Invitation for Nominations for 1967 American Institute of Nutrition Awards

Nominations are requested for the 1967 annual awards administered by the American Institute of Nutrition to be presented at the next annual meeting. Nominations may be made by anyone, including members of the Nominating Committees and non-members of the Institute.

The following information must be submitted: (1) Name of the award for which the candidate is proposed. (2) A brief convincing statement setting forth the basis for the nomination and, where appropriate, a selected bibliography which supports the nomination. *Seconding or supporting letters are not to be submitted.* (3) Five copies of the nominating letter must be sent to the chairman of the appropriate nominating committee *before October 1, 1966*, to be considered for the 1967 awards.

General regulations for A.I.N. awards. Membership in the American Institute of Nutrition is not a requirement for eligibility for an award and there is no limitation as to age except as specified for the Mead Johnson Award. An individual who has received one Institute award is ineligible to receive another Institute award unless it is for outstanding research subsequent to or not covered by the first award. A Jury of Award composed of A.I.N. members, which makes final selection and remains anonymous, may recommend that an award be omitted in any given year if in its opinion the work of the candidates nominated does not warrant the award. An award is usually given to one person, but, if circumstances and justice so dictate, a Jury of Award may recommend that any particular award be divided between two or more collaborators in a given research.

Presentation of awards will be made at the annual dinner at the annual meeting.

1967 Borden Award in Nutrition

The Borden Award in Nutrition, consisting of $1000 and a gold medal, is made available by the Borden Company Foundation, Inc. The award is given in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of milk or its components. The award will be made primarily for the publication of specific papers during the previous calendar year, but the Jury of Award may recommend that it be given for important contributions made over a more extended period of time not necessarily including the previous calendar year. Employees of the Borden Company are not eligible for this award nor are individuals who have received a Borden Award from another administering association unless the new award be for outstanding research on a different subject or for specific accomplishment subsequent to the first award.

Former recipients of this award are:

1944 – E. V. McCollum
1945 – H. H. Mitchell
1946 – P. C. Jeans and Genevieve Stearns
1947 – L. A. Maynard
1948 – C. A. Cary
1949 – H. J. Deuel, Jr.
1950 – H. C. Sherman
1951 – P. György
1952 – M. Kleiber
1953 – H. H. Williams
1954 – A. F. Morgan and A. H. Smith
1955 – A. G. Hogan
1956 – F. M. Strong
1957 – no award
1958 – L. D. Wright
1959 – H. Steenbock
1960 – R. G. Hansen
1961 – K. Schwarz
1962 – H. A. Barker
1963 – Arthur L. Black
1964 – G. K. Davis
1965 – A. E. Harper
1966 – R. T. Holman

NOMINATING COMMITTEE:

P. H. WESWIG, *Chairman*
E. L. HOVE
K. E. HARSHBARGER

Send nominations to:

DR. P. H. WESWIG
Department of Agricultural Chemistry
Oregon State University
Corvallis, Oregon 97331

1967 Osborne and Mendel Award

The Osborne and Mendel Award of $1000 and an inscribed scroll has been established by the Nutrition Foundation, Inc., for the recognition of outstanding recent basic research accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the

most significant published contribution in approximately the calendar year preceding the annual meeting of the Institute, or who has published recently a series of papers of outstanding significance. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration.

Former recipients of this award are:

1949 – W. C. Rose
1950 – C. A. Elvehjem
1951 – E. E. Snell
1952 – Icie Macy Hoobler
1953 – V. du Vigneaud
1954 – L. A. Maynard
1955 – E. V. McCollum
1956 – A. G. Hogan
1957 – G. R. Cowgill
1958 – P. György
1959 – Grace A. Goldsmith
1960 – N. S. Scrimshaw
1961 – Max K. Horwitt
1962 – William J. Darby
1963 – James B. Allison
1964 – L. Emmett Holt, Jr.
1965 – D. M. Hegsted
1966 – H. H. Mitchell

NOMINATING COMMITTEE:

M. O. SCHULTZE, *Chairman*
ALEX BLACK
L. E. HOLT, JR.

Send nominations to:

DR. M. O. SCHULTZE
Department of Biochemistry
University of Minnesota
St. Paul, Minnesota 55101

1967 Mead Johnson Award for Research in Nutrition

The Mead Johnson Award of $1000 and an inscribed scroll is made available by Mead Johnson and Company to an investigator who has not reached his 46th birthday during the calendar year in which the Award is given. Selection by the Jury of Award will be based primarily on a single outstanding piece of research in nutrition published in the year preceding the annual meeting or on a series of papers on the same subject published within not more than the three years preceding the annual meeting.

Former recipients of this award are:

1939 – C. A. Elvehjem
1940 – W. H. Sebrell, Jr.
J. C. Keresztesy
J. R. Stevens
S. A. Harris
E. T. Stiller
K. Folkers
1941 – R. J. Williams
1942 – G. R. Cowgill
1943 – V. du Vigneaud
1944 – A. G. Hogan
1945 – D. W. Woolley
1946 – E. E. Snell
1947 – W. J. Darby
P. L. Day
E. L. R. Stokstad
1948 – F. Lipmann
1949 – Mary S. Shorb
K. Folkers
1950 – W. B. Castle
1951 – no award
1952 – H. E. Sauberlich
1964 – J. S. Dinning
1965 – J. G. Bieri
1966 – M. Daniel Lane

NOMINATING COMMITTEE:

P. L. DAY, *Chairman*
A. R. KEMMERER
L. M. HENDERSON

Send nominations to:

DR. P. L. DAY
National Heart Institute
National Institutes of Health
Bethesda, Maryland 20014

1967 Conrad A. Elvehjem Award for Public Service in Nutrition

The Conrad A. Elvehjem Award for Public Service in Nutrition, consisting of $1000 and an inscribed scroll, is made available by the Wisconsin Alumni Research Foundation. The award is bestowed in recognition of distinguished service to the public through the science of nutrition. Such service, primarily, would be through distinctive activities in the public interest in governmental, industrial, private, or international institutions but would not exclude, necessarily, contributions of an investigative character.

The first recipient of this award was:

1966 – C. Glen King

NOMINATING COMMITTEE:

E. E. HOWE, *Chairman*
F. W. QUACKENBUSH
R. E. SHANK

Send nominations to:

DR. E. E. HOWE
Merck Institute for Therapeutic Research
Rahway, New Jersey 07065

Invitation for Nominations for 1967 American Institute of Nutrition Fellows

The Fellows Committee of the American Institute of Nutrition invites nominations for Fellows in the Society. Eligible candidates are active or retired members of the Society who have passed their sixty-fifth birthday (by the time of the annual meeting) and who have had distinguished careers in nutrition. Up to three Fellows may be chosen each year.

Nominations may be made to the Chairman of the Fellows Committee by any member of the Society, including members of the Committee.

Nominations (in 5 copies) are due by October 1. A supporting statement giving the reason for the nomination is desirable.

Final selection will be made by the Fellows Committee and a suitable citation will be presented at the Annual Dinner in April.

Fellows Committee:

S. L. Hansard, *Chairman*
Paul E. Johnson
W. H. Griffith
Agnes F. Morgan
Richard M. Forbes

Send nominations to:

Dr. S. L. Hansard
Department of Animal Science
Louisiana State University
Baton Rouge, Louisiana 70803

The following persons have been elected previously as Fellows of the Society:

J. B. Brown (1964)
Thorne M. Carpenter (1958)
George R. Cowgill (1958)
Henrik Dam (1964)
Eugene F. DuBois (1958)
R. Adams Dutcher (1961)
Ernest B. Forbes (1958)
Casimir Funk (1958)
Wendell H. Griffith (1963)
Paul György (1965)
Albert G. Hogan (1959)
Icie Macy Hoobler (1960)
Paul E. Howe (1960)
J. S. Hughes (1962)
C. Glen King (1963)
Max Kleiber (1966)
Samuel Lepkovsky (1966)
Leonard A. Maynard (1960)
Elmer V. McCollum (1958)
Harold H. Mitchell (1958)
Agnes Fay Morgan (1959)
John R. Murlin (1958)
Leo C. Norris (1963)
Helen T. Parsons (1961)
Lydia J. Roberts (1962)
William C. Rose (1959)
W. D. Salmon (1962)
Arthur H. Smith (1961)
Genevieve Stearns (1965)
Harry Steenbock (1958)
Hazel K. Stiebeling (1964)
Raymond W. Swift (1965)
Robert R. Williams (1958)
John B. Youmans (1966)

Invitation for Nominations for Honorary Membership in the American Institute of Nutrition

The Committee on Honorary Memberships of the American Institute of Nutrition invites nominations for Honorary Members.

Distinguished individuals of any country who are not members of the American Institute of Nutrition and who have contributed to the advance of the science of nutrition shall be eligible for proposal as Honorary Members of the Society.

Nominations may be made to the Chairman of the Committee on Honorary Memberships by two members of the Society.

Nominations (in three copies) are due by October 1. A supporting statement giving the reason for the nomination is desirable but not necessary.

Final selection of nominees will be made by the Council of the American Institute of Nutrition and such nominations submitted to the Society at the spring meeting. Election requires a two-thirds majority of the ballots cast.

Honorary members pay no membership fees but are eligible to subscribe to the official journal(s) at member's rates.

