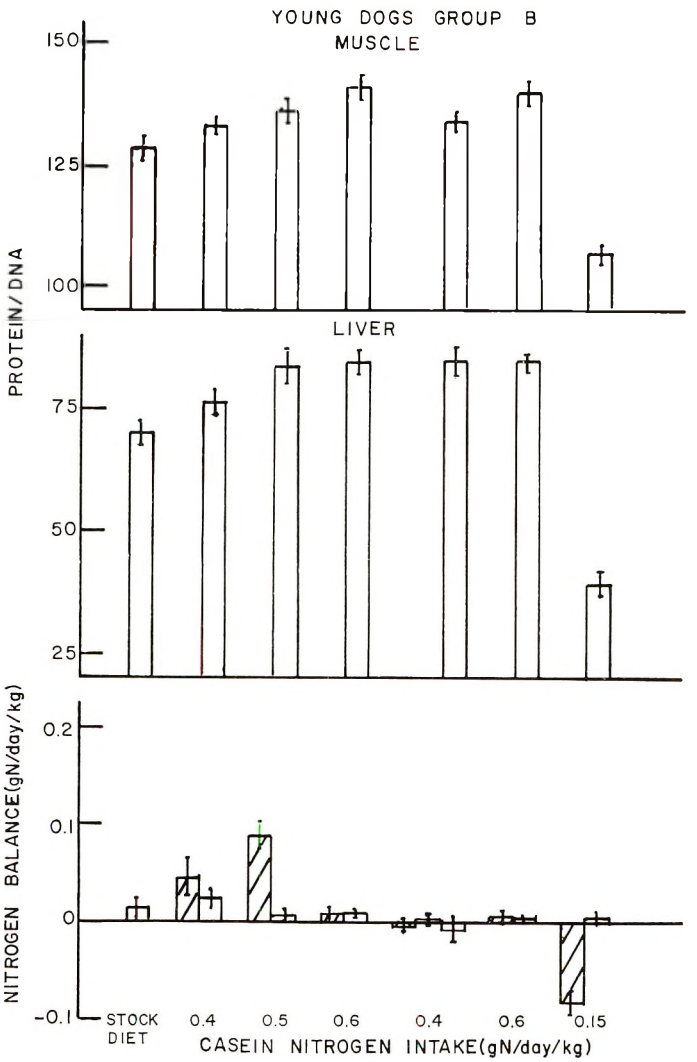

Fig. 2 Nitrogen balance (g N/day/kg) and protein-to-DNA ratios (mg/mg) of liver and muscle from a group of young dogs (B) fed at various levels of casein nitrogen (g N/day/kg). Bars with slanted lines are the nitrogen balance values when the dogs were initially changed to a new diet. Each value is the mean of 6 dogs and the line in the middle of each bar is the standard error of the mean.

0.15, 0.20 or 0.30 g of casein nitrogen/day/kg. When the animals in group A (fig. 1) were shifted from a stock diet to 0.3 g N/day, the dogs went into marked negative balance. After the dogs had been fed this diet for 4 weeks, they were in nitrogen equilibrium, but the cellular proteins of liver and muscle were reduced below the values recorded for the stock diet. The protein which was lost from these tissues has been associated with the so-called "protein reserves" of the animal (2, 17). The feeding of 0.6 g N/day/kg to these depleted animals resulted in an initial marked positive balance which returned to nitrogen equilibrium after maintaining the dogs with this diet for 4 weeks. Subsequent tissue biopsies on these repleted animals indicated that the liver and muscle cellular proteins were increased above the levels recorded for the stock diet. These higher levels of liver and muscle reserve proteins could be maintained when the dogs were shifted to diets which supplied 0.5 or 0.4 g of casein N/day/kg. The feeding of 0.2 g N/day/kg to these animals with maximal protein reserves resulted in a marked loss of body nitrogen (negative nitrogen balance) and decrease in liver and muscle cellular proteins. Subsequent repletion with 1.2 g casein N/day/kg filled the protein reserves of liver and muscle to the maximal level noted in dogs maintained with 0.4, 0.5 or 0.6 g N/day/kg.

The dogs in group B were slightly depleted at the start of the experiment. Feeding 0.4 g of casein nitrogen/day/kg slowly repleted the protein reserves of these dogs (fig. 2), but 0.5 g N/day/kg was needed for rapid repletion of these proteins. Once optimal filling of liver and muscle cellular protein was obtained, the dogs could be maintained in nitrogen equilibrium when the diet was shifted from 0.5 to 0.6 or 0.4 g N/day/kg. Feeding 0.15 g N/day/kg to these animals, with optimal protein reserves, resulted in an initial severe negative balance and loss of tissue proteins. After the dogs had received this low protein intake for 4 weeks, their nitrogen equilibrium, returned to normal as reported previously (1), but liver and muscle proteins were significantly reduced below optimal levels.

Data obtained using old dogs from 12 to 13 years of age has demonstrated that they were placed in nitrogen equilibrium and maintained with 0.2, 0.3, 0.4, 0.5, or 0.6 g of casein nitrogen/day/kg of body weight (fig. 3). The liver and muscle cellular protein, however, were definitely lower in these older dogs fed 0.2 or 0.3 g of casein nitrogen/day/kg. When the dogs were shifted from a stock diet to 0.3 g N/day/kg, the animals went into a marked negative balance (fig. 3). After receiving this diet for 4 weeks, the dogs were in nitrogen equilibrium, but the cellular proteins of liver were reduced significantly below the values recorded for the stock diet. The feeding of 0.4 g N/day/kg resulted in marked positive balance, even after 4 weeks of this diet. This was reflected in an increased concentration of liver and muscle protein, but these values were still below those recorded for the stock diet. After 8 weeks of receiving 0.6 g N/day/kg the dogs were in nitrogen equilibrium. Under these conditions, the concentration of liver and muscle cellular proteins was increased significantly above the values noted with the stock diet. Then, the feeding of 0.2 g N/day/kg resulted in marked negative balance. The cellular proteins of the liver and muscle were very low when nitrogen equilibrium was obtained with this diet. Following this diet, 16 weeks were required to obtain nitrogen equilibrium with 0.4 g N/day/kg. Under these conditions the concentration of cellular proteins in liver and muscle was less than that noted when the dogs obtained nitrogen equilibrium when fed 0.6 g casein N/day/kg. Feeding 0.5 g N/day/kg to these dogs with optimal filling of their tissue proteins resulted in a slight but not significant initial negative balance and subsequent decrease in protein reserves. Since an unbalanced protein (23) or very high protein intakes (8) would produce a high protein-to-DNA ratio in liver, this ratio in muscle gave a better correlation with magnitude of protein reserve of the animal.

A comparison of liver and muscle protein- to -DNA ratios in dogs that were maintained in nitrogen equilibrium by various concentrations of dietary casein (figs. 1–3) revealed that old dogs (open

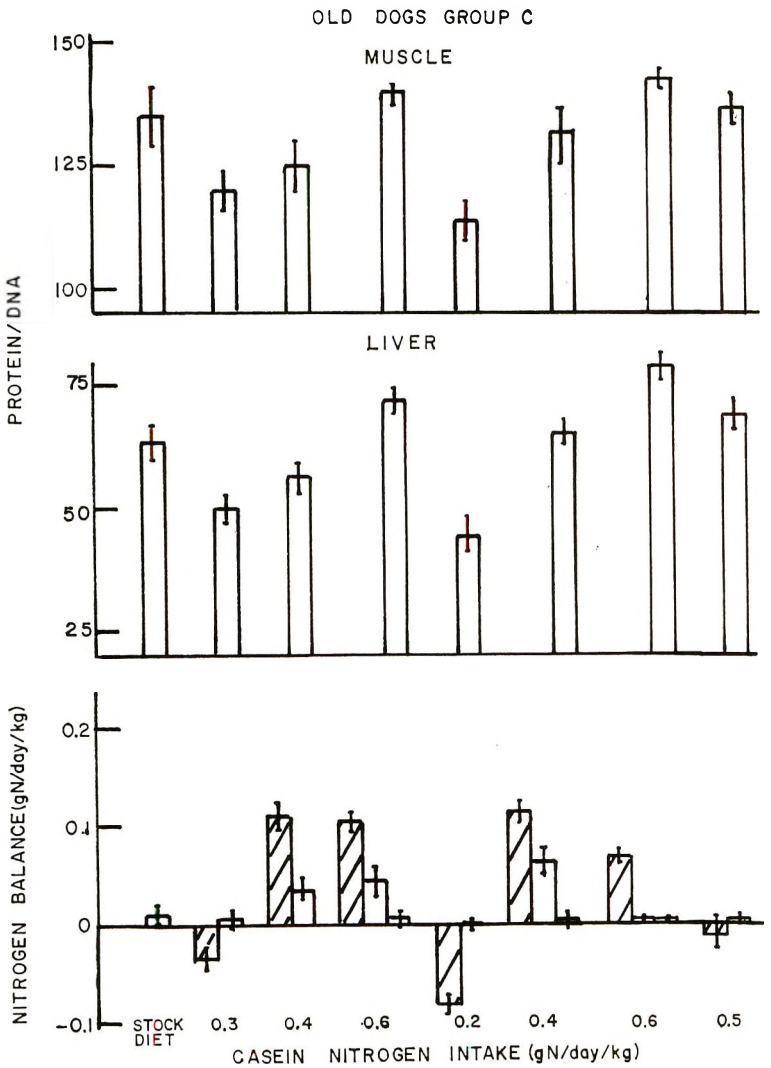

Fig. 3 Nitrogen balance (g N/day/kg) and protein to DNA ratios (mg/mg) of liver and muscle from old dogs (group C) fed at various levels of dietary casein nitrogen (g N/day/kg). Bars with slanted lines are the nitrogen balance values when the dogs were initially changed to a new diet. Each value is the mean of 7 dogs and the line in the middle of each bar is the standard error of the mean.

circle, fig. 4) require 0.6 g N/day/kg to obtain maximal protein reserves, whereas young animals (solid circles) obtained optimal reserves on 0.4 g N/day/kg. The consumption of larger quantities of dietary casein did not increase the levels of this reserve protein. Thus, these results indicated that young dogs required approximately 7 mg of protein nitrogen/basal kcal and old dogs 10 mg of nitrogen /basal kcal to maintain maximal filling of the protein reserves. Previous data had demonstrated (2–4, 7) that optimal filling of the protein reserves was necessary for the animal to respond maximally to certain stresses, whereas excessive protein intakes could be harmful and even reduce the magnitude of muscle cellular proteins (7).

The RNA-to-DNA ratios (fig. 4) of liver and muscle followed directly the changes in cellular protein content of these tissues.

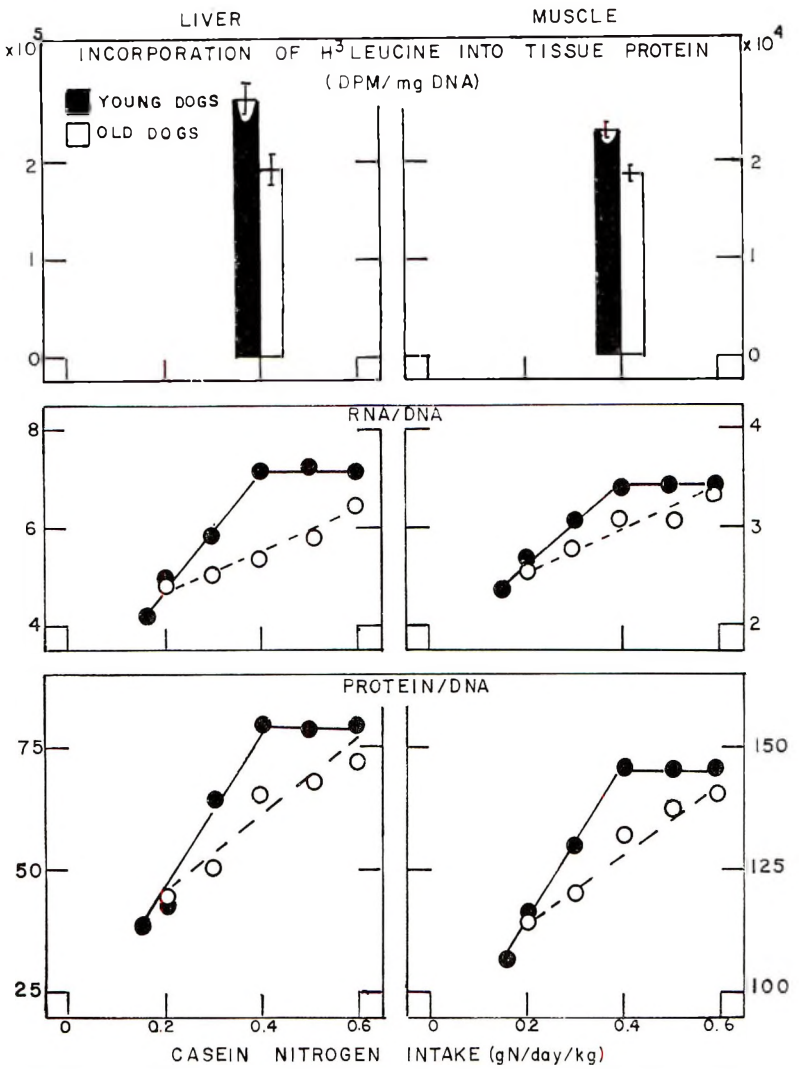

Fig. 4 The protein-to-DNA and RNA-to-DNA ratios (mg/mg) of liver and muscle from young (solid circles) and old (open circles) dogs that reached nitrogen equilibrium at various levels of dietary casein nitrogen (g N/day/kg) (figs. 1–3). The upper graph is the 2-hour incorporation of ^{3}H leucine into liver and muscle proteins of young (solid bar) and old (open bar) dogs that obtained nitrogen equilibrium with 0.4 g of casein nitrogen/day/kg of body weight. These values are expressed as disintegrations per minute of tissue protein per milligram of DNA. Each value is the mean of 7 dogs in the group of old dogs and 6 dogs in the young group. The line in the middle of the bar graph is the standard error of the mean.

These results and the significantly greater incorporation of leucine by protein of liver and muscle from young dogs suggested that these animals possessed a more efficient mechanism for anabolism of cellular proteins than did the older dogs. The data indicated, also, that rate of protein anabolism was a function of the protein intake, and that the rate of protein biosynthesis was correlated with the RNA content of the cell. Breuer and Florini (18) have presented data to indicate that muscles from older rats had a lower rate of in vitro protein synthesis and concen-

tration of cellular polyribosomes than observed in muscles from younger animals that were fed a similar diet. These results support the concept that older animals had a decreased rate of protein anabolism as compared with that of younger adults. Similarly, the higher essential amino acid requirements of older men (10–13) might be associated with a lower rate of protein anabolism.

Development of a theoretical kinetic model for protein metabolism could help in understanding the tissue protein changes which were noted with shifts in intakes of dietary nitrogen. Tissue protein in an adult animal was continually being replaced (19, 20) and degradation of these proteins appeared to follow first order kinetics (21). Hence, the simplest model for change in cellular protein content of a tissue is:

$$dp/dt = K - kP \qquad (1)$$

where P is the protein content of a cell, K the rate constant for protein synthesis, k the first order rate constant for protein degradation, and dp/dt the change in cellular protein content per unit of time. On integration this equation gives:

$$P = \left(1 - \frac{K}{k}\right) e^{-kT} + \frac{K}{k} \qquad (2)$$

From equation 2 it was evident that P would increase or decrease when the rate of protein synthesis (K) was elevated or lowered. An increase or decrease in K, denoted as K' and K'', respectively, will result in an elevation or lowering in P along an exponential time course until a new level of cellular protein, which is equal to $\frac{K'}{K}$ or $\frac{K''}{K}$ times the original concentration of P, was achieved. These conclusions would be valid as long as k remains constant. Waterlow et al. (22) had suggested that when animals were fed at various levels of dietary protein, the rate of protein catabolism (k) did remain relatively constant. This might mean that the rate of synthesis of reserve proteins would decrease as the dogs were shifted from a diet which supplies 0.6 g of casein nitrogen/day/kg to 0.2 g N/day/kg, and this would result in a negative balance that would continue until the concentration of cellular protein was equal to the new rate of protein anabolism (equation 1), thus explaining how animals were maintained in nitrogen equilibrium with diets that supply various intakes of dietary nitrogen. Therefore, nitrogen equilibrium was not an indicator of the magnitude of the protein reserves or a measure of the protein requirements of the animals. Since feeding excess dietary nitrogen would not increase the rate of protein synthesis (K) beyond that which was needed to maintain maximal levels of cellular RNA and protein, then changes in nitrogen balance could be used to estimate the protein requirements of an individual. If at a given protein intake (P') an animal was in nitrogen equilibrium, and feeding more dietary protein would not produce positive balance, but a slight reduction in nitrogen intake would result in negative balance, then this original protein intake (P') would represent the nitrogen requirement that was necessary to maintain maximal protein reserves in this individual.

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Use of Free Amino Acid Concentrations in Blood Plasma of Chicks to Detect Deficiencies and Excesses of Dietary Amino Acids

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ABSTRACT A crystalline amino acid diet (reference diet) which contained each amino acid at the requirement level previously established by chick growth assay was used to study the effect of suboptimal and superoptimal dietary concentrations of amino acids on the free amino acid content of chick blood plasma. A comparison of plasma amino acid concentrations was made between chicks fed the reference diet and chicks fed the diet altered to create 1) amino acid deficiencies, 2) amino acid excesses and 3) combinations thereof. The feeding procedure used assured an equal intake of all nutrients except for the amino acid under study among experimental groups prior to the time that blood samples were taken. Also the frequency at which the chicks ate during the feeding period was controlled. Diets containing suboptimal amounts of either lysine or valine resulted in a marked lowering of the limiting amino acid and an increase in most of the other amino acids in plasma. A diet in which lysine and valine were demonstrated by growth assay to be first and second limiting, respectively, resulted in a decrease in these 2 amino acids in plasma, with lysine showing the greater decline. Most of the other amino acids in plasma increased as a result of this treatment. In some instances, plasma histidine appeared to be lowered as a result of the above mentioned deficiencies. A dietary excess of lysine resulted in a striking increase in plasma lysine and relatively small changes in other amino acids. However it did appear that arginine, glutamic acid and a mixture of glutamine and asparagine decreased as a result of the excess lysine. A diet containing suboptimal amounts of valine and threonine and superoptimal amounts of lysine, tyrosine and isoleucine resulted in marked changes in the corresponding amino acids in plasma; the former two decreased and the latter three increased. Relatively small changes were observed in other amino acids in plasma.

The recognition that the amino acid composition of dietary protein influenced the concentrations of free amino acids in blood plasma (1–4) led to a number of studies designed to examine the effect of either suboptimal or superoptimal dietary concentrations of amino acids on plasma amino acid levels (5–14). Although apparently conflicting results can be found among several of these studies, a major portion of the data supports the conclusion that increasing or decreasing the level of an amino acid in the diet is accompanied by a corresponding change in the level of that amino acid in plasma. It appears reasonable to assume, therefore, that with improvement in techniques it should be possible to detect amino acid deficiencies or excesses from plasma amino acid concentrations.

Methods have been explored for not only detecting amino acids that are limiting or in excess in the diet but for determining the specific order in which each amino acid becomes limiting for growth or maintenance. Longenecker and Hause (10) attempted to predict the order in which the amino acids in a protein become limiting for the adult dog by determining the ratio of the change in the plasma level of an amino acid after feeding, to the dog's requirement for that amino acid and then ranking the ratios in order of magnitude. Although their method showed some promise, interpretation of their results was made difficult by the fact that proof of the actual limiting order of amino acids in the proteins tested was lacking. A modification of the plasma amino acid ratio method was applied to the chick by Hill and Olsen (15). They tested 6 different diets with known or suspected amino acid deficiencies. Plasma amino acid ratios

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revealed the first limiting amino acid in all 6 diets. Prediction of the second limiting amino acid was successful for two, or at the most four, of the diets. Prediction of the third limiting amino acid appeared to be unsuccessful in most cases.

The present investigations were carried out in an effort to determine whether known dietary amino acid deficiencies or excesses or combinations thereof were reflected in plasma amino acid concentrations. Use was made of a crystalline amino acid diet developed at this laboratory for the chick (16) which supplied each amino acid at the requirement level previously determined with the diet. The assays were designed to permit a comparison of plasma amino acid levels between chicks fed the complete amino acid diet (reference diet) and chicks fed the same diet altered to create the desired dietary regimens. A feeding technique was used that assured an equal intake of all nutrients, except for the amino acid under investigation, between experimental groups prior to the time that blood was taken; also the frequency at which the chicks ate during the feeding period was controlled. Furthermore, the feeding method resulted in maximal intake of the test diets on a voluntary basis even though they were severely deficient in or contained an excess of one or more amino acids.

EXPERIMENTAL

Male chicks originating from the mating of New Hampshire males to Columbian females were fed a corn-soybean meal ration for the first 7 days after hatching. On the seventh day, approximately twice the number of chicks required for an experiment were selected on the basis of uniformity of weight. The chicks were then put in electrically heated wire cages (modified rat cages) designed for feeding chicks individually. Initially, 2 chicks were placed in each cage because the chicks learned to eat sooner than when a single chick was confined to a cage. The chicks were fed an isolated soybean protein-glucose diet until they were 11 days of age. At this time the chicks were weighed (chicks were fasted overnight prior to weighing) and assigned to experimental treatments in a manner to insure that each experimental group had the same mean weight and a similar weight distribution. One chick was placed in a cage at this time. All chicks were fed the amino acid reference diet (table 1) until the fourteenth day. On the fourteenth day all chicks were subjected to the fasting and feeding procedure (described later) that was to be used in giving the test diets on the following day except that all chicks received the amino acid reference diet. This procedure was solely for the purpose of training the chicks to eat in the manner described. At the end of the training period the chicks were returned to the reference diet ad libitum. On the morning of the fifteenth day the chicks were fasted for 4 hours. At the end of the fasting period each chick in each treatment was fed 0.8 g of the appropriate experimental diet once every 30 minutes for 12 consecutive feedings (except in exp. 1 where the number of feedings was varied). This amount of diet (0.8 g) was based upon preliminary work which showed this was approximately the maximal amount of a lysine-

TABLE 1

Composition of standard reference diet

	%
L-Arginine·HCl	1.33(1.10) [1]
L-Histidine·HCl·H_2O	0.62(0.48)
L-Lysine·HCl	1.40(1.12)
L-Tyrosine	0.63
L-Tryptophan	0.225
L-Phenylalanine	0.68
L-Cystine	0.35
DL-Methionine	0.55
L-Threonine	0.85
L-Leucine	1.20
L-Isoleucine	0.80
L-Valine	1.04
L-Glutamic acid	12.00
Glycine	1.60
L-Proline	1.00
Corn oil	15.00
Salt mixture [2]	5.37
Cellulose [3]	3.00
Antiacid [4]	1.00
$NaHCO_3$	1.00
Choline chloride	0.20
Cornstarch	50.155
Vitamins [5]	+
Total	100.00

[1] Values in parentheses expressed as the free base.
[2] Klain et al. (24); ($ZnCO_3$ substituted for $ZnCl_2$).
[3] Solka Floc, Brown Company, Chicago 3, Illinois.
[4] Antiacid absorbent (an aluminum hydroxide-magnesium trisilicate preparation), Warner-Chilcott Laboratories, Morris Plains, New Jersey.
[5] Klain et al. (24).

TABLE 2

Effect of a lysine deficiency and length of feeding period on the concentrations of free amino acids in blood plasma

	Length of feeding period								
	2 hours			4 hours			6 hours		
	Standard reference diet [1] (A)	Lysine-deficient diet [2] (B)	$\frac{(B)-(A)}{(A)} \times 100$	Standard reference diet (A)	Lysine-deficient diet (B)	$\frac{(B)-(A)}{(A)} \times 100$	Standard reference diet (A)	Lysine-deficient diet (B)	$\frac{(B)-(A)}{(A)} \times 100$
	μg/ml plasma			*μg/ml plasma*			*μg/ml plasma*		
Lysine	62	44	−29	76	19	−75	35	20	− 43
Valine	48	54	+13	51	66	+29	38	71	+ 88
Threonine	199	233	+17	185	230	+24	120	264	+120
Isoleucine	11	15	+36	12	19	+58	9	29	+222
Tyrosine	20	25	+25	18	21	+17	10	24	+140
Phenylalanine	14	16	+14	13	16	+23	10	18	+ 80
Cystine	31	49	+58	26	39	+45	19	46	+142
Methionine	16	23	+44	17	21	+23	9	23	+156
Leucine	15	21	+40	19	26	+37	15	39	+160
Arginine	25	36	+44	44	46	+ 5	16	69	+331
Histidine	26	19	−27	30	28	− 7	15	36	+140
Glycine	98	117	+19	126	114	− 9	77	109	+ 42
Serine	86	93	+ 8	81	89	+10	41	74	+ 80
Glutamic acid	102	119	+17	164	149	− 9	78	111	+ 42
Glutamine and asparagine	102	111	+ 9	101	101	0	71	123	+ 73
Alanine	252	257	+ 2	255	294	+15	196	255	+ 30

[1] See table 1.
[2] Standard reference diet (table 1) containing 0.70% L-lysine·HCl.

deficient diet (the amino acid reference diet containing lysine at 50% of the requirement) that chicks would voluntarily consume when fed in the above manner. It was later observed that this was also approximately the maximal amount of the complete diet that the chicks would consume under the same conditions. Except where noted otherwise, 8 chicks received each dietary treatment.

Thirty minutes after the last feeding, approximately 3 ml of blood were taken by heart puncture from each chick. The blood from each chick was centrifuged separately in a heparinized tube. An equal amount of plasma was then taken from each tube and pooled according to treatment. The plasma was deproteinized with picric acid according to the method described by Stein and Moore (17).

The amino acid content of the deproteinized plasma was determined by ion exchange chromatography according to the manual procedure of Moore et al. (18) in experiment 1 and with an automatic amino acid analyzer[2] in all other experiments. In using the instrument, a standard mixture of amino acids was analyzed with each new batch of ninhydrin and recoveries remained at 100 ± 3% throughout the analysis reported herein.

Effect of a single amino acid deficiency and length of feeding period

Experiment 1. In this study the effect of a lysine deficiency and also the influence of the number of feedings given prior to taking blood samples on plasma amino acid levels was investigated. The lysine-deficient diet contained 0.56% L-lysine which was equal to 50% of the requirement. This level of lysine supported about one-third the rate of gain from 8 to 14 days as did the lysine adequate diet. Twenty-four chicks were fed each of the test diets as described earlier except that 8 chicks from each treatment were removed and bled at 2, 4 and 6 hours after feeding was initiated.

The results are shown in table 2. In addition to the observed amino acid levels in plasma, the percentage change in each value observed with the deficient diet above or below the corresponding value observed with the reference diet is shown.

[2] Beckman Model 120B automatic amino acid analyzer, Palo Alto, California.

For convenience in referring to this and other tables, the first 11 amino acids listed will be referred to as essential amino acids although cystine and tyrosine are not usually classified as such. Similarly the remaining amino acids will be referred to as non-essential amino acids although glycine and glutamic acid are usually classified as essential for the chick. The results show that at 2 hours after the start of feeding, plasma from chicks fed the lysine-deficient diet contained a lower level of lysine and histidine and higher levels of all the other amino acids than did plasma from chicks fed the reference diet. The comparison between the 2 diets after 4 hours shows that lysine was markedly decreased and most of the other amino acids were increased in the plasma of chicks fed the lysine-deficient diet. The levels of arginine, histidine and most of the non-essential amino acids appeared to be affected relatively little by the diet modification. Comparison of the 2 diets after 6 hours indicates that lysine was again decreased in the plasma of chicks fed the deficient diet. Without exception all the other amino acids were increased in plasma of chicks fed the deficient diet. The non-essential amino acids were, for the most part, affected to a lesser degree than the essentials.

Plasma amino acid levels of chicks fed the reference diet tended to be lower 6 hours after feeding was initiated than for either 2 or 4 hours. In contrast most of the values obtained with the lysine-deficient diet were slightly higher than those at either 2 or 4 hours. Since the differences between the plasma amino acid levels resulting from the diets adequate in lysine and deficient in lysine appeared to be greatest after 6 hours of feeding, this period was adopted for use in subsequent assays.

Effect of single and multiple amino acid deficiencies

Experiment 2. Although it was possible to adjust the level of two or more amino acids in the test diet so as to create a known order of limitation, it was not possible to be certain of the true order of limitation. Therefore, before studying the effect of a first and second limiting amino acid on plasma amino acid levels, a growth assay was conducted to determine the order of limitation after reducing lysine to 50% and valine to 65% of the requirement. The design and results are shown in table 3. The addition of lysine to the deficient basal diet gave the expected improvement in growth but not maximal growth. Adding valine alone did not improve growth significantly. The addition of both lysine and valine improved growth above that obtained with lysine alone. These data offer conclusive proof that lysine and valine were first and second limiting, respectively.

Experiment 3. After confirming the limiting order of lysine and valine, the effect of single deficiencies and a multiple deficiency involving these 2 amino acids on plasma amino acid levels was investigated. The levels of lysine and valine used were the same as those in experiment 2. In addition to the above treatments, 8 chicks were removed from the experiment and bled at the end of the 4-hour fast routinely imposed prior to feeding the test diets. The purpose of this treatment was not to obtain fasting plasma amino acid levels to be used as a "base" as Longenecker and Hause (10) did, but only to gain some insight as to the effect of the relatively short fasting period used in the present studies.

The results of this study are shown in table 4. The single deficiency of lysine had essentially the same effect on plasma amino acid levels as observed in experi-

TABLE 3

Effect of the dietary level of lysine and valine on chick growth

	Treatment	Gain/chick/day [1]	Gain / Feed
		g	
1	Basal [2]	4.72 ± 0.24	0.42
2	Basal + 0.70% L-lysine·HCl	10.11 ± 0.64	0.63
3	Basal + 0.36% L-valine	5.06 ± 0.20	0.43
4	As 2 + 3	14.56 ± 0.15	0.73

[1] Average ± SE of triplicate groups of 3 chicks each for the period 8 to 14 days of age.
[2] Standard reference diet (table 1) modified to contain 0.70% L-lysine·HCl and 0.68% L-valine.

TABLE 4

Effect of single and multiple amino acid deficiencies and fasting period on the concentration of free amino acids in blood plasma

	Standard reference diet [1]	Lysine-deficient diet [2]	Valine-deficient diet [3]	Lysine-valine-deficient diet [4]	Fasting level [5]
	μg/ml plasma	*μg/ml plasma*	*μg/ml plasma*	*μg/ml plasma*	*μg/ml plasma*
Lysine	86	15(−83) [6]	66(−23)	11(−87)	55(−36)
Valine	51	79(+55)	12(−76)	32(−37)	36(−29)
Threonine	198	313(+58)	231(+17)	253(+28)	192(−3)
Isoleucine	15	32(+113)	20(+33)	26(+73)	11(−27)
Tyrosine	15	34(+127)	28(+87)	32(+113)	13(−13)
Phenylalanine	10	20(+100)	18(+80)	19(+90)	11(+10)
Cystine	9	17(+89)	11(+22)	16(+78)	23(+155)
Methionine	29	44(+52)	36(+24)	39(+34)	8(−72)
Leucine	18	38(+111)	23(+28)	34(+89)	14(−22)
Arginine	57	76(+33)	52(−9)	65(+14)	10(−82)
Histidine	40	31(−22)	26(−35)	32(−20)	14(−65)
Glycine	115	121(+5)	119(+3)	116(0)	42(−63)
Serine	68	78(+15)	79(+16)	78(+15)	112(+65)
Proline	64	89(+39)	74(+16)	81(+27)	31(−52)
Glutamic acid	74	86(+16)	88(+19)	70(−5)	43(−42)
Glutamine and asparagine	136	156(+15)	138(+1)	153(+13)	143(+5)
Alanine	255	279(+9)	319(+25)	266(+4)	143(−44)

[1] Table 1, and diet 4, table 3.
[2] Diet 3, table 3.
[3] Diet 2, table 3.
[4] Diet 1, table 3.
[5] Blood was withdrawn at end of 4-hour fast.
[6] Percentage change from value obtained with standard reference diet.

ment 1, namely, lysine was markedly reduced and most of the other amino acids were increased. As observed in 2 instances in experiment 1, plasma histidine decreased. An uncomplicated deficiency of valine resulted in a marked lowering of plasma valine and an increase in most of the other amino acids in plasma except lysine and histidine which appeared to be lowered. Arginine, glycine and the mixture of glutamine and asparagine showed only minor changes. The multiple deficiency involving both lysine and valine effected a marked reduction in plasma lysine and a somewhat lesser reduction in valine. This treatment resulted in an increase in all of the essential amino acids except histidine which was decreased. Again some of the non-essentials were changed very little. It is noteworthy that valine was reduced considerably more when it was first limiting than when it was second limiting (76% vs. 37%) even though the intake of valine was the same in both cases.

A comparison of plasma amino acid levels at the end of the 4-hour fast with those after feeding (reference diet) shows that fasting lowered the level of most of the amino acids. Methionine was lowered considerably, whereas cystine was increased markedly, which may be the result of a conversion of the former to the latter. Also a conversion of glycine to serine may account for the lowering of the former and increase in the latter in plasma.

Effect of an excess of a single amino acid

Lysine was selected as the amino acid to be fed in an excessive amount in the following study since it is one of the more "toxic" amino acids to the chick (19). Also, relatively small supplemental amounts of this amino acid have been shown to result in a growth depression in chicks receiving a casein-glucose diet.[3]

Experiment 4. A growth study was conducted to determine the effect of adding 1.00, 2.00 and 4.00% L-lysine (as L-lysine·HCl) to the reference diet. The results are presented in table 5. Even the lowest supplemental level of lysine resulted in a marked growth depression (44%).

[3] Unpublished data, H. M. Scott, 1961.

TABLE 5

Effect of excess L-lysine·HCl on chick growth

	Treatment	Gain/chick/day [1]	Gain as % of diet 1	Gain / Feed
		g		
1	Basal [2]	14.33 ± 0.34	100	0.77
2	Basal + 1.25% L-lysine·HCl	8.06 ± 0.39	56	0.55
3	Basal + 2.50% L-lysine·HCl	6.61 ± 0.97	46	0.51
4	Basal + 5.00% L-lysine·HCl	5.45 ± 0.15	38	0.46

[1] Average ± SE of triplicate groups of 3 chicks each for the period 8 to 14 days of age.
[2] Standard reference diet (table 1).

Experiment 5. The effect of an excess of 1.00% lysine on plasma amino acid levels was investigated in this study. The results are shown in table 6. Chicks fed excess lysine had 3 times as much lysine in plasma as chicks fed the reference diet. Several amino acids appeared to decrease as a result of feeding excess lysine, notably glutamic acid, arginine and glutamine plus asparagine.

Effect of an amino acid mixture containing both suboptimal and superoptimal amounts of certain amino acids

Experiment 6. Since an intact protein may contain several amino acids that are high and at the same time a number of other amino acids that are low in concentration in relation to the animal's requirement, it seemed worthwhile to examine the effect on plasma amino acids of feeding an amino acid mixture simulating these conditions. The mixture used was formulated to contain 65% of the required amounts of valine and threonine and 150% of the required levels of lysine, tyrosine and isoleucine. To obtain an estimation of the differences in amino acid levels between pooled samples of plasma from chicks fed the same diet, 16 chicks were fed the standard reference diet, and 2 pooled samples of plasma (each from 8 chicks) were obtained.

The results are shown in table 7. The amino acids that were deficient in the modified diet (valine and threonine) were decreased markedly in plasma and those that were in excess (lysine, tyrosine and isoleucine) were markedly increased. The other amino acids appeared to be affected relatively little, with most of the differences being of questionable significance especially when the magnitude of the differences between the 2 pooled samples of plasma from chicks fed the reference diet is considered.

DISCUSSION

In each of the studies in which a deficiency of a single amino acid was tested (exps. 1 and 2), the limiting amino acid, without exception, decreased in blood plasma. Most of the other amino acids increased in the plasma of the deficient chicks. However, a few amino acids, histidine in particular, were inconsistent in this respect. In some instances the level of histidine was depressed. It is obvious that erroneous conclusions regarding the dietary adequacy of histidine could be drawn

TABLE 6

Effect of excess L-lysine·HCl on the concentrations of free amino acids in blood plasma

	Standard reference diet [1] (A)	Standard reference diet + 1.25% L-lysine·HCl (B)	(B) − (A) / (A) × 100
	μg/ml plasma	*μg/ml plasma*	%
Lysine	71	217	+206
Valine	59	57	− 3
Threonine	213	224	+ 5
Isoleucine	16	17	+ 6
Tyrosine	19	15	− 21
Phenylalanine	15	15	0
Cystine	13	13	0
Methionine	35	32	− 8
Leucine	20	20	0
Arginine	44	30	− 32
Histidine	27	24	− 11
Glycine	106	99	− 7
Serine	79	68	− 14
Proline	69	60	− 13
Glutamic acid	119	69	− 42
Glutamine and asparagine	163	111	− 32
Alanine	318	256	− 19

[1] See table 1.