Committee on Honorary Memberships:

PAUL GYÖRGY, *Chairman*
GRACE GOLDSMITH
R. W. ENGEL

Send nominations to:

DR. PAUL GYÖRGY
University of Pennsylvania
Pennsylvania General Hospital
Philadelphia, Pennsylvania 19104

The following persons have been elected previously as Honorary Members of the Society:

Kunitaro Arimoto
W. R. Aykroyd
Frank B. Berry
Edward Jean Bigwood
Frank G. Boudreau
Robert C. Burgess
Harriette Chick
F. W. A. Clements
David P. Cuthbertson
Herbert M. Evans
Joachim Kühnau
Toshio Oiso
H. A. P. C. Oomen
Lord John Boyd Orr
Conrado R. Pascual
V. N. Patwardhan
B. S. Platt
Emile F. Terroine
Jean Tremolieres
Eric John Underwood
Artturi I. Virtanen

Tissue and Intracellular Distribution of Radioactive Thiamine in Normal and Thiamine-deficient Rats [1]

MESBAHEDDIN BALAGHI [2,3] AND W. N. PEARSON

Division of Nutrition, Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee

ABSTRACT The distribution of radioactive ^{14}C-thiazole-labeled thiamine in intracellular fractions of rat liver, brain, kidney, and testes was studied in adult rats of the Sprague-Dawley strain that had been injected with physiological levels of thiamine for a long period of time. In all tissues 60 to 70% of the radioactivity was noted in the mitochondrial plus soluble fractions. About 20% of the radioactivity was present in the nuclear fractions from liver, kidney and testes but only 9% was found in this fraction from brain. Liver and kidney microsomal fractions contained 7 to 8% of the radioactivity, but 20 to 24% of the radioactivity in brain and testes appeared in this fraction. It is considered that mitochondrial contamination of the nuclear fractions prepared from liver, kidney and testes contributed some of the observed radioactivity in this fraction. The high thiamine content of the mitochondrial and microsomal fractions of brain may likewise be related to contamination with "pinched-off" nerve endings. During thiamine depletion the thiamine in the intracellular compartments depleted uniformly, i.e., no one compartment lost thiamine preferentially. The failure to observe a preferential depletion of thiamine from the soluble fraction of the cell was interpreted to mean that transketolase has a higher thiamine requirement than other enzymes requiring this co-factor or that the apoenzyme level itself was reduced.

The tissue distribution of thiamine, its relation to the state of thiamine deficiency, and its urinary excretion have been well-studied (1-5). Several investigators have more recently determined the intracellular distribution of thiamine in rat liver (6-8). No information is available, however, about the intracellular distribution of thiamine in tissues other than liver, and nothing is known about the effects of advanced thiamine deficiency upon this distribution pattern. In the present communication the tissue distribution and intracellular distribution of radioactive thiamine compounds in normal rat and the changes which occur during a progressive thiamine deficiency are reported.

METHODS

For determination of the tissue distribution of thiamine 6 adult rats of the Sprague-Dawley strain were fed a thiamine-deficient diet for 10 days. The composition of this diet has been described previously (9). The animals were then injected daily with 40 μg of ^{35}S-labeled thiamine[4] (specific activity 15,000 dpm/μg) for a period of 8 weeks. At the end of this period the animals were killed and the organs were processed in the following manner. Tissues were blended in a small Waring Blendor with 10 volumes of 0.1 N hydrochloric acid and then steamed in an autoclave for 30 minutes. This usually gave a clear solution, an aliquot of which was taken for scintillation counting. When analyzing the entire carcass for radioactivity, the carcass was first passed twice through a meat grinder. Then 1500 ml of 0.2 N hydrochloric acid were added, and the preparation was steamed for 30 minutes in an autoclave and passed through 2 layers of cheesecloth. The material trapped by the cheesecloth was placed in a beaker (cloth included) 1500 ml of 0.1 N hydrochloric acid added, and again steamed for 30 minutes and strained through cheesecloth. The combined extracts were stirred for 30 minutes and du-

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[1] Supported in part by Public Health Service Research Grant no. AM-02565 and no. AM-07709 from the National Institute of Arthritis and Metabolic Diseases.

[2] Present address: Imperial Iranian Army Nutrition Committee, Army Medical Department, Aziz Khan Crossroad, Hafez Avenue, Tehran, Iran.

[3] A portion of these data was taken from a thesis submitted by Mesbaheddin Balaghi to the faculty of the Graduate School of Vanderbilt University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry.

[4] Nuclear Chicago Corporation, Chicago.

plicate aliquots were taken for scintillation counting. Each sample was corrected for radioactive decay and the radioactivity was converted to micrograms of thiamine on the assumption that the specific activity of the thiamine pool was the same as that of the injected thiamine, a justified assumption considering the long period of injection. The method of Hogeboom (10) was used for fractionation of the tissues. All operations were carried out at 5° or below. Each particulate fraction was resuspended in 5 ml of sucrose solution and 1-ml fractions were taken for scintillation counting.

In a second study, the effects of thiamine deficiency on the distribution of thiamine compounds in the intracellular fractions was carried out in a group of ten adult rats which had received 2-^{14}C-thiazole-labeled thiamine[4] (specific activity 5,600 dpm/μg) injections daily for 11 months. Although different levels of thiamine were injected during the first 7 months the mean daily intake was approximately 40 μg. For the last 4 months of the 11-month period each rat was injected with 40 μg of the radioactive thiamine daily. On the first day of the experiment described here (day 0) the thiamine injections were discontinued and a pair of animals selected at random was killed. The total content of thiamine compounds in liver, brain and kidney was determined by the measurement of radioactivity. The same extraction procedure used with the ^{35}S thiamine studies was employed. The remainder of the tissues was then fractionated into nuclear, mitochondrial, microsomal, and soluble fractions according to the procedure of Hogeboom (10). The radioactivity in each fraction was measured by liquid scintillation counting. On experimental days 10, 20, 27, and 37, pairs of rats were selected at random, killed and the organs were processed as above.

RESULTS

Table 1 shows the distribution of ^{35}S-labeled thiamine among the rat tissues. The mean total body content of each animal was 447 μg, and since the mean body weight of the animals was 412 g, this represents an average of 1.08 μg of thiamine compounds/g of body weight. The carcass (the animal body after removal of the organs) contained 61% of the total body radioactivity. This was the lowest concentration on a weight basis. Among the organs, liver contained 22%, the testes and gastrointestinal tract about 5% each, heart and brain about 1.7% and 1.5%, respectively. The lungs and spleen each contained less than one per cent of the total body content. The last column in table 1 shows the levels of radioactivity in the tissues expressed as micrograms of thiamine per gram. The liver with 9.3 μg/g had the highest concentration, followed by heart, 6.6 μg/g and testes 6.5 μg/g. These values are in general agreement with those of other workers (3-5, 11). Brain and kidney contained moderate amounts of thiamine and the gastrointestinal tract and lungs only small quantities. The carcass, with a concentration of 0.77 μg/g, had the lowest concentration but as the preparation contained bone and hair, the true thiamine level in muscle was probably somewhat higher. Although we did not make such studies, the bulk of the thiamine contained in the tissues was no doubt in the diphosphate form as demonstrated by deCaro et al. (11).

TABLE 1

Distribution of ^{35}S-labeled thiamine in various organs of the rat [1]

Organ	No. of rats	Total vitamin B_1 in organ	Vitamin B_1/g fresh tissue
		μg	μg
Liver	6	98.8	9.3
Heart	6	7.8	6.6
Brain	6	6.7	3.8
Testes	5	23.5	6.5
Kidney	6	9.5	3.8
G.I. tract	6	22.9	1.7
Lungs	5	2.9	1.5
Spleen	6	1.3	1.8
Carcass	6	273.9	0.77

[1] The rats received 40 μg of thiamine ^{35}S for 8 weeks prior to killing. The values were obtained by scintillation counting and represent the average of the corresponding number of rats (5 or 6 as shown). The radioactivity was converted to micrograms of thiamine.

Table 2 shows the radioactivity of the nuclear, mitochondrial, microsomal and soluble fractions expressed as the percentage of that recovered from the liver, brain, kidney and testes. In all tissues about 60 to 70% of the radioactivity is in the mitochondrial plus soluble fractions. In liver

TABLE 2

Intracellular distribution of ^{35}S-labeled thiamine in various organs of the rat [1]

Subcellular fraction	% of total organ content			
	Liver	Brain	Kidney	Testes
Nuclear	17.9	9.1	22.3	17.2
Mitochondrial	29.2	37.9	37.1	23.1
Microsomal	8.5	24.7	7.3	20.7
Soluble	44.2	28.1	33.1	38.1

[1] The rats received 40 μg of thiamine ^{35}S for 8 weeks prior to killing. The values were obtained by scintillation counting and each value is the average of 6 rats.

and kidney and testes about 20% of the radioactivity was observed in the nuclear fraction but only 9% in the nuclear preparation from brain. The liver and kidney microsomal fraction contained 7 to 8% of the radioactivity, whereas in brain and testes 20 to 24% of the radioactivity was found in this fraction.

Because the nuclear fraction prepared by the method used contains some unbroken cells, some mitochondria, some erythrocytes, and some connective tissue, the extent of contamination with mitochondria was determined by assaying the succinic dehydrogenase activity of the nuclear and mitochondrial fractions of liver and kidney (12). Because this enzyme system is tightly bound to the mitochondria, and is not solubilized even after fragmentation of these particles, the ratio of succinic dehydrogenase activity of the nuclear and mitochondrial fraction gives information about the extent of contamination of the nuclear fraction with the mitochondria. The ratios obtained for the succinic dehydrogenase activity of the mitochondrial fraction were 5.2 for liver and 6.1 for kidney. From these ratios it may be calculated that about one-third of the radioactivity in the liver and kidney nuclear fractions was caused by mitochondrial contamination. The percentage of total radioactivity in the nuclear fractions after correction for this contamination is accordingly 11.9 for liver and 16.3 for kidney instead of 17.9 and 22.3 as shown in table 2. Since other contaminants of the nuclear fraction have but a slight effect, we consider that the presence of some form of thiamine in the nucleus has been confirmed.