TABLE 7

Effect of an amino acid mixture deficient in valine and threonine and containing an excess of lysine, isoleucine, and tyrosine on the concentrations of free amino acids in blood plasma

	Standard reference diet [1] (A)		Modified diet [2] (B)	(B) minus (A)	$\frac{(B)-(A)}{(A)} \times 100$
	μg/ml plasma		*μg/ml plasma*	*μg/ml plasma*	*%*
Lysine	73 [3]	(7) [3]	154	+ 81	+111
Valine	53	(4)	10	− 43	− 81
Threonine	243	(36)	133	−110	− 45
Isoleucine	13	(3)	38	+ 25	+192
Tyrosine	19	(1)	35	+ 16	+ 84
Phenylalanine	13	(0)	15	+ 2	+ 15
Cystine	13	(1)	14	+ 1	+ 8
Methionine	31	(3)	30	− 1	− 3
Leucine	18	(2)	20	+ 2	+ 11
Arginine	44	(8)	44	0	0
Histidine	30	(4)	30	0	0
Glycine	102	(7)	110	+ 8	+ 8
Serine	77	(12)	85	+ 8	+ 10
Proline	64	(5)	62	− 2	− 3
Glutamic acid	91	(12)	70	− 21	− 23
Glutamine and asparagine	173	(7)	138	− 35	− 20
Alanine	294	(40)	249	− 45	− 15

[1] See table 1.
[2] Standard reference diet (table 1) modified to contain 0.55% L-threonine, 0.68% L-valine, 2.10% L-lysine·HCl, 0.95% L-tyrosine, and 1.20% L-isoleucine.
[3] Mean of duplicate pooled samples, each obtained from 8 chicks, with difference between the samples given in parentheses.

from the plasma amino acid levels observed in these studies. Despite the inconsistency of histidine, in most cases histidine did not decrease as much as the amino acid known to be limiting. The observation that most of the amino acids, except for the limiting one, tended to accumulate in plasma could be explained on the basis that an insufficient amount of the limiting amino acid at the sites of protein synthesis (resulting from a deficiency in the diet and therefore a reduced level in the extracellular fluid) limited protein synthesis and resulted in a reduced uptake from the extracellular fluid of all amino acids except the limiting one. The feeding method used in these studies might be expected to be conducive to an accumulation of these "excess" amino acids since the intake of all amino acids (except the one that was deficient) was equalized and held at a maximal rate.

When a multiple deficiency involving lysine and valine (exp. 2) was tested, the limiting order of these was shown in plasma by a greater decrease in lysine than in valine. Again histidine decreased but not as much as either lysine or valine. As observed in the case of the single deficiencies, most of the other amino acids in plasma increased.

A dietary excess of lysine (exp. 5) was revealed in plasma by a large increase in plasma lysine. Apparently a depression of certain other amino acids may be associated with an excess of lysine. Hier (20) observed a similar effect when excessive amounts of leucine, isoleucine or methionine were administered to dogs. More recently Jones (21) reported that a reduction in plasma arginine occurred when excess lysine was fed to chicks.

When the test diet contained valine and threonine at levels below, and lysine, tyrosine and isoleucine in amounts in excess of the requirement (exp. 6), each deficiency or excess could be detected in plasma since the limiting amino acids decreased and the excess amino acids increased. Valine and threonine were each present in the modified diet in amounts equal to 65% of their respective requirement levels, yet the percentage depression of plasma valine (81%) was much greater than that of threonine

(45%). However, it was not established by growth assay whether these 2 amino acids were equally limiting. The levels of lysine, tyrosine and isoleucine were each increased 50% in excess of their requirements in the modified diet, but the percentage increases in plasma varied from 84% for tryrosine to 192% for isoleucine. Thus it appears that under conditions as imposed in these experiments the percentage change of the level of an amino acid in plasma relative to the level attained with the standard reference diet will reveal whether an amino acid is deficient or in excess (provided the standard is correct) but may not necessarily reflect the exact limiting order of the amino acids.

The method used in the present studies has been applied to the study of intact proteins by Smith and Scott (22–23) who were able to detect amino acid deficiencies known to exist in a number of intact proteins. Their studies also emphasize that the method is dependent upon how well the amino acid reference diet meets, but does not exceed, the amino acid requirements of the chick.

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Comparison of Neutral Fat and Free Fatty Acids in High Lipid-Low Carbohydrate Diets for the Growing Chicken [1,2]

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ABSTRACT Three experiments were conducted with day-old chicks a) to determine if they require an exogenous source of carbohydrate when fed diets high in lipids, and b) to study the comparative nutritional properties of soybean oil (SO) and soybean oil fatty acids (SOFA). Semipurified diets based on isolated soybean protein were used. The reference high carbohydrate diet supplied in percentage of metabolizable calories: glucose, 45; protein, 32; and SO, 23. For the high lipid diets, both glucose and SO were equicalorically replaced with the lipid under study; supplemental carbohydrate added to these diets replaced an equicaloric amount of lipid. Diets supplying the non-protein calories as SO permitted growth nearly equal to the high carbohydrate controls; addition of 3% glycerol or up to 6% glucose calories did not improve it. In contrast, chicks fed the high SOFA diet grew slowly and developed foot dermatitis and beak deformities. This syndrome was not prevented by feeding the fatty acids as the methyl or ethyl esters. The supplementation of these diets with 6% glucose markedly improved growth but did not prevent the development of the foot and beak abnormalities. The high SO diet induced moderate ketonemia; the high SOFA diet induced hypoglycemia and severe ketonemia, which were prevented completely by the supplementation with 6% glucose. β-Hydroxybutyric acid is the major ketone substance in the ketonemic chick. Apparent lipid absorbability was of the order of 90% for all diets except those containing the ethyl esters of SOFA in which absorbability was approximately 80%.

Carbohydrates are nutrients of diverse chemical complexity which are usually plentiful in the diets of man and domestic animals. Physiologically, carbohydrates are of the greatest significance as a source of energy for body tissues, particularly for the brain which depends critically on circulating glucose for its metabolism (1). However, the available evidence suggests that carbohydrates may not be required in the diet of man and other vertebrates, in contrast with certain insects (2, 3). This non-essentiality of exogenous carbohydrate is due to the ability of the body to synthesize glucose from a variety of precursors, among which certain amino acids are of major importance.

The dietary carbohydrate requirement of the growing chicken has received little study. Reports from several laboratories (4–6) have shown that the chicken can grow normally when fed diets very high in fat, provided that a proper balance is maintained between energy and all other nutrients. Recently, Renner (7) reported that fat can completely replace carbohydrate in the diet without affecting rate of growth or nitrogen retention in the chick. However, when the neutral fat of the high fat diet was replaced by the corresponding mixture of fatty acids, growth was markedly retarded (8). Supplementation of this high fatty acid diet with small amounts of either glycerol or glucose restored growth essentially to normal rate. These data clearly show that the chick exhibits under certain conditions a dietary requirement for carbohydrate, which can be met by glycerol. Our experimental approach differs in several respects from that used by Renner and co-workers, and has produced even more striking evidence

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[2] Reported in part at the 1964 and 1965 meetings of the American Institute of Nutrition: Brambila, S., and F. W. Hill 1964 Comparison of neutral fat vs. free fatty acids in a low carbohydrate diet for chicks, Federation Proc., 23: 550 (abstract); Hill, F. W., and S. Brambila 1965 Properties of high lipid diets based on free fatty acids, Federation Proc., 24: 501 (abstract).

of the nature of extreme carbohydrate deficiency in this species. In the present work we have studied the ability of the young chick to utilize diets in which virtually all the non-protein calories are supplied by lipid, and the comparative effects of carbohydrate supplementation of such diets based respectively on neutral fat and fatty acids. Experimental criteria included rate of growth, blood glucose, blood ketone bodies, lipid absorbability and nitrogen retention.

EXPERIMENTAL

Animals. Male chicks of a meat strain (Arbor-Acres) were used in all experiments. They were reared in battery brooders with thermostatically controlled electric heating units and raised wire screen floors, located in a laboratory room with automatically controlled temperature. Each diet was randomly assigned to duplicate groups of 10 chicks each. The experimental diets and water were given ad libitum from the first day. Individual body weights and group food consumption were recorded at weekly intervals.

Diets. The composition of the high carbohydrate reference diet is shown in table 1. The level of soybean oil used in this diet amply provides for essential fatty acids and other fat-associated properties which are necessary for maximal growth and metabolic efficiency (6, 9, 10). Chromic oxide was added as an inert index substance to facilitate the determination of fat absorbability and nitrogen retention (11, 12).

TABLE 1

Composition of high carbohydrate reference diet

	% *dry matter*
Glucose [1]	49.75
Isolated soybean protein [2]	32.00
Soybean oil	10.00
DL-Methionine	0.70
Glycine	1.00
Mineral mixture [3]	5.20
Vitamin mixture [4]	1.03
Chromic oxide	0.30
Antioxidants [5]	0.02

[1] Anhydrous Cerelose, Corn Products Company, Argo, Illinois.

[2] C-1 Assay Protein, Archer-Daniels-Midland, Minneapolis.

[3] Mineral mixture supplied in mg/100 g diet: $CaHPO_4 \cdot 2H_2O$, 2040; $CaCO_3$, 1000; Na_2HPO_4, 700; KCl, 700; NaCl, 400; $MgSO_4$, 300; $MnSO_4 \cdot H_2O$, 25; ferric citrate, 20; $ZnCO_3$, 13; $CuSO_4$, 1.0; KIO_3, 1.0; Na_2MoO_4, 0.8; cobaltous acetate, 0.18; and Na_2SeO_3, 0.022.

[4] Vitamin mixture supplied in mg/100 g diet: niacin, 8; Ca pantothenate, 4; pyridoxine, 2; riboflavin, 1.0; thiamine, 1.0; folacin, 0.3; menadione, 0.3; biotin, 0.04; cyanocobalamin, 0.005; choline chloride, 200; vitamin A palmitate, 1000 IU; vitamin D_3, 150 ICU; *d*-α-tocopheryl acetate, 3.

[5] 0.01% each of butylated hydroxytoluene and diphenyl-p-phenylenediamine.

For the preparation of the high lipid diets, both glucose and soybean oil of the reference diet were replaced with an equicaloric amount of the lipid under study (29.60 g soybean oil; 32.20 g soybean oil fatty acids (SOFA); 32.80 g SOFA methyl esters; 30.60 g SOFA ethyl esters), and enough cellulose [3] to give a mealy texture to the diet (20 g). In each diet, protein supplied 32% of the total metabolizable energy, which was approximately equivalent to 74 g protein per 1000 kcal; glucose or lipid, or both, provided the remaining 68%. In the high carbohydrate reference diet, glucose supplied 45% and soybean oil, 23% of total metabolizable energy.

The total metabolizable energy supplied by each diet, as well as the relative caloric contribution of individual dietary ingredients, was estimated on the basis of the following metabolizable energy values: (in kcal/g) glucose, 3.64 (13); glycerol, 4.10; isolated soybean protein, 3.83 (14); soybean oil, 9.24 (15, 16); SOFA, 8.50; SOFA methyl esters, 8.10; SOFA ethyl esters, 8.50. The metabolizable energy content of glycerol and lipids other than soybean oil were estimated on the basis of their respective heats of combustion (17) and percentage absorbability by the chick. Glycerol is known to be well utilized by the chicken (18); in order to estimate its metabolizable energy it was assumed to be 95% absorbable. SOFA has been reported to be 90% absorbed (19); the methyl and ethyl esters of SOFA were also assumed to be 90% absorbed, which proved to be a high estimate for the ethyl esters, as will be shown later. The caloric contribution of methanol in the methyl esters was disregarded; that of ethanol was estimated as outlined above.

Preparation of lipids. The fatty acids used in these studies either were obtained through commercial channels,[4] or were prepared in the laboratory in all-glass

[3] Solka Floc BW-200, Brown Company, Boston, Massachusetts.

[4] Wecoline S, kindly supplied by Drew Chemical Company, Inc., New York.

equipment by alkaline hydrolysis of degummed soybean oil. For this purpose, 5 kg oil were mixed under constant stirring with 10 liters of a 10% solution of NaOH in 70% ethanol previously warmed at 60 to 65°. When the suspension became a solution the stirring was stopped and the mixture allowed to stand overnight at 50 to 55°. This solution, which contained the SOFA in the form of sodium salts, was acidified by adding 750 ml concentrated H_2SO_4 in 10 liters water. The supernatant layer was then repeatedly washed with a warm, aqueous solution of 2% NaCl to remove glycerol and traces of mineral acid. The mixture of fatty acids was finally dried over anhydrous Na_2SO_4, filtered through paper and kept refrigerated under nitrogen. Yields were always 95% or more on the basis of stoichiometric calculation. Samples of each batch were dissolved in ethanol and titrated with standard base using phenolphthalein as indicator. By assuming an average fatty acid molecular weight of 282, the product obtained was consistently found to contain not less than 97% free fatty acids.

The monoesters were prepared by direct esterification of SOFA with an equal volume of the corresponding anhydrous alcohol which contained 5% concentrated H_2SO_4 by weight as a catalyst. This mixture was left overnight at room temperature after which water was added. The oily, supernantant layer, which still contained 10 to 20% free fatty acids, was separated, dried with anhydrous Na_2SO_4 and esterified once more by the same procedure. Finally, the mixture of esters was repeatedly washed with warm, aqueous solutions containing 2 to 3% NaCl and 1% $NaHCO_3$, dried with anhydrous Na_2SO_4, filtered and stored under nitrogen at low temperature.

The fatty acid composition of SOFA, as well as that of the monoesters, was essentially identical to that of soybean oil as determined by gas-liquid chromatography of the fatty acids as methyl esters, conducted with an Aerograph dual column gas chromatograph using 230 × 0.635 cm columns packed with 10% diethylene glycol-succinate on Chromosorb W. The yield was always above 90% of theoretical amount. The presence of free fatty acids in the monoesters was of the order of 1% as determined by titration with standard base.

ANALYTICAL PROCEDURES

Nitrogen retention and fat absorbability. Excreta were collected daily on 3 consecutive days at the selected stage of the experiment. The methods used for processing excreta, and for the determination of chromic oxide, nitrogen and lipid in food and excreta have been described previously (20–22). As determined in this study, nitrogen retention is the difference between nitrogen intake and output in combined excreta and is expressed as percentage of nitrogen intake.

Blood glucose and ketone bodies. Blood samples were obtained either from the wing vein or by heart puncture using heparin as the anticoagulant. Blood glucose was determined colorimetrically by the ferricyanide micro method of Folin and Malmros (23) using sodium lauryl sulfate instead of gum ghatti as emulsifier (24).

Blood acetone plus acetoacetate were determined colorimetrically as acetone (25). Total ketone bodies were determined according to the method of Greenberg and Lester as modified by Michaels et al. (26). This procedure is based on the conversion of acetoacetate and β-hydroxybutyrate to acetone, which is then determined colorimetrically as 2,4-dinitrophenylhydrazone.

Liver glycogen. Glycogen was determined colorimetrically by the anthrone method (27).

Thin-layer chromatography. Lipids from abdominal fat and intestinal contents were extracted with a mixture of chloroform-methanol (2:1) as described by Entenman (28); non-lipid contaminants were removed by partitioning with water. Neutral lipids were fractionated in thin layers of silica gel [5] applied in 0.5 mm thickness to 20 × 20 cm glass plates using a Stahl-Desaga applicator. The chromatograms were developed with heptane:ethyl ether:acetic acid 70:30:1 (v/v/v). For the separation of methyl or ethyl esters

[5] Silica Gel H, Research Specialties Company, Richmond, California.

from triglycerides the same mixture of solvents but in the proportion 90:10:1 was preferred (29). Visualization of spots was accomplished by using iodine vapors as indicator. The different classes of compounds were identified by the introduction of appropriate standards in each plate.

Statistical analysis. The results were analyzed by analysis of variance (30) and treatment differences were evaluated by Duncan's multiple range test (31).

RESULTS

Experiment 1. The effects of carbohydrate supplementation in high lipid diets on growth, lipid absorbability and nitrogen retention are shown in table 2. The equicaloric substitution of soybean oil for glucose in the reference diet caused a slight decrease in rate of growth which approached statistical significance ($P > 0.05$), and which was not improved by the addition of up to 6% glucose or 3% glycerol calories. The growth of chicks fed the high fatty acid diet was markedly depressed and only partially overcome by the addition of 3% glucose or glycerol calories, which is approximately equivalent to the theoretical amount necessary to esterify completely the dietary fatty acids into triglycerides. In this experiment, glycerol appeared to be superior to glucose; in subsequent experiments this has not been observed consistently. The diet supplying the lipid calories as the mixture of neutral fat and SOFA (1:1) produced unexpectedly good growth which cannot be attributed to its glycerol contribution. The glycerol furnished by this diet, which was bound as glyceride, was equivalent to 1.5% of metabolizable energy calories.

Data on lipid absorbability (table 2) confirm and extend previous observations (16, 19). The apparent absorbability of both soybean oil and SOFA was high and independent of their dietary level.

The chicks receiving the fatty acid diets, with or without glucose supplementation, showed a sharp decrease in nitrogen retention at the fourth week (table 2); a similar decrease was not produced by the diets high in neutral fat or by the diet containing the mixture of soybean oil and SOFA.

All the chicks fed diets with SOFA developed a dry, scaly dermatitis of the feet and a beak deformity characterized by thick, shortened mandibles.

Experiment 2. The SOFA preparation used in the first experiment was prepared commercially by acid hydrolysis of soybean oil and distillation of the fatty acids under reduced pressure. To determine whether the severe growth depression it produced might have been due to contaminants or the method of preparation, it was compared with SOFA prepared in

TABLE 2

Growth and food utilization of chicks fed high neutral fat and high fatty acid diets with and without carbohydrate (exp. 1)

Treatment [1]	Avg wt, 4 weeks	ME intake (calculated)	Apparent lipid absorbability		Nitrogen retention	
			2 weeks	4 weeks	2 weeks	4 weeks
	g	*kcal/chick*	%	%	%	%
Glucose (45) + soybean oil (23) (reference diet)	589 [a 2]	2733	93	91	62	60
Soybean oil (SO) (68)	538 [ab]	2657	95	95	60	59
Glucose (3) + SO (65)	527 [b]	2645	—	—	—	—
Glucose (6) + SO (62)	542 [ab]	2638	94	95	62	57
Glycerol (3) + SO (65)	529 [b]	2605	—	—	—	—
Soybean oil fatty acids (SOFA) (68)	211 [c]	1162	93	91	59	47
Glucose (3) + SOFA (65)	260 [cd]	1329	—	—	—	—
Glucose (6) + SOFA (62)	243 [c]	1319	93	90	64	40
Glycerol (3) + SOFA (65)	301 [d]	1470	—	—	—	—
SO (34) + SOFA (34)	493 [b]	2431	94	95	61	61

[1] Numbers in parentheses are % metabolizable calories supplied by ingredient; remainder (32) was protein.
[2] Values are averages of duplicate groups. Figures with differing superscript are significantly different ($P < 0.05$).

our laboratory by alkaline hydrolysis (see description under Preparation of Lipids). The results of this experiment are summarized in table 3, and indicate clearly that there was no difference attributable to source of fatty acids.

As in the first experiment, the chicks fed the high fatty acid diets showed greatly retarded growth. Supplementation with 6% glucose calories significantly improved rate of growth, which nonetheless was below that of the chicks fed the reference diet or the high neutral fat diet. The addition of glucose also increased the absolute intake of fatty acids.

Measurements of lipid absorbability and nitrogen retention confirmed the results of the previous experiment, and showed that the poor growth of the chicks fed high levels of fatty acids was not due to impaired absorbability of lipids or nitrogen retention, at least up to 2 weeks of age.

Growth was slightly reduced when the soybean oil of the reference diet (10% by weight; 23% of metabolizable energy) was replaced by SOFA. This was barely significant for the laboratory preparation ($P = 0.05$) but not significant for the commercial sample ($P > 0.05$).

As before, all the chicks fed diets with high levels of SOFA developed foot dermatitis and beak deformity.

Experiment 3. To test the hypothesis that the poor growth, dermatitis of the feet and beak abnormalities associated with the diets high in SOFA were due to feeding the fatty acids in the free rather than esterified form, we prepared and fed the corresponding methyl and ethyl esters.

The data in table 4 show that both the methyl and ethyl esters of SOFA retarded growth when fed in the high carbohydrate diet ($P < 0.05$). The growth of chicks fed the high lipid-no carbohydrate diets was depressed equally by SOFA and the monoesters. The addition of 6% glucose calories markedly improved the growth of the chicks fed SOFA, and, to a lesser extent, that of the chicks receiving monoesters.

Feeding the fatty acids as esters did not prevent the development of dermatitis or the beak abnormality. Although supplementation of the diets with glucose improved growth and general condition of the chicks, it had no effect on dermatitis or the beak abnormality.

Table 4 also shows that the methyl esters were as well absorbed as the free fatty acids; the apparent absorbability of the ethyl esters, however, was appreciably lower. The data on nitrogen retention suggest that the monoesters had an adverse effect on the amount of nitrogen retained by the chicks.

TABLE 3

Growth and food utilization of chicks fed 2 different preparations of soybean oil fatty acids (exp. 2)

Treatment [1]	Avg wt, 2 weeks	ME intake (calculated)	Lipid intake	Apparent lipid absorbability	Nitrogen retention
	g	*kcal/chick*	*g/chick*	%	%
High glucose (45) + lipid (23)					
Soybean oil (reference diet)	262 [a 2]	915	23	94	64
SOFA (a) [3]	241 [ab]	850	22	—	—
SOFA (b) [4]	239 [b]	836	21	—	—
High lipid (68)					
Soybean oil	209 [c]	814	60	96	61
SOFA (a)	112 [d]	439	35	92	61
SOFA (b)	121 [d]	407	33	—	—
Glucose (6) + lipid (62)					
SOFA (a)	162 [e]	558	41	93	64
SOFA (b)	177 [e]	584	43	—	—

[1] Numbers in parentheses are % metabolizable calories supplied by ingredient; remainder (32) was protein.
[2] Values are averages of duplicate groups. Figures with differing superscript are significantly different ($P < 0.05$).
[3] Soybean oil fatty acids, acid hydrolysis followed by distillation.
[4] Soybean oil fatty acids, alkaline hydrolysis.

Data from all 3 experiments on blood glucose, blood ketone bodies and liver glycogen are given in table 5. The high SOFA diet induced hypoglycemia, severe ketonemia and extremely low hepatic glycogen. Statistical analysis of the pooled data on blood glucose indicated that, as compared with the high carbohydrate control group, the high neutral fat diet did not significantly ($P > 0.05$) reduce the level of circulating glucose. The hypoglycemia induced by the high fatty acid diet was significant ($P < 0.05$) and was prevented by supplementation with 6% glucose calories.

Determination of blood acetone and acetoacetate in experiment 1 showed a small but significant increase in these substances induced by the high neutral fat (soybean oil) diet, and a further increase by the SOFA diet. In the third experiment, total blood ketones including β-hydroxybutyrate were determined. The high soybean oil diet produced a threefold increase, and the high SOFA diet produced a tenfold increase in blood ketones over the high carbohydrate reference diet. Supplementing either high lipid diet with glucose at 6% of calories brought the blood ketone level to that of the high

TABLE 4

Growth and food utilization of chicks fed methyl and ethyl esters of soybean oil fatty acids (exp. 3)

Treatment [1]	Avg wt, 2 weeks	ME intake (calculated)	Lipid intake	Apparent lipid absorbability	Nitrogen retention
	g	*kcal/chick*	*g/chick*	%	%
High glucose (45) + lipid (23)					
Soybean oil (reference)	225 [a] [2]	786	20	—	—
SOFA [3]	224 [a]	818	21	89	56
SOFA methyl esters	203 [b]	750	21	87	55
SOFA ethyl esters	200 [b]	782	20	75	52
High lipid (68)					
Soybean oil	215 [ab]	793	58	—	—
SOFA	119 [c]	426	33	94	52
SOFA methyl esters	119 [c]	424	35	94	48
SOFA ethyl esters	124 [cd]	526	40	82	44
Glucose (6) + lipid (62)					
Soybean oil	218 [ab]	772	52	—	—
SOFA	180 [e]	631	45	—	—
SOFA methyl esters	138 [df]	493	37	—	—
SOFA ethyl esters	150 [f]	581	41	—	—

[1] Numbers in parentheses are % metabolizable calories supplied by ingredient; remainder (32) was protein.
[2] Values are averages of duplicate groups. Figures with differing superscript are significantly different ($P < 0.05$).
[3] Soybean oil fatty acids.

TABLE 5

Selected blood and liver constituents for 2-week-old chicks in the absorptive condition [1]

Treatment [2]	Blood glucose			Blood acetone + acetoacetate [3]	Blood total ketone bodies [3]	Liver glycogen
	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 3	Exp. 3
	mg/100 ml			*mg/100 ml*	*mg/100 ml*	*mg/100 g*
High glucose reference diet	191	191	222	1.7 [a]	4.0 [a]	3780 [a]
High soybean oil (SO) (68)	188	191	194	2.5 [b]	14.5 [b]	1360 [b]
Glucose (6) + SO (62)	199	—	193	1.7 [a]	3.0 [a]	1965 [c]
High SOFA [4] (68)	160	158	161	4.0 [c]	41.9 [c]	70 [d]
Glucose (6) + SOFA (62)	172	182	188	1.9 [ab]	4.6 [a]	465 [d]

[1] Values are averages of 10 chicks. Figures with differing superscript are significantly different ($P < 0.05$).
[2] Numbers in parentheses are % metabolizable calories supplied by ingredients; remainder (32) was protein.
[3] Expressed as acetone.
[4] Soybean oil fatty acids.

carbohydrate diet. These data show that the major blood ketone in carbohydrate-deficient chicks is β-hydroxybutyric acid, and this has been confirmed in further experiments.

DISCUSSION

The results of this series of experiments confirm previous reports that the chick is able to utilize very high levels of neutral fat in the diet (6, 7). Diets in which virtually all the non-protein calories (68%) were supplied by soybean oil promoted rapid growth, essentially equal to that of chicks fed a high glucose diet. In agreement with the report of Renner and Elcombe (8), the substitution of SOFA for soybean oil in a diet virtually devoid of digestible carbohydrate, resulted in severe retardation of growth to about half-maximum. However, in contrast with their observations we were unable to restore growth to normal by the addition of small amounts of glucose or glycerol to the high SOFA diet, although such supplements significantly improved rate of growth. This discrepancy between work in the 2 laboratories probably reflects differences in experimental methods. In our studies, all experimental diets were fed from hatching throughout the experimental period; Renner and Elcombe fed their chicks a high soybean oil diet for 4 or 7 days before commencing the feeding of the SOFA diets. Another difference which may be important was that the SOFA diets used by Renner contained a small amount of soybean oil, whereas our SOFA diets did not. In this respect, the good growth produced by the high lipid diet containing an equicaloric mixture of soybean oil and SOFA may be particularly significant; this effect of the intact oil may be due either to improvement in the rate of SOFA absorption, or to the addition of some essential component or property of the oil.

The growth-depressing properties of high levels of SOFA in the diet cannot be explained in terms of impaired absorbability of the fatty acids, because they were as well absorbed as the intact neutral fat. However, the total food intake and therefore the total amount of lipid absorbed and utilized was much greater when soybean oil was fed than when the diet was based on SOFA. A possible explanation for the very poor growth with SOFA is that the rate of its absorption, transport or a combination of these, or utilization, may be limiting, and that this in turn limits food intake. The marked improvement produced by replacing half the SOFA with soybean oil could be due to enhanced rate of SOFA absorption, brought about by the presence of the products of soybean oil digestion.

The mechanism whereby supplemental carbohydrate increases the growth of chicks fed fatty acid diets is not readily apparent. If it is assumed that intestinal fatty acids must be esterified into triglycerides at the intestinal mucosa before they are transported to other body tissues (32), then dietary carbohydrate may be increasing the utilization of the fatty acids by supplying the glycerol necessary to esterify them.

The growth-depressing properties of the free fatty acids were not overcome by feeding them as the corresponding methyl or ethyl derivatives. However, analysis of duodenal contents of chicks fed monesters revealed the presence of large amounts of free fatty acids, suggesting that they are hydrolyzed before intestinal absorption occurs. If this is the case, then it is reasonable to expect no large differences between diets based on free fatty acids or monoesters since they would be equivalent at the gut level. Also relevant is our inability to detect methyl esters by thin-layer chromatography in the abdominal fat of chicks fed high levels of SOFA methyl esters for 2 consecutive weeks.

The excreta of chicks fed the ethyl derivatives of SOFA contained large amounts of unhydrolyzed esters suggesting that their reduced absorbability was probably due to incomplete hydrolysis in the intestine.

Dermatitis of the feet and shortened, thickened mandibles were consistently observed in chicks fed diets containing high levels of SOFA. To our knowledge, this observation has not been reported previously. Though similar in outward appearance to the dermatitis produced in chicks by deficiency of pantothenic acid or biotin, the condition is probably not related to

these nutrients because they were provided at high levels in the experimental diets (approximately 5 times the minimal requirement).

Data on blood glucose and ketone bodies indicate that the chicks fed the high SOFA diet were in a physiological state of carbohydrate insufficiency which was completely prevented by supplementation with 6% glucose calories. Under these restricted experimental conditions, we postulate that the chick exhibits a requirement for exogenous carbohydrate. Contrary to the generally held view, our results show that the chicken is susceptible to ketosis. The magnitude of the ketonemia induced by the high fatty acid diet is comparable to that reported for ketotic cows, ewes and sows (33) and for fasting ducks (34). The evidence obtained in our work also shows that β-hydroxybutyrate is the predominant ketone body in the ketonemic chick.

The observation that a comparatively small amount of carbohydrate in the high fatty acid diet completely prevents hypoglycemia and ketonemia, but is unable to restore normal growth, shows that the fatty acids themselves are growth-depressing. We have presented experimental evidence (35) suggesting that this growth-inhibiting property of SOFA may be due to the lack of a nutrient required by the chick under these conditions and which is present in intact soybean oil.

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Some Aspects of Tryptophan and Niacin Metabolism in Young Women Consuming a Low Tryptophan Diet Supplemented with Niacin [1,2]

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ABSTRACT The metabolic use of supplements of niacin was studied in 6 college women transferred from a nutritionally adequate control diet of ordinary foods to a semipurified diet low in tryptophan and niacin. Criteria of evaluation were nitrogen balance, blood pyridine nucleotide levels, and urinary excretion of 6 tryptophan and 2 niacin metabolites. Nitrogen loss occurred with a tryptophan intake of 25 mg; nitrogen storage occurred on intakes of 160 or 185 mg. Niacin supplementation had no apparent effect on the amounts of nitrogen excreted. Conversely, the amount of nitrogen excreted did not correlate with metabolite excretion. Blood pyridine nucleotide levels decreased following the second period in which the diet was limiting in tryptophan and niacin, and 10 mg of niacin in the presence of 160 mg tryptophan re-established control levels. Additional niacin did not increase values further. The excretion of tryptophan metabolites tended to respond to the level of the tryptophan ingested, decreasing when tryptophan was limiting and increasing slightly when the tryptophan intake was increased. Niacin supplementation had no apparent effect on tryptophan metabolite excretion. Under these experimental conditions it appears that supplements of niacin added to a diet containing tryptophan at a level just sufficient to establish and maintain nitrogen balance are used first to establish and maintain the levels of the blood pyridine nucleotides and only then is there an increase in the excretion of urinary niacin metabolites.

The metabolic use of tryptophan in young adult women consuming a low niacin, low tryptophan, semipurified diet supplemented with gradually increasing levels of tryptophan has been reported by Vivian et al. (1) and Brown et al. (2). Under the conditions of the experiment it appeared that tryptophan was used first to establish and maintain nitrogen equilibrium, second for the synthesis of blood pyridine nucleotides and finally, when blood pyridine nucleotides had reached nearly normal levels, there was an increase in the urinary excretion of the niacin metabolites, N^1-methylnicotinamide and N-methyl-2-pyridone-5-carboxamide. The urinary excretion of tryptophan metabolites intermediate on the pathway from tryptophan to nicotinic acid followed, in general, the level of tryptophan furnished in the diet. Even at the lowest level of tryptophan intake (25 mg) these metabolites did not disappear completely from the urine. Under similar experimental conditions and using the same criteria of evaluation, the metabolic use of supplements of niacin with a diet low in niacin and tryptophan was investigated. Evidence was obtained which indicated that niacin was apparently used first for blood pyridine nucleotide synthesis and that niacin supplementation did not affect the urinary excretion of nitrogen or the measured tryptophan metabolites.

EXPERIMENTAL

Subjects. Six American college women of the Caucasian race participated in the study while engaging in their usual activities. They ranged from 21 to 32 years of

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[2] Data in this paper were taken in part from a thesis submitted by Virginia M. Vivian in partial fulfillment of the degree of Doctor of Philosophy.

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[4] American Cancer Society — Charles S. Hayden Foundation Professor of Surgery in Cancer Research.

age, from 166 to 175 cm in height and from 56.4 to 65.9 kg in weight. The subjects were determined to be in good health by physical examinations prior to and immediately following the experiment and had no known history of any metabolic disease.

Diets. The general plan of administration of the diet, composition and preparation of ordinary foods, amino acid mixtures, mineral mixtures, vitamin capsule, et cetera, have been described (1). The experimental periods with the dietary content of niacin and tryptophan are shown in table 1 and figures 1 and 2. For convenience in discussion the serial intervals of dietary experiences of the subjects are called "periods" and these are identified in table 1. A diet of ordinary foods was given for 6 days (period 1) to establish control values. A 3-day period (period 2) was provided for transition to the semipurified regimen (periods 3–9). The nitrogen content was approximately 10 g in all periods and supplements of niacin were given as aqueous solutions added to the amino acid solutions.

TABLE 1

Niacin and tryptophan intake during the periods of study

Period	Duration	Serial days of study	Niacin intake		Tryptophan intake		Niacin equivalents
	days		*mg*	*μmole*	*mg*	*μmole*	*mg*
1	6	1–6	10.0	82	680	3,333	21
2 [1]	3	7–9	—	—	—	—	—
3	6	10–15	2.5	21	25	123	3
4	6	16–21	2.5	21	185	907	6
5	2	22–23	3.5	29	185	907	7
6	5	24–28	2.5	21	25	123	3
7	5	29–33	5.0	41	160	784	8
8	5	34–38	10.0	82	160	784	13
9	7	39–45	15.0	123	160	784	18

[1] The content of the diet varied for each day of the transition period.

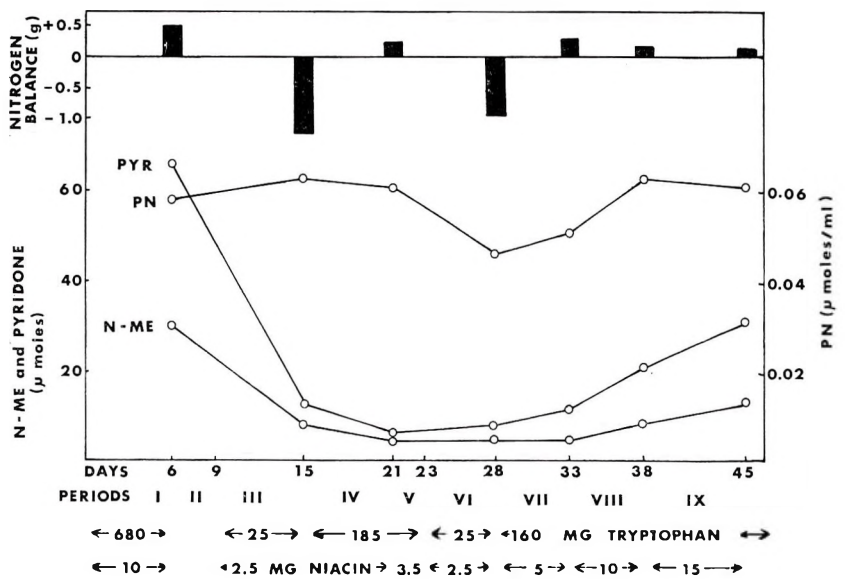

Fig. 1 The mean urinary excretion of N-methyl-2-pyridone-5-carboxamide (PYR) and N[1]-methylnicotinamide (N-Me), mean blood pyridine nucleotides (PN), and mean nitrogen balance per period during which various levels of tryptophan and niacin were ingested. The values represent the means of the last 2 days of each period for the 6 subjects.

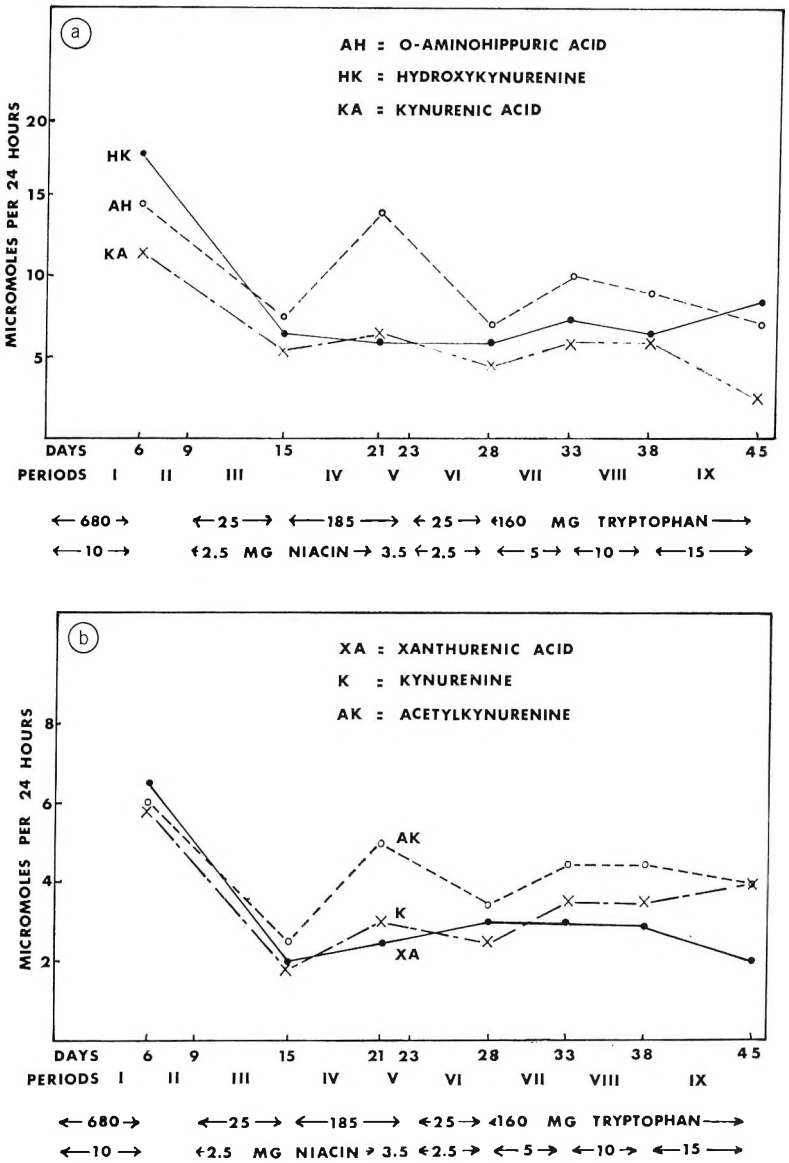

Fig. 2 The mean urinary excretion of hydroxykynurenine, o-aminohippuric acid, kynurenic acid, acetylkynurenine, kynurenine and xanthurenic acid per period during which various levels of tryptophan and niacin were ingested. The values represent the means of the last 2 days of each period for the 6 subjects.

Methods. Methods used for collection and preparation of urine, feces and food composites, for daily determinations of urinary creatinine, nitrogen and N-methyl-2-pyridone-5-carboxamide (pyridone) excretion and for blood pyridine nucleotide (PN) values measured on the last 2 days of each period have been described previously (1). N^1-Methylnicotinamide (N-Me) was determined by a procedure in which ion exchange resins were used to remove interfering substances from the urine before fluorescence was developed (3). Methods used for determination of 6 urinary metabolites of tryptophan (kynurenine, hydroxykynurenine, kynurenic

acid, *o*-aminohippuric acid, xanthurenic acid and acetylkynurenine) were those used in a previous study from these laboratories (2).

RESULTS

The values presented in figure 1 are the mean values for the last 2 days of each period (except periods 2 and 5) for all subjects for nitrogen balance, urinary excretion of N-Me and pyridone, and blood PN levels. Preliminary examination of data indicated that the ranges of excretions were similar to those observed in our related human metabolism studies and that the first few days of each period were required for adjustment to treatment. Nitrogen equilibrium was established for all subjects with the control diet of ordinary foods. Nitrogen loss was observed when the tryptophan intake was 25 mg; nitrogen retention occurred with intakes of 160 and 185 mg.

Blood PN values remained relatively constant in periods 1, 3 and 4 (fig. 1). The values decreased in period 6, the second period in which the diet supplied 25 mg tryptophan and 2.5 mg niacin. When the intakes of tryptophan and niacin were increased to 160 and 5 mg, respectively, in period 7, there was a slight rise in PN levels. When the niacin intake was increased to 10 mg, the PN values were the same as those observed in the first 3 periods. Increasing the niacin intake to 15 mg did not alter the PN levels.

The excretion of the pyridone and N-Me had decreased sharply by the end of period 3 (tryptophan intake, 25 mg; niacin intake, 2.5 mg) and continued to decrease in the next period even though the tryptophan intake was increased to 185 mg (fig. 1). When the tryptophan intake was again reduced to 25 mg in the next period, the excretion levels of niacin metabolites were not appreciably altered from the low values of the preceding periods. When tryptophan and niacin intakes were both increased in period 7 (160 and 5 mg, respectively), N-Me values remained essentially unaltered although the pyridone values increased slightly. Pyridone excretion continued to increase as the niacin intake was increased in the last 2 periods. N-Me excretion first increased when the niacin intake was 10 mg and continued to increase when 15 mg was provided. At the conclusion of the study the urinary excretion values of the 2 niacin metabolites were approximately 50% of the values observed in the control period.

The mean values for the excretion of the 6 urinary tryptophan metabolites studied for the last 2 days of each period for all subjects are shown in figure 2. The excretion of each of these metabolites had sharply decreased by the end of period 3. Excretion of the metabolites (except hydroxykynurenine) increased slightly when the dietary tryptophan content was increased in period 4 and decreased again when the tryptophan intake was lowered in period 6, except for xanthurenic acid excretion which continued to increase in this period. Xanthurenic acid values remained at these same levels when the tryptophan and niacin intakes were increased in period 7, whereas excretion of the other 5 metabolites increased. In period 8 (niacin increased to 10 mg) excretion of hydroxykynurenine and o-aminohippuric acid decreased slightly, whereas the values for the other 4 metabolites remained unchanged. When the niacin intake was further increased to 15 mg, hydroxykynurenine excretion increased, o-aminohippuric acid, kynurenic acid and xanthurenic acid excretion decreased and kynurenine and acetylkynurenine values showed little change. At the end of the study the excretion of these metabolites remained well below the levels observed during the ingestion of ordinary foods with the higher tryptophan intake.

DISCUSSION

A daily minimal requirement of 157 mg of tryptophan for young adult women was reported by Leverton et al. (4). In this experiment, after nitrogen equilibrium had been established with 160 mg of tryptophan and 5 mg niacin, supplements of niacin had no apparent effect on the amounts of nitrogen excreted. This observation is in agreement with that of Horwitt et al. (5), who reported that variations in tryptophan and niacin intakes above 280 mg and 5.5 mg, respectively, had no apparent effect on nitrogen excretion.

Vivian et al. (1) had previously reported that blood PN levels decreased in a 6-day period in which the diet furnished 25 mg tryptophan and 2.5 mg niacin and continued to decrease in the subsequent period even though the tryptophan intake was increased to 170 mg. These results were interpreted as evidence that the tryptophan intake in the 2 periods was inadequate to maintain normal blood PN levels. In the current study, however, the PN values were not lowered in the corresponding periods (3 and 4, fig. 1). The observed stability of the PN levels may have reflected a better pre-study nutritional status of these subjects. Therefore, in an effort to reduce PN levels, the experimental plan was altered by terminating period 5, instituting a second period (period 6) in which the tryptophan and niacin intakes were low and reducing the intake of tryptophan from 185 to 160 mg in subsequent periods. By the end of period 6 the average PN values had decreased to the same lower levels observed in the earlier study. When 160 mg tryptophan and 5 mg niacin were fed in period 7, PN levels increased. Although both niacin and tryptophan were increased simultaneously, it is probable that the PN response is largely attributable to the increase in niacin as Vivian et al. (1) had demonstrated that 170 mg of tryptophan was used to establish nitrogen equilibrium and did not alter the pyridine nucleotide levels. When the niacin intake was raised to 10 mg (period 8), PN values were comparable to those of the control period and the increase to 15 mg was not associated with a further response. The PN levels of these 2 periods are in good agreement with the reported blood PN values of subjects eating various normal diets as compiled from the literature by Morley and Storvick (6). Thus, under the present experimental conditions, 10 mg of niacin in the presence of 160 mg of tryptophan was adequate to re-establish and maintain control PN levels.

In period 1 the average pyridone excretion was slightly more than twice that of the N-Me. At the end of period 4 the average excretion of N-Me was only slightly less than that of the pyridone. When the niacin content of the diet was increased, the level of pyridone excretion increased more rapidly than that of the N-Me. These observations were similar to those of Rosenthal et al. (7) who reported that following the administration of graded doses of nicotinamide, the excretion of pyridone increased more rapidly than N-Me. Goldsmith et al. (8) reported that an average of 56.9% of supplemental niacinamide was excreted as these 2 metabolites, pyridone accounting for 70 to 90% of the increase and N-Me for 10 to 30%. In the present study 30 to 40% of each niacin addition (5 mg) was accounted for by an increase in the excretion of niacin metabolites, but each metabolite contributed the same percentage of increase.

Several investigators have suggested using the ratio of urinary pyridone to N-Me as a tool in measuring niacin status (9–12). A low ratio has been interpreted to indicate that a deficiency of niacin in the diet or body tissues, or both, exists. In the present study the excretion of pyridone always remained higher than that of the N-Me and hence the ratio was never less than one. In a previous study by Vivian et al. (1) which was conducted with a diet low in tryptophan and niacin, using a procedure similar to this one, the ratio of pyridone to N-Me excreted was less than one for all subjects in the depletion period. It should be emphasized that the N-Me values in the present study were determined by the revised method of Vivian et al. (3) and were lower than those reported by Vivian et al. (1) in the earlier study using another analytical method. Therefore, the ratio of pyridone to N-Me excreted will depend upon the analytical procedures which are used.

When tryptophan and niacin were furnished in limited amounts, there was little variation among the subjects in the amounts of pyridone and N-Me excreted. When larger amounts of the limiting components were supplied, variation in excretion among subjects became more apparent. This observation is in agreement with data from similar studies (1, 13) and may be interpreted to represent individual differences in the metabolism of tryptophan and niacin when these components were

supplied in some excess of the individual requirement.

The excretion values of N-Me and pyridone in period 9 (fig. 1) are approximately 50% of the values in the control period (period 1). Calculated on the basis of niacin equivalents, assuming 60 mg of tryptophan to be equivalent to 1 mg of niacin (5, 8), the food in period 1 supplied 21 niacin-equivalents and in period 9 about 18 (table 1). Thus, on nearly comparable intakes of niacin-equivalents there was a 50% difference in the excretory levels of the 2 niacin metabolites. One possible explanation of this observation is that the difference in excretion levels may indicate tissue niacin repletion. Also, the difference in the individual contribution of the niacin and tryptophan to the niacin-equivalents, that is, 11 equivalents from tryptophan and 10 from niacin in period 1 (table 1) and 3 from tryptophan and 15 from niacin in period 9, may account for the observed difference in the excretion of the niacin metabolites.

The excretion of the 6 measured tryptophan metabolites, although sharply decreased when the tryptophan intake was restricted, did not decrease to zero, indicating that some conversion of tryptophan to niacin occurred even when the intake of the amino acid was extremely low. Some tryptophan might have been available from catabolic release from body protein during negative nitrogen balance. However, these data are in agreement with the suggestion of Vivian (13) that 3% of tryptophan is always converted to niacin regardless of protein metabolism status. Increasing the niacin intake to 5 or 10 mg (periods 7 and 8) produced relatively little change in the excretion of these metabolites. However, when the niacin intake was increased to 15 mg, the excretion of the quinoline derivatives decreased, whereas the excretion of the kynurenines increased. This pattern somewhat resembles that observed when there is a disturbance in kynurenine transaminase, such as occurs in nutritional pyridoxine deficiency (14). Since an ample supply (3.5 mg) of pyridoxine was provided, there is no evident explanation for this excretion pattern. However, since the low excretion levels of these tryptophan metabolites border on the limits of sensitivity of the analytical methods used, the changes may not be of significance.

Under these experimental conditions it appears that the addition of supplements of niacin to a diet containing tryptophan at a level just sufficient to establish and maintain nitrogen balance are used first to establish and maintain the levels of the blood pyridine nucleotides, and only then is there an increase in the excretion of urinary niacin metabolites.

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Relationship of Fractions of Soybeans and a Crystalline Soybean Trypsin Inhibitor to the Effects of Feeding Unheated Soybean Meal to Chicks [1]

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ABSTRACT Several experiments were conducted to study the effects of fractions isolated from unheated soybean meal on pancreatic hypertrophy, fat absorption, utilization of non-fat components of the diet, and growth rate of chicks, to determine whether these measurements were affected by specific components of the meal. Unheated soybean meal was fractionated into a water-insoluble residue, pH 4.4 insoluble proteins, salts insoluble at pH 8, and whey proteins recovered by dialysis and lyophilization of the portion of the meal soluble at pH 4.4. When fed to chicks at levels of 1.3 to 1.9% of the diet, the soybean whey proteins markedly depressed growth rate, caused pancreatic hypertrophy, reduced fat absorption, and lowered metabolizable energy value of the diet. Whey proteins were batch separated on DEAE cellulose into 2 fractions, one high in hemagglutinin activity and one high in trypsin inhibitor activity. Neither fraction when fed alone affected growth rate, fat absorption or metabolizable energy value of the diet to the same extent as a combination of the 2 fractions. A diet containing 0.6% of a crystalline Kunitz soybean trypsin inhibitor depressed growth rate of chicks and metabolizable energy value of the diet, caused pancreatic hypertrophy, but had no other undesirable effects. These experiments suggest that multiple factors are present in unheated soybean meal which cause the detrimental effects observed.

Studies by Brambila et al. (1) and Nesheim et al. (2) showed that the absorption of dietary triglycerides and dietary free fatty acids was markedly depressed in chicks fed diets containing unheated soybean meal, being most pronounced up to 3 weeks of age. Chicks 4 weeks of age or older showed little impairment of fat absorption. Supplementation of the diet containing unheated soybean meal with crude or crystalline trypsin preparations (1), or with sodium taurocholate (3) restored fat absorption to normal in 2-week-old chicks. Despite the effectiveness of trypsin supplementation, trypsin inhibitors per se do not appear to be responsible for poor fat absorption as feeding a highly purified trypsin inhibitor from soybeans did not result in poor fat absorption (2).

Reports by several investigators (4–6) have indicated that unheated soybean meal decreased chick growth rate, reduced the dietary metabolizable energy value, and produced pancreatic hypertrophy. Some of these effects could be caused by feeding chicks one of the trypsin-chymotrypsin inhibiting proteins in the soybean (2).

The objective of the present investigation was to study the effects of fractions of unheated soybean meal on pancreatic hypertrophy, decreased fat absorption, decreased utilization of the non-fat portion of the diet, and poor growth of chicks in an attempt to determine whether these effects were caused by specific components of the meal.

METHODS AND PROCEDURES

Male Rhode Island Red × Barred Plymouth Rock chicks purchased from a commercial hatchery were fed the basal diet (2) which contained heated soybean meal (50%), soybean oil (15%), and glucose as major components with methionine (0.3%), vitamin and mineral supplements.

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When unheated soybean meal was included in the diet it replaced a portion of heated soybean meal. The total amount of soybean meal was 50% of all diets. Solvent-extracted dehulled, soybean flakes obtained from a commercial soybean processing plant, at the step prior to removal of the solvent, were used as "unheated" meal in all experiments and for fractionations. The solvent was removed by evaporation into the air at ambient temperature. In experiments 3 and 4, the heated soybean meal was prepared by autoclaving the raw meal at 107° for 30 minutes according to procedures described by Renner and Hill (4), while in the remaining experiments commercially processed, dehulled soybean meal was used.

Fat absorption and metabolizable energy (ME) value of diets were measured according to procedures described by Renner and Hill (7) and Hill and Anderson (8). The metabolizable energy values reported in the tables are adjusted to a common degree of fat absorption using a value of 9.4 kcal/g as gross energy of unabsorbed fat, to allow a comparison of the utilization of non-fat components of the diets under conditions where fat absorption was not equal in all groups of chicks studied. The metabolizable energy values are all corrected to nitrogen equilibrium so that differences in nitrogen retention also do not affect comparisons of ME values of various diets.

Fresh pancreas weights were determined immediately after killing the chicks. Pancreas weights given in the tables have been adjusted for differences in body weight among the treatments by analysis of covariance (9).

Hemagglutinin activity was determined by the method of Liener (10).

Trypsin inhibitor activity of soybean meal and various fractions was determined by measurement of the inhibition of hydrolysis of benzoyl-DL-arginine *p*-nitroanilide (11) by trypsin. The crystalline trypsin inhibitor [3] used in experiment 4 is called the Kunitz inhibitor (12) in this paper.

Chromatographic analysis of fractions from soybeans and trypsin inhibitor preparations was made on diethyl-amino-ethyl (DEAE) cellulose using gradient elution procedures described by Rackis et al. (13). Protein analysis of the column effluent was conducted on individual fractions by the procedure of Lowry (14), or automatically by analyzing column effluent directly by the Lowry method with an autoanalyzer.[4]

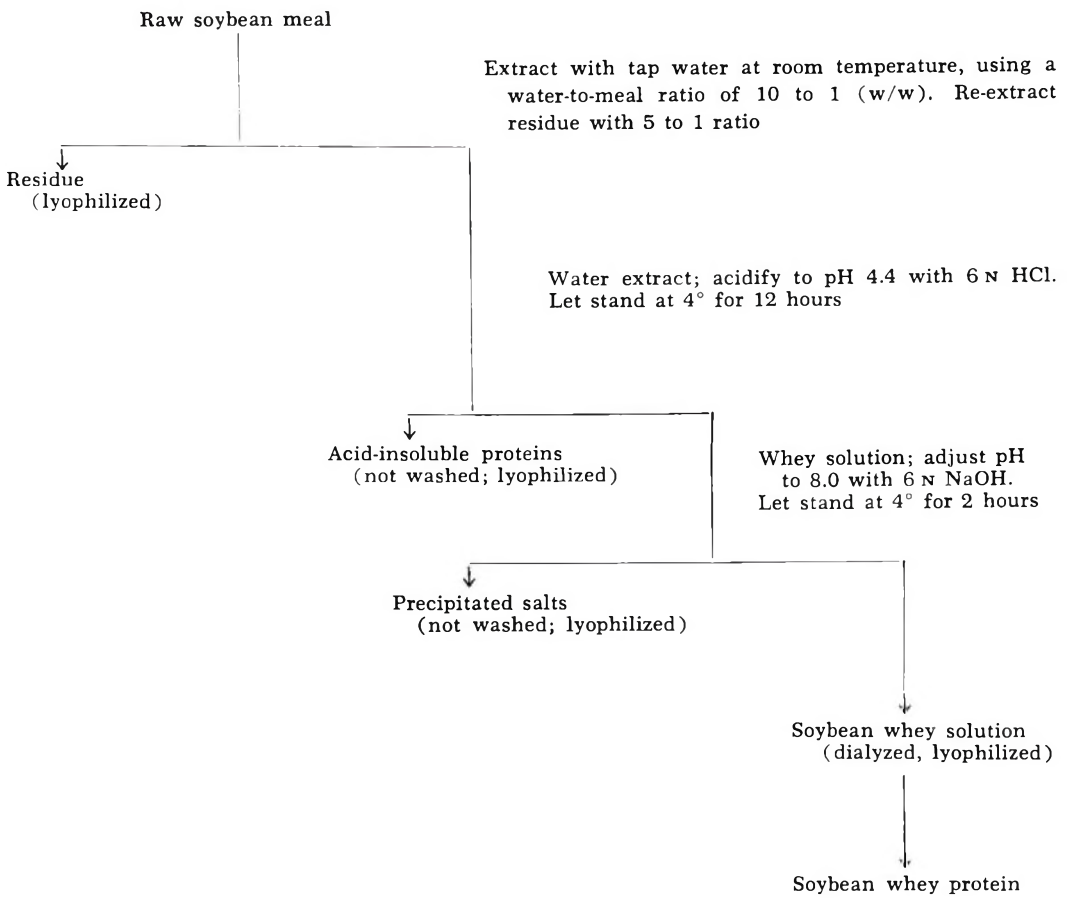
Batch fractionation of soybean whey proteins on DEAE cellulose was conducted as follows: DEAE cellulose (300 g) was suspended in 3 liters of distilled water and the pH adjusted to 7.6 with a saturated solution of KH_2PO_4. The suspension was poured on a large Büchner funnel and filtered. The cellulose was resuspended in 2 liters of solution of 0.034 M sodium chloride in 0.01 M phosphate buffer, pH 7.6. The suspension was filtered, and washed with the sodium chloride-buffer solution twice more. After washing, the DEAE cellulose was left on the filter and 75 g of soybean whey proteins dissolved in 750 ml of 0.034 M sodium chloride in 0.01 M phosphate buffer were added to the DEAE cellulose-packed funnel. The soybean whey protein was washed into the DEAE cellulose on the funnel with one liter of the 0.034 M sodium chloride-buffer solution. The proteins not retained by the DEAE celluose at this salt concentration were washed from the funnel with three one-liter washings of 0.034 M sodium chloride-buffer solution. These proteins were called fraction 1. The receiving flasks were changed and the remainder of the proteins were eluted from the cellulose with three one-liter quantities of 0.5 M sodium chloride in 0.01 molar phosphate buffer. These proteins were called fraction 2. The eluted fractions were dialyzed at 4° against distilled water until barely detectable amounts of chloride ion remained in the dialyzate, and the dialyzate was frozen and lyophilized. This procedure was repeated 4 times to prepare a sufficient quantity for a feeding experiment. The 8 individual preparations were chromatographed on DEAE cellulose. The elution patterns for a given fraction were found to be similar for each preparation. The respective fractions were combined.

[3] Obtained from Nutritional Biochemicals Corporation, Cleveland, as 5X crystalline soybean trypsin inhibitor, lot no. 3358.

[4] Technicon Chromatography Corporation, Chauncey, New York.

FIGURE 1

Fractionation of raw soybean meal



EXPERIMENTAL

In experiment 1 unheated soybean meal was fractionated into 4 components (fig. 1) to determine whether any of the biological activities could be concentrated in a single component. The procedure used to prepare these fractions is essentially that used by Rackis et al. (13). Fractionation of duplicate 50-g samples of unheated meal by this procedure yielded the following distribution: water-insoluble residue 58.1%, pH 4.4 insoluble proteins 19.6%, pH 8 insoluble material 0.42%, and total solids in soybean whey solution 21.8%. After dialysis and lyophilization, the whey proteins recovered from the soybean whey solution represented 2% of the original meal extracted. The design and results of this experiment are included in table 1.

Diets were formulated which contained the residue, pH 4.4 insoluble proteins, or the pH 8 insoluble residue in the same proportions as would be present in a diet containing 15% unheated soybean meal. The fractions were added to the diet at the expense of glucose. The soybean whey proteins were added to the basal diet to provide the same amount of trypsin inhibitor activity as provided by 11.5% unheated soybean meal. The soybean whey protein fraction contained most of the trypsin inhibitor activity of the unheated soybean

TABLE 1
Effect of soybean fractions on growth and fat absorption (exp. 1)

	Dietary treatment [1]	Body wt, 2 weeks	Adjusted pancreas wt	Feed consumption	Fat absorption	ME value of diet
		g	*g*	*g/chick*	%	*kcal/g*
1	Heated soybean meal	167^{a} [2]	0.47^{a} [2]	182	91^{a} [2]	3.68^{a} [2]
2	15% Unheated soybean meal	107^{e}	—	231	58^{c}	—
3	9.3% Residue	134^{bcd}	0.72 [3]	169	80^{ab}	—
4	3.14% pH 4.4 Insoluble proteins	151^{ab}	—	204	81^{ab}	—
5	0.07% pH 8 Insoluble salts	160^{a}	—	164	81^{ab}	—
6	Combination of treatments 3, 4 and 5	141^{bc}	—	187	82^{ab}	—
7	11.5% Unheated soybean meal	131^{cd}	0.84^{b}	213	65^{bc}	3.28^{b}
8	1.3% Whey proteins	118^{de}	0.78^{b}	211	55^{c}	3.24^{b}

[1] Each diet was fed from hatching to 2 weeks of age to 3 replicate groups of 8 Rhode Island Red × Barred Plymouth Rock male chicks except diet 8, which was fed to 3 replicate groups of 7 chicks. All diets contained 50% soybean meal; in treatments 2 and 7 unheated meal replaced heated meal. Fractions in rest of treatments replaced glucose.

[2] Values not followed by same letter are significantly different ($P < 0.05$), by Duncan's multiple range test (30).

[3] Pancreas weights from 24 chicks in treatments 1 and 7, 21 chicks in treatment 8, and 12 chicks in treatment 3. Treatment 3 not included in statistical analysis.

meal. Quantitative recovery of undenatured soybean whey proteins was difficult. Therefore, equating the soybean whey proteins and unheated soybean meal appeared to be more valid on the basis of trypsin inhibitor activity than on fractionation yield.

Growth rate was significantly depressed only when chicks were fed the diets containing unheated soybean meal, residue, or soybean whey proteins. The absorption of fat by chicks fed the residue, acid-insoluble proteins, and the pH 8.2 insoluble salts was not significantly depressed compared with that of chicks fed the basal diet. The small effects on fat absorption of these fractions were not additive because their combination (treatment 6) resulted in no poorer fat absorption than the addition of any one fraction alone. Fat absorption was significantly depressed only in the chicks that consumed diets containing the soybean whey proteins or unheated soybean meal. Pancreas size of chicks fed the residue was increased compared with that of chicks fed the basal diet. Measurements of pancreas weight were not made on chicks fed the acid-insoluble proteins and the insoluble salts.

Chicks fed the diet containing the soybean whey proteins not only absorbed fat poorly, but growth rate and utilization of the non-fat portion of the diet were decreased and pancreas size increased, compared with the chicks receiving heated soybean meal. These effects were as great or greater than those of feeding a diet containing unheated soybean meal.

To aid in the preparation of large quantities of soybean whey, the method described in figure 1 was modified to precipitate the proteins from soybean whey with acetone.

Two volumes of cold acetone were added to the undialyzed soybean whey solution. A small amount of saturated sodium chloride solution was added to induce flocculation of the protein. The precipitate was suspended in distilled water and dialyzed at 4° for 48 hours. Any insoluble material remaining after dialysis was discarded. Upon lyophilization, the supernatant solution yielded soybean whey proteins equivalent to about 1% of the original weight of unheated soybean meal extracted. Experiment 2 was conducted to determine the effect of feeding whey prepared in this manner on chick growth rate and ability to absorb fat. The soybean whey proteins were added to the diet at a level equivalent to 15% unheated soybean meal in trypsin inhibitor activity. The results are shown in table 2.

Chicks fed the diet containing the soybean whey proteins grew more slowly and absorbed fat poorly compared with the chicks fed a similar diet containing no whey proteins. The use of acetone in the isolation procedure did not appear to destroy the biological activity of the whey. This experiment confirmed the effects of feeding soybean whey as previously re-

TABLE 2

Effect of acetone-precipitated soybean whey proteins on chick performance (exp. 2)

	Dietary treatment [1,2]	Body wt gain [3]	Apparent fat absorption	Feed consumption	Feed/gain
		g	*%*	*g/chick*	
1	Heated soybean meal	106	90	136	1.29
2	18% Unheated soybean meal	86	74	136	1.58
3	1.5% Acetone-precipitated soybean whey proteins	78	69	141	1.85

[1] Each diet was fed to 3 replicate groups of 9 Rhode Island Red × Barred Plymouth Rock male chicks.
[2] All diets contain 50% soybean meal and 15% soybean oil. The unheated soybean meal replaced an equal weight of heated soybean meal in the basal diet. The soybean whey proteins replaced an equal weight of glucose.
[3] Body weight gain from 8 to 17 days of age (fed experimental diets 9 days).

ported. The soybean whey proteins used in the next experiment were prepared by acetone precipitation.

The earlier experiments showed that dialyzed, lyophilized soybean whey proteins contained components which could duplicate the effects observed when unheated soybean meal was fed to chicks. To separate factors responsible for these biological effects, further fractionation of soybean whey was attempted. Rackis et al. (13) showed that soybean whey proteins could be fractionated into a number of components by chromatography on DEAE cellulose. Components of soybean whey were separated by elution from the DEAE cellulose by varying concentrations of sodium chloride in buffer. By this procedure the major trypsin inhibitors of soybean whey were eluted from the DEAE cellulose by relatively high concentrations of sodium chloride, whereas many other components including the hemagglutinin activity were eluted at relatively low salt concentration. Therefore, batch fractionation of soybean whey into 2 components, called fraction 1 (F_1) and fraction 2 (F_2) was accomplished by the procedure outlined under Methods. The DEAE cellulose chromatography of these 2 fractions on an analytical column is shown in figure 2. The fractionation procedure was largely successful in separating the whey protein into 2 fractions; however, a small amount of overlapping does occur. Fraction 1 contained 44% of the original protein of the whey and fraction 2 contained 56%. Fraction 2 contained 1.8 trypsin inhibitor units/μg, whereas fraction 1 contained 0.5 units/μg. The hemagglutinin activity of fraction 1

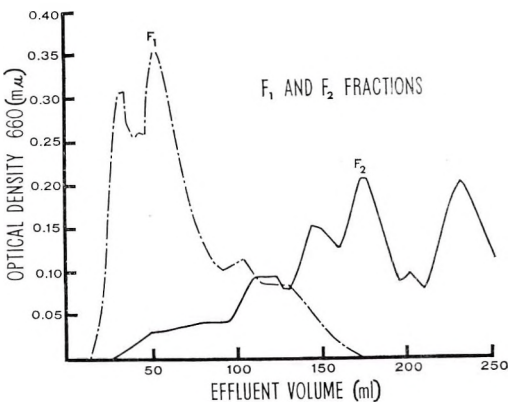


Fig. 2 Chromatography of Fraction$_1$ and Fraction$_2$ preparations from soybean whey on DEAE cellulose. Proteins eluted from 1.5 × 17 cm column by gradient elution 0 to 0.3 M sodium chloride in 0.01 M pH 7.6 phosphate buffer with 160-ml mixing chamber.

was 43 units/mg of dry matter, whereas fraction 2 contained only 2 hemagglutinin units/mg.[5] The batch fractionation had effectively separated the hemagglutinin activity of the soybean whey and had concentrated the trypsin inhibitor activity in one fraction and the hemagglutinin in another.

In experiment 3, these 2 fractions were fed to chicks separately and in combination (table 3) in the proportions that would be present if the soybean whey proteins were 1.9% of the diet. Unheated soybean meal was fed to provide the same trypsin inhibitor activity as the 1.9% combined soybean whey proteins.

[5] The hemagglutinin measurements reported were made by Dr. Walther Sambeth, whose assistance is gratefully acknowledged.

Chicks fed the diet containing fraction 1 grew at the same rate as those fed the control diet. Pancreas weights were significantly increased. This is probably a response to the trypsin inhibitor proteins present in fraction 1. The addition of fraction 1 to the diet significantly reduced the utilization of the non-fat portion of the diet as indicated by the lower adjusted metabolizable energy value.

The apparent decrease in fat absorption by chicks fed the diet containing fraction 1 was not statistically significant, but does suggest that fraction 1 may contain a small amount of a component responsible for decreasing fat absorption.

Chicks fed the diet containing fraction 2 grew significantly more slowly than the controls. Fat absorption and metabolizable energy value of the diet were also significantly depressed by the F_2 fraction. The decreased utilization of the non-fat portion of the diet as indicated by the low adjusted metabolizable energy value may be at least partially attributed to the large amount of trypsin-chymotrypsin inhibiting activity in fraction 2. The Kunitz trypsin inhibitor which is present in fraction 2 is known to decrease utilization of the non-fat portion of the diet (table 4).

All of the measurements made, except pancreatic hypertrophy, were affected more by the combination of fractions 1 and 2 than by either fed separately. The effects on growth rate and fat absorption in particular appear to be greater than the sum of the effects of the 2 fractions. The combined fractions were as effective in reduc-

TABLE 3

Effects of feeding diets containing soybean whey protein fractions (exp. 3)

	Dietary treatment [1,2]	Body wt, 12 days	Feed consumption	Feed/gain	N retention	Adjusted pancreas wt [3]	Fat absorption	Adjusted ME value
		g	*g/chick*		*% of intake*	*g*	*%*	*kcal/g*
1	Heated soybean meal (HSBM)	146[a 4]	140	1.31	54[a]	0.49[a 5]	91[a]	3.73[a]
2	HSBM + 0.836% fraction 1	142[a]	136	1.32	48[ab]	0.57[b]	77[ab]	3.58[b]
3	HSBM + 1.064% fraction 2	127[b]	132	1.50	43[b]	0.91[d]	70[b]	3.41[c]
4	HSBM + 0.836% fraction 1 + 1.064% fraction 2	108[c]	142	2.06	30[c]	0.78[c]	40[c]	3.15[d]
5	41.8% Unheated soybean meal	92[d]	139	2.62	24[d]	0.82[c]	16[d]	3.13[d]

[1] Each value (except pancreas weights) represents average of determinations on 3 replicate groups of 10 Rhode Island Red × Barred Plymouth Rock male chicks. Diets were fed from hatching to 12 days of age.
[2] All diets contain 15% added soybean oil and 50% of soybean meal. In diet 5 the unheated soybean meal replaced an equal weight of heated soybean meal. In diets 2, 3 and 4, the fractions replaced an equal weight of glucose monohydrate.
[3] Each value represents the average of 24 chicks.
[4] Duncan's multiple range test (30): Values not followed by the same letter are significantly different ($P < 0.01$).
[5] Duncan's multiple range test: Values not followed by the same letter are significantly different ($P < 0.05$).

TABLE 4

Comparison of the soybean trypsin inhibitors in their effects on chick performance (exp. 4)

	Dietary treatment [1,2]	Body wt	Adjusted pancreas wt [3]	Fat absorption	Feed consumption	Adjusted ME value	Feed/gain
		g	*g*	*%*	*g/chick*	*kcal/g*	
1	Heated soybean meal	177[a 4]	0.58[a 4]	95[a 4]	185	3.79[a 4]	1.34
2	Ethanol-soluble pH 4.8 soluble proteins, 0.39%	168[ab]	0.90[b]	91[a]	178	3.78[a]	1.39
3	Kunitz soybean trypsin inhibitor, 0.6%	158[bc]	1.05[b]	85[ab]	189	3.55[b]	1.59
4	Raw soybean meal, 7%	151[bc]	0.91[b]	84[ab]	184	3.68[ab]	1.64
5	Raw soybean meal, 12.5%	143[cd]	1.00[b]	67[bc]	194	3.59[b]	1.85
6	Raw soybean meal, 18%	125[de]	1.00[b]	50[c]	194	3.40[c]	2.13

[1] Each value except pancreas weight data represents the average of determinations on 3 replicate groups of 8 Rhode Island Red × Barred Plymouth Rock male chicks. Diets were fed from hatching to 2 weeks of age.
[2] All diets contain 15% soybean oil and 50% soybean meal; in diets 4, 5 and 6 unheated meal replaced heated soybean meal and in treatments 2 and 3 the inhibitor preparations replaced glucose.
[3] Each value represents the average of 21 chicks.
[4] Duncan's multiple range test (30): values not followed by the same letter are significantly different ($P < 0.01$).

ing the adjusted metabolizable energy value of the diet as the amount of unheated soybean meal which provided the same level of trypsin inhibitor activity.

The results of this experiment may be interpreted in several ways. The combination of the fractions may have been no more detrimental than the effects which would be observed upon feeding the same high level of either fraction alone. There is some overlap of proteins contained in the 2 fractions (fig. 2), and the combination may have increased the dietary concentration of a highly active component. However, it is possible that certain components in one fraction are made more effective by action of components in the other fraction.

The combination of fraction 1 and fraction 2 appeared to decrease fat absorption to a greater extent than the sum of the effects of either fraction alone. The overall digestibility of the diet was markedly reduced by the combined fractions. Possibly some of the most active components of the fractions were protected longer from proteolytic digestion and thus exerted greater effects on the chick.

Effects of ingested trypsin inhibitor. Nesheim et al. (2) reported previously that a crystalline Kunitz soybean trypsin inhibitor preparation depressed chick growth rate and metabolizable energy value of the diet and caused pancreatic hypertrophy. Further studies of the trypsin inhibitors of soybean whey were conducted. A crude trypsin inhibitor was prepared from soybeans by a method similar to that of Bowman (15). The defatted meal was extracted with 60% ethanol (solvent-to-meal ratio, 1:10). The pH of the extract was adjusted to 4.4 to precipitate proteins insoluble at this pH, then dialyzed and lyophilized. The extraction of the meal with 60% ethanol left most of the Kunitz inhibitor behind in the residue. The 60% ethanol-soluble proteins were chromatographed on DEAE cellulose and found to contain as a major component a trypsin inhibiting protein with chromatographic behavior and chymotrypsin inhibiting properties which suggested that it was identical with the inhibitor isolated by Rackis (16) called the A_1 inhibitor. A small amount of Kunitz trypsin inhibitor was present and other trypsin inhibitor proteins were observed. The latter are probably similar to those subsequently isolated by Rackis et al. (17) and called B_1 and B_2. This preparation along with a commercial crystalline Kunitz inhibitor was fed to chicks in experiment 4, which was designed to study the relative effects of feeding different trypsin inhibitors from soybeans. The Kunitz inhibitor was found to be nearly homogeneous by chromatography on DEAE cellulose with analytical procedures described by Rackis et al. (16). Only a small amount of denatured inhibtor appeared to be present. Although the 60% ethanol-soluble inhibitor preparation was not pure, it had more trypsin inhibiting activity per unit weight than the Kunitz inhibitor (1.5 times), possibly because of the lower molecular weight of the A_1 inhibitor. The trypsin inhibitor activity per unit weight of the A_1 inhibitor was reported by Rackis to be 1.6 times that of the Kunitz inhibitor, whereas the B_1 and B_2 inhibitors have 1.8 and 2.0 times, respectively, the activity of the Kunitz inhibitor (17). The design and results of experiment 4 are shown in table 4. The trypsin inhibitors were added to the control diet at levels to provide an amount of activity equivalent to a diet containing 12.5% unheated soybean meal. To obtain an estimate of effects of slightly higher or lower levels of unheated soybean meal compared with those of the inhibitors, 7 and 18% unheated soybean meal were also fed.

The Kunitz trypsin inhibitor depressed growth, caused pancreatic hypertrophy and depressed metabolizable energy value of the diet, but had little effect on fat absorption. The comparable level of unheated soybean meal (12.5%) depressed growth rate and fat absorption to a greater extent than the inhibitor. The metabolizable energy value of the diets containing the 12.5% raw soybean meal or the Kunitz trypsin inhibitor were decreased and the pancreas weight increased to the same extent. The ethanol-soluble trypsin inhibitors had no significant effect on growth rate, fat absorption, or metabolizable energy value of the diet but did produce pancreatic hypertrophy.

These data indicate that not all of the inhibitor proteins in soybeans have similar

effects when fed to chicks. A highly purified Kunitz soybean trypsin inhibitor, however, did significantly affect growth rate and utilization of the diet. Neither inhibitor preparation had a significant effect on the absorption of fat by chicks. This confirms previous observations (2).