The consistent occurrence of about 20% of the total tissue radioactivity in the microsomal fraction of brain and testes was also unexpected. The thiamine content of the microsomal fraction of liver and kidney are about twice those reported by Goethart (6). We have no independent measure of the "purity" of our preparation of microsomes but the method of Hogeboom (10) is generally considered to yield preparations of endoplasmic reticulum of excellent purity.

Table 3 shows the decrease in the thiamine compounds in liver, brain and kidney brought about by thiamine deficiency. In 37 days the total content of thiamine compounds in the tissues decreased as a result of thiamine deficiency from 68.1 μg to 1.3 μg in liver, from 5.5 μg to 1.2 μg in brain, and from 12.2 μg to 0.6 μg in kidney. The same data are presented in graphic form in figure 1. Each point on the curve shows the average concentration of thiamine compounds in the tissue as micrograms per gram of the tissue (calculated on the basis of radioactivity) for 2 animals killed on that particular day. The concentrations decreased (in μg/g fresh tissue) from 7.9 to 0.25 in liver, from 3.3 to 0.75 in brain and from 4.9 to 0.37 in kidney. The rate of decrease in the concentration of the thiamine compounds was highest in liver and lowest in brain. Although the liver had the highest thiamine concentration at the beginning of the thiamine deprivation, the order was reversed during the course of the experiment and on day 37, its concentration was the lowest. The rate of disappearance of the radioactivity from the kidney is similar to that for the liver, but the brain shows a different pattern. This difference is not

TABLE 3

Effect of progressive thiamine deficiency on the thiamine content of various organs of the rat [1]

Organ	Thiamine content in entire organ				
	Days of thiamine deprivation				
	0	10	20	27	37
	μg	μg	μg	μg	μg
Liver	68.1	25.2	14.0	4.9	1.3
Brain	5.5	4.8	2.8	2.1	1.2
Kidney	12.2	4.2	2.2	1.2	0.6

[1] The rats received 40 μg of thiazole-^{14}C-labeled thiamine daily for 16 weeks prior to the experiment. The values were obtained by scintillation counting of tissue homogenates, and each value is the average of 2 rats.

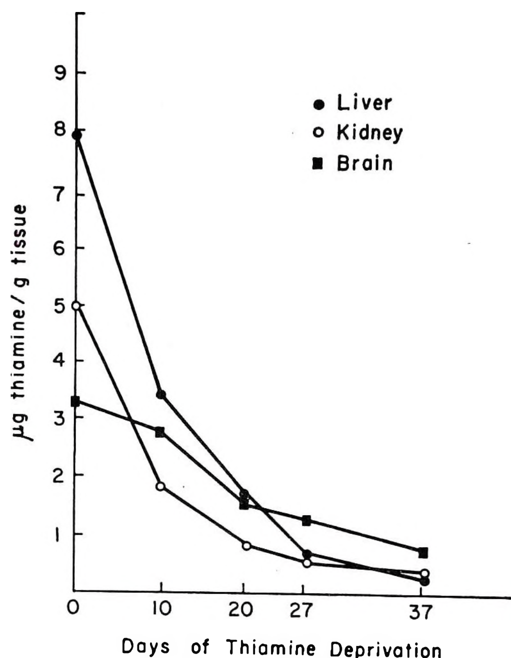

Fig. 1 Decrease of thiamine compounds in rat tissues during progressive thiamine deficiency. The tissue levels were estimated from liquid scintillation measurements of the ^{14}C radioactivity.

only quantitative, but also qualitative since the shape of the brain thiamine-loss curve is different from the shapes from liver and kidney. Whether the blood-brain barrier, the membrane properties of the central nervous system, or different rates of turnover are responsible for this observed difference cannot be decided from these experiments. The slower rate of thiamine excretion (or breakdown) in brain tissue may be an important protective mechanism. Salcedo et al. (5) obtained similar results on the disappearance of thiamine from the tissues of the thiamine-deficient rat as have deCaro et al. in a more recent study (11). The changes that occur in the percentage distribution of thiamine among subcellular fractions of liver, brain, and kidney tissues during thiamine deprivation are shown in table 4. The only consistent alteration is a slight increase in the percentage of radioactivity in the microsomal fraction especially in the liver and kidney tissues. Thus, a 37-day deprivation of thiamine which caused a 52-fold decrease in total thiamine compounds in liver, a 20-fold decrease in kidney, and a 4-fold decrease in brain, did not appreciably change the distribution pattern of these compounds among the cellular fractions of these tissues.

DISCUSSION

The radioisotopic technique used in this study might be expected to give somewhat higher tissue values than other methods

TABLE 4

Effect of thiamine deficiency on the subcellular distribution of thiamine compounds in rat tissues [1]

Tissue	Subcellular fraction	% of total radioactivity of corresponding tissue homogenate [2]				
		Days of deprivation				
		0	10	20	27	37
Liver	Nuclear	22.8	24.1	24.0	22.1	15.4
	Mitochondrial	24.0	16.0	18.9	21.0	24.1
	Microsomal	8.1	7.0	11.2	13.8	13.0
	Soluble	44.8	52.7	45.8	42.9	47.4
Brain	Nuclear	10.0	13.7	12.6	9.9	14.1
	Mitochondrial	39.9	45.8	42.8	43.2	45.0
	Microsomal	15.1	12.9	18.5	20.8	19.9
	Soluble	34.8	27.4	25.9	26.0	20.8
Kidney	Nuclear	29.4	35.5	27.5	25.4	29.1
	Mitochondrial	28.0	26.3	29.3	30.3	24.3
	Microsomal	6.6	6.3	7.2	10.5	11.1
	Soluble	35.9	31.7	35.7	33.5	35.3

[1] The rats were injected with 40 μg of thiazole-^{14}C-labeled thiamine for 16 weeks prior to initiation of this experiment.

[2] Each value is the average of fractions from 2 rats and was obtained by scintillation counting.

since it is theoretically less specific than methods demanding biological activity or conversion of thiamine to thiochrome. It is of interest, therefore, that the tissue levels reported here do not differ markedly from those reported by other workers using somewhat different dietary regimens and animal, chemical or microbiological methods for thiamine assay (1-5, 11). It appears, consequently, that the large number of urinary thiamine metabolites observed in this laboratory (9, 13) having neither microbiological nor thiochrome activity do not accumulate to any extent in the tissues.

The amount of thiamine present in the liver nuclei in the present study is less than that reported by Nose et al. (8) but greater than that reported by Goethart (6) and Dianzani and Dianzani (7). Whether these differences represent a function of the tissue preparation or of the thiamine methodology remains to be determined, but it is known that even the most advanced techniques now in use do not yield absolutely pure nuclear preparations (14). It is unlikely, however, that contamination accounts for all of the thiamine present in nuclear preparations and it would be worthwhile to consider possible thiamine functions in this organelle.

It is generally conceded that there is no ATP formation in nuclei via oxidative phosphorylation or closely related pathways. This conclusion is based largely on the lack of members of the respiratory chain in the nuclei and the calculation of extremely small P:O ratios in respiring preparations (14). Thus, the presence of thiamine in the nucleus cannot be explained in terms of a need for this compound in the conventional processes of oxidative carbohydrate metabolism. In addition, the presence of the hexose monophosphate shunt, a metabolic pathway which would require the presence of thiamine pyrophosphate, has not been established in the nucleus.

The apparent absence of the major thiamine-requiring metabolic systems in the nucleus leaves 2 alternative explanations for its presence. The first is a new metabolic function for thiamine; the second is that its presence is merely fortuitous. Because there is no current evidence for the former the second explanation appears to be more plausible. It is entirely possible, for example, that thiamine might be phosphorylated in the cytoplasm (16) and then transported into the nucleus.

The presence of a large percentage of the cellular thiamine in the mitochondria is not unexpected since it is required for the proper functioning of the tricarboxylic acid cycle. Likewise, the occurrence of thiamine in the soluble cell fraction may be rationalized by the presence of the hexose monophosphate shunt.

The distribution of thiamine in the intracellular fractions of the brain deserves special comment. Both mitochondrial and microsomal thiamine levels were significantly higher than those in liver and kidney. It is known that both mitochondrial and microsomal preparations from brain as conventionally prepared are contaminated with "pinched-off" nerve endings produced during tissue homogenization (17, 18). Whether such contaminants are, in fact, a rich source of thiamine seems to be worth examining, particularly since Gray and Whittaker (17) have succeeded in preparing pure preparations of nerve-ending particles.

It has been shown that the 3 thiamine-dependent mammalian enzymes (transketolase, the pyruvate dehydrogenase system and the oxoglutarate dehydrogenase system) show different degrees of sensitivity to thiamine deficiency. In most tissues transketolase is the most sensitive (19) and oxoglutarate dehydrogenase is the least sensitive (20). Since transketolase is present in the soluble cell fraction, it is reasonable to assume that the reason for the early inactivation of this enzyme in thiamine deficiency is that the content of the soluble fraction of the cell is preferentially depleted, to protect the mitochondrial enzymes. This is apparently not the case since, in these studies, thiamine deprivation reduced the contents of all 4 fractions proportionately. Thus, the reason for the consecutive inactivation of enzymes in thiamine deficiency must be due to other considerations, such as a difference in the thiamine pyrophosphate requirement of each enzyme or depletion of the transketolase apoenzyme itself. The latter is a

strong possibility in the rat because Brin[5] has observed that the transketolase activity of the tissues of thiamine-deficient rats cannot be restored in vitro by addition of thiamine pyrophosphate.