Trypsin inhibitors are widely distributed in nature, and differ markedly in their molecular size and occurrence. Egg white is a particularly rich source of naturally occurring trypsin inhibitors. The ovomucoid of egg white has long been known to possess this activity, and has been shown to be a complex of proteins with varying degrees of trypsin and chymotrypsin inhibiting properties (18). Experiment 5 was conducted to measure the relative effects of feeding lyophilized raw egg white, ovomucoid, and raw soybean meal on an equivalent trypsin inhibitor basis. The ovomucoid was prepared from egg white by the method of Lineweaver and Murray (19). In this experiment (table 5) chicks fed raw soybean meal grew more slowly than control chicks fed the heated meal. Growth rate, fat absorption and nitrogen retention were not affected by feeding the egg white or ovomucoid. Compared with the pancreases of chicks fed the heated soybean meal, those of chicks fed egg white and ovomumcoid were enlarged, but not as much as those of chicks fed unheated soybean meal. This suggests that pancreatic hypertrophy per se is not related to other effects of feeding unheated soybean meal such as poor growth rate, fat absorption or dietary energy utilization.

DISCUSSION

The results of these experiments show that the effects of feeding unheated soybean meal to chicks can be duplicated by a small fraction of the meal, which appears to consist of those proteins which are soluble in aqueous solution at pH 4.4. Added in small quantities to the control diet, these soybean "whey proteins," as shown by the poor fat absorption and low metabolizable energy value of the diet for chicks, can markedly influence the utilization of other dietary components. It has been shown (20) that this low metabolizable energy value is almost entirely due to poor digestion of protein. The whey proteins must interfere with the digestion of other proteins, either dietary or endogenous, to cause such a marked decrease in overall digestibility. If all the depression in metabolizable energy came from lowered protein digestibility (assuming 4.2 kcal/g as the ME of protein), protein digestibility in the diet containing the Kunitz inhibitor (table 4) would be about 12% less than in the control diet. The undigested protein required to cause such a metabolizable energy depression with diets containing soybean whey (tables 1 and 3) would amount to 40 to 55% of the dietary protein. This would be consistent with the impaired proteolysis found by other investigators (21) in intestinal tracts of chicks fed unheated soybean meal. Only a slight depression in protein digestibility has been observed in rats (22).

Recently, Lepkovsky et al. (23) reported that nitrogen digestibility was markedly

TABLE 5

Comparison of the effects of ingestion of ovomucoid, raw egg white, and raw soybean meal by chicks (exp. 5)

	Dietary treatment [1,2]	Body wt, 2 weeks	Apparent fat absorption	Adjusted pancreas wt [3]	N retention	Feed consumption	Feed/gain
		g	*%*	*g*	*% of intake*	*g/chick*	
1	Heated soybean meal	152[a 4]	91[a 4]	0.54[a 4]	55[a 4]	171	1.51
2	Raw egg white, 8.6%	154[a]	84[a]	0.72[b]	45[a]	177	1.54
3	Ovomucoid, 1.06%	150[a]	82[a]	0.71[b]	48[a]	180	1.62
4	Unheated soybean meal, 20.8%	115[b]	25[b]	0.82[c]	24[b]	207	2.80

[1] Each value except for pancreas weight data is average of determinations on 3 replicate groups of 9 Rhode Island Red x Barred Plymouth Rock male chicks. Diets were fed from hatching to 2 weeks of age.
[2] Raw egg white and ovomucoid added to diet at expense of glucose; the unheated soybean meal replaced an equal weight of heated meal.
[3] Average of determination of 15 chicks/treatment.
[4] Values not followed by same letter are significantly different ($P < 0.05$) by Duncan's multiple range test (30).

depressed in chickens with ileostomies by feeding unheated soybean meal, suggesting that it might contain an "unavailable" nitrogen fraction. These experiments indicated that the poor utilization of nitrogen was due to depression in digestibility by components of unheated soybean meal, not by some undenatured proteins of unheated meal resistant to proteolysis. Lepkovsky and associates also observed that the utilization of the non-nitrogenous fraction of the diet was unaffected by feeding unheated soybean meal.

The response of feeding the Kunitz soybean trypsin inhibitor (table 4) shows that a homogeneous protein preparation can interfere with utilization of the diet and cause the pancreas to hypertrophy. This undoubtedly is not the only factor in soybeans harmful to chicks, since the pure inhibitor preparation did not depress growth rate or fat absorption to the same extent as unheated soybean meal. This points to the multiplicity of factors in unheated soybeans that cause the net effects observed with the feeding of unheated soybean proteins.

The results of the experiment with the F_1 and F_2 fractions of soybean whey also suggest that multiple factors are present in the whey which act similarly or synergistically to cause the effects observed in chicks. Thus any attempt to isolate one component of the soybean whey responsible for all the effects produced might not be successful since another component might be needed for full expression of the effect.

The mechanism by which unheated soybean meal causes poor fat absorption in young chicks is unknown. Inhibition of lipase is not the cause. Nesheim et al. (2) showed that dietary soybean fatty acids which are normally well utilized by the young chick were poorly absorbed when unheated soybean meal was fed. Preliminary investigations [6] had established that the poor fat absorption was not the result of a protein or sulfur amino acid deficiency, nor due to retarded growth or retarded development of the young chick's normally increasing ability to absorb fat. Garlich and Nesheim (3) detected no bile deficiency in chicks fed unheated soybean meal. A histological investigation of the gross and microscopic morphology of the intestinal epithelium revealed no abnormality such as that present in humans with the non-tropical sprue malabsorption syndrome (24).[7]

The results of the experiments with soybean whey and fractions 1 and 2 indicate that the factor responsible for poor fat absorption can be concentrated. The component may be a protein, or a non-protein compound which remains closely associated with the proteins during the isolation procedure. Proof of the existence of one specific component which impairs fat absorption awaits further experimentation.

The results obtained by feeding whey are in direct contrast to those obtained by Saxena et al. (25), who reported that the residue from extraction of soybean meal alone actively depressed growth rate of chicks, whereas the soybean whey did not depress growth rate or cause hypertrophy of the pancreas. The levels of whey fed are not specified in the paper of Saxena et al. nor are values given for trypsin inhibitor activity. The lack of pancreatic response suggests that the whey prepared by these workers may have been fed at levels too low to elicit a response, or may have been partially denatured during preparation. The results obtained in our experiments with soybean whey agree well with studies reported by Rackis et al. (26) with rats.

Commercial preparations of soybean trypsin inhibitor should be used only after careful assay, preferably by measurement of inhibiting activity and chromatography in DEAE cellulose, as recommended by Rackis et al. (16). During the studies reported here, samples of crystalline soybean trypsin inhibitor purchased from 2 commercial sources were completely lacking in trypsin inhibiting activity.

The residue remaining after extraction of defatted soybean meal was also growth-depressing although considerably less than the soybean whey. This preparation also caused hypertrophy of the pancreas. Several investigators have indicated that unheated soybean meal contained a growth-

[6] Garlich, J. D. 1964 Studies on the effects of feeding diets containing raw soybean meal or certain of its constituent proteins on fat absorption and protein digestion by the chick. Ph.D. Thesis, Cornell University.

[7] See footnote 6.

depressing factor in the water-insoluble residue (25, 26), reported to be low in trypsin inhibitor activity but still causing pancreatic hypertrophy. In these studies a very low level of raw soybean meal (7%, table 4) caused nearly maximal pancreatic response. "Bound" trypsin inhibitor activity in the residue would be difficult to measure because of the insolubility of this component of soybean meal. Recently Bielorai and Bondi (27) reported that even after 6 extractions of raw soybean meal with dilute HCl, the residue still significantly inhibited the tryptic digestion of casein and fish meal. Although another component of soybean meal causing pancreatic hypertrophy may be the explanation of the results with "residue" fractions, more trypsin inhibitor activity may be released from these fractions during digestion.

Pancreatic hypertrophy certainly appears to occur in response to many dietary substances containing trypsin inhibitors. This has been found true for many legumes containing trypsin inhibitors (28),[8] and also for ovomucoid which contains quite different inhibitor proteins. Highly purified inhibitors from soybeans have been shown to cause pancreatic response in this study and in reports by Lyman and Lepkovsky (29). Although this does not indicate that pancreatic response in the absence of trypsin inhibitors cannot occur, a hypertrophic response in the pancreas of a chick or rat in response to a dietary supplement certainly suggests that a trypsin inhibitor is present. The effect on pancreatic response of trypsin inhibitors from such varied sources suggests that pancreatic secretion may be regulated by some mechanism effected by proteolytic activity in the intestinal tract.

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Metabolism of Polyunsaturated Fatty Acids and Serum Cholesterol Levels in the Rat [1]

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ABSTRACT Male albino rats were fasted for 4 days and then fed various fats in order to study the effect of the fat upon blood ketone and liver glycogen levels. The fats included coconut oil, lard, and linseed, safflower and tung oils. The ingestion of the more saturated fats, and tung oil with its conjugated double bonds, was associated with higher blood ketone levels than the other more unsaturated fats. The opposite was true for liver glycogen levels except for coconut oil which may have spared glycogen by its more rapid oxidation. The results obtained are in line with the speculation that the polyunsaturated fatty acids during their utilization might be so degraded that the 3-carbon fragments between the double bonds formed would be more glycogenic in nature than the ketolytic 2-carbon fragments produced when saturated fatty acids are oxidized. The speculation was advanced that such degradation, if true, might play some part in affecting blood cholesterol and lipid levels.

Lower blood lipid and cholesterol levels are generally accepted to be associated with diets containing either a low fat content or large amounts of polyunsaturated fatty acids (PUFA) than with diets containing an excess of saturated fatty acids (1). The nature and degree of unsaturation appear to be important factors involved, with total unsaturation instead of essential fatty acids being the more important factor (2). The linolenic acid of linseed oil, while as effective as linoleic acid in reducing blood lipid levels, cannot completely replace linoleic acid as a source of essential fatty acids (3). The highly unsaturated eleostearic acid of tung oil, containing conjugated double bonds, markedly elevates the blood cholesterol level (4).

Sinclair [3] reported less ketosis in patients eating high fat diets which contained more PUFA than saturated acids. The speculation was advanced that the PUFA, and their more highly unsaturated metabolic products, might be so degraded that the 3-carbon fragments between the double bonds might be more glycogenic in nature than the ketolytic 2-carbon fragments formed when saturated fatty acids are oxidized. If this should be true, the nature of the utilization of these 3-carbon fragments might in some way aid in the lowering of blood cholesterol and lipids after the ingestion of appreciable amounts of PUFA.

In line with the above suggestion, Peifer [4] reported that fatty acids more highly unsaturated than linoleic acid were more effective as hypocholesterolemic agents. Since some metabolic products of linoleic and linolenic acids recovered from the liver have been found to contain five or more double bonds (5), the degradation products of these fatty acids might allow approximately one-half of the acid to be metabolized as 3-carbon fragments. The opposite would be true of eleostearic acid with its conjugated double bonds.

Some evidence is available which suggests higher liver glycogen levels after the ingestion of corn oil than after its substitution by the hydrogenated oil (6). The more rapid oxidation of short-chain fatty acids was proposed as responsible for the higher levels of plasma ketone bodies than present during utilization of long-chain saturated fatty acids. However, all saturated fatty acids used appeared to induce higher ketone levels than the polyunsaturated acids (7).

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[3] Sinclair, H. (England), personal communication.

[4] Peifer, J. J. 1964 Hypercholesterolemia changed by specific types of unsaturated fatty acids. Sixth International Congress of Biochemistry, p. 117 (abstract).

A study was undertaken to learn whether 2 sources of PUFA, linseed and safflower oils, might cause less ketosis and be more effective in promoting glycogen formation than the more saturated fats, lard and coconut oil. Changes in liver carbohydrate and blood ketone bodies after diets rich in these fats might aid in clarifying whether any such differences in the degradation of fatty acids might occur. If true, such metabolic differences might account, at least in part, for the effects produced since the end result would be equivalent to replacing a part of the dietary fat with a glycogenic material.

EXPERIMENTAL

White male rats, weighing between 250 and 350 g, were pretreated in order to develop an initial ketosis. After access for 3 days to only a 10% glucose [5] solution and water, ad libitum, the rats were fasted 4 days. Coconut oil was prefed during the last 2 days in an attempt to increase the ketosis (1.25 ml two times a day by feeding needle). Of the 6 or 8 rats used in each of 12 groups, 2 to 4 rats were then fed various fats in order to learn the effect of each on blood ketone and liver glycogen levels. The fats fed included lard, linseed oil, safflower and tung oils.[6] Feedings at the rate of 1.5 ml of the fat 3 times daily were given for 2.5 days. The tung oil feedings had to be reduced to 1 ml for only 2 days. Tung oil has been used previously as a source of unsaturated fatty acids containing conjugated double bonds (4). Blood for ketone values was drawn daily from each rat's tail until the time that the rat was decapitated. Duplicate 2-g samples of liver were quickly removed and replaced in hot 30% KOH for glycogen determinations. The above procedure was modified slightly during the latter part of the study in that the pretreatment involved only a 4-day fast with no fat feedings.

The glycogen was separated according to the method of Good and Kramer (8) and the hydrolyzed glucose was determined by the method of Nelson (9) as modified by Somogyi (10). Acetone values of the blood were obtained according to the directions of Natelson (11) using 0.2 ml of blood and color development with salicylaldehyde after distillation from dichromate-acid mixture.

RESULTS

The blood ketone levels of the rats fed coconut oil during the preperiod were elevated markedly (table 1). As a result of this high initial ketone level, the final ketone body level was less than the initial value when linseed oil was fed. The difference between the initial and final levels in the rats fed lard and those given linseed oil was about 40% (from +21 to −20%, respectively). The results with coconut oil and tung oil were similar despite the reduced feedings of tung oil and shorter period of intake. The latter oil was so toxic that none of the rats survived until this change in procedure was adopted.

The results from the first 7 groups of rats prompted a repetition of this part of

[5] Cerelose, Corn Products Company, Argo, Illinois.
[6] Appreciation is expressed to U. S. Department of Agriculture Research Service and H. P. Dupy for the tung oil.

TABLE 1

Blood ketone levels after fast and fat feedings

Fat fed	No. rats	Blood ketones			
		Initial	Final	Difference	Change
		mg/100 ml	*mg/100 ml*		%
Coconut oil [1]	10	2.85 ± 0.38 [2]	4.86 ± 0.69	2.01	+71 [3]
Lard	9	5.17 ± 0.11	6.27 ± 2.07	1.10	+21
Linseed oil	10	4.65 ± 1.76	3.71 ± 1.78	0.94	−20
Tung	9	2.70 ± 1.07	5.15 ± 0.92	2.45	+91
Lard	5	5.13 ± 0.54	7.50 ± 1.05	2.37	+46
Safflower oil	8	5.31 ± 1.20	6.02 ± 0.68	0.71	+13
Linseed oil	8	4.35 ± 0.66	4.45 ± 1.02	0.10	+ 2

[1] From prefeeding period when coconut oil was fed.
[2] Mean values with SD.
[3] (+) or (−) to indicate increase or decrease in levels.

TABLE 2
Glycogen content of liver after fast and feeding various fats [1]

Fat fed	No. rats	Liver glycogen	Relative change
		%	%
Lard	16	0.49 ± 0.02 [2]	100
Coconut oil	10	2.65 ± 0.72	541
Linseed oil	16	0.86 ± 0.09	175
Safflower oil	17	1.25 ± 0.34	255
Tung	7	0.26 ± 0.14	53

[1] Livers obtained from rats killed after blood ketone determinations.
[2] With SD.

the study since linseed oil appeared to be more effective than lard in reducing ketosis. The tung oil was replaced with safflower oil because of the high content of linoleic acid in safflower oil. Again the rats fed lard had approximately 40% higher blood ketone body levels than those fed linseed oil (second part, table 1). The results with linseed oil and safflower oil, fats with a high content of polyunsaturated fatty acids, were similar in causing little or no change in existing ketone levels. Results confirmed data obtained when coconut oil was fed in the preperiod although different ketone levels were obtained.

The glycogen content of the livers after the various fat feedings is listed in table 2. An appreciably higher glycogen level was present in the livers of rats fed linseed oil and safflower oil — up to 2.5 times the amount in those after ingestion of lard. The low glycogen content of rats fed tung oil is not unexpected when the physical state of the animals is considered. However, the higher glycogen level of rats fed coconut oil was quite unexpected.

DISCUSSION

Considerable evidence is available to support the contention that, under certain conditions, the ingestion of polyunsaturated fatty acids will reduce the blood level of cholesterol and total lipids (12). No satisfactory mechanism has been found to fully explain such changes. However, several theories have been advanced as partial explanations as to why PUFA ingestion is associated with lower blood lipid and cholesterol levels. One theory involves the idea that the lowered cholesterol may be the result of decreased cholesterol synthesis induced by the metabolism of the PUFA as a result of increased efficiency of systems involved (1). In line with this theory, Sinclair [7] has suggested that the possible differences in the metabolic degradation of the PUFA and the more saturated acids might aid in accounting for their effects on serum cholesterol lowering by affecting its synthesis or metabolism.

Although giving no information about a possible altered synthesis or metabolism of cholesterol as a result of PUFA ingestion, the data obtained in this study are in agreement with Sinclair's speculation that differences in the degradation of PUFA and saturated fatty acids might have an effect upon cholesterol metabolism by a possible replacement of a ketogenic material with a glycogenic one. In both groups of rats, the higher blood ketone body levels after the ingestion of fats containing more saturated fatty acids suggest the possibility of different mechanisms for the metabolism for the 2 types of fatty acids (12). This is also true if the liver glycogen levels after feeding safflower oil and linseed oil are compared with the level after feeding lard. The unexpectedly higher liver glycogen content of rats fed coconut oil might be explained as the result of carbohydrate sparing during the more rapid oxidation of the short chain fatty acids (7) as evidenced by the much higher ketone levels in the blood. Such an explanation for the higher glycogen values after feeding linseed oil and safflower oil do not appear justified since they produced even less ketone bodies than did the lard-fed rats. However, Lynn and Brown (13) have reported unsaturated fatty acids to be more readily oxidized than saturated ones and this might be a factor in the results obtained. Further studies are in progress.

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Effects of Strain, Age and Diet on the Response of Rats to the Ingestion of *Cycas circinalis* [1]

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ABSTRACT *Cycas circinalis* fruits obtained from Guam were dried in the laboratory and the nut meats were ground and incorporated in diets fed to rats in order to study the properties of the toxic factors in these nuts. Growth depression curves were linear when the percentages of cycad in diets were plotted against logarithms of body weight changes during a 5-day bioassay period. The toxicity of cycad decreased as a result of soaking and incubation in water, heating and storage. The decrease in toxicity was probably due to enzymatic and chemical degradation of cycasin, the toxin in cycad. Younger rats appeared to be more severely affected by cycad toxicity than older rats, while strain, sex and origins of rats had no influence on susceptibility. Increasing concentrations of casein (protein) and methionine in diets did not appreciably alter the toxicity response of animals fed cycad. Rats fed 2% cycad diets for 2 weeks died with typical symptoms of cycasin toxicity even though they had been fed the basal ration (without cycad) following the cycad diet.

Cycas circinalis is one of 9 species of cycads growing in tropical or subtropical areas. Cycads resemble tree ferns and represent an evolutionary transition between ferns and Gymnosperms or evergreens (1). They are of medicinal, economic and nutritional importance in the areas where they are indigenous (2). Although some species of cycad are widely distributed, *Cycas circinalis* is indigenous primarily to Guam and Oceania.

As early as 1770, observations suggested that cycads contained toxic substances. Gastrointestinal and paralytic symptoms have been observed in animals and men that have ingested parts of the plant (3). The first sign of paralysis in animals is a staggering gait with a crossing of the hind limbs when resting. In this position, the animal sways from side to side, and hence the term, "wobbles," used by the Australians to describe the condition (3). Cattle and sheep have been reported to die when grazing in pasture where the predominant source of forage was cycad (4).

Some of the first animal studies confirmed the development of gastrointestinal symptoms and locomotor disturbances (3). Severe liver damage was a common pathological finding. In some animals, chronic feeding of cycad resulted in a locomotor disturbance that was progressive, irreversible and exaggerated by exercise. However, no neurological basis for the paralysis was found (5).

Present interest in *Cycas circinalis* stemmed from a suggested correlation between its use as a human food and a high incidence in Guam of amyotrophic lateral sclerosis. This disease is 100 times more prevalent in Guam than in the United States or Europe (3). It is characterized by neural and muscular degeneration, appears usually between the ages of 40 and 50, progresses rapidly and terminates fatally within 2 to 5 years (6).

The use of cycad by the natives in Guam and other Southwestern Pacific Islands stems from the fact that this plant survives adverse climatic conditions. It is one of the few foods available during droughts

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TABLE 1

Composition of cycad flour and white flour (wheat)

	Cycad	White wheat flour [1]
	%	%
Nitrogen	2.0	1.8
Water	6.5	12.0
Fat	0.8	1.0
Ash	2.6	0.4
Carbohydrate (known)	45.0 [2]	76.1
Unknown	34.0	

[1] See reference (8).

[2] The known carbohydrates as a percentage of cycad flour are: free glucose, 1.2–2.1; glucose (freed by acid hydrolysis), 39.0; free fructose, 0.7–0.9; fructose (freed by acid hydrolysis), 1.4; cycasin, 2.0; and trace quantities of xylose, primeverose(?), *myo*-inositol, sequoyitol and pinitol (personal communication from Drs. N. K. Richtmyer and S. S. Chernick, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health). Determination of total carbohydrate by the anthrone method (7) showed cycad flour to contain 52% carbohydrate.

and following devastating typhoons. The seed or nut meat is the part of the plant most frequently used. Since its toxicity is well recognized by the natives in Guam, the seed is treated to eliminate its toxicity. The seed (approximately 4 cm in diameter) is quartered and the center, or nut meat, is soaked in vats of water. The water may be changed every 3 or 4 days during soaking which lasts from a few days to 3 weeks. The nut slices are dried in the sun and then ground into a powder. The latter is used for thickening soups, puddings and porridge and in tortillas (3).

The proximate composition of the dried unwashed cycad is comparable to that of wheat flour (table 1). The 34% not identified is thought to be carbohydrate. With the exception of glucose and fructose, the other carbohydrates are present in trace amounts only.

In 1941, Cooper isolated from *Macrozamina spiralis*, a species of cycad indigenous to Australia, a glycoside which was lethal to animals (9). Cycasin, a glycoside similar to the one isolated by Cooper except for the sugar moiety, was isolated from *Cycas revoluta* by Nishida and coworkers (10) in 1955 and from *C. circinalis* by Riggs (11) in 1956. The aglycone common to these glycosides is methylazoxymethanol.[4] It is considered responsible for the toxicity of the glycosides (12). The aglycone is toxic only after hydrolysis of the glycoside by bacterial β-glucosidases in the intestinal tract. The absence of toxicity in germfree rats fed cycasin confirms the essential role of intestinal bacterial enzymes (13). Furthermore, Nishida and co-workers (14) noted that toxic symptoms appeared only after oral ingestion of the glycoside but not following intravenous or subcutaneous injection.

The present paper reports the stability of the toxic factor(s) of cycad and variations in the response of rats of different sexes, ages, strains and colonies. Reported, as well, are the effects of dietary changes and discontinuation of cycad feeding on the toxic response.

EXPERIMENTAL

Cycad materials. Cycad nuts were shipped to our laboratory immediately after collection from various parts of Guam.[5] Each lot of nuts was coded with the year, month and day of delivery (e.g., 623-6 for 1962, March 6). These materials had not been soaked as described earlier, and will be referred to as unwashed cycad. The unwashed material was used because it should contain a high concentration of the toxic factor and thus produce more definitive results. The nuts were husked, cut open, thinly sliced by machine and dried under vacuum at 40°. The dried slices were ground into flour in a Wiley Mill.

Bioassay. Male weanling rats of the Sprague - Dawley and Osborne - Mendel strains were used except where indicated. Previous to the feeding of experimental rations, all animals were fed a basal grain ration until a weight increase of 5 to 6 g/day was achieved. The average body weight of the animals at the beginning of the experimental feeding trials was approximately 80 g. In most studies, 10 animals were used in each experimental group. In some of the preliminary work, 3 or 5 animals were used. All were housed individually in suspended wire cages. Feed and water were given ad libitum.

Natural grain rations were fed as the

[4] $CH_3-\overset{O}{\overset{\uparrow}{N}}=N-CH_2OH$.

[5] We are sincerely grateful to Drs. Leonard T. Kurland and Marjorie G. Whiting of the Institute of Neurological Diseases and Blindness, Bethesda, Maryland, for making the cycad nuts and crystalline cycasin available to us.

basal diet.[6] Cycad materials were thoroughly mixed with appropriate proportions of the basal diet and refrigerated at 5° prior to feeding.

Growth rates and liver lesions were used to indicate the relative toxicity of each new lot of cycad flour. Weights were recorded on the first and fifth day of the assay period. On the fifth day, the animals were etherized and killed. The livers were removed, fixed in 4% acetate-buffered-formalin and later examined for pathlogical lesions. Some animals were maintained for longer periods.

RESULTS

Animals fed the unwashed flour as a percentage of their basal ration gained less weight than controls fed the basal ration only. Growth depression of animals varied with the lot of flour used, but all flours caused a linear response when weight changes were plotted logarithmically against the percentage of unwashed cycad flour included in the ration (fig. 1). The growth depressions for all bioassays are plotted as the logarithm of the changes in weight + 40 (to avoid negative numbers). The corresponding actual body weight changes (in grams) are also given. Zero per cent cycad indicates the growth response of control rats. Vertical lines represent standard errors calculated according to Mantel (15). Data were analyzed by analysis of variance (16) and mean differences by Duncan's multiple range test (17).

Decrease and loss of cytoplasmic basophilia from the centrolobular zones and necrosis of individual liver cells in these zones were characteristic of the animals fed experimental rations. With increasing concentration of cycad flour, there was a corresponding increase in the severity of the liver damage. These lesions were identical with those previously reported (18) as the early changes resulting from cycad toxicity.

Preliminary studies suggest that most of the toxic symptoms associated with the feeding of unwashed cycad can be attributed to cycasin, the major glycoside in cycad. Based on body weight changes for 90 male Sprague-Dawley rats fed various levels of unwashed cycad flour (lot no. 628–10) or cycasin in a 5-day bioassay, the cycad flour contained approximately 2.3% cycasin (dry-weight basis) (fig. 2). The break in the curve at the 1% cycad level is unexplainable. This was the first assay (in approximately 25) where the curve showed this break. Apart from that, the slope of the main part of this curve parallels that of the previous assays run under comparable conditions.

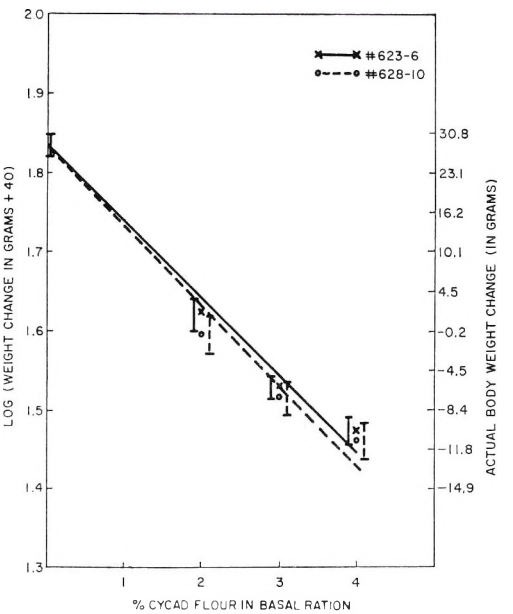

Fig. 1 Growth depression of rats fed either of 2 lots of unwashed cycad flour for 5 days.

The assay could not be repeated since the supply of crystalline cycasin was exhausted. The value for the cycasin content of unwashed cycad flour agrees with that secured in other bioassays. It is also similar to the value of 2.3% reported by Matsumoto and Strong (12) for the chemically determined cycasin content of lot no. 632-6.

[6] The percentage composition of this grain ration was: ground corn, 60.7; soybean meal (50% protein), 28.0; alfalfa meal (17% protein), 2.0; fish meal (12.5% protein), 2.5; dried whey (67% lactose), 2.5; limestone (38% Ca), 1.6; dicalcium phosphate (18.5% P, 22–25% Ca), 1.75; iodized salt, 0.5. Supplementary minerals, vitamins and antibiotics were added to provide per kilogram of feed: (in mg) Mn, 121; Fe, 95; Cu, 7; Zn, 4; I_2, 4; Co, 2; choline chloride, 400; Ca pantothenate, 6; riboflavin, 3; niacin, 33; menadione, 2; DL-methionine, 500; penicillin, 2; streptomycin, 8; arsenilic acid, 968; (in μg) vitamin B_{12}, 7; (in international units) vitamin A, 8010; vitamin D_2, 750; vitamin E, 5 (Alpha Tocopherol Powder, Nutritional Biochemicals Corporation, Cleveland).

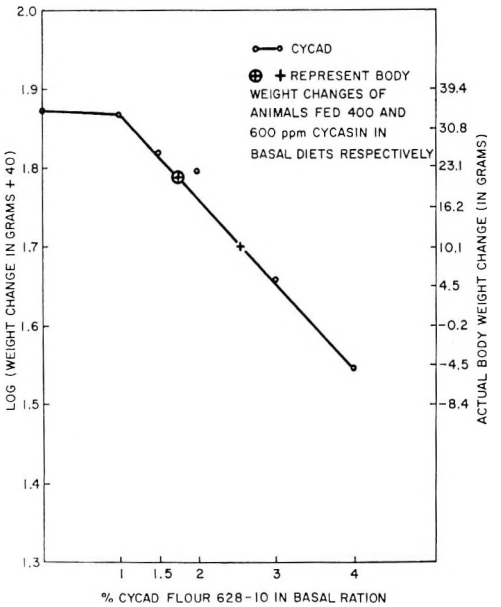

Fig. 2 Determination of cycasin concentration in unwashed cycad flour. Groups of rats were fed the basal ration containing the indicated levels of cycad. The 5-day weight changes of these animals were used in plotting the curve. Other groups of rats received the basal ration containing either 400 or 600 ppm of crystalline cycasin. From the location on the curve of the points for the rats fed crystalline cycasin, the concentration of the cycasin in the cycad flour was calculated.

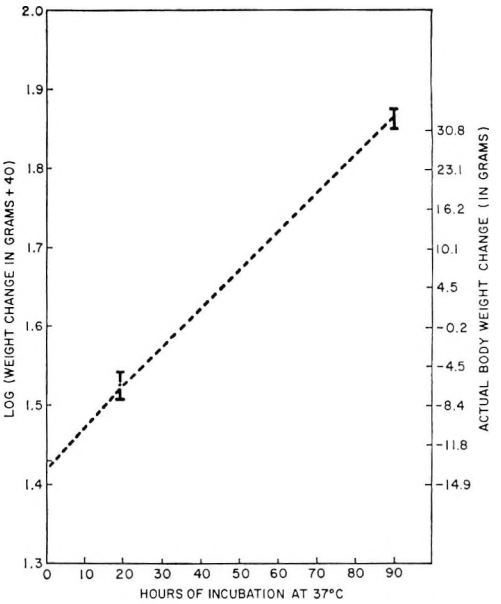

Fig. 3 Growth of rats fed 15% cycad flour no. 628-1, incubated for varying intervals at 37° (5-day bioassay).

Stability of toxic factor

Water treatment. Reports from Guam suggest that the water in which cycad nuts were soaked is lethal to animals.[7] This implies that the toxic factor is water-soluble, it can be leached out of the nut and it is stable in solution. To test this hypothesis, 624 g of unwashed cycad nut slices, of known toxicity (see no. 623-6, fig. 1), were soaked in 1.5 liters of tap water at 37° for 5 days. The solids were removed by filtration, dried at room temperature and ground. The filtrate was concentrated under vacuum to 66 ml. Diets containing either 5% ground solids, an aliquot amount of the concentrated filtrate, or both, were fed to groups of 10 weanling rats. Growth responses for the 5-day assay averaged 31, 32, and 33 g, respectively. These increases were similar to gains of control animals, indicating the absence of the toxic factor in both the residue and the filtrate prepared in our laboratories. These data, contrary to reports from Guam, suggest that a degradation of the toxic compound has occurred during the soaking process.

To study the rate of loss of toxicity, pastes made from unwashed cycad flour (lot no. 628-1) and water were incubated at 37° for varying lengths of time, dried under vacuum at 50°, and reground into flour. The growth of animals fed a diet containing 15% non-incubated flour was less than that of animals fed diets 15% of which was the incubated flour. The treatment caused a progressive decrease in the toxicity of the pastes with time. All toxic activity was lost some time prior to 90 hours of incubation (fig. 3). Large volumes of gas generated during the first 16 hours of incubation are believed to be breakdown products (probably nitrogen) associated with the loss of toxicity.

That the toxic component of cycad nuts is destroyed during incubation in the moist state is evident. Experiments are presently underway to test the nutritional safety of

[7] Personal communication from Dr. M. G. Whiting, National Institute of Neurological Diseases and Blindness, National Institutes of Health, Bethesda, Maryland.

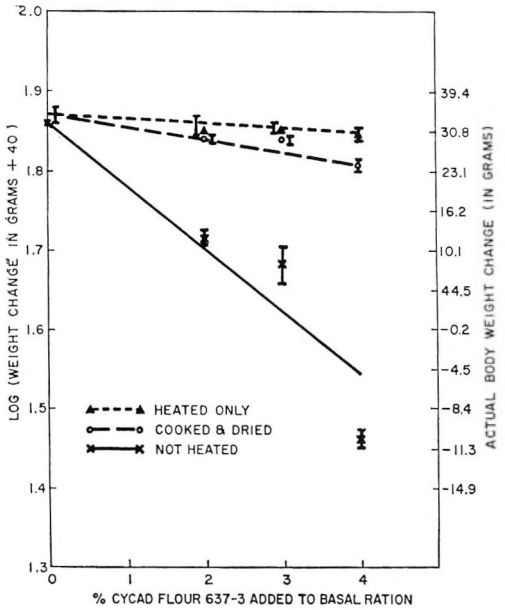

Fig. 4 Growth of rats fed heat-treated, unwashed cycad flour for 5 days.

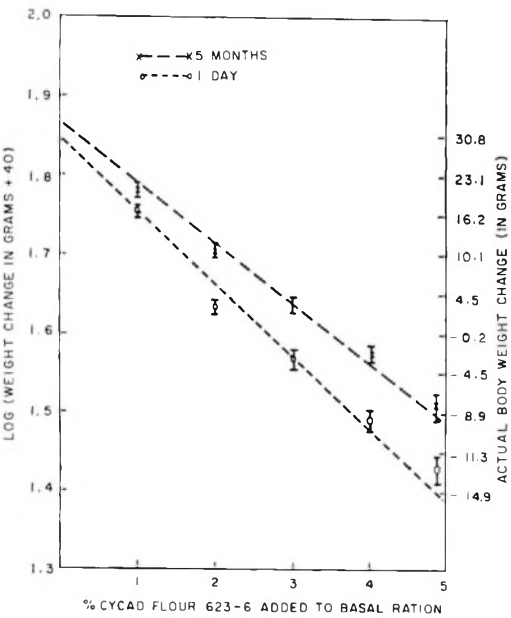

Fig. 5 Growth of rats fed unwashed cycad flour no. 623–6, stored at 5° for varying intervals (5-day bioassay).

the cycad prepared for food by the natives in Guam.

Heat treatment. Cycad flour is used as an ingredient in the cooked dishes of the Guamanian people. To determine whether cooking aids in destroying any residual toxicity that might be present in cycad flour, the following experiment was carried out.

Toxic, unwashed flour (lot no. 637-3) was cooked at 90° for one hour with 9 volumes of tap water, dried in thin layers at 37° for 30 hours and ground in a Wiley Mill. An amount of the same flour was heated at 90° for one hour without the addition of water. The growth response of animals fed either of these heat-treated flours at levels of 2, 3, or 4% of the ration was in most cases, similar to the growth response of control animals fed only the basal ration (fig. 4). With the exception of the group fed 4% cooked flour (moist heat), the livers from these animals were microscopically normal. This latter group showed some loss of cytoplasmic basophilia from the centrolobular areas. These data indicate that the toxic factor in unwashed cycad flour is largely destroyed by moist or dry heat at 90°. This instability of the toxic factor to heat adds a margin of safety to the consumption of washed cycad flour when it is used in cooked foods.

Storage. Growth curves from successive animal feeding trials indicated a loss in the toxicity of the cycad flour during storage. Flours stored at − 20° retained more toxicity than flours stored at 5°. One lot of unwashed flour (no. 623-6), stored at 5°, was assayed 4 times over a period of 5 months. Figure 5 represents the data obtained from the first and fourth assays. The curves from the second and third assays lie between those shown. As an extension of the preceding work, an additional assay was carried out 10 months after preparation of the cycad flour. That assay was completed at Michigan State University, and the first 4 assays were completed at the National Institutes of Health. The sample stored at 5° for 10 months appeared much more toxic than the original sample. The apparent increase in the toxicity of this batch of cycad flour probably was associated with some change in experimental conditions. For this reason, a study was undertaken to determine the effect of different rations and different strains and ages of rats on the bioassay.

TABLE 2
Average weight changes of rats of different strains and from different colonies fed indicated rations for 5 days [1]

Avg wt change	Level of cycad	Males	Females
	%		
	Osborne-Mendel [2]		
Gain, g	0	29.6	24.2
Gain, g	2 [3]	11.2	6.4
Δ, g		−18.4	−17.8
Δ, % of zero level		62.2	73.6
	Sprague-Dawley [4]		
Gain, g	0	34.6	26.4
Gain, g	2 [3]	14.2	9.4
Δ, g		−20.4	−17.0
Δ, % of zero level		59.0	64.4
	Osborne-Mendel (NIH) [5]		
Gain, g	0	31.1	
Gain, g	2 [3]	− 0.1	
Gain, g	4 [3]	−16.4	
	Osborne-Mendel (Camm) [2]		
Gain, g	0	32.4	
Gain, g	2 [3]	4.0	
Gain, g	4 [3]	−12.3	

[1] Five rats/group.
[2] Camm Research Institute, Wayne, New Jersey.
[3] Lot no. 623–6.
[4] Spartan Research Animals, Inc., Haslett, Michigan.
[5] National Institutes of Health, Bethesda, Maryland.