LITERATURE CITED

1. Brodie, J. B., and F. L. McLeod 1935 Quantitative experiments on occurrence of vitamin B_1 in organs. J. Nutrition, *10:* 179.
2. Leong, P. C. 1937 LIII. Vitamin B_1 in the animal organism. 1. The maximum storage of vitamin B_1 in the tissues of the rat. Biochem. J., *31:* 367.
3. Schultz, A. S., R. F. Light, L. J. Crasas and L. Atkin 1939 The concentration of vitamin B_1 in the tissues of the rat. J. Nutrition, *17:* 143.
4. Ferrebee, J. W., N. Weissman, D. Parker and P. S. Owen 1942 Tissue thiamine concentrations and urinary thiamine excretion. J. Clin. Invest., *21:* 401.
5. Salcedo, J., Jr., V. A. Najjar, L. E. Holt, Jr. and E. W. Hutzler 1948 The relation between urinary excretion and tissue concentrations of thiamine in rats. J. Nurition, *36:* 307.
6. Goethart, G. 1952 The thiamine pyrophosphate content of centrifugally-prepared fractions of rat liver homogenate. The influence of a thiamine-deficient diet. Biochim. Biophys. Acta, *8:* 479.
7. Dianzani, M. U., and M. A. Dianzani Mor 1957 Displacement of thiamine pyrophosphate from swollen mitochondria. Biochim. Biophys. Acta, *24:* 564.
8. Nose, O., M. Kawakami, K. Hirano and J. Okuno 1960 Distribution of thiamine in the subcellular components of rat liver. Bitamins, *20:* 425.
9. Neal, R. A., and W. N. Pearson 1964 Studies of thiamine metabolism in the rat. I. Metabolic products found in urine. J. Nutrition, *83:* 543.
10. Hogeboom, G. H. 1955 Fractionation of cell components of animal tissues. In: Methods in Enzymology, vol. 1, eds., S. P. Colowick and N. O. Kaplan. Academic Press, New York.
11. deCaro, L., G. Rindi and L. de Giuseppe 1961 Contents in rat tissue of thiamine and its phosphates in dietary thiamine deficiency. Internat. Ztschr. Vitaminforsch., *31:* 333.
12. Slater, E. C., and W. D. Bonner 1952 The effect of fluoride on the succinic oxidase system. Biochem. J., *52:* 185.
13. Balaghi, M., and W. N. Pearson 1966 Metabolism of physiological doses of 2-^{14}C-thiazole-labeled thiamine by the rat. J. Nutrition, in press.
14. Siebert, G., and G. B. Humphrey 1965 Enzymology of the Nucleus. In: Advances in Enzymology, vol. 27, ed., F. F. Nord. Interscience Publishers, New York, p. 239.
15. Allfrey, V. G., A. E. Mirsky and H. Stern 1955 The chemistry of the cell nucleus. In: Advances in Enzymology, vol. 16, ed., F. F. Nord. Interscience Publishers, New York, p. 411.
16. Leuthardt, F., and H. Nielsen 1952 Biological phosphorylation of thiamine. Helv. Chim. Acta, *35:* 1196.
17. Gray, E. G., and V. P. Whittaker 1962 The isolation of nerve endings from brain. An electron microscopic study of cell fragments derived from homogenization and centrifugation. J. Anat. (London), *96:* 79.
18. Gillis, C. N., N. J. Giarman and D. X. Freedman 1964 Retention of 5-hydroxytryptamine by subcellular fractions of rat brain homogenates. Biochem. Pharmacol., *13:* 1457.
19. Brin, M., S. S. Shohet and C. S. Davidson 1958 The effect of thiamine deficiency on the glucose oxidative pathway of rat erythrocytes. J. Biol. Chem., *230:* 319.
20. Wright, R. C., and E. M. Scott 1954 Pyruvate and alpha-ketoglutarate metabolism in thiamine deficiency. J. Biol. Chem., *206:* 725.

[5] Personal communication from Dr. Myron Brin, Department of Biochemistry, Upstate Medical Center, State University of New York, Syracuse, New York.

Excretion of Metabolites of ^{14}C-Pyrimidine-labeled Thiamine by the Rat at Different Levels of Thiamine Intake [1]

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ABSTRACT The effect of the level of intake of radioactive thiamine on the quantitative and qualitative aspects of the urinary excretion of thiamine metabolites was studied in rats. Rats were maintained with different levels of ^{14}C-pyrimidine-labeled thiamine (8, 20, 30, 50, 100 μg/day) and urinary metabolites were partitioned by column and paper chromatography. Five peaks of radioactivity were obtained. The percentage of total radioactivity in three of these peaks remained constant at all intake levels. The percentage of radioactivity declined in one peak and increased in another as intake increased. Virtually no thiamine appeared in the urine at intake levels of less than 100 μg/day or at tissue levels of less than 75% of saturation. At the lower levels of intake, the thiamine content of the urine as assayed microbiologically was only about 2% of that predicted from the radioactivity. About 13% of the radioactivity of the intake appeared daily in the feces. The thiamine values found for tissues by microbiological assay were somewhat higher than those obtained by radioactivity measurement. The microbiologically inactive products of thiamine metabolism observed in the urine are apparently not present in appreciable quantity in the tissues.

In an early report, Neal and Pearson (1) demonstrated the presence of at least 22 different metabolites of pyrimidine ^{14}C-labeled thiamine in rat and rabbit urine. Two of these compounds were identified as thiamine, and 2-methyl-4-amino-5-pyrimidine carboxylic acid, but the remaining compounds are still unidentified (2). Since these studies were made at a thiamine intake level (100 μg/day) considerably in excess of the actual needs of the animal, the excretion patterns of these urinary metabolites have now been determined at various intake levels and the relationship between urinary excretion and body stores has been examined.

EXPERIMENTAL PROCEDURE

Twelve weanling male rats of the Sprague-Dawley strain were used. They were housed in pairs in stainless steel metabolism cages constructed to permit the separate collection of urine and feces. Twenty-four-hour urine specimens were collected under toluene in glass bottles containing 1 ml of 2 N acetic acid. No other preservative was used because microbiological assays were to be carried out. Each day's collection of urine and feces (6 samples) was pooled and stored at −20° prior to analysis.

The rats were fed a thiamine-deficient diet throughout the experiment, which lasted approximately 10 months. The diet had the following percentage composition: vitamin-free casein, 18; cottonseed oil, 5; sucrose, 70; non-nutritive fiber, 3; and salt mixture,[3] 4. The following vitamins were added: (mg/100 g of basal diet) riboflavin, 0.8; niacin, 4.0; Ca pantothenate, 4.0; biotin, 0.04; folic acid, 0.20; pyridoxine, 0.50; *p*-aminobenzoic acid, 10.0; choline chloride, 100.0; α-tocopheryl acetate, 4.0; and menadione, 0.5. Vitamins A and D were added at 400 and 40 IU/100 g of diet, respectively.

The rats were fed diet and water ad libitum throughout the experiment. After they had been fed the deficient diet for 2

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[3] Hubbel, R. B., L. B. Mendel and A. J. Wakeman 1937 A new salt mixture for use in experimental diets. J. Nutrition, *14:* 273.

weeks and a growth plateau had been reached, daily intraperitoneal injections of an aqueous solution of ^{14}C-pyrimidine-labeled thiamine were begun. This compound had a specific activity of 0.296 $\mu c/\mu mole$ and its synthesis has been described previously (1). Radioactivity measurements of aliquots of the daily pooled urine samples were made in a Packard Tri-Carb liquid scintillation counter. The scintillation fluid used for counting these aqueous samples was composed of a mixture of naphthalene, 100 g; 2, 5-diphenyloxazole, 7 g; 1, 4 di(2, 5 phenyloxazole) benzene, 0.3 g, and sufficient *p*-dioxane to make the volume to one liter. A quenching connection was made by counting a duplicate sample to which had been added a known amount of thiamine-^{14}C. The radioactivity values were converted to an equivalent number of micrograms of thiamine for convenience in expressing results. Feces were pooled by 8 to 10-day periods and were extracted by autoclaving (15 minutes at 121°) in 5 times their weight of 0.1 N HCl. The extract was then filtered (Whatman no. 1) and the residue was autoclaved again with the same volume of HCl. After filtration, both extracts were combined for the determination of radioactivity. This was determined by the same scintillation counting procedure used for urine with an internal standard being used for correction of quenching.

For column chromatography of the urine, the procedure, previously described in detail, was used (1). This entailed the absorption of the radioactive metabolites on activated charcoal, recovery of the charcoal by centrifugation, and elution of the metabolites with a pyridine/ethanol/water mixture. The eluates were reduced in volume under vacuum, the pH adjusted to 5.5 and chromatography carried out on columns of Amberlite CG 50 (1.0 $\times$ 40 cm; 200-400 mesh H^+ form). The metabolites were eluted first with 200 ml of water and then with 200 ml of a pyridine-acetic acid-water mixture (75/10/915 by volume). Five-milliliter fractions were collected and were checked for radioactivity by plating 0.5-ml aliquots on aluminum planchets, drying, and counting in a Nuclear-Chicago D-47 gas-flow detector equipped with a model M5 sample changer. No attempts were made to correct for internal absorption during this screening procedure. The peaks of radioactivity detected by this method were subsequently pooled and quantitatively assayed for radioactivity using scintillation counting and an internal quenching standard.

The radioactive peaks were subjected to ascending paper chromatography after being reduced in volume under vacuum with subsequent lyophilization. Acid-washed sheets of Whatman no. 1 or 3 paper were used with a solvent composed of *n*-propanol/water/1 M acetate buffer, pH 5.0 (70/20/10 by volume). Metabolites were detected by radioautography, by fluorescence or quenching under ultraviolet light or by a combination of these methods. Radioautographs were made by exposing the chromatogram to Eastman No-Screen X-ray Film for periods varying from 2 to 30 days with development in the conventional manner.