TABLE 3
Effect of age on weight changes of young Osborne-Mendel rats fed unwashed cycad flour no. 623–6 [1,2]

Cycad in diet	Age and weight at start of experiment	
	25 days (75 g)	37 days (130 g)
%	g	g
0	28.2	32.4
2	2.0	4.0
3	− 6.2	− 6.7
4	−10.3	−12.3

[1] Ten rats/group, average for 5 days.
[2] All levels of cycad were significantly different from controls ($P < 0.01$), 3 and 4% cycad were different from 2% cycad ($P < 0.01$), responses due to age were not significantly different ($P > 0.05$).

Rations. Various commercial rations were fed as the basal diet. The very poor growth associated with one ration [8] was traceable to a coccidiostat [9] which apparently reduced the food intake and thereby the growth of the rats. Apart from this complication, three other grain rations used[10] had no apparent effect on the bioassay.

Strain, sex and colony variations. Body weight losses of Osborne-Mendel and Sprague-Dawley rats fed at various levels of unwashed cycad flour indicated that there was no difference between the strains in susceptibility to the toxicity (table 2). This was true for both males and females and for body weight changes expressed on either an absolute or a relative basis. In addition, male Osborne-Mendel rats obtained from National Institutes of Health lost approximately the same amounts of body weight as did rats of the same strain purchased from a commercial outlet (table 2).

Age of Animals. Newborn rats appeared to be much more susceptible to cycasin toxicity than weanling animals. In one assay, when newborn rats were permitted to suckle a dam fed a natural grain ration containing 400 ppm cycasin, all pups were dead by the fifth day. However, when the same ration was fed to weanling rats, 50% of them survived for more than 7 months. In a third trial, weanling animals 25 days old (75 g) lost a greater percentage of their body weight than animals 37 days old (130 g) when both groups were fed diets containing 2, 3, or 4% unwashed cycad flour (table 3). Although these differences in absolute body weight losses were not statistically important ($P > 0.05$), all three of the preceding trials showed a similar trend, suggesting that younger animals are more susceptible to cycad toxicity than older ones.

Dietary effects. Proteins provide the amino acids essential for cell regeneration and are generally regarded as being potentially able to ameliorate the effects of toxic substances, especially those affecting the liver (19). Since one of the primary effects of cycad toxicity is centrolobular liver necrosis, rations containing different levels of casein were studied for the effect of this protein on cycad toxicity.

Three trials using weanling Sprague-Dawley male rats and diets adequate in

[8] Chick Startena, Ralston Purina Company, St. Louis, Missouri.

[9] Zoalene (3,5-dinitro-o-toluamide), The Dow Chemical Company, Midland, Michigan.

[10] M-1 (see footnote 6), Rockland Mouse/Rat Diet, Tehland, Inc., Monmouth, Illinois, Michigan State University Pig Grower Ration no. 1; a nutritionally adequate natural grain pig ration prepared locally.

TABLE 4

Average final body weights of rats fed for 5 days diets containing different concentrations of casein and unwashed cycad flour [1]

Cycad [2]	Trial [3]	Average final body weight		
		Casein, %		
		12	18	24
%		g	g	g
0	A	90.8	—	110.2
	B	68.6	69.6	70.8
	C	77.4	—	93.2
1	A	—	—	—
	B	67.0	68.0	71.8
	C	74.4	—	86.6
2	A	79.2	—	91.6
	B	66.6	68.4	69.0
	C	68.6	—	75.6
3	A	—	—	—
	B	67.6	64.0	63.0
	C	63.4	—	63.8

[1] Five weanling male rats/group; initial body weights for all groups were comparable within each trial.

[2] Trial A unwashed cycad flour no. 623–6; trials B and C unwashed cycad flour no. 632–4A.

[3] Trials A and C rats fed ad libitum; trial B rats pair-fed.

minerals and vitamins [11] showed that as the levels of cycad increased for each given level of protein, the body weight gains of the animals decreased (table 4). The weight gains of rats were slightly greater for higher levels of casein when the levels of cycad were zero, 1 or 2%. These data therefore indicate that increasing the casein (protein) from 12 to 24% of the diets did not ameliorate the growth-depressing effects of cycad.

Methionine, because of its lipotropic activity has been considered the effective principle of high protein diets in modifying experimental hepatic toxicities (19). Preliminary investigation with groups of 3 rats each indicated that the addition of 1.0 or 0.5% DL-methionine, to a grain ration containing 2% unwashed cycad flour, exerted a beneficial effect on the growth of the animals, but a similar fortification of a 3% unwashed cycad diet with methionine had a negligible effect. In a later study, feeding groups of 10 weanling rats a grain diet containing 2% unwashed cycad and 1% DL-methionine [12] exaggerated the growth depression over the 3-week experimental period. Weight gains for animals fed 2% cycad flour averaged 124 g; those fed the 2% cycad fortified with methionine gained 110 g and control animals 139 g. The mean weight changes calculated from these data are significantly different from each other ($P < 0.01$). Thus, methionine, added to a ration containing adequate protein, has no beneficial effect on the short-term growth response of rats receiving cycad.

Although neither protein nor methionine appreciably influence the acute cycad toxicity, there is a possibility that they may have an effect on the chronic toxicity of cycad and on the incidence of tumors. Studies are underway to test this possibility.

Irreversibility of toxic response. To determine whether the lesions induced by the intake of unwashed cycad were progressive or reversible, 2 groups of male, weanling, Osborne-Mendel rats were fed diets containing 2% unwashed cycad flour (lot no. 623–6).

After 18 days, the 19 animals showing the greatest loss of weight were fed the basal ration free of cycad. The remaining 26 continued to be fed the 2% cycad ration. During the following week, the rats in the latter group lost weight at such a rapid rate that it became imperative to reduce the level of cycad in their ration to 1%.

The slopes for the cumulative mortality curves of the rats fed the 2% cycad ration for 18 days and thereafter only the basal ration paralleled that of the group fed the 2% cycad ration (middle vs. top curve, fig. 6). The difference in the position of the curves is probably attributable to the extra week the rats in the latter group were fed the 2% cycad ration. That level of cycad usually produced a high mortality in weanling rats 3 or 4 weeks after starting the cycad feeding.

[11] The source of casein was High Protein Casein, General Biochemicals, Inc., Chagrin Falls, Ohio. Each diet contained: (in per cent) salt mixture (L. G. Wesson, Science, 75: 339, 1932), 4; vitamin mixture, 2.2; corn oil, 5; and sucrose to make 100. The vitamin mixture contained per kilogram of mixture: (in grams) vitamin A conc (200,000 units/g), 4.5; vitamin D conc (400,000 units/g, 0.25; α-tocopherol, 5.0; ascorbic acid, 45.0; inositol, 5.0; choline chloride, 75.0; riboflavin, 1.0; menadione, 2.25; p-aminobenzoic acid, 5.0; niacin, 4.5; pyridoxine·HCl, 1.0; thiamine·HCl, 1.0; Ca pantothenate, 3.0; dextrose, 847.5; and (in milligrams) biotin, 20; folic acid, 90; and vitamin B_{12}, 1.35. The salt mixture and vitamin mixture (Vitamin Diet Fortification Mixture) were obtained from Nutritional Biochemicals Corporation.

[12] Nutritional Biochemicals Corporation, Cleveland.

Almost all rats fed the 1% cycad ration were dead by the 80th day of the study. The rats transferred from the cycad to the basal ration showed an 80% mortality. These results indicate that insofar as mortality is concerned, cycad feeding for a period of more than 2 weeks resulted in an increased death rate which continued at an unabated rate for the next 60 days. Under these circumstances the toxicity once established appears to be irreversible.

Absence of cycad toxic factor in liver. The liver plays an important role in the detoxification of harmful substances and is one of the primary targets of cycad toxicity. To determine whether this organ accumulates the cycad toxic substance, a heifer was fed a commercial ration to which had been added 2% unwashed cycad flour (lot no. 623–6). After 43 days of feeding, the heifer gained very little weight and showed symptoms suggestive of neurological disturbances (occasional difficulty in walking and standing). The heifer was killed at this time because it appeared to be close to death. The liver obtained at autopsy was freeze-dried and ground to a fine powder.[13] Groups of 10 weanling rats were fed this dried liver or liver from a normal heifer at levels of 2, 5, or 10% of their basal grain ration. Weight gains over a 5-day assay period were similar for all groups. Average gains (in grams) for animals fed the liver from the cycad-fed heifer compared with those for the animals fed the liver from a control heifer were, respectively, 31.8 vs. 32.3 (2%), 32.9 vs. 24.9 (5%), and 28.4 vs. 30.2 (10%). These data suggest that the compound responsible for the toxicity of cycad flour was not stored in concentrations great enough to produce toxicity in rats fed 10% of the dried liver in their rations.

DISCUSSION

The reliance on the growth bioassay in estimating the toxicity of cycads under various conditions stems from a good correlation between growth rate and the degree of liver lesions (13). Although growth followed the food consumption pattern, records indicated that the animals consumed near normal amounts of feed during the first day but markedly less thereafter.[14] Liver changes were recognizable as early as 24 hours after cycad feeding was initiated (13). Food consumption merely reflected this rapidly developing metabolic disturbance.

Bioassays have shown that the toxicity of the cycad nuts is related to their stage of maturity. The green nuts are more toxic than the riper or brown-colored ones. This toxicity probably is attributable to

[13] Liver examined by Dr. A. Allen, Laboratory Aids Branch, National Institutes of Health, Bethesda, Maryland. Liver was atrophied with loss of hepatic cells adjacent to edematous interlobular connective tissues.

[14] Unpublished data.

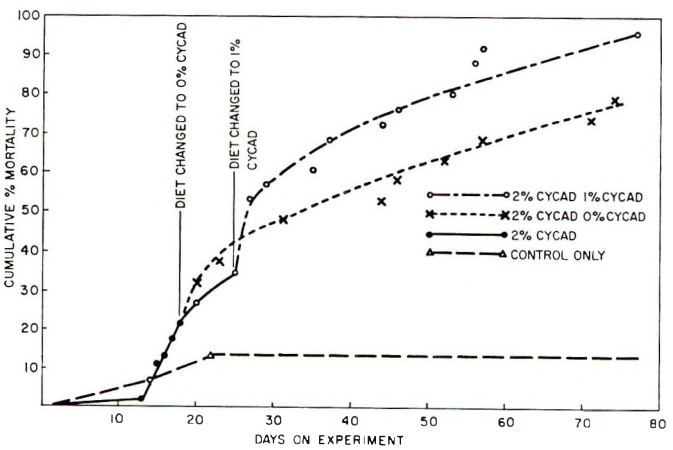

Fig. 6 Mortality of rats fed 2% unwashed cycad flour for 18 or 25 days and thereafter zero or 1% cycad flour.

the glycoside, that is, the cycasin content of the nut.[15] Comparative assays suggest that the flour prepared from the green cycad nuts contains as much as 2 to 4% cycasin. Whether all the toxicity is traceable to the glycoside has been questioned by Matsumoto and Strong (12) who secured evidence for the presence of the free aglycone in *C. circinalis*. The aglycone, methylazoxymethanol, is a water-soluble compound released from cycasin by the action of a β-glucosidase.

The toxic material in the dry unwashed cycad flour shows some destruction on storage. This loss occurs even at temperatures as low as −20°. The mechanism(s) involved in the destruction of the toxic substance has not been elucidated. The fact that the rate of destruction during incubation shows a logarithmically linear progression with time suggests a first order reaction (fig. 3). On the basis of studies on the rate of gas evolution, Williams[16] originally concluded that enzymatic reactions were involved in the destruction of toxicity occurring at 37°. Attempts to isolate the enzyme systems involved in these reactions convinced him that non-enzymatic mediated reactions were responsible for the gas evolution. The latter is presumably associated with the loss of toxicity of the cycad.

The preceding observations suggest that the washing process carried out in Guam facilitates a chemical and perhaps an enzymatic reaction which results in the destruction of the toxic substance(s) in the cycad. Temperatures on the island, ranging from 20 to 32° (20) would facilitate either one of these reactions. Additional evidence against the "leaching" process is the physical barrier to water penetration in the cycad seed slices. They are approximately 1-cm thick and even after 24 to 48 hours of soaking, show little signs of water uptake. The foam reported on the surface of the "washing" vat suggests the presence of a reaction involving the loss of a gas when the nuts are kept in water.

The toxic factor is labile to both moist and dry heat. Our observations suggest that dry heat is slightly more effective in destroying the toxicity than is moist heat.

Protein frequently protects against liver injury (19) and high dietary intakes of protein are known to modify the effects of some toxic chemicals (21). Study of the influence of protein on cycad toxicity has been limited to the acute phase manifested by weight changes. Under such circumstances, there is no pronounced effect of protein on the growth responses of the rats. The same was true for methionine added to a natural grain ration. Whether these observations have any bearing on the long-term effect that might be associated with levels of cycad that produce tumors remains for future study.

The rapid turnover of body tissue and cellular components may account for the increased sensitivity of younger animals to the toxicity of cycad. Within as short a span as 24 hours after ingestion of unwashed cycad, the livers of rats showed morphologic changes (13). The cumulative response caused by the ingestion of the flour at the 2% level for 18 days resulted in damage that was irreversible. Feeding trials indicated, however, that the toxic compound was not stored in high concentrations in the liver of animals fed cycad. Thus, the question arises whether, after the initial liver damage takes place, detoxification of the toxic compound occurs in the liver or at a different site.

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Effects of Zinc, Cadmium, Silver and Mercury on the Absorption and Distribution of Copper-64 in Rats

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ABSTRACT The effects of high levels of zinc, cadmium, silver, and mercury on the absorption and distribution of ^{64}Cu were studied. A technique for placing doses of ^{64}Cu and the accompanying antagonist directly into in vivo ligated segments of the rat gastrointestinal tract was used. The following results were obtained: Zinc affected copper uptake from the stomach and from the duodenum in the same manner and to about the same extent. In both cases, high levels of zinc depressed ^{64}Cu uptake, but did not produce any change in the tissue distribution pattern. Cadmium depressed ^{64}Cu uptake severely, caused increases in the relative proportions of ^{64}Cu observed in the blood, heart, and spleen, and decreased the proportion retained by the liver. Silver had little effect on ^{64}Cu uptake, but a significantly greater proportion of the absorbed isotope was deposited in the liver, and significantly less was retained by the blood of the silver-treated rats. Mercury produced a moderate, but not statistically significant, lowering of ^{64}Cu uptake, an increase in the relative percentage of ^{64}Cu noted in the kidney, and a decrease in the proportions of ^{64}Cu retained by the blood and by the liver.

A number of cations affect the utilization of copper. Zinc has been one of the most widely studied of these cations, and the effects of high dietary levels of this element on copper metabolism have been well documented for both rats and chicks (1–6). In spite of the large amount of work that has been done on the zinc-copper antagonism, the mechanism by which zinc interferes with the normal utilization of copper is still unknown. Previous work on the absorption of ^{64}Cu and ^{65}Zn conducted in this laboratory (7) demonstrated that ^{64}Cu was absorbed to about the same extent from the stomach as from the duodenum. By contrast, ^{65}Zn was absorbed rapidly from the duodenum but was not absorbed from the stomach to any significant degree. This phenomenon of both elements being absorbed from one site (duodenum), whereas only ^{64}Cu was absorbed readily from a second site (stomach), led to speculation that these differences might reflect some fundamental differences between the absorptive processes in the stomach and in the duodenum. The first experiments reported here were designed to test one aspect of such an hypothesis; namely, does excess zinc affect the uptake of copper from either the stomach or the duodenum and, if so, is uptake from the 2 segments affected in the same manner.

The results of these first experiments stimulated an interest in other cations known to affect the utilization of copper. Hill et al. (8) have shown that copper is involved in the cadmium toxicity observed in chicks. In a subsequent publication, these workers (9) reported that silver tended to accentuate the effects of copper deficiency, whereas mercury had no deleterious effect on copper-deficient chicks but had an adverse effect on copper-adequate ones. These observations provided a basis for a second series of experiments designed to compare the effects of zinc, cadmium, silver, and mercury on the absorption and distribution of ^{64}Cu in rats.

MATERIALS AND METHODS

Male rats of the Sprague-Dawley strain were used in these experiments. They were housed in stainless steel batteries with raised wire floors and were fed a chow diet.[1] This diet contained, by chemical

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[1] Big Red Dog Chow, Agway, Inc., Syracuse, New York. Trade names and company names are included for the benefit of the reader and do not imply any endorsement or preferential treatment of the product listed by the U. S. Department of Agriculture.

analyses, 7 to 10 ppm of copper and 45 ppm of zinc.

^{64}Cu and the cation to be tested were administered to the animals by placing them directly into the stomach or into a 7.0-cm segment of intestine immediately distal to the pylorus. The essential features of this dosing procedure were as follows: After an overnight fast, rats were anesthetized with ether and the peritoneal cavity was opened by a 2.5- to 3.5-cm midline incision. When the injection was to be made into the stomach, a ligature was placed loosely around the pylorus and the needle was inserted through the ligature and into the stomach. The ligature was tightened about both the pylorus and the needle; the injection was made, the needle was withdrawn, and the ligature was tied. A virtually identical procedure was used when the dose was given in the duodenal segment, with the exception that the first ligature was placed around the pylorus and tied; then the injection was made through a second ligature placed about 7.0-cm distal to the pylorus. After the injection had been made, the abdominal incision was closed with sutures and stainless steel wound clips.

In experiments designed to compare the effect of zinc on the absorption of ^{64}Cu from the stomach to that from the duodenum ^{64}Cu was received as $Cu(NO_3)_2$, was taken up in 0.9% saline solution, and the pH was adjusted to 4.5 to 5.5. This solution was subsequently diluted with an aqueous solution of $ZnCl_2$, so that the 0.3-ml doses administered to the rats contained 0.01 μmoles of copper and either 0.5 or 5.0 μmoles of zinc. A similar procedure was used for those experiments designed to test the effects of zinc, cadmium, mercury, and silver on ^{64}Cu uptake. Differences in preparation of the second group of solutions were as follows: The ^{64}Cu was taken up in distilled water rather than in saline solution; the zinc, cadmium, mercury, and silver were added as the nitrates rather than as the chlorides; and the 0.4-ml doses contained 0.01 μmoles of copper and enough of the metal in question to give metal:copper ratios of 0, 150, 300, or 600. All rats in the second series of experiments were dosed via the duodenum.

Male rats weighing 200 to 400 g were used in a randomized block design. Replication was on the basis of body weight, and any individual experiment consisted of one complete replication; that is, every treatment was included in each run. Experiments were run for 3 hours after dosing; the rats were then re-anesthetized and decapitated. Samples of blood, heart, kidney, liver and spleen were counted in a Nuclear Chicago Model DS-202 well-type scintilation detector attached to a Nuclear Chicago Model 132 computer-analyzer. Values for blood were estimated by multiplying counts per minute per gram of blood by 0.07 times body weight. Values for kidneys were obtained by doubling the count observed for one kidney. Data were subjected to an analysis of variance, and individual means were compared using a multiple range test (10). Statements of significance are based on odds of at least 19 to 1.

RESULTS

Effect of dosing site and zinc level. Results of these studies are presented in table 1. As had been observed previously (7), ^{64}Cu uptake from the stomach was slightly higher than from the duodenum in both the controls and the zinc-treated rats; however, these differences were not statistically significant. With a zinc-to-copper ratio of 500, a significant depression ($P \leq 0.05$) in ^{64}Cu uptake was noted. A 58% depression in absorption was noted when the isotope was placed in the stomach as compared with a 60% depression when the isotope was placed in the duodenal segment. The added zinc apparently affected ^{64}Cu absorption from the stomach and from the duodenal segment in about the same manner and to about the same extent.

Effect of zinc, cadmium, silver and mercury. In tables 2, 3, 4, and 5, the percentages of the administered doses that were recovered in the 5 sampled tissues (blood, heart, kidney, liver, and spleen) are presented. These figures are used as indexes of ^{64}Cu absorption. The remaining portions of each table are devoted to the relative distribution of ^{64}Cu among the sampled tissues. The data were expressed in this manner in an attempt to separate the effects of the various ions on absorption

TABLE 1

Effect of zinc level and dosing site on the absorption of ^{64}Cu

Injection site	Zn/Cu	% of administered dose/tissue [1,2]				
		Blood	Heart	Kidneys	Liver	Sum [3]
Stomach	0	2.47^{a}	0.07^{a}	2.71^{ab}	7.27^{ab}	12.45^{ab}
	50	2.70^{a}	0.07^{a}	3.40^{a}	8.91^{a}	15.08^{a}
	500	1.10^{b}	0.04^{bc}	0.86^{c}	3.29^{cd}	5.29^{cd}
Duodenum	0	1.84^{ab}	0.04^{ab}	1.54^{bc}	5.35^{bc}	8.92^{bc}
	50	2.28^{a}	0.05^{ab}	1.88^{bc}	6.12^{ac}	10.32^{ac}
	500	0.70^{b}	0.02^{c}	0.87^{c}	1.90^{d}	3.32^{d}

[1] Each value is the mean of 7 observations.
[2] Values in any column which are not followed by the same letter are significantly different ($P \leq 0.05$).
[3] Percentage of administered dose recovered in blood, heart, kidneys, and liver.

TABLE 2

Effect of zinc on the absorption and distribution of ^{64}Cu

Zn/Cu	% of dose in sampled tissues [1]	Relative distribution [2,3]				
		Blood	Heart	Kidneys	Liver	Spleen
		%	%	%	%	%
0	21.89^{a}	24.47^{a}	0.42^{a}	17.09^{a}	58.06^{a}	0.42^{a}
150	11.80^{ab}	22.51^{a}	0.43^{a}	17.58^{a}	59.08^{a}	0.39^{a}
300	14.80^{ab}	23.02^{a}	0.37^{a}	21.44^{a}	54.64^{a}	0.53^{a}
600	9.33^{b}	20.65^{a}	0.29^{a}	19.68^{a}	58.93^{a}	0.30^{a}

[1] Percentage of administered dose recovered in blood, heart, kidneys, liver, and spleen.
[2] Each value is the mean of 5 observations.
[3] Values in any column which are not followed by the same letter are significantly different ($P \leq 0.05$).

TABLE 3

Effect of cadmium on absorption and distribution of ^{64}Cu

Cd/Cu	% of dose in sampled tissues [1]	Relative distribution [2,3]				
		Blood	Heart	Kidneys	Liver	Spleen
		%	%	%	%	%
0	21.89^{a}	24.47^{a}	0.41^{a}	17.09^{a}	58.06^{a}	0.42^{a}
150	3.10^{b}	20.90^{a}	0.39^{a}	18.20^{a}	60.03^{a}	0.47^{ab}
300	1.93^{b}	23.34^{a}	0.52^{a}	22.10^{a}	53.46^{a}	0.58^{ab}
600	1.01^{b}	33.22^{b}	0.82^{a}	16.54^{a}	48.23^{a}	0.85^{b}

[1] Percentage of administered dose recovered in blood, heart, kidneys, liver, and spleen.
[2] Each value is the mean of 5 observations.
[3] Values in any column which are not followed by the same letter are significantly different ($P \leq 0.05$).

TABLE 4

Effect of silver on the absorption and distribution of ^{64}Cu

Ag/Cu	% of dose in sampled tissues [1]	Relative distribution [2,3]				
		Blood	Heart	Kidneys	Liver	Spleen
		%	%	%	%	%
0	21.89^{a}	24.47^{a}	0.42^{a}	17.09^{a}	58.06^{a}	0.42^{a}
150	14.12^{a}	18.07^{ab}	0.33^{a}	15.26^{a}	65.85^{ab}	0.48^{a}
300	12.71^{a}	16.96^{ab}	0.42^{a}	16.92^{a}	65.36^{ab}	0.34^{a}
600	19.32^{a}	11.54^{b}	0.26^{a}	15.91^{a}	71.87^{b}	0.47^{a}

[1] Percentage of administered dose recovered in blood, heart, kidneys, liver, and spleen.
[2] Each value is the mean of 5 observations.
[3] Values in the same column which are not followed by the same letter are significantly different ($P \leq 0.05$).

TABLE 5

Effect of mercury on the absorption and distribution of ^{64}Cu

Hg/Cu	% of dose in sampled tissues [1]	Relative distribution [2,3]				
		Blood	Heart	Kidneys	Liver	Spleen
	%	%	%	%	%	%
0	21.89[a]	24.47[a]	0.41[a]	17.09[a]	58.06[a]	0.42[a]
150	14.61[a]	24.70[a]	0.33[a]	20.29[a]	54.22[a]	0.37[a]
300	17.20[a]	21.12[a]	0.38[a]	29.86[ab]	48.25[a]	0.38[a]
600	13.08[a]	18.72[a]	0.43[a]	36.38[b]	44.41[a]	0.49[a]

[1] Percentage of administered dose recovered in blood, heart, kidneys, liver, and spleen.
[2] Each value is the mean of 5 observations.
[3] Values in any column which are not followed by the same letter are significantly different ($P \leq 0.05$).

of ^{64}Cu from their effect on ^{64}Cu once it has been absorbed. The relative distribution values were obtained by dividing the counts per minute recovered in a particular tissue by the sum of the counts per minute recovered in all 5 sampled tissues.

The data of table 2 demonstrate the effect of zinc on the uptake of ^{64}Cu from the duodenum. The major zinc effect was a moderate depression in total uptake of ^{64}Cu. Differences between the controls and the rats that received the highest level of zinc were statistically significant. This is consistent with the results reported in table 1. Zinc had no significant effects on the relative distribution of ^{64}Cu among the sampled tissues in either series of experiments.

The effects of cadmium on the uptake of copper from isolated duodenal segments are demonstrated by the data of table 3. Cadmium produced a severe depression of copper uptake at all levels tested. A comparison between the 0:1 and the 600:1 cadmium:copper treatments indicates that the relative ^{64}Cu content of the blood and spleen was increased ($P \leq 0.05$) by cadmium. Cadmium treatment caused an upward trend in the relative percentage of ^{64}Cu observed in the heart and a downward trend in the relative proportion of ^{64}Cu observed in the liver. The proportion of the absorbed dose recovered in the kidneys was not affected.

The influence of silver on the absorption and distribution of ^{64}Cu is demonstrated by the data in table 4. There was no significant depression of ^{64}Cu uptake by the levels of silver used in the experiments, but the relative distribution of ^{64}Cu among the sampled tissues was affected. There was a depression in the retention of ^{64}Cu by the blood and an increase in the proportion of ^{64}Cu observed in the liver of the silver-treated rats. These differences were statistically significant for comparisons between no silver and a silver-to-copper ratio of 600:1. The relative proportion of the absorbed isotope in the heart, kidneys, and spleen did not appear to be altered by treatments with silver.

Mercury did not produce a significant depression of ^{64}Cu uptake (table 5), but, as with cadmium and silver, it did produce some changes in the partitioning of ^{64}Cu among the sampled tissues. A comparison between the 0:1 and the 600:1 treatments reveals a significant increase in the percentage of ^{64}Cu observed in the kidneys of the mercury-treated rats. This increase in kidney ^{64}Cu was accompanied by decreases in the percentages of ^{64}Cu in the blood and in the livers of these mercury-treated rats. The ^{64}Cu content of the heart and of the spleen was not affected by mercury.

DISCUSSION

The ions used in these studies were chosen partially because of certain characteristics that they share with one another and with copper. However, one of the more notable facets of the observations reported here was that the effects were so varied. In fact, about the only common feature was that each of the four cations did affect copper metabolism. To facilitate discussion, effects of the various ions are summarized in table 6. Comparisons in this table are based on differences between the controls and the treatments having metal-to-copper ratios of 600:1.

In the animals treated with high levels of zinc, ^{64}Cu was taken up more slowly than in controls. Once the ^{64}Cu had been

TABLE 6

Summary of the effects of zinc, cadmium, silver and mercury on the absorption and distribution of ^{64}Cu

	Effect on absorption [1]	Effect on relative distribution				
		Blood	Heart	Kidneys	Liver	Spleen
Zinc	— —	0	0	0	0	0
Cadmium	— —	+ +	+	0	—	+ +
Silver	0 or —	— —	0	0	+ +	0
Mercury	—	—	0	+ +	—	0

[1] The symbols, + + or — —, indicate statistically significant differences. One + or — indicates a trend in which the difference between the highest metal treatment and the control was not statistically significant. 0 indicates that the metal in question produced no discernible effect.

absorbed, however, its distribution among sampled tissues in zinc-treated animals was not appreciably different from that in control rats. Magee and Matrone (5) administered ^{64}Cu, intragastrically, to rats that were being fed high-zinc diets. They noted a 20% smaller ^{64}Cu content in the liver and an increased urinary excretion of ^{64}Cu in the zinc-fed rats. These workers also reported a depression in the liver-copper concentrations in zinc-fed rats. Thus, at least 2 facets to the zinc toxicity syndrome are indicated. The primary effect of high levels of zinc in an acute short-term situation of the type reported here seems to be one of impeding the uptake of ^{64}Cu. By contrast, chronic effects would include the depressed liver-copper concentrations and increased urinary excretion observed by Magee and Matrone (5).

Cadmium depressed the uptake of ^{64}Cu much more severely than any of the other ions tested. This is in accord with studies of Hill et al. (8, 9) in that their results indicate that cadmium was more toxic than zinc, silver, or mercury when fed at the same levels.

Cadmium ions at low concentrations uncouple the oxidative phosphorylation associated with mitochondrial oxidation (11). If cadmium interferes with energy generation and, consequently, protein synthesis, it could prevent incorporation of "direct-reacting" serum copper (12) into ceruloplasmin (13), hepatocuprein (14), and other copper proteins and thus account for the observations reported here. Cadmium could also depress ^{64}Cu uptake by interfering with normal passage of ^{64}Cu through the intestinal epithelium. Currently, available information does not provide an adequate basis for choosing from among various plausible explanations.

Major effects of silver were a decrease in the relative proportion of ^{64}Cu in blood and an increase in the proportion deposited in the liver. The liver is exposed to or contains "direct-reacting" serum copper (15), ceruloplasmin copper (13), hepatocuprein copper (14), and several copper-containing enzymes. The increase in liver copper could be due to an increase in any or all of these forms. Silver can reversibly inactivate some copper-containing enzymes (16), bind sulfhydryl groups (17), and is concentrated in the liver (18). These properties provide a means whereby silver could affect the normal pathways of copper metabolism.

At the highest level of mercury, there was a moderate decrease in ^{64}Cu uptake, an increased retention in the kidneys, and a decrease in the relative proportions of ^{64}Cu retained by blood and by liver. Mercury characteristically accumulates in the kidney (19) and induces renal failure (20). This could be responsible for the increased retention of ^{64}Cu in this organ. Hill et al. (9) reported that mercury was more damaging to chicks fed diets supplemented with copper than to chicks fed copper-deficient diets. This would be expected if renal failure were the critical factor.

A similar increase in the relative proportion of ^{64}Cu in the kidney was observed in experiments in which rats were dosed with ^{64}Cu, then killed at various intervals.[2] However, quantitative comparisons indicate that the effects of mercuy are not due

[2] Unpublished observations, D. R. Van Campen.

to a simple time lag in the overall absorption-distribution process.

Probably, the most salient feature of the results reported here is the diversity of responses produced by ions that were chosen partially on the basis of common chemical characteristics. If these individualistic responses prove to be specific and indigenous to the cations which produced them, they could provide useful tools for elucidating mechanisms involved in copper metabolism.

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A Chemically Defined Liquid Diet for the Chick [1]

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ABSTRACT A chemically defined liquid diet has been developed which allowed 6.0 g gain/chick/day when fed ad libitum during the first week of life. It was fed at a concentration of 1 g solids/5 ml solution. Similar performance was achieved when the diet was fed in the solid form. The liquid diet produced sustained growth (8.9 g/day) for 3 weeks and an earlier modification produced growth (11.8 g/day) for 7 weeks.

The successful development of a water-soluble, chemically defined diet for the rat was accomplished by Greenstein and associates in 1960 (1). A major advantage of this type of diet is that it can be rendered sterile by filtration, whereas sterilization by steam or irradiation results in chemical alteration of dietary constituents. These are important considerations when the objective is to perform gnotobiotic studies using nutritionally defined diets.

Others have reported similar but unsuccessful attempts using chicks. Pipkin et al.[2] and Kopfler and Wilkinson (2) reported that chicks receiving a liquid diet developed a major osmotic pressure disturbance resulting in a critical loss of tissue water as indicated by dehydration and mortality. Feeding a solution containing 19% glucose caused death; however, a solution containing only 8% glucose allowed 80% livability. In these studies the liquid diet was administered to the chick continuously by use of a peristaltic pump.

Since the present studies were begun, Johnston and Gray (3), reported achieving 4.95-g gain/chick/day during zero to 11 days of life by administering a water-soluble synthetic diet with a similar force-feeding technique as described above.

The present report describes the steps involved in development of a chemically defined liquid diet for ad libitum feeding of chicks, and presents data showing comparative results with liquid and solid "synthetic" diets.

EXPERIMENTAL METHODS

Male chicks (Ledbrest × Cobb White Rock) were reared in electrically heated battery brooders with raised wire-mesh floors in a conventional room held between 21 to 22°. The liquid diet was provided in a glass candlestick holder dish fitted with a round wood dowel to prevent chicks from walking through the diet. To encourage food consumption, each chick's beak was dipped into the diet twice daily for the first 2 days.

All components of the diet were of highest purity available commercially. Mineral sources and sucrose were of reagent grades. Amino acids [3] and vitamins were of high purity. The monocalcium salt of fructose-1,6-diphosphate was used to avoid precipitation of phosphates as reported previously (1).

The liquid diets (table 1) were prepared as follows: For the quantity of diet listed, 400 ml of distilled water [4] were placed in an Erlenmeyer flask with continuous magnetic stirring and no heating. The amino acids, fructose-1,6-diphosphate (calcium), and sucrose were added in order and allowed to dissolve; then the remaining ingredients (beginning with potassium acetate) were added in the or-

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[2] Pipkin, G. E., W. S. Wilkinson and J. G. Lee 1962 Concentration of solids — a major factor in livability of day-old chicks force-fed liquid diets. Federation Proc., *21*: 388 (abstract).

[3] Amino acids were purchased from General Biochemicals, Inc., Chagrin Falls, Ohio (to conform to specifications outlined in National Research Council Publication 719, "Specification and criteria for biochemical compounds") and from Mann Research Laboratories, 136 Liberty St., New York 6 (M.A. grade).

[4] Diet preparation is more rapid when nearly the total final quantity of water is used due to increased rate of solubility. All diets shown, excepting diet 9 (due to high content of glutamic acid) would, however, achieve complete solution at a concentration of 1 g solids in 2 ml solution if desired.

TABLE 1

Composition of liquid diets

	1	3	6	7	8 [1]	9 [2]
	g	*g*	*g*	*g*	*g*	*g*
Sucrose	71.0	71.0	71.0	71.0	71.0	71.0
L-Arginine·HCl	1.33	1.33	1.33	1.33	1.55	1.33
L-Histidine·HCl·H_2O	0.50	0.50	0.50	0.50	0.58	0.41
L-Lysine·HCl	1.25	1.25	1.25	1.40	1.44	1.40
L-Phenylalanine	1.00	1.00	1.25	1.31	0.67	0.68
L-Tryptophan	0.22	0.22	0.22	0.22	0.20	0.225
DL-Methionine	0.75	0.75	0.75	0.90	0.40	0.55
L-Threonine	0.65	0.65	0.65	0.65	0.78	0.65
L-Leucine	1.20	1.20	1.20	1.20	1.30	1.20
L-Isoleucine	0.80	0.80	0.80	0.80	0.80	0.80
L-Valine	0.82	0.82	1.00	1.00	0.95	0.82
L-Proline	1.00	1.00	1.00	1.00	1.00	1.00
Glycine	1.60	1.60	1.20	1.20	1.20	1.60
L-Diglutamic acid monocalcium·H_2O	5.35	4.00	4.00	4.00	4.00	4.00
L-Glutamic acid sodium·H_2O	2.00	2.00	2.00	2.00	2.00	2.00
L-Glutamic acid	—	1.00	1.00	1.00	1.00	7.10
L-Alanine	2.00	2.00	1.00	1.00	1.00	—
L-Serine	2.00	2.00	0.50	0.50	0.50	—
L-Aspartic acid	1.80	—	—	—	—	—
L-Asparagine	—	2.00	1.00	1.00	1.00	—
L-Glutamine	—	—	1.00	1.00	1.00	—
L-Tyrosine ethyl ester·HCl [3]	—	—	—	—	0.904	0.854
Cystine ethyl ester·HCl [3]	—	—	—	—	0.506	0.536
Fructose-1,6-diphosphate, calcium	3.67	3.00	3.00	3.00	3.00	3.00
Potassium acetate	—	1.00	1.00	1.00	1.00	1.00
Sodium acetate	—	0.50	0.50	0.50	0.50	0.50
Potassium chloride	0.67	—	—	—	—	—
Magnesium sulfate (anhydrous)	0.220	0.220	0.220	0.220	0.220	0.220
Trace minerals [4]	0.101	0.101	0.101	0.101	0.101	0.101
Vitamins [5]	0.220	0.220	0.220	0.220	0.220	0.220
Total solids	100.151	100.161	97.691	98.051	98.821	101.196

[1] In diet no. 8, essential amino acid levels as given in ration 1 of Dobson et al. (6) were used.
[2] In diet 9, amino acid mixture of Dean and Scott (5) designated as "final mixture" was used.
[3] Equimolar quantities of the water-soluble forms of cystine and tyrosine were used.
[4] Trace minerals: (in milligrams) $FeSO_4(NH_4)_2SO_4·6H_2O$, 41.8; $MnSO_4·H_2O$, 34; $ZnSO_4·7H_2O$, 22; $CuSO_4·5H_2O$, 1.67; KI, 0.65; $NaMoO_4·2H_2O$, 0.5; $NaSeO_3$, 0.218. The amount of each compound shown was added separately as an aqueous solution in a volume of 1 ml. Ferrous ammonium sulfate solution was prepared freshly each mixing time because of apparent oxidation.
[5] The vitamins were provided by a vitamin solution (final volume 1 ml): added in order (in milligrams) biotin, 0.05; pyridoxine·HCl, 0.625; thiamine·HCl, 2.5; ascorbic acid, 6.25; niacin, 6.25; (at this point 2.4 mg $NaHCO_3$ were added to raise solution pH to 4.5); vitamin B_{12}, 0.0025; menadione sodium bisulfite (63% USP), 0.25; riboflavin phosphate, 1.5; and Ca p-pantothenate, 2.5. Separate additions were folic acid, 0.5 mg in 2.5 ml in fine suspension and choline chloride 0.200 g/ml.

der shown and according to the table footnotes. Final diet concentration in all diets in experiment C-181 was the amount of diet (about 100 g) shown in table 1 in 500 ml solution. The pH values of diets 3, 6, 7, 8, and 9 were 5.1, 5.2, 5.2, 5.1, and 4.4, respectively.