Tissues were extracted with 10 volumes of 0.1 N HCl (w/v) for determination of radioactivity and for microbiological assay. All organs but the carcass were homogenized in an appropriate amount of 0.1 N HCl in a Waring Blendor and were then steamed in an autoclave for 30 minutes. After filtration, the pH was adjusted to 6.8 to 7.0 and the samples diluted to an appropriate volume for assay. The carcass, pelt and bones were passed through an electric meat grinder twice prior to extraction. After steaming for 30 minutes in 0.2 N HCl the mixture was rapidly filtered through a cotton gauze pad and the residue retained by the gauze was autoclaved again (gauze included) this time in an equal volume of 0.1 N HCl. The latter was again filtered and the filtrates were combined. Prior to assay the filtrate was defatted by extracting twice with one volume of petroleum ether. No radioactivity or microbiologically active material was found in the petroleum ether layer. Aliquots of the tissue extracts were assayed for radioactivity by scintillation counting using the scintillation solution and the internal standard procedure as described previously.

Microbiological assays for thiamine activity were made with *Lactobacillus viri-*

descens ATCC 12706 using the procedure of Deibel et al. (3). When appropriate, the acid extracts of tissue or feces were treated with Mylase[4] to convert the phosphorylated forms of thiamine to the free compound. Urine samples were assayed directly without treatment.

RESULTS AND DISCUSSION

After being rendered thiamine-deficient, 12 weanling male rats were injected with 8 μg of radioactive thiamine daily for 66 consecutive days. This period was followed successively by 20 μg for 51 days, 30 μg for 72 days, 50 μg for 54 days and 100 μg for 54 days. Upon termination of the 20-μg period of injection (day 117 of the experiment) and on termination of each dose level thereafter, 2 rats were killed for tissue analysis. The growth curve during the entire experiment is shown in figure 1. "True percentage growth rates" were calculated over various segments of the curve using the method of Brody (4). It should be pointed out that the number of animals had decreased from 12 to 2 by the end of the experiment.

At the 8-μg level of intake the rats grew at a reasonable, although reduced, rate (K = 2.5%) for about 40 days. A reduced rate of growth was then evident (K = 0.4%; body weight about 240 to 250 g) which continued until the 66th day. The weights of the rats at this growth plateau agree well with the results of Shibata et al. (5) who found that daily oral doses of 2.5 and 5.0 μg of thiamine resulted in weight plateaus of 60 to 80 g and 140 to 160 g, respectively. At this point, increasing the dose to 20 μg produced a period of accelerated growth of about 20 days (K = 0.7%). A rate reduction again occurred (K = 0.46%) for the next 37 days. Here, the dose was increased to 30 μg daily but growth did not begin again until 10 days of injection after which time it continued slowly for about 50 days. Increasing the dose to 50 μg did not initiate further growth until after 30 days of injection after which time growth proceeded slowly. The inflection of the growth curve at day 260 is wholly irrelevant, being due to the killing of the smaller pair of the remaining rats.

The mean daily excretion of thiamine metabolites calculated from the ^{14}C activity of the urine and by microbiological assay is compared in table 1. The percentage of thiamine-^{14}C intake excreted in the urine decreased from 38% of intake at the 8-μg level to 21% of intake at the 50-μg level. This was followed by an abrupt increase to 27% at the 100-μg level of intake which resulted from the appearance of appreciable quantities of free thiamine in the urine for the first time.

No detectable urinary thiamine at the low levels of intake (< 100 μg) was found

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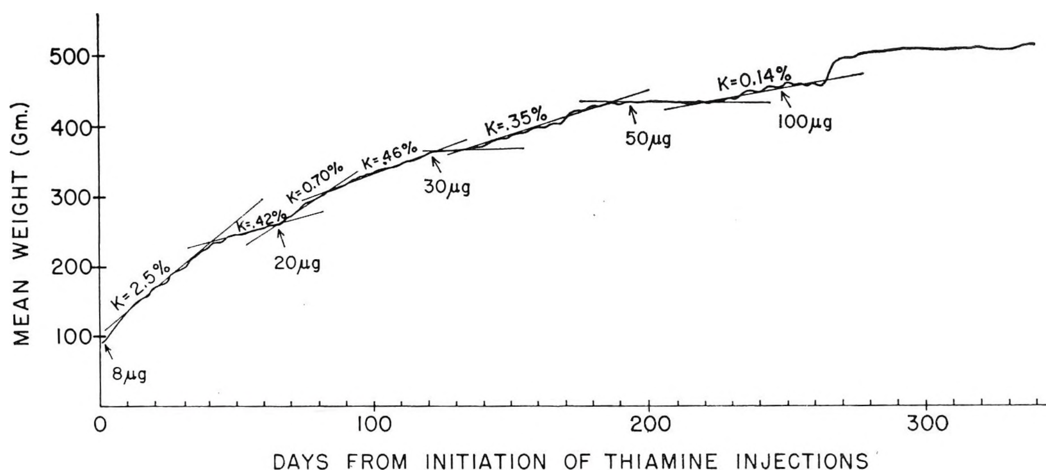

Fig. 1 Growth response of rats to various thiamine intakes.

TABLE 1

Relation between thiamine intake and excretion of urinary metabolites

Mean rat wt [2]	No. of rats [3]	Level of intake	No. of days injected	Mean ^{14}C excretion calculated as thiamine	Mean micro-biological thiamine [1]	Urinary ^{14}C	Urinary micro-biological thiamine
g		*μg/day*		*μg/rat/day*		*% of intake*	*% of intake*
217	12	8	66	3.1	0.17	38.0	2.1
314	12	20	51	7.1	0.24	36.0	1.7
394	10	30	72	10.0	0.65	33.0	2.2
437	8	50	54	10.7	0.64	21.0	1.3
493	6	100	54	27.2	15.0	27.0	15.0

[1] Determined by *Lactobacillus viridescens* assay.
[2] This represents the mean weight of the rats during the particular period of injection.
[3] Pairs of rats were killed for tissue assays at various stages of the experiment.

by assay with the thiochrome procedure. In fact, the presence of non-specific fluorescence often gave negative values. Small amounts of thiamine activity were noted with the more sensitive microbiological assay procedure. This did not exceed 2% of intake, however, until the 100-μg dosage level was reached when a thiamine microbial assay value of 15% of intake was recorded.

A schematic representation of a typical column chromatographic radioactivity pattern found at all levels of intake below 100 μg is shown in figure 2. Five peaks were detected—two were eluted with distilled water and three were eluted with the pyridine-acetic acid mixture. At the 100-μg level, peak 4 (which contained thiamine) is increased considerably. Depending upon the level of tissue saturation with thiamine, it may or may not exceed peak 3 in size. The last peak (no. 5) is of particular interest because this was not detected in our earlier work (1). We ascribe this to 2 reasons. First, our entire previous study was carried out at an intake level of 100-μg of thiamine daily. At this level the body stores were fully saturated with thiamine and a considerable portion of the intake appeared in peak 4. The large size of this peak may well have obscured and prevented the detection of peak 5. In addition, as will be discussed later, the percentage of the total urinary radioactivity contained in peak 5 decreased as intake increased. Thus, peak 5 would have been relatively small at the high intake level in the earlier studies.

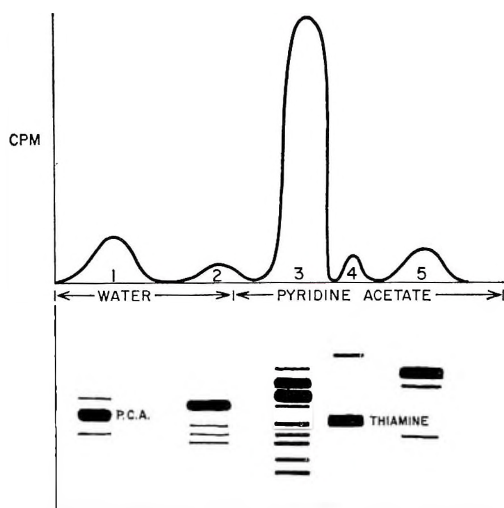

Fig. 2 Upper figure: Schematic representation of the column chromatographic separation of ^{14}C-pyrimidine-labeled urinary metabolites of thiamine on Amberlite CG-50. Lower figure: Schematic representation of autoradiograms of column peaks chromatographed in propanol·H_2O acetate buffer solvent.

A schematic representation of the pattern of radioactive metabolites in each peak obtained by paper chromatography and radioautography is also shown in figure 2. The quantitative aspects of this pattern are typical for rats whose body stores of thiamine are not saturated and are excreting relatively small amounts of thiamine. Of the 6 bands having the most radioactivity, only thiamine and pyrimidine carboxylic acid (PCA) have been identified. The 2 major unidentified radioactive bands in peak 3 are of considerable interest since, if thiamine itself is excluded, they are the principal quantitative end-products of thiamine metabolism in rat urine.

In table 2, the percentage distribution is shown of radioactivity in the 5 chromatographic peaks at the 5 levels of thiamine intake used. At the two lowest levels of intake (8 μg, 20 μg) it was necessary to pool 10 consecutive daily urine collections in order to obtain sufficient radioactive material for fractionation. At the 30-, 50- and 100-μg intake levels, 5-day urine collections were pooled and fractionated.

Peak 1, the principal component of which is pyrimidine carboxylic acid, accounted for about 20% of the intake at all dosage levels. Likewise, peak 3, which contains at least nine unidentified radioactive bands, consistently accounted for about 60% of the total urinary radioactivity. We have no explanation for the low value obtained at the 20-μg level (48%), but this represents the average of only 2 determinations. The thiamine-containing peak (no. 4) was not detected at the 8-μg level of intake and only a trace appeared when the intakes were 20, 30 or 50 μg, respectively. At the 100-μg level, 6.6% of the radioactivity appeared in this peak. In the previous study, approximately 50% of the urinary radioactivity was present as ^{14}C-thiamine (1). The reason for the low percentage in the present study is probably related to degree of tissue saturation. In our early study the rat tissues were fully saturated with thiamine. In the present study, even after 54 days of injection with 100 μg of thiamine, the tissues were not fully saturated.