To facilitate study of liquid diet variables, the fat-soluble vitamins and essential fatty acid were provided in a corn oil supplement by dropper. Each milliliter of supplement contained 4 mg vitamin A palmitate (1,000,000 IU/g), 10 μg vitamin D_3 (40,000 ICU/mg), and 10 mg *d*-α-tocopheryl acetate (1,360 IU/g) in refined corn oil and provided 4,000 IU vitamin A, 400 ICU vitamin D_3, and 13.6 IU vitamin E. A dosage of 0.25 ml/chick was administered on the second and fifth days of the experiment. This amount of corn oil was calculated to be approximately equivalent to 0.5% of dietary intake on a solids basis. As an exception, 2 treatments provided 0.5% of ethyl linoleate containing the same supplementation of vitamins A, D, and E given above in an

oil-in-water emulsion produced by adding 1.5% polyoxyethylene 20 sorbitan monooleate [5] to the liquid diet as described (1).

The composition of the stock diet (VNS-1) follows: ground yellow corn, 55; soybean meal (dehulled), 30; fish solubles (100% equivalent dried on soybean meal), 3; dehydrated alfalfa meal (17% protein), 3; dried whole whey, 3; corn oil (refined), 2; methionine hydroxy analogue, calcium (90%), 0.05; dicalcium phosphate, 2; calcium carbonate, 1; salt, 0.5; and trace mineral mixture (supplying diet in ppm: manganese 90, zinc 30, iron 30, copper 3, iodine 1.8, and cobalt 0.3, in calcite carrier), 0.15; and vitamin mixture (supplying per kg diet: vitamin A, 16,500 IU; vitamin D_3, 2,750 ICU; vitamin E acetate, 44 IU; menadione sodium bisulfite — 63% USP, 4.5 mg; choline chloride, 250 mg; vitamin B_{12}, 44 μg; biotin, 0.55 mg; folic acid, 4.4 mg; pyridoxine·HCl, 8.8 mg; riboflavin, 11 mg; thiamine·HCl, 22 mg; Ca D-pantothenate, 22 mg; and niacin, 66 mg), 0.30.

Statistical analyses of variance are reported by means of Duncan's multiple range test (4). Additional experimental details are given in table footnotes.

RESULTS

Preliminary experiments. The first liquid diet formulated (table 1, no. 1) was patterned after a typical purified chick diet, but incorporating an earlier version of Dean and Scott's final amino acid mixture (5) and other necessary features of a soluble diet (1). In a series of short-term (2- to 7-day) studies with chicks 1 to 2 weeks of age, it was determined that (a) diet dilution of 1 g to 2.5 to 3.5 ml produced better gains than 1:2 when no supplementary water was given to drink, (b) the weight loss occurring during the first 24 to 48 hours upon feeding the liquid diet could be diminished by providing water to drink or by force-feeding the liquid diet, and (c) sucrose as the dietary carbohydrate supported somewhat better growth than glucose. In a more successful trial, chicks 14 days of age fed diet 1 at 1:2.5 dilution gained 11.6 g/day during the second to sixth day on experiment, whereas chicks fed the stock diet gained 14.7 g/day.

The following studies were conducted with chicks from one day of age, and diet dilution was 1 g in 2 ml with water supplied ad libitum. Diet 2 (not shown) was modified from diet 1 and included reduced calcium, phosphorus, and chloride and increased sodium and potassium levels. Growth during zero to 12 days of age was approximately as good with diet 2 as diet 1. Diet 3 was modified from diet 2 by replacing 1.8% aspartic acid with 2.0% asparagine. This change improved daily gain during 7 to 12 days of age from 4.0 to 5.1 g. At 12 days of age, 4 superior chicks from diet 3 and from the stock diet were selected for a long-term study. At 50 days of age, the former chicks averaged 631 g and the latter 1168 g. All chicks appeared normal, although livers of the former chicks contained 34.6% ether extract compared with 10.1% for the controls.

In other studies with newly hatched chicks, neither addition of chlortetracycline·HCl (22 mg/100 g solids) nor feeding separate supplements of cystine and tyrosine improved chick growth. A most significant observation was the helpfulness of greater diet dilution in alleviating a growth lag during zero to 3 days of life. Diet dilutions of 1:5 or 1:7.5 resulted in better growth (with little or no lag) than dilutions of 1:2 or 1:3.5 during zero to 7 days, with water to drink.

Since plasma free amino acids of chicks fed diet no. 3 were low in phenylalanine and valine, and high in glycine, alanine, and serine as compared with chicks fed the stock diet, diet no. 6 was formulated with adjustment of the levels of these amino acids (plus addition of glutamine and reduction of asparagine). In 2 experiments prior to that shown in this paper diet 6 produced significantly greater growth than diet 3.

Experiment C-181. An experiment was designed to compare results achieved above with those obtained with solid purified and natural diets during the first week of life. A diet emulsion and other amino acid patterns were also included. Data are shown in table 2. Liquid diet 6 again

[5] Emulsifier used was polyoxyethylene 20 sorbitan monooleate (Tween 80), Atlas Chemical Industries, Inc., Chemicals Division, Wilmington, Delaware.

produced a greater rate of gain than diet 3. The feeding of ethyl linoleate and fat-soluble vitamins as an emulsion using polyoxyethylene sorbitan monooleate in diet no. 6 depressed growth rate (the same comparison with solid diet also showed growth depression, but non-significant). Diet 7, which contained higher levels of phenylalanine, methionine, and lysine than diet 6, did not materially influence growth. Liquid diet 8, with the essential amino acid mixture of Dobson et al. (6) and diet 9, with the entire amino acid mixture of Dean and Scott (5) produced lower rates of growth as compared with diets 6 and 7.

The inclusion of 15% corn oil in diet 6 in solid form improved the rate of growth significantly with sucrose as carbohydrate. Performance with added oil was similar with either sucrose or starch. A similar level of growth was achieved with the Dean and Scott diet or with diet 6 amino acids substituted into the latter diet. Although excellent growth rate was achieved with these diets, chicks fed the natural stock diet VNS-1 grew at a significantly faster rate.

DISCUSSION

The research reported here represents 15 experiments conducted over a 2-year period. Since the problem was to get chicks to grow with the liquid diet, most of the effort was spent keeping feeders supplied and making daily weighings of each chick, rather than measuring food consumption. This is not to minimize the value of feed intake data, but special procedures are required to obtain accurate consumption with liquid diets due to evaporation.

In earlier experiments considerable effort was given to force-feeding, but with the use of greater diet dilution the chicks usually began eating immediately. Occasionally a chick or a group of 3 chicks was delayed in the onset of diet consumption for some unknown reason. This factor was a major source of experimental error among replicates.

Earlier studies used chicks 1 to 2 weeks of age in view of the well-known advantage of pre-selecting animals of known growth rate with consequent experimental error reduction. The observation, how-

TABLE 2

Comparison of liquid, solid and stock diets (exp. C-181) [1]

Treatment	Gain, 0–7 days	Avg daily gain	Multiple range test [2]
	g	*g*	
Liquid diets (1:5 dilution)			
No. 3	28.1	4.0	ab
No. 6	42.0	6.0	cd
No. 6, emulsion [3]	29.4	4.2	ab
No. 7	45.1	6.4	de
No. 8	26.5	3.8	a
No. 9	25.3	3.6	a
Solid diets			
No. 6 [4]	41.0	5.9	cd
No. 6, emulsion [3]	35.8	5.1	bc
No. 6, 15% corn oil-sucrose [5]	55.7	8.0	f
No. 6, 15% corn oil-starch [6]	58.6	8.4	f
Dean and Scott (5) diet [7]	56.5	8.1	f
No. 6 amino acids in Dean and Scott (5) diet	52.6	7.5	ef
Stock diet, VNS-1	68.8	9.8	g

[1] Each treatment contained triplicate groups of 3 chicks each; initial average weight, 42.2 g.
[2] Treatment means not followed by the same letter are significantly different at ($P < 0.05$).
[3] Emulsion of 0.5% ethyl linoleate containing vitamins A, D, and E in aqueous diet with addition of 1.5% polyoxyethylene (20) sorbitan monooleate as emulsifier (see Methods section for details).
[4] Diet 6 fed in the solid form was supplemented with 0.5% of the corn oil-fat soluble vitamin mixture given by dropper to chicks fed the liquid diets.
[5] Fifteen per cent refined corn oil replaced an equivalent amount of sucrose.
[6] As in footnote 4 except pearl cornstarch replaced sucrose.
[7] A starch-amino acid diet identified as diet A "final mixture" in Dean and Scott (5), with the exception that vitamin A palmitate replaced vitamin A acetate.

ever, that such animals experienced a 24 to 48 hour period of weight loss because of not eating the diet readily introduced a new source of variation which negated the main advantage of using the older chicks. Diet cost considerations and the obvious advantage of starting germfree chicks with the liquid diet at hatching were additional reasons for turning to the day-old chick for further study. Yolk reserves did not permit performance during the first week which would not continue, since no let-down in growth rate was observed in subsequent weeks.

In the early stages of this study, the inclination was to use a diet of 1 g solids in a total of 2 ml and provide water to drink as had been done with rats (1). However, since chicks during the first 2 weeks voluntarily consume approximately 2.5 parts of water to 1 part of feed (8) and because of early difficulty in achieving solubility with glutamic acid, it was felt worthwhile to include all of the needed water in the diet. This practice was discontinued when it was noted that chicks suffered less initial weight loss with water to drink, and because of recognizing the improbability of predicting water needs in a relatively uncontrolled environment. The observation that chicks grew optimally at diet concentrations of 1:5 (g solids/ml) and 1:7.5 during the first week was unexpected and indicates wide tolerance of chicks to diet concentration. In a recent study chicks fed 1:5, 1:4, and 1:3 concentrations during each of the first 3 weeks, respectively, grew slightly faster than chicks given 1:5 continuously (free choice water to drink in either case). Daily gains were 8.9 and 7.8 g, respectively, in contrast with 15.8 g for chicks fed the stock diet.

The inclusion of asparagine and glutamine was adopted since recent studies with rats (8, 9) showed beneficial effects of these compounds. The availability of plasma free amino acid data was helpful in guiding the adjustments between diets 3 and 6: chicks fed diet 3 contained 2.1, 2.5, and 2.7 times the plasma free levels of serine, glycine, and alanine, respectively, than those fed the stock diet and therefore it was considered possible to reduce the amounts of these amino acids while on a similar basis, increasing the levels of other amino acids. Other workers (1, 3) preparing soluble diets used esters of cystine or tyrosine, or both, in their diets to circumvent the insoluble nature of these amino acids and it is helpful to know that under the test conditions of this study methionine and phenylalanine could completely meet the needs of these "dispensable" amino acids.

In assessing performance of the liquid diet, it is not sufficient to compare only with a natural diet, since factors of diet form as well as composition are involved. Results indicate that diet 6 performed equally well in solution or as a solid. The level of growth of solid diet 6 (5.9 g/day during the first week) was good considering that the diet contained only 0.5% corn oil with vitamins A, D, and E; most other similar chick diets contain 9 to 15% vegetable oil following recognition of the concept (10, 11) that increased energy intake was required with free amino acid diets. The substitution of 15% corn oil for sucrose improved growth rate significantly, but it cannot be stated whether this is a specific effect of dietary fat, nutrient density, or energy-nutrient balance. Also, sucrose and starch performed similarly in solid diet 6; the presence of small quantities of protein in starch has been pointed out as a possible source of needed protein or peptide-like activity in free amino acid diets for chicks. Notwithstanding the small although significant improvement in growth with the stock diet over growth shown with the solid amino acid diets, the latter "synthetic" diets supported excellent growth during the first week, in agreement with others (12, 13).

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Biliary Secretion of Metabolites of Retinol [1] and of Retinoic Acid in the Guinea Pig and Chick [2]

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ABSTRACT The secretion of metabolites of ^{14}C-retinol and ^{14}C-retinoic acid into the bile of the guinea pig and chick was studied. Within 6 hours after the intraportal injection of retinol or retinoic acid, the bile of the chick contained 5 and 20%, respectively, of the given dose whereas guinea pig bile contained 20 and 30%, respectively, of the total dose. This radioactivity in the bile was resolved by anion exchange chromatography into 3 fractions. The fractions contained non-ionic compounds, acidic substances like retinoic acid, and more polar acidic derivatives. About 12% of the injected dose of retinol was stored in the liver of both the chick and the guinea pig as retinyl ester, whereas retinoic acid was rapidly lost from the tissues of both animals. Since appreciable amounts of metabolites of retinol and retinoic acid are secreted in the bile of the guinea pig and chick as well as of the rat, this route for the removal of metabolic products of retinol from the liver may well be common in higher vertebrates.

Although retinol was identified as a growth factor for animals over 50 years ago, many aspects of the metabolism of this vitamin remain obscure. That retinol may be converted reversibly to its aldehyde, retinal, and may be further oxidized irreversibly to retinoic acid is well known, but the subsequent fate of retinoic acid is less well defined. Metabolites of retinoic acid have been observed, however, in the stomach [3] and colon [4] of rats, and metabolites of retinol and retinoic acid appear as well in the plasma, urine, liver and feces of chickens and rats (2–4).[5,6] Recently, polar metabolites of retinol and retinoic acid have been observed in large amounts in the bile of an isolated perfused rat liver preparation (5) and in the bile of rats in vivo (6, 7). In the present report, studies on retinol metabolism were extended to the chick and guinea pig. The rate of secretion of metabolites of retinol and retinoic acid in the bile, the chromatographic properties of these metabolites and the storage of labeled retinyl ester in the liver are considered and compared with previous results obtained with bile duct-cannulated rats.

EXPERIMENTAL METHODS

The experiments were carried out with fasted (10 to 12 hours) albino male guinea pigs weighing 300 to 500 g (University stock) and White Leghorn chicks weighing 150 to 200 g (University stock). The animals were anesthetized with ether and the bile duct of the guinea pigs was cannulated below the cystic duct. In chicks, 2 bile ducts were cannulated: a) the hepato cysticus, which courses from the right lobe of the liver to the gallbladder, from which the cystic duct passes to the termination of the duodenum, and b) the hepato enteric duct from the left lobe of the liver (8). Pertinent data relating to bile duct-cannulated guinea pigs, chicks and rats are compared in table 1.

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[1] In accordance with the definitive rules for the nomenclature of vitamins which were approved by the Commission in the Nomenclature of Biological Chemistry of the International Union of Pure and Applied Chemistry (1), the terms retinyl ester, retinol, retinal and retinoic acid are used in place of vitamin A ester, vitamin A, retinene and vitamin A acid, respectively.

[2] This investigation was supported by a grant-in-aid from the National Institutes of Health (A-1278). Part of this work was carried out in an undergraduate senior research program by one of the authors (M.B.S.).

[3] Rogers, W. E., M-L. Chang and B. C. Johnson 1963 A biologically active metabolite of vitamin A. Federation Proc., 22: 433 (abstract).

[4] Sundaresan, P. R., and G. Wolf 1963 Evidence for the participation of a vitamin A-derivative in ATP-sulfurylase action. Federation Proc., 22: 293 (abstract).

[5] Krishnamurthy, S., and J. G. Bieri 1962 Metabolism of vitamin A acid in the chick. Federation Proc., 21: 475 (abstract).

[6] Zile, M., and H. F. DeLuca 1964 A biologically active metabolite of vitamin A acid. Federation Proc., 23: 294 (abstract).

TABLE 1

Data for the bile duct-cannulated animals studied

Criteria	Rat (Rolfsmeyer) [1]	Guinea pig (albino, University stock)	Chick (White Leghorn)
Average weight, g	250	325	200
Average age, days	90	90	30
Anesthesia sensitivity (diethyl ether)	+	+ + +	+ +
Liver weight, g	7–8	12–15	6–7
Gallbladder	−	+	+
Bile flow — first 2 hours, ml/hr	0.5	1.3–1.8	0.1–0.2
Survival time, hours after operation (H_2O ad libitum only)	24+	16–20	8–10

[1] Data for rats taken from references 5 and 6.

Identical experiments were carried out in which either 6, 7-^{14}C-retinol (35 to 60 μg/animal, specific activity, 7.6 μc/mg) or 6, 7- ^{14}C-retinoic acid (15 μg/animal, specific activity, 19.9 μc/mg) was used as the substrate. A solution of 1 ml of the appropriate substrate suspended in 5 to 10% polyoxyethylene sorbitan monooleate (Tween 80) in Krebs-Ringer bicarbonate buffer, pH 7.4, was injected into the portal vein. After closing the incision, the animal was placed in a restraining cage and the bile was collected for different intervals.

At predetermined times the animals were killed, various organs were homogenized in 20 volumes of $CHCl_3$:methanol (2:1) and the extracts were filtered, but in contrast with the method of Folch et al. (9) were not washed with aqueous $CaCl_2$. Instead the filtrates were directly evaporated to dryness under reduced pressure, and the residue was taken up in a small volume of methanol. The methanolic extracts of organs and the methanolic solutions of bile were chromatographed on columns (2.5 cm × 5 cm) of Bio-Rad AG2-X8 [7] anion exchange resin (200 to 400 mesh) in the acetate form. The resin bed was equilibrated with methanol before use. After placing the methanolic extracts on top of the column, non-ionic material was washed through with 70 to 100 ml of methanol. The methanol elution was followed in a step-by-step fashion with similar amounts of increasing concentrations of acetic acid in methanol (1%, 15 to 40% and 100%). Ten to twelve fractions containing 8 to 10 ml each were collected with each eluant used. A total of 45 fractions was collected, monitored for 350 mμ absorbing material with a Coleman Universal Spectrophotometer and further analyzed for radioactivity. Occasionally livers were homogenized in hexane:ethanol (3:1)) and washed with 1% aqueous $CaCl_2$ solution. The hexane:ethanol phase was then evaporated to dryness under reduced pressure, and the residue was taken up in hexane. The hexane extract of liver was then chromatographed on deactivated Al_2O_3 to isolate retinyl ester.

Radioactive solutions were plated as infinitely thin samples on aluminum planchets, and were assayed in a windowless gas flow Geiger-Müller counter (Nuclear-Chicago). Each sample was counted for a total of 1000 counts or more.

RESULTS

Production of bile metabolites from injected ^{14}C-retinol and ^{14}C-retinoic acid. Radioactive metabolites of ^{14}C-retinol (fig. 1) and of ^{14}C-retinoic acid (fig. 2) were rapidly secreted into the bile of both species after the intravenous injection of either compound. The chick, whose bile flow was only about 0.1 that of the guinea pig (table 1), secreted less radioactive metabolite than the guinea pig, but both animals secreted more radioactivity into the bile when retinoic acid was injected. Six hours after the injection of ^{14}C-retinol, 5% of the total injected radioactivity appeared in the bile of the chick, and 20% in that of the guinea pig. By 16 hours the guinea pig had secreted 25 to 30% of the radioactivity in the bile. Six hours after the injection of ^{14}C-retinoic acid, however, 20%

[7] Bio-Rad Laboratories, Richmond, California.

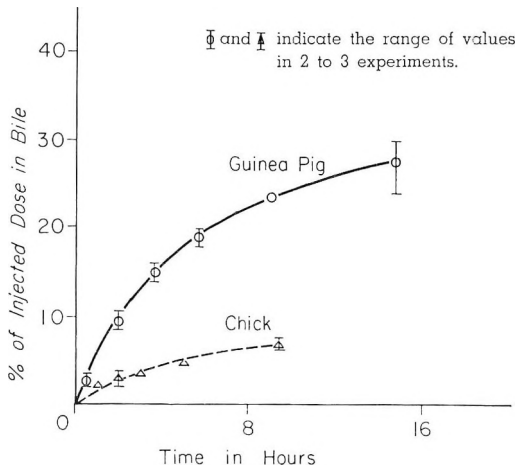

Fig. 1 The appearance of radioactivity in the bile after the intraportal administration of ^{14}C-retinol into the chick and guinea pig. After bile duct cannulation, 35 to 60 μg of ^{14}C-retinol dissolved in 0.05 ml of Tween-80 and 1.0 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, were injected into the portal vein. Bile collection was made for desired intervals and the radioactivity in each sample was determined.

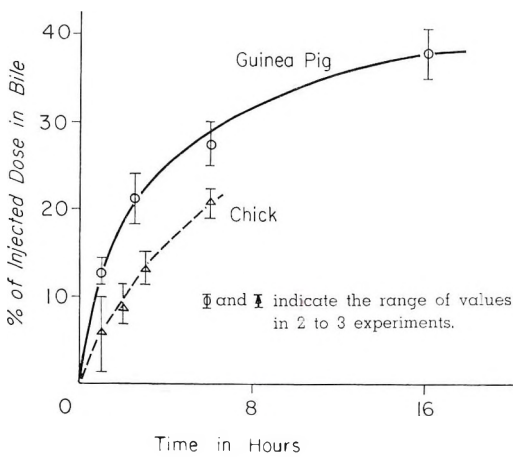

Fig. 2 The appearance of radioactivity in the bile after the intraportal administration of ^{14}C-retinoic acid into the chick and guinea pig. Conditions were similar to those cited in figure 1, except that 15 μg of ^{14}C-retinoic acid were injectcd.

of the dose appeared in the bile of the chick, and 30% in that of the guinea pig. All experiments were conducted with similar dosages of retinol (35 to 60 µg) and of retinoic acid (15 µg).

Anion exchange chromatography of bile metabolites. The radioactivity in bile was separated into 3 major fractions by anion exchange chromatography (fig. 3). After the injection of ^{14}C-retinol into guinea pigs or chicks, fraction I contained 20 to 25% of the total radioactivity of bile recovered from anion exchange columns. In guinea pigs or chicks injected with ^{14}C-retinoic acid, however, fraction I contained only 10% of the radioactivity of bile. When either retinol or retinoic acid was injected into guinea pigs, fraction II contained 25 to 35% and fraction III 45 to 65% of the radioactivity of the bile. But the bile of

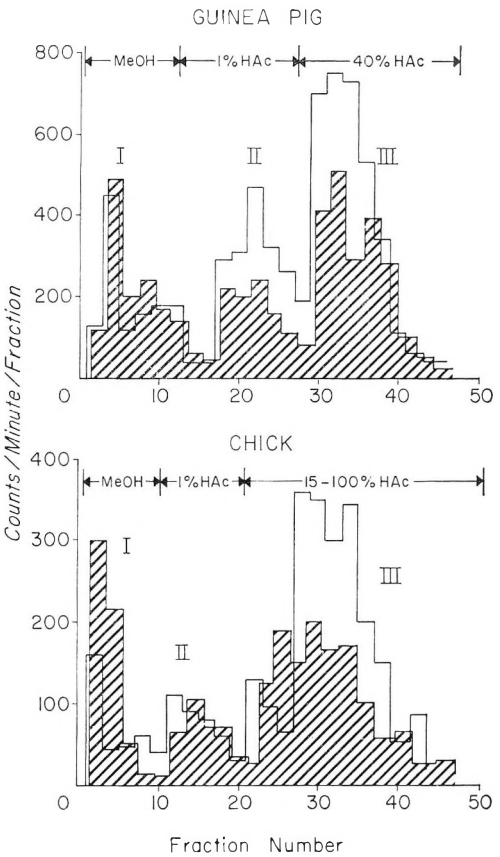

Fig. 3 The separation of labeled ^{14}C components of bile by ion exchange chromatography after the intraportal injection of ^{14}C-retinoic acid (outlined form) or ^{14}C-retinol (shaded form) into the guinea pig and chick. All chromatographic procedures were carried out on bile collected between 0.5 to 3 hours after injection. Fractions were eluted batch-by-batch with the following solvents: fraction I, methanol; fraction II, 1% acetic acid in methanol; fraction III, 15% or 40% acetic acid in methanol and 100% acetic acid.

chicks injected with ^{14}C-retinol or ^{14}-retinoic acid contained only 10 to 20% of the total recovered radioactivity in fraction II, whereas 60 to 75% appeared in fraction III. Only 5 to 7% of the radioactivity of bile was extractable with *n*-hexane, regardless of the substrate used or the species investigated.

Metabolites of retinol and retinoic acid in tissues. The guinea pig retained 14% of the injected dose of ^{14}C-retinol as ^{14}C-retinyl ester during the first 6.5 hours, whereas the chick stored 12% as liver retinyl ester in 2.5 hours after injection (table 2). Thereafter the retinyl ester in guinea pig liver decreased somewhat with time, but that in chick liver remained constant. The ^{14}C-retinyl ester was isolated by alumina chromatography. In addition, 1 to 4% of the radioactive dose was noted in the liver as unidentified metabolites which were more polar than the ester on alumina columns. The intestine of the chick contained 2.5% of the total injected radioactivity, about 70% of which was ^{14}C-retinyl ester.

The radioactivity observed in the liver and intestines of animals injected with ^{14}C-retinoic acid decreased with the time after injection. Methanolic extracts of the liver of either animal given retinoic acid contained the majority of the radioactivity (50 to 75%) in fraction II (fig. 4). Guinea pig intestine, however, contained about equal amounts in fraction II (48%) and fraction III (48%), and chick intestine contained approximately 60% of the radioactivity in fraction II and 25% in fraction III. After retinoic acid injection, fraction I contained 15% or less of the dose in every case. The intestinal contents usually contained about 1% of the radioactive dose, regardless of the compound injected or the animal used. Chick pancreas did not contain any radioactivity.

DISCUSSION

The production of bile metabolites of retinol compounds is a pathway common to at least 3 different species in both the growing animal and the adult. The rat (6), the guinea pig and the chick secrete significant amounts of the radioactivity of injected ^{14}C-retinol and ^{14}C-retinoic acid in the bile. A comparison of the amount of labeled metabolites secreted in the bile of various species is difficult, however, in view of the fact that the liver weight and the bile flow varied in all cases. Since the conversion of retinol and retinoic acid to bile metabolites in the rat occurs mainly in the liver (5), however, the amount of liver tissue acting on the injected substance must certainly be considered. The rate of bile flow, however, will influence the time course of secretion, but may af-

TABLE 2
Metabolites of retinol and retinoic acid in tissues

Animal	Compound administered	Time	% of radioactive dose			
			Liver		Intestine	
			Retinyl ester	Other	Wall	Contents
		hours				
Guinea pig	retinol	4.5	4.5	1.9	—	—
		6.5	14.0	4.0	—	—
		24.0	9.0	3.5	—	—
	retinoic acid [1]	2.0	—	2.6	2.0	0.9
		5.0	—	2.6	1.6	1.6
		16.0	—	1.6	1.0	1.0
Chick	retinol	2.5	12.5	2.5	1.7	0.8
		7.5	11.0	1.0	1.8	1.4
		9.5	12.2	1.2	1.6	1.3
	retinoic acid [1]	1.0	—	5.0	—	0.9
		2.0	—	2.5	2.3	4.7
		5.0	—	2.5	3.9	0.9
		6.0	—	1.1	1.0	—

[1] When ^{14}C-retinoic acid was administered, the majority of the radioactivity in tissues was retinoic acid which appeared in fraction II.

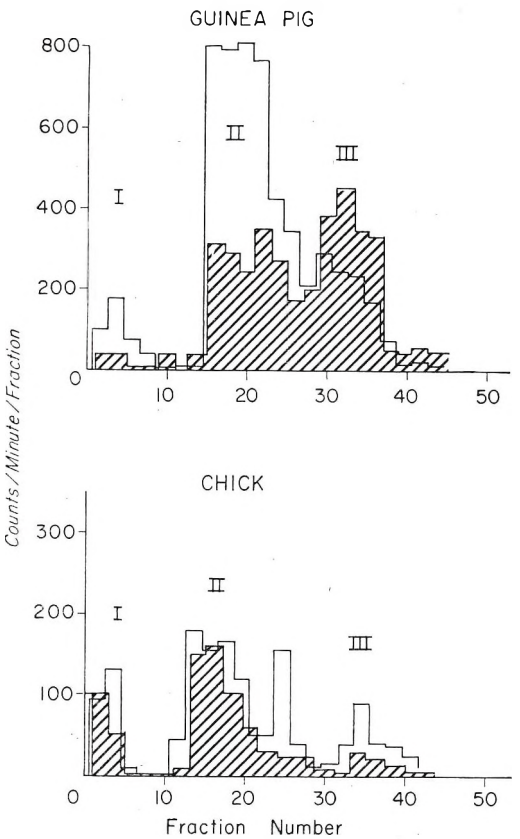

Fig. 4 The chromatographic separation of labeled lipid components of liver (outlined form) and intestine (shaded form) 2 hours after the administration of ^{14}C-retinoic acid to the guinea pig and chick. Fractions were eluted by the solvents cited in figure 3.

fect less the total amount secreted in the bile. In the present work, *all* bile was collected during a given time interval, and all intervals were summed to obtain the total amount secreted. Hence, although the chick appeared to produce smaller quantities of bile metabolites than either the rat or the guinea pig, the differences are less marked when the amount of metabolite produced per gram of liver is considered. Since small doses of labeled substrates were given, the secretion of metabolites of retinol in the bile appears to be a normal physiological event in several species rather than the expression of a unique detoxification and excretion process which occurs only in the rat. In fact, when chylomicra containing ^{14}C-labeled retinol are administered to a rat, an appreciable portion of ^{14}C (9%) is excreted in the bile (7).

The bile metabolites of retinol and retinoic acid produced by the chick and the guinea pig have been separated into 3 major fractions by anion exchange chromatography. These fractions correspond closely to those obtained from rat bile after the administration of similar doses of labeled retinol and retinoic acid (6, 10). In the latter case, these fractions have been partially characterized: fraction III contains retinoyl glucuronide (11) and presumably other polar compounds (7), fraction II consists mainly of free retinoic acid and fraction I appears to contain mainly esters of retinoic acid.[8] Since the 3 radioactive fractions obtained from the bile, liver and intestine of the chick and guinea pig have chromatographic properties identical with those of the rat, it is likely that retinol is metabolized in a similar manner in all 3 species. However, complete identification of the various metabolites is necessary before more refined comparisons can be made.

All 3 species, when injected with ^{14}C-retinoic acid, secrete two to four times more ^{14}C in bile than when injected with ^{14}C-retinol. This rapid and continuous excretion of retinoic acid metabolites is accompanied by a decline in the amount of ^{14}C observed in the animals' tissues. The disappearance of ingested retinoic acid from tissues has been observed repeatedly in the past (2, 12, 13),[9] but the mode of excretion was not known. In the chick Krishnamurthy et al. (2) reported that 80% of the retinoic acid present in the liver 3 hours after feeding a given dose of ^{14}C-retinoic acid had disappeared by 6 hours, and in the rat ^{14}C-retinoic acid disappears from the liver and intestine at similar rates (10).

Both the rat and the chick store injected retinol better than the guinea pig under the experimental conditions used. The rat stores 20 to 25% of the injected dose as liver retinyl ester (6), whereas the guinea pig and chick store 12%. When retinol

[8] Unpublished data, P. E. Dunagin, Jr., R. D. Zachman and J. A. Olson, 1966.

[9] Yagishita, K., P. R. Sundaresan and G. Wolf 1964 A biologically active vitamin A metabolite. Federation Proc., 23: 294 (abstract).

storage is expressed in terms of retinyl ester per gram of liver, however, the relative values are: rat, 3; chick, 2; guinea pig, 1. This low value for the guinea pig is in keeping with the fact that its normal content of liver retinyl ester is among the lowest in the animal kindom (14). Worker (15) has shown that guinea pig liver rapidly destroys retinol and has suggested that the low storage value for this species may be due to a rapid catabolism of vitamin A. Our observation that retinyl ester decreased more rapidly from 6.5 to 24 hours in the guinea pig than in the rat or chick is in accord with this suggestion. The storage of 12% of the injected dose of ^{14}C-retinol as retinyl ester in chick liver in the present work compares favorably with the results of previous storage studies, in which 8% of an intraduodenally administered dose of retinyl acetate was observed in the liver as retinyl ester within 4 hours (16).

The presence of metabolites of retinol and retinoic acid in the intestinal tract (2) [10] and feces (2, 3) of the rat and chick has generally been attributed to the direct action of the intestinal wall or intestinal bacteria on the administered compound. It appears likely, however, that the majority of these metabolites are initially secreted into the gut from the liver via the bile, although they subsequently may be altered by the action of intestinal bacteria.

ACKNOWLEDGMENTS

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[10] See footnotes 4 and 9.

Dietary Requirement for Asparagine and its Metabolism in Rats [1]

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ABSTRACT The effects of asparagine and glutamine on rat growth and the metabolism of ^{14}C-asparagine in the rat were studied. Asparagine was found to be required for maximal growth of young rats at different stages of growth. However, rats "adapted" to an asparagine-free diet within 4 to 8 days. Dietary glutamine was found to be a partially effective substitute for dietary asparagine. A level of 0.4 to 0.6% asparagine added to an otherwise complete diet supported optimal growth in rats. Sufficient response to a lower level of asparagine was obtained to indicate that the response obtained when a small amount of intact casein is added to a protein-free diet may be due solely to its asparagine content. More than 80% of either dietary or injected ^{14}C-asparagine was incorporated into tissues of the rats. Less than 20% of either dietary or injected ^{14}C-aspartic acid was incorporated into the tissues and the major part of the doses was lost as $^{14}CO_2$.

Previous studies at this laboratory (1,2) were concerned with the development of amino acid diets capable of supporting the level of rat performance obtained when high quality protein diets are fed. It was shown that growth and feed intakes of rats fed amino acid or protein hydrolysate diets were improved by the addition of asparagine to the diets (2). The present report deals with studies of the effects of dietary supplements of asparagine and glutamine during different phases of growth and at different times following the initiation of amino acid diet feeding, studies of the quantitative dietary requirement for asparagine and studies on the metabolism of ^{14}C-labeled asparagine.

EXPERIMENTAL

Experiment 1. Earlier studies (2) indicated that the greatest response to asparagine supplementation occurred in the first few days after amino acid diet feeding was initiated. This experiment was designed to determine whether this effect was due to a change in tissue requirements for asparagine with age or stages of growth or to metabolic "adaptations" in response to the absence of dietary asparagine. Because studies by Levintow et al. (3) showed that asparagine is derived from glutamine in cultured mammalian cells and because intact proteins may contain glutamine as well as asparagine, the effects of glutamine supplementation were studied to determine whether glutamine may be an effective dietary substitute for asparagine.

The composition of the basal amino acid and the 20% casein control diets used are shown in tables 1 and 2. Three additional treatments consisted of the basal amino acid diet supplemented with either 2% asparagine, 4% glutamine or 2% asparagine plus 4% glutamine. Fresh diets were prepared daily to minimize possible decomposition of glutamine in the diets containing glutamine. Each diet was fed to a group of 5 male rats of the Sprague-Dawley strain [3] housed in individual wire-bottom cages in a constant-temperature room (23 ± 1°). The rats were fed a commercial diet [4] for several days prior to the experiment and weighed an average of 61 g at the start of the experiment. The experimental diets and tap water were supplied ad libitum and feed consumption and weight gains were recorded daily. The casein and amino acid basal diets were fed

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[2] Present address: Animal Science Department, Texas A & M University, College Station, Texas.
[3] Purchased from Holtzman Company, Madison, Wisconsin.
[4] Big Red Laboratory Animal Feed, Agway, Inc., Feed Store, Ithaca, New York.

TABLE 1

Composition of experimental diets (exps. 1, 2 and 3)

	% of diet
Supplemented casein,[1] supplemented casein hydrolysate [2] or amino acid mixture [3]	20–22
Hydrogenated vegetable oil [4]	14.0
Mineral mixture [5]	4.0
Vitamin mixture [6]	2.2
Antioxidant [7]	0.0125
Sucrose [8]	

[1] "Vitamin-free" Casein was purchased from General Biochemicals, Inc., Chagrin Falls, Ohio, fed at a level of 20% of the diet by weight and supplemented with DL-methionine to provide a supplemental level in the diet of 0.26%.

[2] A "salt-free" acid hydrolysate preparation purchased from General Biochemicals, Inc. The hydrolysate was fed at a level of 22% of the diet and supplemented with the following essential amino acids and sodium bicarbonate to furnish as % of diet: DL-threonine, 1.0; L-tryptophan, 0.3; L-phenylalanine, 0.3; DL-methionine, 0.26; L-cystine, 0.2; $NaHCO_3$, 1.6.

[3] See table 2 for composition of amino acid mixtures.

[4] Crisco, Procter and Gamble Company, Cincinnati.

[5] Jones and Foster mixture purchased from General Biochemicals, Inc., furnishing the following salts per 100 g of diet: NaCl, 0.557 g; KH_2PO_4, 1.556 g; $CaCO_3$, 1.526 g; $MgSO_4$, 0.229 g; $FeSO_4 \cdot 7H_2O$, 0.108 g; $CuSO_4 \cdot 5H_2O$, 20 μg; $MnSO_4 \cdot 2H_2O$,180 μg; KI, 320 μg; $CoCl_2 \cdot 6H_2$, 0.8 μg; $ZnCl_2$, 12 μg.

[6] "Vitamin Fortification" Mixture, Nutritional Biochemicals Corporation, Cleveland, furnishing the following amounts of the vitamins/100 g of diet: vitamin A, 1980 IU; vitamin D, 220 IU; and (in milligrams) α-tocopherol, 11; ascorbic acid, 99; inositol, 11; choline chloride, 165; menadione, 5; p-aminobenzoic acid, 11; niacin, 9.9; riboflavin, 2.2; pyridoxine·HCl, 2.2; thiamine·HCl, 2.2; Ca pantothenate, 6.6; and (in micrograms) biotin, 44; folic acid, 198; vitamin B_{12}, 3.

[7] Santoquin, Monsanto Chemical Company, St. Louis.

[8] Sucrose added as required to complete the mixture to 100%.

TABLE 2

Amino acid mixtures fed in experiments 1 and 3 [1]

	Experiment 1	Experiment 3
	% of diet	% of diet
Arginine·HCl	0.80	0.36
Histidine·HCl·H_2O	1.00	0.60
Isoleucine	3.00 [2]	0.75
Leucine	2.00	1.20
Lysine·HCl	1.85	1.68
Methionine	0.80(DL-)	0.60
Cystine	0.30	0.30
Phenylalanine	1.30(DL-)	0.90
Tyrosine	0.80	0.45
Threonine	1.50(DL-)	0.75
Tryptophan	0.50(DL-)	0.225
Valine	2.80(DL-)	1.05
Alanine	0.60(DL-)	0.20
Glutamic acid	4.00	4.21
Glycine	0.40	0.40
Proline	0.50	0.65
Serine	0.50(DL-)	0.30
Asparagine	—	0.65
Aspartic acid	—	0.65
$NaHCO_3$	1.60	0.80

[1] Amino acids were purchased from General Biochemicals, Inc., Chagrin Falls, Ohio.

[2] A mixture of 50% L-isoleucine and 50% D-alloisoleucine.

for 21 days. The amide-containing diets were fed for 16 days. For the next 4 days, rats previously receiving the amide-supplemented diets were fed the amino acid basal diet. The data were tested statistically by Duncan's method (4) at the 0.05% level.

Experiment 2. In previous studies, levels of 0.6 to 2.0% asparagine increased growth rates. There was no indication that amounts added in excess of 0.6% were beneficial. Several workers have observed that 5% intact casein added to amino acid diets greatly improve rat performance (5–7). Since casein is believed to contain about 4% asparagine (8, 9), dietary levels of asparagine lower than 0.6% must be effective in promoting rat growth if the asparagine in casein is solely responsible for the growth response from adding casein to an otherwise complete diet. In this experiment levels of zero, 0.2, 0.4, 0.6 and 0.8% asparagine were fed with an amino acid-supplemented 22% casein hydrolysate diet.

The diet, which is similar to one used in a previous study (2), is shown in table 1. Amino acids were added to provide the essential amino acids in the pattern found in intact casein using information obtained from an analysis of the hydrolysate. The experimental procedures were similar to those outlined for experiment 1 except that 6 rats of an average weight of 71 g were assigned to each diet and the diets were fed for 14 days.

Experiment 3. This experiment deals with studies of the metabolism of ^{14}C-labeled dietary asparagine in rats. In studies by Krotkov et al. (10), intraperitoneal or intravenous doses of ^{14}C-asparagine were recovered partially as respired $^{14}CO_2$ within 3 hours after administration to rats. Studies by Tower et al. (9) indicated that a portion of a massive dose of unlabeled asparagine administered either orally or intravenously to humans was probably catabolized to urea. Fridhandler and Quastel (11) reported that asparagine was hydrolyzed to aspartic acid and ammonia during absorption by intestinal segments of guinea pigs. Sansom and Barry (8) showed by the use of double-labeling techniques that free asparagine in blood plasma of goats

was the major precursor of asparagine residues found in milk and plasma proteins.

Rats were allowed to consume an amino acid diet containing uniformly labeled ^{14}C-asparagine or ^{14}C-aspartic acid or were given intraperitoneal injections of the labeled amino acids. Aspartic acid was chosen for comparative purposes because of the structural similarity to asparagine and because of the differences shown in the growth responses of rats to asparagine and aspartic acid (2). The 2 routes of administration were chosen to distinguish between possible metabolic effects related to absorption and other effects. The labeling of respired CO_2 was measured during a 3-hour period following administration of the labeled amino acids after which the rats were killed and tissue samples were taken for studies of the labeling of body fluids and tissues.

The labeled amino acids were fed as components of the complete diet shown in table 1 with a fat level of 10%, and a mixture of L-amino acids shown in table 2. The labeled acids [5] were admixed with the diet by adding them in solution to a slurry of the sucrose and amino acid portions of the complete diet which was subsequently lypholized, reground and then mixed with the fat, mineral and vitamin portions of the diet. Portions of each labeled diet were assayed in triplicate using a vibrating reed electrometer to determine the activity of CO_2 produced upon combustion of samples by the Van Slyke wet-combustion technique (12).

Four individual experiments of the following design were conducted, two each with asparagine- and aspartic acid-labeled diets. A male Holtzman rat (Sprague-Dawley strain) was fed ad libitum the 15% casein diet shown in table 1 with a fat level of 10% and was permitted to grow to a weight in excess of 100 g. Unlabeled diet of the same composition as the labeled diet was offered to the rat for 24 hours prior to feeding the labeled diet. The unlabeled diet was removed in the late afternoon of the day of the experiment and a feed cup containing the labeled diet was placed in the rat's cage 4 to 5 hours later at a time previously determined to coincide with the normal feeding period of the rat.

As soon as the rat appeared to have finished its meal, it was transferred to a respiration chamber connected in series with a vibrating reed electrometer with a 250-ml flow-through ion chamber as described by Johnson et al. (13). Air was drawn through the system at 200 ml/minute. After allowing the apparatus to equilibrate, the activity of respired CO_2 was measured for 3 hours from the time the labeled diet was placed before the rat. Samples of tissues were lypholized and assayed for ^{14}C following wet-combustion. Samples of the

[5] Both were uniformly labeled with ^{14}C and were purchased from the Nuclear-Chicago Corporation, Chicago.

TABLE 3

Growth responses to asparagine and glutamine in rats at different stages of growth [1] (exp. 1)

Diet description	Days 1 to 4		Days 5 to 16		Days 17 to 20	
	Wt gain	Feed intake	Wt gain	Feed intake	Wt gain	Feed intake
	g/day	*g/day*	*g/day*	*g/day*	*g/day*	*g/day*
20% Casein	4.90[a]	10.45[a]	6.91[a]	13.58[a]	5.45[a]	15.20[a]
Basal amino acids	2.75[c]	9.10[b]	6.50[a]	13.00[a]	5.40[a]	15.60[a]
Basal amino acids, 2% asparagine days 1 to 16 and omitted days 17 to 20	4.35[a]	10.05[a]	6.22[a]	13.30[a]	3.85[b] [2]	14.90[a]
Basal amino acids, 4% glutamine days 1 to 16 and omitted days 17 to 20	3.60[b]	9.70[ab]	6.64[a]	13.67[a]	5.20[a]	15.60[a]
Basal amino acids, 4% glutamine and 2% asparagine days 1 to 16 and omitted days 17 to 20	3.90[ab]	9.45[ab]	6.27[a]	13.33[a]	3.65[b]	15.20[a]

[1] Treatment means given as daily averages of 5 rats. Means without a common letter in their superscript are significantly different ($P < 0.05$).

[2] Average weight gains on days 17 and 18 were 3.2 g and 2.2 g, respectively, returning to near the means of the control groups on days 19 and 20

liver and upper gastrointestinal tract were homogenized and extracted with 50% methanol at − 20° as outlined by Simmons and Mitchell (14) and the extract was assayed for ^{14}C.

Rats weighing 180 g that had been fed a commercial rat diet [6] were used in 2 experiments in which solutions of labeled asparagine and aspartic acid were injected into the peritoneal cavity. The injections consisted of 0.8 ml of 0.9% NaCl, 0.8 μc of the labeled amino acid and 0.8 mg of the unlabeled amino acid serving as a carrier. Respiratory $^{14}CO_2$ was recorded for 3 hours following the injection with the apparatus described above.

RESULTS AND DISCUSSION

Experiment 1. The effects of asparagine and glutamine supplementation of an amino acid diet on early growth of rats and the effects of their removal from the diet at a later stage of growth are shown in table 3. In the first 4 days of the experiment, growth rates were increased significantly by either asparagine or glutamine supplementation, with the effect from asparagine addition being significantly greater than that from glutamine addition. Feed intakes were increased significantly by asparagine supplementation but not by glutamine supplementation during the first 4 days of the experiment. There were no significant differences in weight gains or feed intake during the 5- to 16-day period. Weight gains were depressed significantly when either asparagine alone or asparagine in combination with glutamine was removed from the diet after the sixteenth day of the experiment. No effect on growth was noted when glutamine alone was removed from the diet. There was no significant effect on feed intake when either asparagine or glutamine was omitted from the diet after day 16 of the experiment.

As shown in previous studies (2), the effect of omitting asparagine from the diet is limited to the first few days of a given experiment, with rats consuming a diet devoid of asparagine growing at rates comparable to those of rats receiving asparagine in the latter part of the experiment. This appears to represent a response to the diet since weight gains were similarly depressed when asparagine was removed from the diet of rats after 16 days of an experiment. This suggests that an "adaptation" takes place whereby asparagine is provided from endogenous sources to meet tissue requirements in response to a lack of dietary asparagine. Ravel et al. (15) have reported a somewhat analogous phenomenon in *Lactobacillus arabinosus* where asparagine furnished in the nutrient medium appeared to depress asparagine synthesis by the bacteria through repression of the synthesis of asparagine synthetase and inhibition of its activity.

The results of this experiment indicate that dietary glutamine may partially substitute for the asparagine requirement under the conditions imposed. The effects of feeding asparagine and glutamine did not appear to be additive or, in other words, no evidence was obtained for the existence of a dietary glutamine requirement in the presence of adequate dietary asparagine. These data may be interpretated to indicate that glutamine serves as a substrate for asparagine synthesis in the intact animal as has been shown in cultured mammalian cells (3).

Experiment 2. The results of studies on the quantitative asparagine requirement for maximal growth of rats are shown in table 4. In the first 8 days of the experiment, growth was increased significantly by the addition of asparagine to the casein hydrolysate diet. Growth rates were significantly higher when a diet containing 0.4% asparagine was fed than when a diet containing 0.2% asparagine was fed and decreased significantly when a diet containing 0.8% asparagine was fed. There were no significant differences in growth rates during the second period of the experiment although growth rates tended to be lower in animals receiving diets containing either 0.2 or 0.8% asparagine. Over the entire experiment, rats receiving diets containing either 0.4 or 0.6% asparagine grew at significantly higher rates than those receiving diets containing 0.2% or no asparagine. The casein hydrolysate diet supplement with either 0.4 or 0.6% asparagine supported growth equal to that obtained when intact casein was fed. There were no significant differences

[6] See footnote 4.

TABLE 4

Asparagine requirement for maximal growth of rats fed casein hydrolysate diets [1] *(exp. 2)*

Diet description	First 8 days		Days 9 to 14		Entire experiment	
	Wt gain	Feed intake	Wt gain	Feed intake	Wt gain	Feed intake
	g	g	g	g	g	g
20% Casein	6.70^{ab}	11.92^{a}	7.06^{a}	14.53^{b}	6.86^{ab}	13.07^{b}
Casein hydrolysate	5.65^{d}	12.15^{a}	7.44^{a}	16.06^{a}	6.42^{b}	13.82^{ab}
Casein hydrolysate, 0.2% asparagine	6.11^{c}	12.40^{a}	6.78^{a}	15.50^{ab}	6.39^{b}	13.73^{ab}
Casein hydrolysate, 0.4% asparagine	6.81^{a}	12.58^{a}	7.64^{a}	16.28^{a}	7.16^{a}	14.17^{a}
Casein hydrolysate, 0.6% asparagine	6.64^{ab}	12.44^{a}	7.58^{a}	16.50^{a}	7.05^{a}	14.18^{a}
Casein hydrolysate, 0.8% asparagine	6.31^{bc}	12.17^{a}	6.92^{a}	15.22^{ab}	6.57^{ab}	13.48^{ab}

[1] Treatment means given as daily averages of 6 rats. Means without a common letter in their superscript are significantly different ($P < 0.05$).

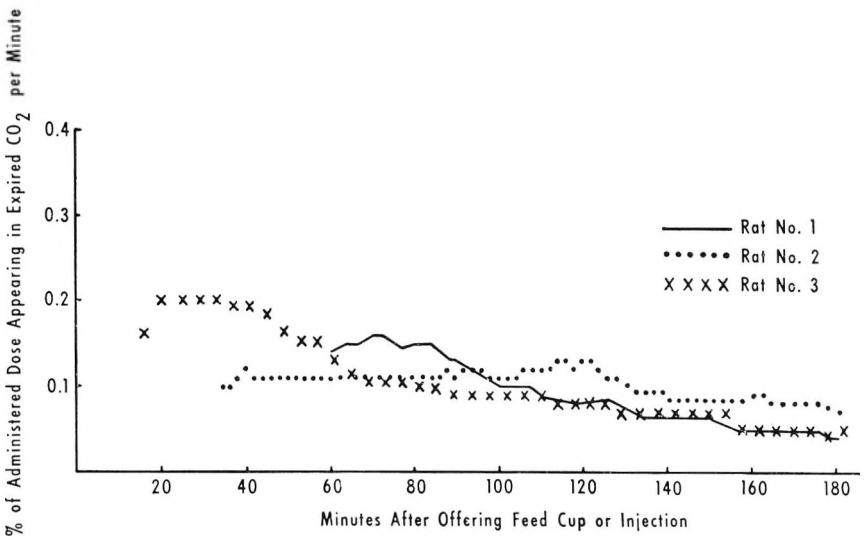

Fig. 1 The $^{14}CO_2$ production of rats fed or injected with L-asparagine-u-^{14}C. Rat no. 1 weighed 116 g and consumed 0.6 g of diet containing 1.2 μc of asparagine-^{14}C in the 45-minute feeding period. The respired $^{14}CO_2$ represents 11.6% of the dose. Rat no. 2 weighed 139 g and consumed 1.25 g of diet containing 2.5 μc of asparagine-^{14}C in the 15-minute feeding period. The respired $^{14}CO_2$ represents 15.2% of the dose. Rat no. 3 weighed 180 g and received intraperioneal injection of 0.8 μc of asparagine-^{14}C with 0.8 mg of unlabeled asparagine dissolved in 0.8 ml of 0.9% NaCl. The respired $^{14}CO_2$ represents 17.2% of the dose.

in feed intake among animals fed the casein hydrolysate diets. The intake of the intact casein diet was significantly lower than that of the hydrolysate diets supplemented with either 0.4 or 0.6 asparagine over the entire experiment, the casein diet apparently being utilized slightly more efficiently for growth. The optimal level of asparagine supplementation appeared to be 0.4 to 0.6% under the conditions imposed. However, the response obtained when 0.2% asparagine was fed indicates that a level of only 5% intact casein may furnish enough asparagine (approximately 0.2%) to account for the growth response when added to an amino acid or protein hydrolysate diet. Furthermore, the results of the previous experiment indicate that the glutamine present in the intact casein could act synergestically with the small amount of asparagine present to provide an additional growth response.

Experiment 3. Figures 1 and 2 show rates of ^{14}C appearance in respired CO_2 of rats following ingestion or injection of ^{14}C-labeled asparagine and aspartic acid, re-

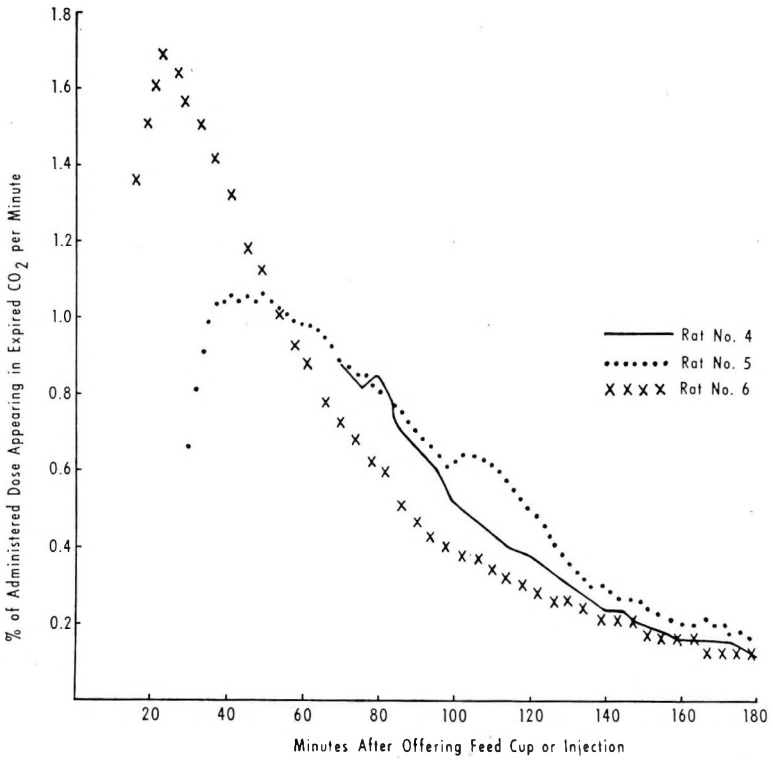

Fig. 2 $^{14}CO_2$ production of rats fed or receiving injection of L-aspartic acid-u-^{14}C. Rat no. 4 weighed 110 g and consumed 1 g of diet containing 1.8 μc of aspartic acid-^{14}C in the 45-minute feeding period. The respired $^{14}CO_2$ represents 45.6% of dose. Rat no. 5 weighed 139 g and consumed 0.55 g of diet containing 1 μc of aspartic acid-^{14}C in the 15-minute feeding period. The respired $^{14}CO_2$ represents 89.9% of dose. Rat no. 6 weighed 180 g and received intraperitoneal injection of 0.8 μc of aspartic acid-^{14}C with 0.8 mg of unlabeled aspartic acid in 0.8 ml of 0.9% NaCl. The respired $^{14}CO_2$ represents 101.9% of dose.

TABLE 5

Percentage of ^{14}C dose retained in various whole tissues of the rat 3 hours after consuming diets containing ^{14}C-labeled asparagine or aspartic acid [1]

Tissue	Rats fed ^{14}C-asparagine		Rats fed ^{14}C-aspartic acid	
	No. 1	No. 2	No. 4	No. 5
	%	%	%	%
Upper GI tract [2]	14.3	9.5	3.3	3.3
Contents of upper GI tract	2.4	3.3	1.0	0.3
Liver	7.0	6.3	2.7	1.3
Kidneys	1.1	1.1	0.3	0.2
Pancreas	2.6	1.7	0.3	
Lower GI tract and contents	2.4		0.8	
Spleen	0.7		0.1	
Lungs	0.6		0.1	
Heart	0.3		0.1	
Remaining carcass	44.8		13.1	

[1] Tissues taken from rats at the end of the $^{14}CO_2$ production experiment 3 hours after feed cups were offered to animals. See legends of figures 1 and 2 for intakes of labeled diet by rats.

[2] Upper GI tract represents portion of gastrointestinal tract including the stomach and the intestine anterior to the cecum. Lower GI tract represents the remainder of the gastrointestinal tract.

spectively. Less than 20% of the asparagine label either fed or injected appeared in respired CO_2 in the 3-hour period, whereas over 80% of the aspartic acid label was recovered in respiratory CO_2. The rate of respiratory $^{14}CO_2$ production of rats receiving ^{14}C-asparagine was low and relatively constant, whereas in rats fed ^{14}C-aspartic acid the appearance of respiratory $^{14}CO_2$ increased to a high level initially and then appeared to decrease exponentially with time after administration.

There were only small differences in the catabolism of either amino acid which may be attributed to the mode of administration. However, the gastrointestinal system appeared to limit maximal rates of $^{14}CO_2$ production probably by delaying the presentation of the amino acids to metabolic processes.

Table 5 shows the percentage of the dose of asparagine-^{14}C or aspartic acid-^{14}C fed to rats appearing in various parts of the rat carcass 3 hours after administration of the label. The analysis of the contents of the stomach and small intestine indicates that the labeled amino acids were nearly completely absorbed, the activity remaining probably being in equilibrium with the body fluids. A relatively large proportion of the asparagine label was retained in the body, whereas only small amounts of the aspartic acid label were retained, which is in agreement with the $^{14}CO_2$ production data. Quantitatively, most of the label retained from either asparagine or aspartic acid was located in the main carcass mass, the stomach and small intestine tissues and in the liver. On the basis of fresh-tissue weight, the label from either amino acid was present in relatively high concentrations in the tissues of the upper gastrointestinal tract, pancreas, liver and spleen. Tissues were fractionated into cold methanol-soluble and -insoluble fractions and activities measured but the results are not presented in the tables. Approximately 3% of the label from either amino acid present in the upper gastrointestinal tissues was extracted with cold 50% methanol. From 10 to 15% of the label in the liver was extracted by this procedure.

Some tentative conclusions regarding the metabolism of dietary asparagine in the rat may be drawn from these data. Asparagine appears to be absorbed intact in the rat. This observation contrasts with that made by Fridhandler and Quastel (11) who reported that asparagine was hydrolyzed to aspartic acid during absorption by the guinea pig intestine, in vitro. Tower et al. (9) reported that asparagine was apparently absorbed intact in humans and suggested that the asparagine hydrolysis observed in guinea pigs could have been due to contamination of the preparation with serum asparaginase. The carbon chain of asparagine appears to be much less subject to metabolic alteration than that of aspartic acid; asparagine and aspartic acid appear to be metabolized by different pathways with little deamidation of asparagine occurring. The appearance of asparagine label in a methanol-insoluble form in relatively high concentrations in tissues known to be very active in protein synthesis suggests that it was incorporated into protein. Although further study is needed to provide unequivocal evidence, it appears that a major role of dietary asparagine is that of providing a substrate for tissue protein synthesis.

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Vitamin B_{12} and the Synthesis of Thymine and Choline in the Chick [1]

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ABSTRACT The presence of large amounts of methionine in the diet of the chick did not dilute out the uptake of formate-^{14}C into liver choline and it was concluded that chicks do not transfer methyl groups from methionine to choline. The uptake of formate-^{14}C into thymine in the chick bursa and into choline in the chick liver was dependent upon the presence of adequate dietary supplements of both methionine and vitamin B_{12}. The data suggest that some step in *de novo* methyl synthesis or the transfer of one-carbon units to choline and thymine, or both, require a cobamide for a role separate from its role in methionine synthesis.

This report is a continuation of the studies in this laboratory on the role of vitamin B_{12} in deoxyribonucleic acid (DNA) biosynthesis and in particular in thymine methyl formation (1–4). A previous study (4) of formate uptake into the nucleic acids of the bone marrow of chicks fed a vitamin B_{12}-deficient diet versus those supplemented with vitamin B_{12} raised 2 questions. In the deficient chickens the formate uptake into thymine methyl was depressed more than the formate uptake into purines. Since the determinations were made on total nucleic acid purines, the question arose whether the primary effect of vitamin B_{12} deficiency involved thymine methyl formation or whether the effect was a general impairment of deoxyribotide formation and only the DNA pyrimidine and purines were affected. The second question concerned the low methionine content of the basal diet (5). In the presence of adequate methionine, when vitamin B_{12} would not be necessary for methionine synthesis from homocysteine, would vitamin B_{12} still be necessary to obtain control level thymine and purine synthesis?

To resolve these questions a similar experiment was carried out on a larger group of chickens and the uptake of formate-^{14}C into DNA thymine, adenine and guanine and into ribonucleic acid (RNA) adenine and guanine was measured in chickens fed a basal diet supplemented by vitamin B_{12} or methionine, or both, or neither. To further clarify the role of methionine in the experiment, the uptake of the formate into the liver choline was also measured.

The formate uptake into the nucleic acids of the bursa of Fabricius rather than into the bone marrow was studied. The bursa of Fabricius in the chicken is a lymphatic organ located as a blind sac extending dorsally from the roof of the cloaca and is one site of embryonic hematopoiesis. It has been called a cloacal thymus and reaches its maximal size at 4 to 5 months and then decreases in size until it disappears at 10 to 12 months (6, 7). This organ represents a good tool for the study of DNA synthesis and was chosen rather than the bone marrow for these studies for the following reasons: 1) The bursa is an actively growing organ at this time. 2) The nucleic acid synthesis in the bursa is depressed to a greater degree by vitamin B_{12} deficiency than in the marrow. 3) The bursa contains a larger amount of material than the marrow. 4) The bursa is easier to remove than the marrow.

EXPERIMENTAL

One-day-old White Leghorn cockerels were divided into 4 groups of 10 to 12 chicks each. The first group was fed the basal high fat, vitamin B_{12}-deficient diet described by Spivey Fox et al. (5). The diet had the following composition: (grams per kilogram of diet) soybean protein,[2]

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[2] Assay Protein C-1 purchased from Skidmore Enterprises, Cincinnati.

300; L-cystine, 3; corn oil, 40; salts A (8), 60; vitamin mix, 5; choline chloride, 1; crude glucose,[3] 396; and hydrogenated vegetable oil,[4] 200. The vitamins added were as follows: (milligrams per kilogram diet) thiamine·HCl, 8; riboflavin, 8; Ca pantothenate, 20; nicotinic acid, 100; pyridoxine·HCl, 8; D-biotin, 0.3; pteroylglutamic acid, 3; vitamin A acetate, 6; vitamin D, 0.04; α-tocopherol, 25; α-tocopheryl acetate, 25; and 2-methyl-1,4-naphthoquinone, 1. The second group received the basal diet supplemented by 0.75% methionine. The third group was fed the basal diet but each chick was given weekly injections of vitamin B_{12} as described previously (4). The fourth group was fed the methionine-supplemented diet and also received the weekly vitamin B_{12} injections. At the end of 6 weeks the chicks were injected intraperitoneally with 10 μc/100 g body weight of formate-^{14}C. After 4 hours the chicks were killed, and the liver was removed and frozen for later vitamin B_{12} and choline determinations. The bursa of Fabricius was removed, weighed and homogenized in 5 ml of 0.4 N perchloric acid at 0° in a Vir-Tis homogenizer. The supernatant fluid was saved, filtered through glass wool and evaporated down to a small volume (approximately 0.5 ml) and subjected to the same hydrolysis and base analysis as described later for the RNA and DNA samples. The bases obtained here are referred to as acid-soluble bases.

The precipitate from the bursa was washed twice with 2% potassium acetate in 95% ethanol. Lipids were extracted from the washed precipitate at 40 to 50° with three 5-ml portions of a 3:1 ethanol: ethyl ether mixture. The remaining precipitate was then suspended in 5 ml of 10% NaCl, pH 7.5 and heated at 100° for one hour. The extraction was repeated a second time for 30 minutes and the combined extracts were filtered through glass wool into 50 ml of cold (5°) 95% ethanol and allowed to stand at 5° overnight or until a precipitate had formed. The precipitate was separated by centrifugation in the cold and was dissolved in 5 ml of 0.1 N NaOH and incubated at 37° overnight in order to hydrolyze the RNA. The sample was then cooled to 0° and 0.04 ml of cold HCl and 0.5 ml of cold 50% trichloroacetic acid (TCA) added to precipitate the DNA. The precipitate was separated by centrifugation in the cold and washed 2 times with cold 5% TCA. The supernatant RNA was evaporated just to dryness under a stream of warm air and then 0.2 ml of 70% perchloric acid (PCA) was added to the RNA sample and to the DNA sample. These, along with the acid-soluble sample obtained earlier, were hydrolyzed at 100° for one hour. The tubes were cooled to 0° and 1.0 ml of cold 3 N KCl was added to precipitate the perchloric acid. After 30 to 60 minutes in the cold (5°) the samples were spotted on Whatman no. 1 chromatography paper and the bases separated by descending chromatography using isopropanol: c. HCl: H_2O (170:41:39). The spots, viewed by ultraviolet light, were eluted from the paper with alcoholic ammonia (73:24:3 ethanol:water:NH_4OH) and the absorption at 250 mμ, 260 mμ and at the wavelength of maximal absorption for each base was read on a Zeiss spectrophotometer. The readings were made against a blank consisting of the eluate from a spot of the same size as the sample cut from the paper at the level of the sample. The identity and purity of the bases was determined by their R_f values on the paper chromatoggraphy and by the ratio of absorption at 280/260 mμ and at 250/260 mμ. The concentration of the bases present was calculated from their molar absorbancy indexes at the wavelength of maximal absorption as measured by standards dissolved in the eluate described and in agreement with previously observed values (9). An aliquot of each sample was evaporated in a planchet for counting at infinite thinness in a windowless flow counter which, under the conditions used, counts ^{14}C at 55% efficiency. The data are recorded as counts per minute per micromole of base. The choline reineckate was isolated from the liver by the method of du Vigneaud (10). The vitamin B_{12} content of the livers was determined by a standard microbiological procedure using *Lactobacillus leichmannii* (11).

[3] Cerelose, Corn Products Company, Argo, Illinois.
[4] Crisco, Procter and Gamble Company, Cincinnati.

RESULTS

The average weights of the chickens at death and the corresponding bursa weights reported as percentage of body weight, and the vitamin B_{12} content of the livers are shown in table 1. The observed differences in weight in the supplemented groups of chicks are small but are repeatable and have been observed in 3 separate experiments. Methionine is required to give maximal growth. However, the growth of the bursa relative to general growth of the chick appears more vitamin B_{12}-dependent.

Table 2 gives the results of the base analysis in the acid-soluble, the RNA, and the DNA samples. A few RNA samples were contaminated by DNA as shown by the presence of thymine. These samples were discarded. Table 3 is another representation of the data in table 2 in which the relationships between the values for the different dietary groups may be seen more easily. The base most affected by the deficient diet is thymine. The reduction in formate uptake into thymine with the deficient diet is greater than the reduction of formate uptake into the other DNA bases, thus indicating a greater effect of the deficiency on thymine formation than on DNA synthesis in general. Thymine is also the only base in which both methionine and vitamin B_{12} supplementations are required to obtain maximal formation from formate. Table 4 shows the results of the liver choline analyses. Here again, both methionine and vitamin B_{12} supplementation were required for maximal formation of choline from formate. The total amount of choline present in the liver was unaffected by the various diets.

DISCUSSION

The effect of the various dietary regimens on the growth of the chicks is in agreement with the earlier work of Spivey Fox et al. (5). The basal diet is high in fat and contains soybean protein which is low in sulfur-containing amino acids (12). The diet was supplemented with 0.3% cystine but there was still a requirement for methionine, and the growth of chicks fed this diet was stimulated by either vitamin B_{12} or methionine.

Why does a high fat diet increase the chick's need for methionine or vitamin B_{12} or for both of these? In the light of recent developments it appears that chicks fed a high fat diet need large amounts of a methylating agent for choline formation in order to metabolize fat. In the absence of large amounts of dietary methionine and in the presence of vitamin B_{12}, it can be postulated that vitamin B_{12} allows the small amount of methionine present in the soybean protein to be used catalytically as a methyl transferring agent, being renewed via the cobamide dependent methylation of homocysteine from 5-methyltetrahydrofolic acid (13, 14). If this was the case, however, it would be expected that a large uptake of the formate into the choline of chicks supplemented by vitamin B_{12} alone would occur and almost no labeling of choline in the chicks fed the methionine supplement. The specific activities of the choline isolated do not support this theory and it is evident that chicks do not use methionine as a direct source of methyl groups for choline. Pool size does not appear to be a factor here as the amount of choline reineckate isolated per

TABLE 1

Effect of experimental diet on body growth, bursa growth and liver vitamin B_{12} content [1]

Dietary supplement	No. of chicks	Body wt	Bursa wt / Body wt	Liver vitamin B_{12}
		g	%	*mμg/g liver*
None [2]	12	239 ± 13 [3]	0.26 ± 0.02	43 ± 14
Methionine	11	528 ± 21	0.33 ± 0.04	23 ± 2
Vitamin B_{12}	10	431 ± 20	0.43 ± 0.02	999 ± 60
Vitamin B_{12} + methionine	12	522 ± 10	0.42 ± 0.03	836 ± 47
		$P < 0.005$	$P < 0.005$	$P < 0.005$

[1] The data in the table were analyzed for variance by an IBM computer. The value of P is given at the bottom of each column.
[2] The basal diet is described in the text.
[3] Mean ± SE.

TABLE 2

Uptake of formate-^{14}C into the nucleic acid bases of the chick bursa of Fabricius [1]

Dietary supplement	Acid-soluble		DNA			RNA	
	Adenine	Guanine	Thymine	Adenine	Guanine	Adenine	Guanine
	count/min/μmole		*count/min/μmole*			*count/min/μmole*	
None	1677 ± 105 [2](10) [3]	958 ± 77 (8)	129 ± 9 (12)	445 ± 64(7)	260 ± 24(6)	616 ± 34(10)	406 ± 31(12)
Methionine	3040 ± 370 (11)	1393 ± 168(9)	244 ± 42(10)	528 ± 68(8)	406 ± 41(6)	974 ± 87(6)	687 ± 63(6)
Vitamin B_{12}	2645 ± 124 (10)	1536 ± 100(10)	219 ± 20(9)	777 ± 62(6)	656 ± 37(8)	1189 ± 92(9)	867 ± 78(7)
Vitamin B_{12} + methionine	3236 ± 273 (12)	1887 ± 240(8)	421 ± 77(10)	693 ± 56(11)	546 ± 45(10)	1265 ± 99(11)	802 ± 82(11)
	$P < 0.005$	$P < 0.01$	$P < 0.005$	$P < 0.01$	$P < 0.005$	$P < 0.005$	$P < 0.05$

[1] All results are given in terms of specific activities and were analyzed for variance by an IBM computer. The value of P is given at the bottom of each column.
[2] Mean ± SE.
[3] Numbers in parentheses indicate number of samples.

gram of liver is approximately the same for each dietary group. These observations are supported by the earlier work of Jukes (15) in which it was noted that methionine cannot substitute for choline in poultry diets. Since the choline is not made from the methionine, the chicks evidently need large amounts of methionine for some other purpose, perhaps simply as a source of this sulfur-containing amino acid for normal protein synthesis and growth.

In the bursa the uptake of formate into the acid-soluble purines and into the RNA purines reflects in general the weight gain of the chicks. That is, supplementation with either vitamin B_{12} or methionine is equally stimulatory (no significant differences at 5% level) and supplementation with both is best. This general effect may reflect the need of the organism for vitamin B_{12} in order to synthesize methionine as described above and to release tetrahydrofolate for purine synthesis as suggested by Buchanan and co-workers (14). The level of cobamide-dependent methionine synthesizing enzyme in the livers of chicks fed diets very similar to those used in this experiment has been reported by Dickerman et al. (13) and the enzyme activity is very low in the vitamin B_{12}- deficient chicks.

The uptake of formate into the purines in the deoxyribonucleic acid, like the growth of the bursa itself, is dependent more upon vitamin B_{12} supplementation than upon methionine supplementation. These are the only nucleic acid bases to show a statistically significant stimulation of formate uptake by vitamin B_{12} supplementation alone over the effect of methionine supplementation alone. This may reflect a dependency of deoxyribose formation upon cobamides as has been observed in bacteria (16) but the differences between the RNA and the DNA purines here are not great enough to be conclusive.

Among the bases of the nucleic acids, thymine formation is by far the most severely affected by the deficient diet and here, as with choline synthesis, both vitamin B_{12} and methionine are necessary to obtain optimal levels of thymine formation. This answers the first question posed by our earlier work (4). The effect of vitamin B_{12} deficiency on thymine methyl formation is greater than the effect on DNA

TABLE 3

Uptake of formate-^{14}C into the nucleic acid bases of the chick bursa of Fabricius given as percentage of control value [1]

Dietary supplement	Acid-soluble		DNA			RNA	
	Adenine	Guanine	Thymine	Adenine [2]	Guanine	Adenine	Guanine
None	52	51	31	64	48	49	51
Methionine	94	74	58	76	74	77	86
Vitamin B_{12}	82	81	52	112	120	94	108
Vitamin B_{12} + methionine	100	100	100	100	100	100	100

[1] This table is another presentation of the data given in table 2 with the values for the fully supplemented group arbitrarily set at 100% and the other values given as the percentage of this control value. Any 2 values sided by the same vertical line are not significantly different from each other at the 5% level as determined by the Duncan multiple range test as modified by Kramer (19).
[2] In this group there is no statistical difference at the 5% level between the value for the methionine supplemented chicks and the value for chicks receiving both supplements.