The percentages of radioactivity in chromatographic peak no. 2 increased with intake. The percentage excretion at the 100-μg intake level was twice that at the 8-μg level. Conversely, the percentage of radioactivity in peak no. 5 declined with increasing intake. In this instance, there was a 7-fold difference in the percentage of excretion at intake levels of 8 μg and 100 μg, respectively.

The total microbiological activity of the urine at the 50-μg level of intake amounted to about 2% of that obtained by radioactivity measurement. This activity was found to be distributed principally among peaks 1, 3, and 4 with virtually no microbiological activity in peaks 2 or 5. The microbiological activity in peak 4 is to be expected since this peak contains free thiamine but there are obviously other thiamine metabolites in urine having trace amounts of activity. *L. viridescens* bioautographic data confirm this impression since most of the radioactive bands separated by paper chromatography have traces of microbiological activity except for the principal metabolites appearing in peaks 2 and 5.

The marked quantitative similarities of the urinary chromatographic radioactivity patterns observed at each level of intake infer that the same metabolites are found in each peak at all intake levels. To verify this conclusion, identical peaks obtained at different intake levels were compared by chromatography on the same sheet of paper. In this way it was found that peaks 2 and 5 showed identical chromatographic patterns at all intake levels indicating that there were no qualitative differences (figs. 3 and 4). Similar studies of peaks 1 and 3 were not possible because the relatively high urinary pigment-to-radioactivity ratios at these relatively low levels of intake prevented clean chromatographic separation on paper. Since the proportions of peaks

TABLE 2

Distribution of radioactive thiamine urinary metabolites in column chromatographic peaks (IRC 50)

Level of thiamine intake	No. of peaks averaged [1]	% of total content of radioactivity				
		Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
μg/day						
8	4	15.2	5.3	58.9	0	20.2
20	2	25.9	6.5	48.0	2.6	16.8
30	8	18.9	8.9	61.4	2.3	9.1
50	5	20.1	9.2	61.7	2.4	6.6
100	7	20.1	10.0	57.8	6.6	3.6

[1] At the 8- and 20-μg levels of intake, pooled 10-day collections were fractionated. At 30-, 50-, and 100-μg levels of intake, pooled 5-day collections were fractionated.

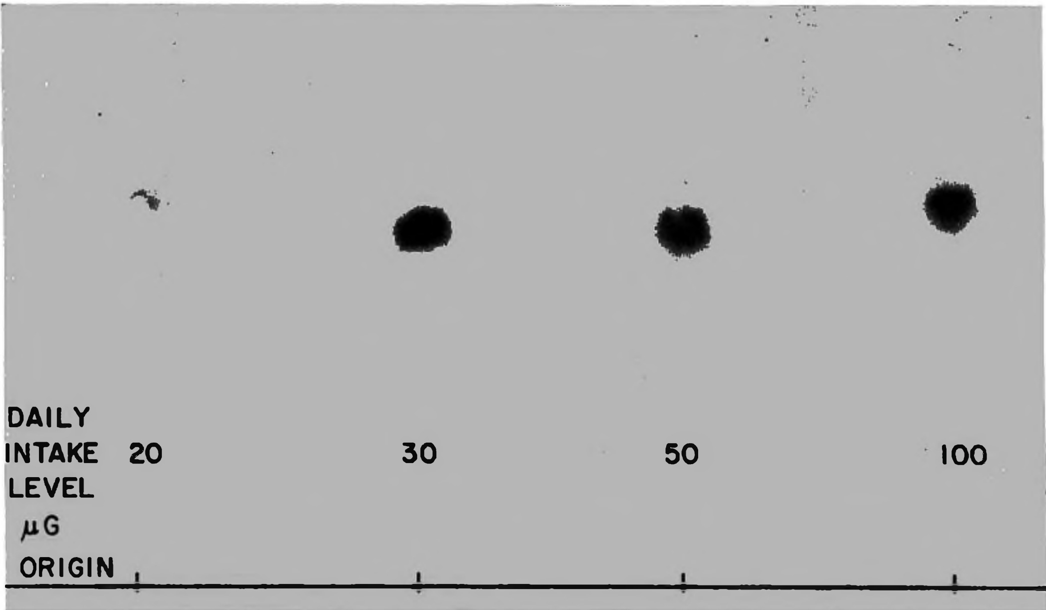

Fig. 3 Autoradiograms of paper chromatograms of urine IRC-50 peak no. 2 made at 4 levels of thiamine intake.

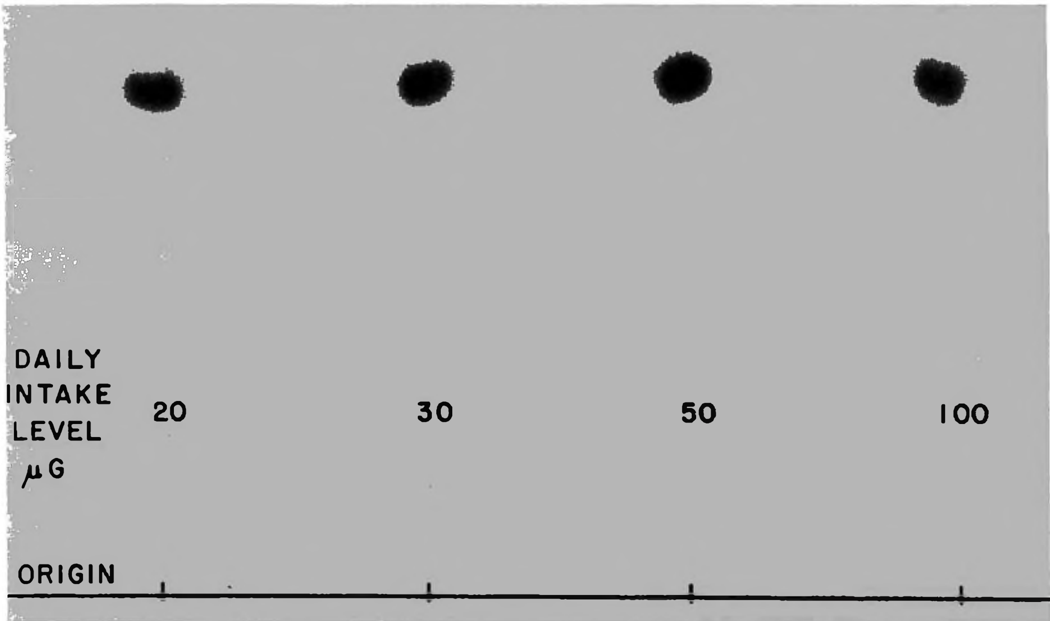

Fig. 4 Autoradiograms of paper chromatograms of urine IRC peak no. 5 made at 4 levels of thiamine intake.

1 and 3 were quite similar at the 4 intake levels it is probable, however, that the qualitative distribution of metabolites in the peaks was also similar.

To determine the effect of thiamine depletion on the urinary excretion pattern, thiamine injections were stopped in 4 animals that had been maintained with a daily dosage of 100 μg of radioactive thiamine for several months. With the animals still fed a thiamine-deficient diet, the excretions of peaks 1, 3 and 4 were followed daily for 30 days at which time the rats were severely deficient. The results of the study are shown in figure 5.

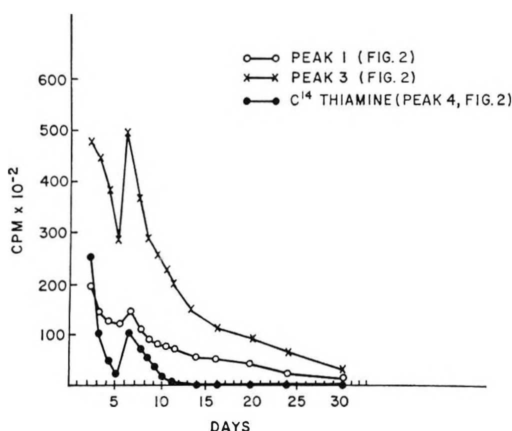

Fig. 5 Daily urinary excretion of ^{14}C-thiamine and metabolites by rats fed a thiamine-deficient diet.

The excretion of thiamine (peak 4) decreased very rapidly initially followed by a slow decline for the next 5 days. By day 10, the excretion of thiamine had for all practical purposes ceased. The excretion of radioactivity corresponding to peak 1 also fell very rapidly initially but then declined gradually thereafter. The radioactivity corresponding to the metabolites in peak 3 was maintained at a high level of excretion for a longer period and then began a rather rapid decline. The increased excretions observed on the sixth day of the experiment are unexplained.

After the thiamine excretion levels (peak 4) had reached levels approaching zero (day 10) the ratios of peak 3 to peak 1 remained quite constant for the duration of the study. This agrees, in general, with our observations at different thiamine intake levels which showed that when the intake level precludes the excretion of thiamine, the percentage of distribution of radioactivity among the urinary peaks is rather constant.

Table 3 shows the fecal excretion of ^{14}C activity at different levels of dietary thiamine intake. In this instance, the total feces collected during 8- to 10-day periods were pooled, extracted and counted. The excretion via this route approximated 13% at all levels of intake. The extract prepared from feces collected during the period of administration of 100 μg of thiamine was lost during processing. Data from previous studies, however, suggest that about 10% of the intake at this level was lost via this route.

To determine the radioactive forms of thiamine in the feces, the extracts were pooled, neutralized with NaOH and subjected to the same charcoal desalting procedure used with urine. After elution of the radioactivity from the charcoal with the pyridine-ethanol-water mixture the eluate, which contained 85 to 90% of the radioactivity on the charcoal, was reduced to low volume and chromatographed on a

TABLE 3

Relation between thiamine intake and fecal excretion of radioactivity

Mean rat weight	Level of intake	No. of samples averaged [1]	Mean daily fecal ^{14}C excretion expressed as thiamine	Mean fecal ^{14}C excretion
g	*μg/day*		*μg*	*% of intake*
217	8	3	1.22	15.2
314	20	6	2.53	12.6
394	30	8	3.97	13.2
437	50	7	5.94	11.9

[1] Pooled 8- to 10-day fecal samples were extracted for counting.

column of Amberlite CG-50 as described for urine. This extract contained considerably more extraneous material than did comparable urine extracts. The bulk of the radioactivity in the eluate appeared in 2 peaks which were in positions similar to peaks 1 and 3 obtained with urine on the same resin. The contents of the tubes in the first peak were pooled, evaporated to low volume and placed on Dowex-1 for further purification. The elution was carried out with 0.001 N HCl as described previously for urine (1) and a single, slightly yellow, radioactive peak was obtained. This was evaporated to dryness, taken up in a small volume of water and chromatographed on paper in *n*-propanol-acetate solvent. Radioautographs revealed a diffuse radioactive spot with an R_f value identical to that of authentic pyrimidine carboxylic acid chromatographed on the same sheet. The unknown spot was eluted, evaporated to low volume and co-chromatographed with authentic radioactive pyrimidine carboxylic acid in 2 other solvents (*n*-propanol/H_2O/1 M acetate; isobutyric acid/NH_4OH/H_2O). The R_f values of the spot obtained from feces extract, authentic PCA, and a mixture of the two were identical confirming the presence of this compound in rat feces. Although precise quantitative data were not obtained, it seems that about 15 to 20% of the total fecal radioactivity appears in this compound.

Treatment of feces peak 1 with mylase and rechromatography on Amberlite CG-50 resin did not yield a radioactive thiamine peak. Thus, radioactive forms of thiamine phosphate esters were not present. Also, the microbiological activity of feces peak 3 was only 4% of the potential thiamine content predicted from its radioactivity.

A radioactive thiamine peak (i.e., peak 4) was not observed in the feces extract. This observation suggests that if radioactive thiamine is excreted into the gut it is reabsorbed or catabolized, or both, rather than being incorporated as such by the intestinal bacteria. The absence of a radioactive thiamine peak also indicates that the catabolic products of thiamine formed by the rat cannot be used as thiamine precursors by the gastrointestinal microflora. Unfortunately, our data do not permit a distinction to be made between the rat and possible bacterial metabolites of thiamine. We have demonstrated previously that the urinary metabolites of thiamine are identical in germfree and conventional rats (1).

The results of the tissue thiamine assays by radioactivity and microbiological assay are shown in figure 6. Assays at all levels of intake except the 8-μg level are presented. The thiamine content of the liver, kidney, heart, gut and carcass increased as intake increased by both methods of assay but the thiamine content of the brain was apparently not related to the level of intake. This is consistent with the reports of Salcedo et al. (6) and deCaro et al. (7) who demonstrated that the thiamine content of the brain is maintained for a long period of time even in the thiamine-deficient animal.

In nearly all cases the thiamine levels obtained microbiologically were somewhat higher than the assays by radioactivity. Thus, the variety of microbiologically in-

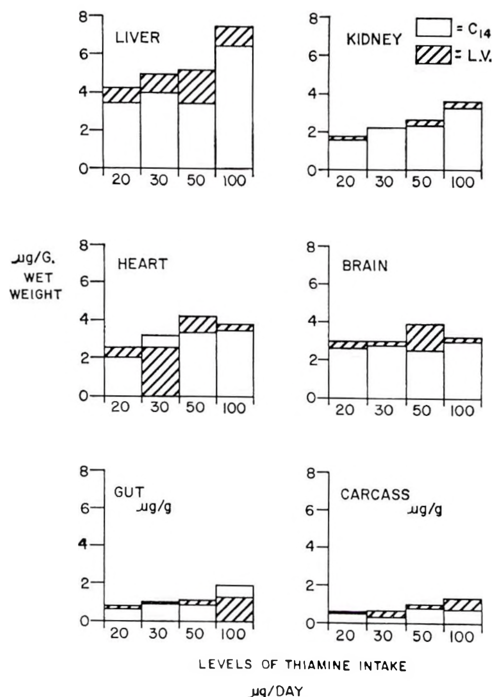

Fig. 6 Mean tissue levels of thiamine as determined by radioactivity and *L. viridescens* (L. V.) assay at 4 levels of intake. Mean values are from pairs of rats at each level of intake.

active thiamine metabolites found in urine do not accumulate in the tissues. Why the microbiological values are higher is not clear but 2 explanations may be considered. It is possible that intestinal synthesis and coprophagy might supply sufficient non-radioactive thiamine to reduce the specific activity of the tissue stores. The feces studies indicate that little radioactive thiamine would be recycled via this route. Conversely it may be presumed that some tissue forms of thiamine may have somewhat more microbiological activity than thiamine itself. Hydroxyethylthiamine, for example, has been reported to be about 80% as active as thiamine for *L. viridescens*, but Skeggs (8) has pointed out that this value was obtained by assay of a synthetic (presumably DL-form) and that the naturally occurring L-form might have twice the activity as thiamine. No decision can be made between these 2 alternatives on the basis of our data. Obviously, both situations might exist.

Attempts to separate tissue thiamine metabolites by column and paper chromatography with the techniques used with urine and feces were not satisfactory. It was discovered, for example, that the acid extraction procedure used resulted in the formation of free thiamine from thiamine phosphate esters and this compound was therefore the principal recognizable metabolite found on chromatography. No evidence for microbiologically inactive radioactive compounds was observed, however, confirming our previous conclusion that these compounds do not accumulate in the tissues.

Although there is a quantitative change in the amounts of peak 2 and peak 5 compounds in urine, the major thiamine metabolites in rat urine do not change qualitatively until relatively high tissue levels are reached. At this point, when the tissues become relatively saturated with thiamine, the urinary pattern is changed abruptly by the appearance of free thiamine. An approximate estimate of the level of tissue saturation required for this "overflow" of free thiamine is instructive. Using liver stores as a general index of tissue saturation, and assuming that 9 μg/g fresh weight of liver represents saturation (7, 9) it may be concluded that free thiamine is not excreted by the rat if the tissues are less than 75% saturated because our rats did not excrete thiamine until their liver stores were 7 μg/g. Rats having liver thiamine levels of less than 7 μg/g did not excrete free thiamine in the urine. Similar conclusions might be drawn from the data on the levels of thiamine observed in the carcass (which represents the greatest reservoir of body thiamine content), kidney and gut. On the other hand, the heart and brain thiamine levels show a somewhat different relationship to intakes and cannot by themselves be used as a valid index of total body stores.

The mechanisms involved in the degradation of thiamine remain to be investigated but it is evident that one pathway is via the removal of the thiazole moiety with a subsequent excretion of the pyrimidine residue as pyrimidine carboxylic acid. Under our conditions about 20 to 25% of that degraded appears to go through this route at several levels of intake as judged by the quantity of pyrimidine carboxylic acid noted in the urine and feces. The major degradation products of thiamine occur in peak 3 but the nature of these metabolites is not yet known.

Since it is probable that the same urinary metabolites of thiamine were excreted at all levels of intake and represented relatively consistent proportions of the intake, it is suggested that a quantitative measurement of metabolites contained in these peaks might offer better insight into tissue thiamine reserves than the conventional urinary thiamine method. This is particularly true when tissue thiamine levels are less than 75% of saturation since under these circumstances free thiamine does not occur in the urine. The principal compounds contained in peaks 1 and 3 appear to be most appropriate for study in this respect but these compounds have either not been identified (peak 3) or practical methods for their determination (PCA, peak 1) have not yet been developed. The direct translation of these observations to man are, at the moment, tenuous but it is encouraging that the gross excretion pattern of ^{14}C pyrimidine-labeled thiamine in man appears to resemble that noted in the rat (1).

LITERATURE CITED

1. Neal, R. A., and W. N. Pearson 1964 Studies of thiamine metabolism in the rat. I. Metabolic products found in urine. J. Nutrition, *83:* 343.
2. Neal, R. A., and W. N. Pearson 1964 Studies of thiamine metabolism in the rat. II. Isolation and identification of 2-methyl-4-amino-5-pyrimidine carboxylic acid as a metabolite of thiamine in rat urine. J. Nutrition, *83:* 351.
3. Deibel, R. H., J. B. Evans and C. F. Niven, Jr. 1957 Microbiological assay for thiamine using *Lactobacillus viridescens.* J. Bacteriol., *74:* 818.
4. Brody, S. 1945 Bioenergetics and Growth. Reinhold Publishing Corporation, New York, p. 504.
5 Shibata, N., S. Yamashita and K. Yasuda 1961 On the minimum requirement and treatment doses of thiamine for rats. J. Biochem. (Japan), *49:* 589.
6. Salcedo, J., V. A. Najjar, L. E. Holt, Jr. and E. S. Hutzler 1948 The relation between urinary excretion and tissue concentrations of thiamine in rats. J. Nutrition, *36:* 307.
7. deCaro, L., G. Rindi and L. de Giuseppe 1961 Contents in rat tissue of thiamine and its phosphates during dietary thiamine deficiency. Internat. Ztschr. Vitaminforsch., *31:* 333.
8. Skeggs, H. 1963 In: Analytical Microbiology, ed., F. J. Kavanagh. Academic Press, New York, p. 521.
9. Balaghi, M., and W. N. Pearson 1966 Tissue and intracellular distribution of radioactive thiamine in normal and thiamine-deficient rats. J. Nutrition, *89:* 127.