TABLE 4

Uptake of formate-^{14}C in chick liver choline [1]

Dietary supplement	Choline reineckate isolated	Choline reineckate, specific activity
	mg/g liver	*count/min/mg*
None	3.29 ± 0.15 [2] (7) [3]	51 ± 26 (8)
Methionine	3.36 ± 0.57 (7)	83 ± 22 (8)
Vitamin B_{12}	3.84 ± 0.29 (5)	63 ± 19 (7)
Vitamin B_{12} + methionine	3.62 ± 0.33 (6)	284 ± 39 (8)
		$P < 0.005$

[1] The specific activities were analyzed for variance and the value of P is given at the bottom of the column.
[2] Mean ± SE.
[3] Numbers in parentheses indicate number of samples.

synthesis in general. Since the formation of choline and of thymine from formate involves *de novo* methyl synthesis followed by a methylation step, the data suggest that this pathway in the chick requires methionine and a cobamide and that the role of the cobamide is distinct from its role in methionine synthesis. This work does not permit a localization of the site of the cobamide requirement. However it appears to lie between the level of purine synthesis, which requires a one-carbon unit at the formate level of oxidation, and the level of thymidylate synthesis, which requires a one-carbon unit at the formaldehyde level of oxidation. The one-carbon unit for the formation of choline in the chick is unknown. Thus the cobamide requirement appears to lie either at the site of reduction of the one-carbon unit from the formate to the formaldehyde level by the enzyme N^5,N^{10}-methylenetetrahydrofolate dehydrogenase or at the site of transfer of the one-carbon units to form thymine or choline. The former site appears to be the most likely as it would be common to thymine and choline synthesis. Much evidence has accumulated in this laboratory for the participation of a cobamide at this site (1–4) but when no difference in the uptake of formate-^{14}C and formaldehyde-^{14}C into the nucleic acid of vitamin B_{12}-deficient chickens was observed (4), the physiological importance of this site was doubted. In view of the recent work of Rachele et al. (17) which showed that formaldehyde injected into rats was quickly oxidized to formate and did not enter the folate pathway at the level of N^5,N^{10}-methylenetetrahydrofolate but at the formate level, further investigation of the relation between cobamide and N^5,N^{10}-methylenetetrahydrofolate dehydrogenase is needed. The work of Lust and Daniel (18) with *Ochromonas malhamensis* also suggests that there is a cobamide require-

ment in *de novo* methyl synthesis. The possibility can not be ruled out, however, of the requirement of a cobamide at the site of transfer of the one-carbon unit to form thymine or choline in view of the well-known double requirement of cobamide and S-adenosylmethionine for the transfer of a methyl group to homocysteine to form methionine in chick liver (13).

A study of the properties in vitro of the appropriate enzymes is now in progress.

ACKNOWLEDGMENTS

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A Study of Some Enzyme Systems in Livers of Rats after Prolonged Ingestion of Alcohol [1]

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ABSTRACT In an effort to determine the reason for the consistently greater quantities of hepatic α-ketoglutaric acid in alcohol-treated rats, 3 enzyme systems which lead to this acid were measured, namely, isocitric (ICDH), succinic (SDH) and glutamic (GDH) dehydrogenases. After an experimental period of 28 weeks, the activities of these enzymes were determined in the livers of control rats and also of rats given a 20% solution of ethanol as the sole drinking fluid. Both groups were fed an adequate purified diet. The results indicated a significant increase in activities for both ICDH and GDH. In contrast, SDH increased only slightly. Based on these observations, a possible explanation was offered for the increase in the hepatic α-ketoglutarate level of the alcohol-treated rats. Moreover, it was postulated that the increase in both enzymes might have been due to an adaptive mechanism induced by alcohol to offset the insult of alcohol on the liver.

In an earlier study of the citric acid cycle in the rat it was observed that α-ketoglutaric acid was present at consistently higher levels in the livers of the alcohol-treated animals than in those of the controls (1). Several mechanisms were proposed that could contribute to the increase of this acid. These included increased activity of some by-pass reactions leading directly to the formation of α-ketoglutarate or, possibly, the occurrence of an alteration(s) in some other enzymatic reactions in the cycle which lead to or follow α-ketoglutarate production, or both of these mechanisms. Although it was not then possible to assess the exact nature of these changes nor to delineate their significance in the process of alcoholism, a suggestion was advanced that one or more of the enzymes involved could be more sensitive to chronic ingestion of alcohol than others, and thus be the earliest to be affected by alcohol treatment.

To investigate these changes further, the activities of 3 enzymes were studied in the livers of rats during chronic ingestion of ethanol. Two of these enzymes were isocitric dehydrogenase (ICDH) and succinic dehydrogenase (SDH), representing enzymes operating before and after α-ketoglutarate in the citric acid cycle, respectively. The third enzyme, glutamic acid dehydrogenase (GDH), operates in a major reaction of α-ketoglutarate metabolism outside of this cycle. The results demonstrate clearly that prolonged treatment with ethanol causes a definite and significant increase in both ICDH and GDH and to a much less extent in SDH.

MATERIALS AND METHODS

Diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) were purchased commercially.[2] Other materials used were chemically pure grades.

Fifty male white rats of the Sprague-Dawley strain, weighing 100 to 120 g, were divided into 2 groups of comparable weight, 25 animals each. The maintenance and feeding procedures were the same as described previously (1) except for the stock diet which was replaced by a purified diet mixture (table 1) during the entire period of the experiment. After 28 weeks, a time when the activities of certain other enzymes are known to reach nearly maximal value (2, 3), the animals of both groups were anesthetized with ether, the abdomens opened and the livers were perfused *in situ* with ice-cold 0.9%

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[2] Obtained from Mann Research Laboratories, Inc., New York.

TABLE 1

Composition of purified diet

	%
Casein (purified)	24
Dextrin (white corn)	34
Sucrose	10
Sucrose, "vitaminized" [1]	10
Corn oil	15
Cod liver oil	1
Salt mixture [2]	4
Dried brewer's yeast	2

[1] "Vitaminized" sucrose contained per 1000 g: (in milligrams) thiamine, 100; riboflavin, 100; pyridoxine·HCl, 100; niacin, 400; pantothenic acid, 400; folic acid, 20; biotin, 10; menadione, 300; and (in grams) inositol, 10; *p*-aminobenzoic acid, 3; sucrose, 965; and (in micrograms) vitamin B_{12}, 10.

[2] Wesson, L. G. 1932 A modification of the Osborne-Mendel salt mixture containing only inorganic constituents. Science, 75: 339.

sodium chloride solution until freed of blood. Finally the livers were removed, washed several times with cold normal saline, pooled according to their respective groups and then used in the preparation of an acetone powder.

The acetone powder was prepared essentially according to the method of Straub (4). The pooled livers, previously cooled to 0°, were freed mechanically from connective tissue and fat and then minced in a meat grinder in the cold. The minced liver was washed 3 times with 10 volumes of ice-cold water with mechanical stirring and strained through cheesecloth. The residue was then homogenized for 5 minutes in a Waring Blendor with 3 volumes of cold 90% acetone and filtered by suction in a large Büchner funnel. The cake of acetone powder thus formed was again homogenized in a Waring Blendor with the same volume of cold 100% acetone for 10 minutes and filtered as before. The last step was repeated twice and the cake was finally suctioned as dry as possible. The acetone powder cake was then broken up and completely dried by spreading on a filter paper under a fan and ground to a fine powder in a mortar and preserved in a closed container in the cold.

Preparation and assay of enzymes

Isocitric dehydrogenase activity. A crude enzyme extract was prepared by mechanically stirring, in 2 small beakers, one gram each of the acetone powders for 30 minutes at room temperature with 20 ml of 0.1 M phosphate buffer (pH 7.4). The mixtures were centrifuged and the clear supernatant solutions were used immediately for the determination of enzyme activity. For every determination a fresh extract was prepared.

The procedure for the determination of ICDH was essentially that of Ochoa (5). For each determination a blank and an experimental cell were used. Both cells contained 2.6 ml of 0.03 M glycylglycine buffer (pH 7.4), 0.1 ml of 0.01 M $MnCl_2$ dissolved in 0.15 M NaCl, and 0.1 ml of 0.03 N TPN dissolved in 0.15 M NaCl. The blank cell contained, in addition, 0.1 ml of 0.15 M NaCl, and the experimental cell contained 0.1 ml of 0.1 M of isocitrate dissolved in 0.15 M NaCl. The reaction mixtures were placed in a constant-temperature bath at 23° after mixing. The reaction was initiated, after taking a zero-time reading, by addition of 0.1 ml of enzyme preparation. The components were mixed and the optical density of the experimental cell was read every 15 seconds for 2 to 3 minutes against that of the blank which was preset at 100% transmission. The measurements were carried out in a Beckman DU Spectrophotometer at a wavelength 340 mμ using rectangular cuvettes of 1.0-cm light path.

Succinic dehydrogenase activity. For the determination of SDH activity the spectrophotometric method of Slater and Bonner (6) was applied to an extract of 20% (w/v) dried acetone powder in phosphate buffer (pH 7.2). The method for the preparation of the crude extract was the same as described for the ICDH.

Glutamic dehydrogenase activity. An aqueous extract of one gram of acetone powder in 20 ml of phosphate buffer (pH 7.6) was used in the spectrophotometric measurement of GDH activity. The reaction mixture contained 2.1 ml of phosphate buffer (pH 7.6), 0.2 ml of DPN solution (5 μmole/ml in water) and 0.5 ml of enzyme preparation. The blank cell, for setting at 100% transmission, contained, in addition, 0.2 ml of water. The reaction mixture was initiated, after taking a zero-time reading, by the addition of 0.2 ml of 0.5 M glutamate in water. Readings were recorded every 15 seconds

at a wavelength of 340 mμ using a quartz cuvette with a 1.0-cm light path.

RESULTS

Typical activity curves for ICDH in liver preparations of normal and alcohol-treated rats are presented in figure 1. The rates of reaction differed, being less in the normal livers as shown by the optical density readings at any given time. The actual activities of ICDH in units were 480 units/g of dried acetone powder for the normal and 2490 units for the alcohol-treated livers. One enzyme unit is arbitrarily defined as the amount of enzyme causing an increase in optical density of 0.01 in one minute calculated for the third 15-second period after the start of the reaction (5).

The results of the spectrophotometric determination of liver SDH are represented in figure 2. The graphs (curves 3 and 4) indicate a slight increase in the activity of the enzyme in the alcohol-treated rats over that of the controls. To ascertain whether the apparent activity was actually enzymatic and not merely due to some "catalytic" action of one of the components of the reaction mixture, portions of the enzyme preparations were boiled prior to the assay. Comparison between curves 1 and 3 and between curves 3 and 4 in figure 2 demonstrates conclusively that nearly all of the activity was due to SDH.

The results of the assay of GDH are shown in figure 3. Again, the graphs show clearly that the activity of the enzyme in the livers of alcohol-treated rats was greater than that in the controls. The activities mentioned above for the 3 enzymes are averages of duplicate experiments on 4 individual extracts prepared freshly for each experiment.

DISCUSSION

It has been observed in laboratory animals as well as in certain human subjects that α-ketoglutaric acid is increased in acute and in chronic alcohol intoxication (1, 7–9). Several possible mechanisms or factors, alone or in combination might be involved: increased activity of liver ICDH or GDH, or both, and decreased activity of SDH or glutamic-oxalacetic

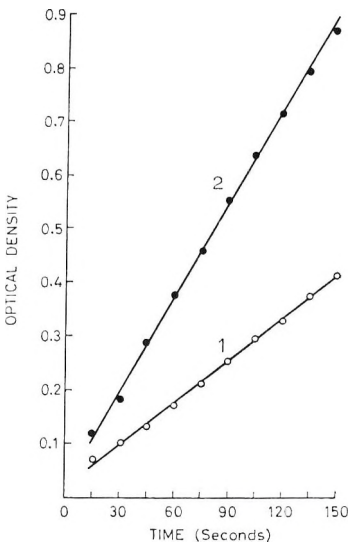

Fig. 1 Isocitric dehydrogenase activity in livers of normal and alcohol-treated rats.
1 Normal livers
2 Alcohol-treated livers

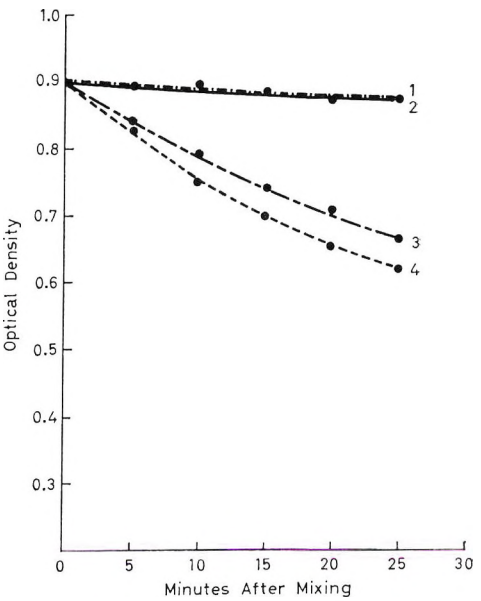

Fig. 2 Succinic dehydrogenase activity in livers of normal and alcohol-treated rats.
1 Crude liver extract from normal rats — boiled
2 Crude liver extract from alcohol-treated rats — boiled
3 Crude liver extract from normal rats
4 Crude liver extract from alcohol-treated rats

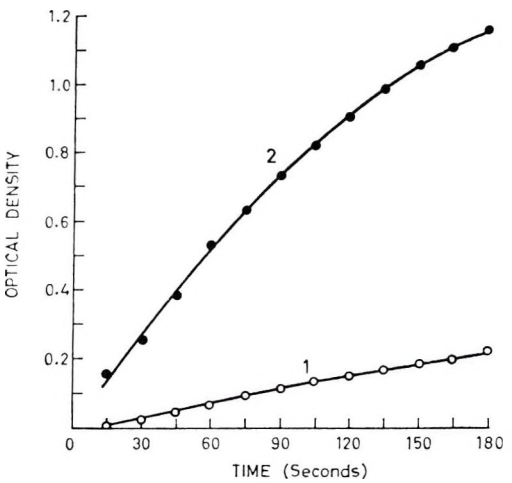

Fig. 3 Glutamic dehydrogenase activity in livers of normal and alcohol-treated rats.
1 Normal livers
2 Alcohol-treated livers

transaminase (GOT), or both. The latter has already been shown to decrease under the influence of alcohol consumption (7).

The data in figure 1 show a fivefold increase in ICDH activity in the alcohol-treated livers over that of the controls (2490:480, respectively). This increase alone in ICDH activity could account, at least in part, for the observed increase in the amount of α-ketoglutarate. However, Figueroa and Klotz (10) reported decreased ICDH in liver tissue obtained by biopsy from patients with alcoholic cirrhosis. The experiment of these investigators, in contrast with the present study, was performed after prolonged ingestion of alcohol and at a time when liver damage was advanced and marked. Presumably, the liver cells at this stage become unable to synthesize protein, thus leading to decreased ICDH, as was observed in their experiment, as well as in decreases in other enzymes (3).

The effect of ICDH described in the present experiment would undoubtedly be accentuated if SDH was elevated only slightly, not changed at all, or actually decreased during the chronic alcohol treatment. In fact, the results represented in figure 2 indicate clearly that only a slight increase has occurred in the SDH activity of the alcohol-treated livers as shown by a comparison between curves 3 and 4. Supporting evidence for this observation in animal experiments as well as from studies on human subjects has been published (11–15). If this is the case it would not be unreasonable to postulate that the net balance of the 2 enzyme reactions is an increase in the level of α-ketoglutarate. This would be particularly true since at the present observed concentration ICDH has a high total turnover as compared with that of SDH (16). Moreover, an increase in α-ketoglutarate level could also result from an increased activity of some by-pass reaction to the citric acid cycle leading directly to the production of α-ketoglutarate such as the "succinate-glycine cycle" (17). In fact, the increased excretion of porphyrins in the alcoholic, biosynthesized presumably by way of this cycle, supports this view (18).

Another striking observation in this study is the effect of ethanol on the activity of hepatic GDH. Data shown in figure 3 indicate that the alcohol-treated liver exhibited an 8.5-fold increase in activity over that of the controls. This appears highly significant from the standpoint that GDH is a key enzyme in amino acid metabolism which, as is known, mediates the interconversion of glutamic and α-ketoglutaric acids. A higher activity of the enzyme would, in turn, increase the utilization of α-ketoglutarate by way of this reaction, thus preventing its excessive build-up as reported in a previous communication (1). Supporting this is the fact that the equilibrium of the reaction is more toward glutamic acid formation. In addition, the formation of this amino acid requires reduced diphosphopyridine nucleotide (DPNH). Increased amounts of DPNH are known to be produced following ingestion of ethanol (3, 19, 20). Consequently, protein synthesis would be promoted under the conditions of the present experiment or at least remain unimpaired. In fact, earlier work (2, 21) did not show any reduction in hepatic protein synthesis during alcohol treatment. It must be emphasized, however, that functions other than GDH could also be involved in protein synthesis under similar experimental conditions (22–24). How-

ever, increased or even normal synthesis of protein in the liver could be of possible significance in the prevention of fatty infiltration or at least in the delay of its development by enhancing lipoprotein formation prior to the release of liproprotein from the liver (21, 25, 26).

Whereas the foregoing reasoning appears tenable it is not altogether clear why the opposite does not take place, namely, a lowered activity of GDH or a reduction in the levels of glutamic acid in favor of excessive production of α-ketoglutarate, or both. However, only higher levels of α-ketoglutarate and not excessive amounts were consistently observed in the alcohol-treated livers (1). In this respect, it has been shown that the enzyme would be inactivated by dissociation under the influence of increased quantities of DPNH (27). The reason for this discrepancy is not apparent. However, a partial explanation may be offered. In the first place the dissociation experiments were carried out in vitro, in contrast with the present in vivo investigation. Thus, under the present experimental conditions it might be that the levels of DPNH did not attain the required inhibitory levels used in the in vitro study. This is not unexpected since DPNH has been shown to be utilized in certain other major metabolic reactions such as fatty acid synthesis (28–30). It is also possible that some other factors(s) not measured in this study had protected the enzyme from the deleterious effect of the increased amounts of DPNH produced during the prolonged and continuous consumption of ethanol.

From the evidence presented above some important points for consideration are raised. Although certain changes in the liver appear to occur following alcohol consumption, no definite conclusion can be drawn at present about their relative importance in the process of alcoholism. Likewise, it is premature to hypothesize whether these results are causes or effects. It is possible that the increase in the 3 enzymes measured was an adaptive mechanism on the part of the liver to meet the continuous metabolic insult of ethanol by providing protein and other metabolites for the regeneration of the deranged cells that are usually observed in the alcoholic liver as well as for the release of accumulated hepatic lipids.

In this respect, the question is raised whether the activities of the enzymes presently studied would follow a course similar to that of ADH (3), namely, whether they increase in the earlier periods of alcohol treatment and then decrease as the administration of ethanol is prolonged. It would also be worthwhile to determine whether there would be any effect of withdrawing alcohol and substituting it with water upon the activities of these enzymes as was the case with ADH (3). These questions need further investigation. It appears reasonable that the explanation of the biochemical role of these factors might provide a better understanding of alcoholism and its associated problems.

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Mellituria and Postprandial Blood Sugar Curves in Dogs after the Ingestion of Various Carbohydrates with the Diet

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ABSTRACT When dextrin-maltose, corn syrup or sucrose furnished 54% of the calories in the diet of beagle dogs there were no signs of intolerance although there was some mellituria. However, when lactose was the dietary carbohydrate, diarrhea and malaise ensued immediately. Fructosuria and galactosuria, but not glucosuria, occurred along with sucrosuria and lactosuria, respectively. Maltose was not detected in the urine. The postprandial blood sugar values obtained by the Somogyi method were higher than those obtained by the glucose oxidase method. This discrepancy may possibly be explained by the presence in the blood of the same non-glucose reducing substances (saccharoids?) observed in the urine. The differences in utilization of the different carbohydrates as shown by mellituria and postprandial blood sugar curves in dogs show similarities to certain human conditions and have nutritional implications with respect to species differences in availability and utilization of carbohydrate calories. The dog may be a useful experimental subject for the study of carbohydrate intolerance as observed in human subjects.

In present day nutritional studies animals of various species are being fed rations of purified ingredients rather than mixtures of foods. The report on the nutritional requirements of dogs by the Committee on Animal Nutrition (1) contains little information about differences in the utilization of various carbohydrates by dogs. It states that up to 65% of the diet as carbohydrate is compatible with a balanced diet, and mentions that excessive amounts of lactose will produce diarrhea. Most of the discussion deals with the utilization of raw versus cooked starches. Cowgill (2) used sucrose, 34.9% by weight (29% of the calories), in his synthetic food mixture for dogs.

In previous work with dogs we noticed that when as little as 14% of the calories was furnished by the lactose in the skim milk of the diets, diarrhea was a common occurrence. In puppies, there was excretion of lactose and galactose in the urine and failure to thrive. Large amounts of other sugars were well tolerated, but non-glucose mellituria occurred frequently. The kind as well as amount of carbohydrate, then, may be as important as the kinds and amounts of fat or protein incorporated into the diet.

The purpose of the present study was to observe the utilization of certain carbohydrate products by the dog as indicated by postprandial blood sugar levels, urinary excretion of sugars, and gastrointestinal disturbances.

EXPERIMENTAL PROCEDURE

Four 17-month-old littermate beagle dogs weighing 5 to 5.5 kg were fed 433 kcal daily for 6 weeks in the form of commercial dog food.[1] Thereafter, each received 4 different experimental rations for 9 to 11 days each. The diets had the following composition as percentage by weight: casein, 21.7; corn oil, 13.0; carbohydrate, 59.0; choline bitartrate, 0.3; Cowgill (2) salt mixture, 1.0; bone meal, 2.0; powdered cellulose,[2] 3.0. Therefore, the percentage of calories derived from protein, fat, and carbohydrate was 19.8, 26.6 and 53.5%, respectively. The commercial dog food used in the preliminary feeding period contained 10.5% protein, 5.0% fat, 8.5% carbohydrate, 3.0% ash, 1.0% fiber, and 72% water by weight; and the percentage

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[1] Calo Pet Food Company, Oakland, California.

[2] Cellu Flour, Chicago Dietetic Supply House, Chicago.

of calories derived from protein, fat, and carbohydrate was 35, 37, and 28%, respectively. In the commercial product the carbohydrate was cereal and vegetable starches, whereas the carbohydrate in the purified diet was either a dextrin-maltose product,[3] sucrose, corn syrup solids, or lactose.[4] The dogs were fed 88 kcal/kg/day in one daily feeding. At the end of each 10-day period a series of heparinized whole blood samples was drawn at intervals during the 5- or 6-hour postprandial period for assay of blood sugar by the method of Somogyi (3) and glucose oxidase (4) method. On the day before or after the blood was taken, the 24-hour urine was collected in 2 portions with thymol as preservative, assayed for total reducing sugar by the Somogyi method (3) and chromatographed on paper for sugar identification. The method of White and Hess (5) was used with a number of modifications.[5] Sensitivity of the chromatography for aldoses was about 2 μg with the exception of 18 μg for maltose and that for sucrose and fructose was 6 and 4 μg, respectively. In other words, with 20 μliters of urine that has been concentrated fivefold, an aldose sugar content of 2 mg/100 ml, and a ketose content of 4 to 6 mg/100 ml in the original urine could be detected.

[3] Dextri-Maltose, Mead Johnson Laboratories, Evansville, Indiana. Product made by the enzymatic action of barley malt on corn flour. Composition: maltose, 56%; dextrins, 42%; and moisture, 2%.

[4] Beta Lactose, The Borden Company, Pharmaceutical Division, New York.

[5] The modifications were as follows: a) An initial step in which 20 ml methanol were added to 10 ml urine and the precipitate centrifuged out after about an hour. b) The supernatant solution (18 or 21 ml) was decanted into a graduated test tube and evaporated to one-third the original volume (6 or 7 ml) with the aid of a water bath and air jet. c) Five milliliters of this solution were de-ionized by means of an electric desalter. The de-ionized urine was quantitatively transferred with washings into a 100-ml round-bottom flask and evaporated to dryness under vacuum on a rotary evaporator. d) The solvent mixture for chromatography was butanol-pyridine-water in the proportions 2:2:1. e) Different aliquots of each urine sample were spotted on 2 different papers for one-dimensional descending separation of the sugars. One paper contained appropriate aliquots (1 to 20 μliters) for aldoses and was sprayed with the aniline reagent, whereas the other paper contained spots (10 to 20 μliters) for detection of ketoses with the orcinol spray. Aliquot size was adjusted and the chromatography repeated when results indicated the desirability of better definition of spots.

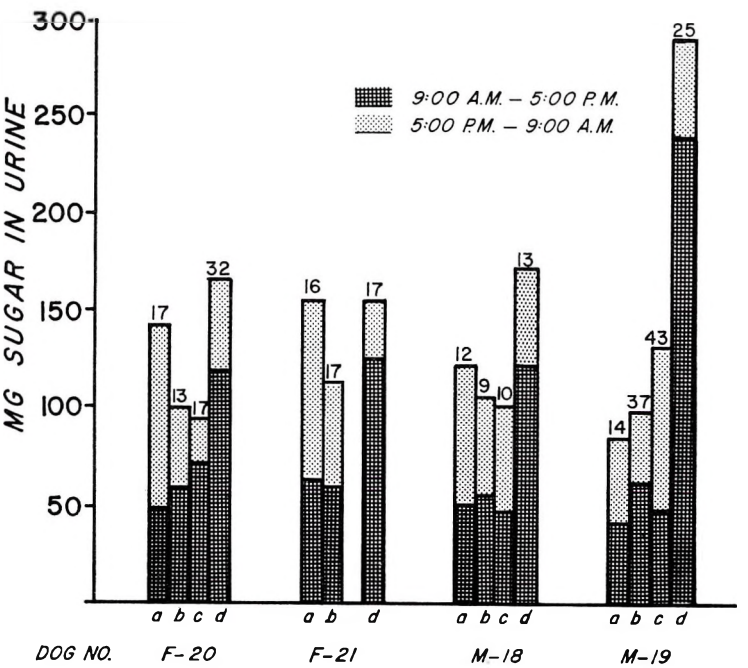

Fig. 1 Amount of sugar in 24-hour collection of urine of beagle dogs fed different carbohydrates in the diet. Height of bar represents total amount of sugar excreted. Letters represent the kind of carbohydrate in the diet: (a) starch (dog food), (b) dextrin-maltose product, (c) corn syrup, and (d) sucrose. The numbers above the bars represent total urine volume times 10^{-1}.

RESULTS

Figure 1 shows the daytime urinary excretion of sugar to be fairly constant (50 mg) for the 4 dogs when their carbohydrate sources were starch, dextrin-maltose, or corn syrup,[6] whereas the daytime urinary excretion increased about 2.5 times when sucrose was the dietary sugar. The sucrose-containing diet tended to yield the greatest amount of total urinary sugar, whereas dextrin-maltose and corn syrup-containing diets produced much lower amounts of urinary sugar.

Table 1 shows that none of the standard sugars [7] were detected in urine when commercial dog food, dextrin-maltose, or corn syrup diets were fed, but sucrose and fructose appeared in the daytime urine when sucrose was the dietary sugar. Ingestion of lactose in the diet provoked immediate diarrhea which was rapidly corrected by changing to commercial dog food.

[6] Percentage composition of corn syrup by weight: dextrin, 37; maltose, 22; dextrose, 8; sucrose, 7; and water, 26.

[7] The standard sugar spots contained 5 μg each of lactose, galactose, glucose, arabinose; 20 μg each of maltose, sucrose and fructose.

TABLE 1

Sugars detected in urine of beagle dogs fed diets containing different carbohydrates

Dietary carbohydrate	Sugar spots						
	Lactose	Maltose	Galactose	Glucose	Sucrose	Fructose	Unknown
Commercial dog food	0	0	0	0	0	0	0
Dextrin-maltose [1]	0	0	0	0	0	0	+
Corn syrup	0	0	0	0	0	0	+
Sucrose	0	0	0	0	d [2]	d	+
Lactose	d,n [3]	0	d,n	d	0	0	+

[1] Dextri-Maltose, Mead Johnson Laboratories, Evansville, Indiana.
[2] *d* indicates present in the 9 AM to 5 PM collection of urine.
[3] *n* indicates present in the 5 PM to 9 AM collection of urine.

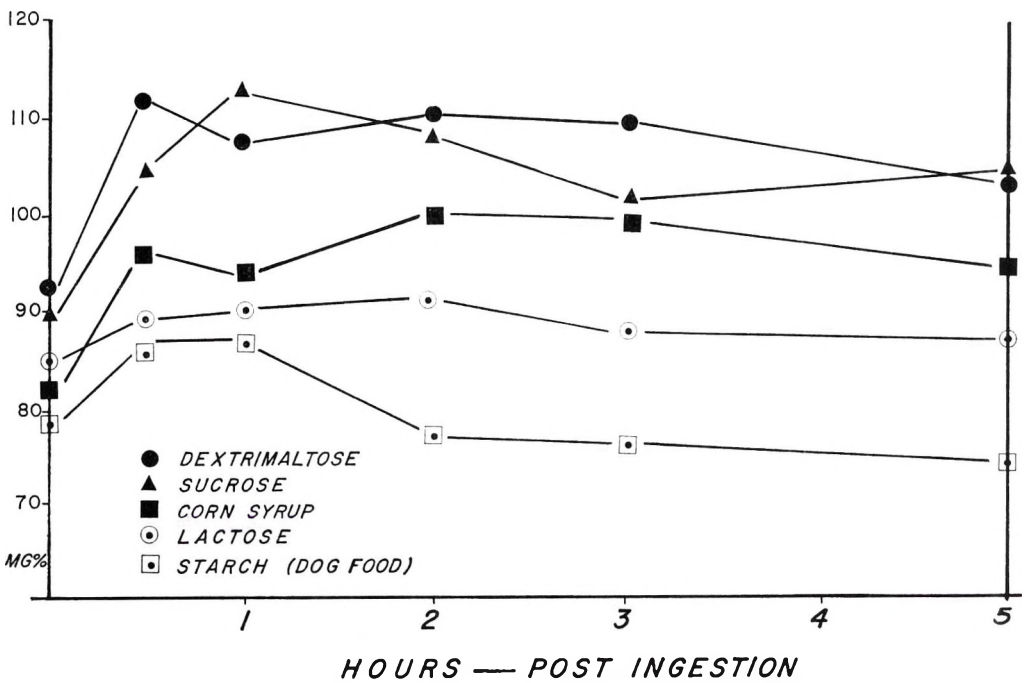

Fig. 2 Blood glucose levels of beagle dogs after the ingestion of diets containing various carbohydrates. Values were determined by the glucose oxidase method and each curve represents the average values for 4 dogs.

It was not possible to get a urine sample from the lactose-fed dogs without fecal contamination; however, galactose, as well as lactose, was in high concentration in both day and night collections, and some glucose also existed in the daytime sample. Almost all urine specimens showed unidentified spots on the paper chromatograms.[8] Postprandial blood glucose curves in figure 2 show different patterns when the dogs were fed diets containing different carbohydrates. Each curve represents the average blood glucose level for the same 4 dogs after they were fed the different diets. Table 2 shows these data for each time interval after the consumption of experimental diets differing only with respect to the carbohydrate constituent and after consumption of the commercial dog food. The curve after the ingestion of commercial dog food is lower than the others — probably because the diet contained only one-half as much carbohydrate as starch which is more slowly digested than compounds in the experimental diets. The lactose-containing diet gave a low, flat curve, reflecting the poor availability as shown by the extensive diarrhea. The dextrin-maltose, sucrose, and corn syrup diets gave higher curves which were still well above fasting levels 5 hours later. Since diarrhea was not manifest in these instances, these curves probably indicate a continuous digestion and absorption of the foodstuffs. Blood sugar values obtained by the glucose oxidase method are consistently lower than those otbained by the Somogyi method. Even though the blood sugar levels for the various diets are quite different (fig. 2), Somogyi values are 5 to 6% higher when the sucrose and corn syrup diets were fed and about 2.4% higher after the ingestion of dextrin-maltose, lactose, and dog food diets (table 3).

TABLE 3

Discrepancy between blood sugar values obtained by Somogyi and glucose oxidase methods (3, 4) after the ingestion of certain carbohydrates by dogs

Dietary carbohydrate	% [1]
Dog food	2.32 [2] (1.8–3.0)
Dextrin-maltose [3]	2.34 (1.8–3.3)
Sucrose	5.95 (4.2–7.4)
Corn syrup	5.26 (3.7–6.9)
Lactose	2.56 (2.0–3.5)

[1] $\% = \frac{\text{Somogyi values} - \text{glucose oxidase values}}{\text{Glucose oxidase values}} \times 100.$

[2] Average of 24 values for each diet: 6 time intervals on the absorption curves for 4 dogs (fig. 2). Numbers in parentheses show the ranges.

[3] Dextri-Maltose, Mead Johnson Laboratories, Evansville, Indiana.

DISCUSSION

Difficulty from feeding lactose to dogs has been reported by McCay (6). He states that diarrhea often results if the dry feed contains 5% skim milk and additional milk is fed. Earlier workers noted the occurrence of ingested sugars in the urine of dogs. Hofmeister (7), for instance, determined "assimilation boundaries" for different sugars. These were the levels of ingestion above which the sugar appeared in the urine. The level for lactose was 0.4 to 0.8 g/kg. Cajori (8) how-

[8] Known compounds run on chromatograms in an attempt to identify them with the spots of the urine which were not the same as the standard sugar spots: galacturonic and glucuronic acids, raffinose, sedoheptulose, sorbose, mannose, xylose, xylulose, fucose, ribose, and ribulose.

TABLE 2

Blood glucose values after the ingestion of certain carbohydrates by dogs [1]

Post-absorption time	Dietary carbohydrate				
	Dog food	Dextrin-Maltose	Sucrose	Corn syrup	Lactose
	mg/100 ml	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
Fasting	77(70–87) [2]	90(87–96)	89(78–97)	81(78–85)	84(79–94)
30 minutes	87(75–99)	112(100–120)	104(90–118)	96(90–106)	88(79–101)
1 hour	87(81–95)	107(95–111)	112(106–118)	94(89–104)	90(82–100)
2 hours	77(67–87)	109(98–116)	109(101–116)	100(93–109)	91(83–103)
3 hours	76(68–87)	109(100–116)	101(97–113)	99(96–107)	87(77–101)
5 hours	74(67–78)	103(97–107)	104(100–111)	94(91–102)	87(74–104)

[1] Each dietary group is composed of the same 4 dogs.

[2] Numbers alone are averages; those in parentheses show the ranges of values of 4 dogs by the glucose oxidase method (4).

ever, claimed that there was enough lactase in the intestinal mucosa for lactose utilization and that there was some lactase in the liver of dogs. Species differences in milk composition hint at the possibility of poor lactose utilization in young dogs because the lactose content of beagle milk is only 8% of the calories (9) whereas that of cow and human milk is 27 and 41%, respectively. Inability to utilize lactose and sucrose has been noted in sea lions whose milk contains glucose (10).

Since all of the experimental diets in our study contained 59% carbohydrate by weight, it appears that diarrhea during the feeding of lactose rations was not simply a response to an hyperosmolar load of sugar in the intestinal lumen. Rather, the situation parallels that of sugar intolerances observed in humans which is traceable to a primary or secondary deficiency of a specific disaccharidase (11–14).

Hofmeister's "assimilation boundary" for sucrose in the dog was 3.6 g/kg. The sucrose-containing diet was well tolerated by the beagles, although sucrose and fructose, but not glucose, appeared in the urine. Similarly, glucose did not appear in the night urine from dogs given lactose in the diet although lactose and galactose did appear. This probably indicates partial hydrolysis of sucrose or lactose during absorption and subsequent utilization of the glucose but excretion of the unused fructose and galactose moieties as well as the unhydrolyzed disaccharides.

Postprandial blood sugar levels indicate good availability of glucose from the dextrin-maltose product, sucrose, and corn syrup. The excretion of sugar in the urine is low when the dextrin-maltose or corn syrup is fed, but high when sucrose is the dietary carbohydrate. This indicates poor utilizability of sucrose by the dog although adverse symptoms were not apparent in this short-term experiment. The blood sugar curve after the ingestion of lactose is flat, thus indicating poor availability of lactose. The small but consistent differences between blood sugar values obtained by the Somogyi method (which measures total reducing sugars) and the glucose oxidase method (which measures glucose specifically) are perhaps accounted for by the presence in the blood of the same reducing substances (saccharoids?) which were unidentified in the urine specimens. Fructose could account for the even greater differences (5.5% versus 2.3%) when sucrose was fed since fructose reacts like glucose in the Somogyi method and fructose was detected in the urine of dogs fed sucrose.

Non-glucose melliturias in humans have been recognized and are the subject of reviews (15–17). Some are benign, whereas others have concomitant manifestations such as failure to thrive, diarrhea, malnutrition, and abnormal blood sugar levels after ingestion of the sugars in question (18). Of particular consequence to the newborn is the lactose utilization defect. Primary intolerance to disaccharides appears to be familial, whereas a secondary intolerance may be acquired. There is rapid remission of all symptoms when the diet is free of the causative saccharide, the basic defect being inadequacy of a specific intestinal enzyme for normal utilization of the dietary carbohydrate.

The presenting symptoms of disaccharidase deficiencies in humans are not unlike those observed in poor sugar utilization in the dogs in this study. It is possible that the dog would be a suitable experimental subject for the investigation of lactase and sucrase deficiencies.

Since the dog and several other species, including the sea lion (10), rat (19), pig (20) and rabbit (21), have varying intolerance to disaccharides, the carbohydrate content of experimental diets must be considered as carefully as that of the other nutrients.

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ERRATUM

Danke, R. J., and A. D. Tillman 1965 Effect of free gossypol and supplemental dietary iron on blood constituents of rats. J. Nutrition, 87: 495–496. In tables 3, 4 and 5 the values for the volume index were erroneously reported 100 times higher than they should have been.

To correct these values in your copy of volume 87, please cut along lines of reprinted sections below and paste over the appropriate lines.

(Table 3)

Vol index	0.90	0.88	0.95	0.88	0.13	3 > 1,2,4**

(Table 4)

Vol index [6]	0.90	1.03	0.98	1.03	0.96	1.04	1.04	1.03	1.00	0.96	0.37

(Table 5)

Vol index	1.24	1.33	1.41	1.71	0.11	1,2 < 3,4*; 1,3 < 2.4**