Effects of Methionine, Menhaden Oil and Ethoxyquin on Serum Cholesterol of Chicks [1]

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ABSTRACT A study was conducted to determine whether either a dietary antioxidant or a fish oil supplement would affect the hypercholesterolemic action of a suboptimal level of dietary methionine in chicks. A 2^3 factorial experiment was conducted with chicks fed a purified diet high in tallow, low in methionine and supplemented with methionine, ethoxyquin, and menhaden oil. Eight comparisons were made to determine how total serum cholesterol or fatty acid composition of tissue lipids were affected by the 3 variables, including interaction of one variable with another. Serum cholesterol determinations showed highly significant differences with high methionine resulting in lowered serum cholesterol values in all comparisons. The menhaden oil supplement also resulted in highly significant reductions of serum cholesterol values in all comparisons. These effects of menhaden oil and methionine supplementation were additive with no evidence of interaction. The reduction of cholesterol values by the added antioxidant was significant only when all 8 groups were compared. The added antioxidant did not counteract the hypocholesterolemic action of the fish oil, in contrast with a reported effect using rats. Fatty acid compositions of tissue lipids were largely determined by composition of the dietary lipids although there were some minor effects ascribable to methionine supplementation.

Dietary factors that affect blood cholesterol levels have been studied extensively. Low protein diets have resulted in hypercholesterolemia in swine (1), chicks (2-6), rats (7) and mice (8). The elevation of blood cholesterol by low protein diets may in large part be due to a lack of methionine (8-10), although some evidence is available (11) that these 2 effects may operate independently.

Marine oils (or their polyunsaturated fatty acids [PUFA]) have been shown to be strongly hypocholesterolemic (12-15). Nimni et al. (16) reported that the antioxidants N,N′-diphenyl-*p*-phenylenediamine (DPPD) and 1,2-dihydro-2,2,4-trimethyl-6-ethoxyquinoline (ethoxyquin) partially counteracted the hypocholesterolemic action of fish oil in rats, even in the presence of vitamin E in the diet.

The purpose of this study was to investigate the action of an antioxidant upon the hypocholesterolemic effect of added fish oil in chicks. The study was also designed to determine whether either the dietary antioxidant or fish oil would affect the action of a suboptimal level of methionine in increasing serum cholesterol, and whether there were any interactions between these dietary factors.

EXPERIMENTAL

A $2 \times 2 \times 2$ factorial experiment was conducted testing all possible combinations. One-day-old male New Hampshire-Columbian Rock crossbred chicks[2] were individually wing-banded, started in an electrically heated battery brooder and fed the basal diet and water for one week. At one week, the chicks were divided into 8 equal weight groups of 10 chicks/group and fed the experimental diets for 6 weeks. The lots were assigned at random to the 8 pens in the top 4 decks of a Jamesway brooder by the use of a random number table. The basal diet contained the following: (in g/kg) glucose,[3] 393; vitamin-free casein,

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[2] Obtained from the Department of Poultry Husbandry, University of Minnesota, St. Paul.

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

[4] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

200; gelatin, 100; non-nutritive bulk,[4] 20; cholesterol, 10; dried ox bile extract, 5; edible tallow, 200; chlortetracycline,[5] 2; minerals,[6] 66.4; and vitamins,[7,8] 3.6.

The basal purified diet was low in methionine plus cystine (0.68% calculated) at the high protein level used (27.5%). The accepted requirement of chicks for methionine ranges from 0.8% (17) to 0.96% (18, 19), or 3.5% of the total protein of the diet. Supplemental methionine (0.5%) was added, making the high methionine diet contain 1.2% methionine plus cystine. The antioxidant used was ethoxyquin[9] at a level of 0.05%. The edible tallow, cholesterol and dried ox bile were used to produce severe hypercholesterolemia in the chicks. The level of menhaden oil used was small, only 3%, since it has been shown that large amounts are not necessary to reduce serum cholesterol (20). All diets were isocaloric. The fatty acid compositions of the supplements are as follows: For the tallow supplement: (% of total fatty acids) 14:0, 3.0; 15:0, 0.9; 16:0, 25.9; 16:1, 3.9; 17:1, 0.6; 18:0, 18.8; 18:1, 40.9; 18:2ω6, 3.5; 20:0, trace; 18:3ω3, 1.1; 20:3ω6, trace; 20:4ω6, trace; 22:3ω6, 0.7; 24:0, 0.6; 24:1ω9, trace. For the 17% tallow and 3% menhaden oil: (% of total fatty acids) 14:0, 4.0; 15:0, 0.9; 16.0, 24.7; 16:1, 5.1; 17:1, 0.8; 18:0, 16.5; 18:1, 36.5; 18:2ω6, 3.4; 20:0, trace; 18:3ω3, 1.3; 21:0, 0.6; 20:3ω6, trace; 20:4ω6, trace; 20:5ω3, 0.3; 22:3ω9, 2.4; 22:3ω6, 0.6; 24:0, 0.6; 22:4ω6, trace; 22:5ω6, trace; 24:1ω9, trace; 22:5ω3, 0.3; 22:6ω3, 1.5. Trace amounts are those less than 0.3% of total fatty acids.

The design of the experiment is shown in table 1. The chicks were allowed feed and water ad libitum and were weighed weekly. The chicks were killed after 6 weeks on trial. Blood samples were taken, and samples of liver, heart, breast muscle and brain (cerebrum plus cerebellum) from each chick were quick-frozen on dry ice and stored at −20°.

Fatty acid analyses were conducted by gas chromatography using a Beckman GC-2A instrument with a hydrogen flame detector. A 183 × 0.6 cm (6′ × 1/4″) column of 10% silanized ethylene glycol succinate on Gas Chrom P, 80-100 mesh, operated isothermally at 175°, was used. The fatty acids were identified by comparisons with standards from The Hormel Institute and NIH,[10] or by comparisons of the calculated equivalent chain lengths (21). In the system of abbreviated notation used the first number represents the number of double bonds, and the number following the ω as the position of the first double bond from the methyl end of the fatty acid molecule.

Total serum cholesterol was determined by the Abel method (22) on individual chick serum samples. The tissue samples were pooled by groups for lipid extraction by chloroform-methanol (2:1), and methyl esters were prepared by the dry HC-methanol procedure.

Free radical determinations were made on individual liver homogenate samples (23) using the stable free radical α, α-diphenyl-β-picrylhydrazyl (DPPH) reagent, which measured a wide variety of DPPH-reactive materials (cysteine, glutathione, tocopherol, hydroquinone, pyrogallol, and other polyhydroxy aromatic compounds).

RESULTS AND DISCUSSION

The results of the experiment (except fatty acid analyses of tissue lipids) are shown in table 1. The growth data demonstrated that the basal diet was low in methionine, as all the low methionine (LM) groups grew poorly, whereas all the high methionine (HM) groups grew well. There were no significant differences in the weight gains within each methionine

[5] Aureomycin, American Cyanamid Company, Princeton, New Jersey.

[6] The final diets contained the following minerals: (g/kg) $CaHPO_4$, 28.4; KCl, 7.0; $CaCO_3$, 10.0; $Na_2HPO_4 \cdot 7H_2O$, 13.2; NaCl, 4.0; $MgSO_4 \cdot 7H_2O$, 3.0; $FeC_6H_5O_7 \cdot 3H_2O$, 0.2; $MnSO_4 \cdot 4H_2O$, 0.25; $ZnSO_4 \cdot H_2O$, 0.3; KIO_3, 0.01; $CuSO_4 \cdot 5H_2O$, 0.02; and $CoSO_4 \cdot 7H_2O$, 0.002.

[7] The final diets contained the following vitamins: (mg/kg) choline xanthate, 2000; biotin, 0.3; thiamine·HCl, 10; Ca pantothenate, 30; riboflavin, 10; pyridoxine·HCl, 10; niacin, 100; folacin, 5; vitamin K, 2; inositol, 250. Also added per kg of diet were vitamin B_{12}, 20 μg; stabilized vitamin A, 25,000 IU; stabilized vitamin D_3, 1000 ICU; and stabilized *d-α*-tocopheryl acetate, 68 IU.

[8] We are indebted for gifts of materials to the following: Merck, Sharp and Dohme, Rahway, New Jersey; Abbott Laboratories, North Chicago, Illinois; American Cyanamid Company, Princeton, New Jersey; Distillation Products Industries, Rochester, New York; Dawe's Laboratories, Inc., Chicago; and the Monsanto Company, St. Louis.

[9] Santoquin, Monsanto Chemical Company, St. Louis.

[10] Standards obtained through the courtesy of the NIH, Lipid Distribution Program, Dr. W. N. Goldwater, Bethesda, Maryland.

TABLE 1

Design and results of 2 × 2 × 2 factorial experiment

Menhaden oil status [1]	No fish oil				3% fish oil			
Ethoxyquin status [2]	− AO		+ AO		− AO		+ AO	
Methionine status [3]	LM	HM	LM	HM	LM	HM	LM	HM
Group no.	4	1	6	5	7	3	2	8
Avg gain in 6 weeks, g	494 ± 35 [4]	801 ± 27	488 ± 34	779 ± 30	459 ± 37	760 ± 42	468 ± 22	845 ± 34
Feed/gain ratio	2.32	1.62	2.34	1.64	2.45	1.71	2.36	1.66
% Lipid extracted, heart [5]	4.67	4.68	5.02	4.94	5.12	4.77	5.24	4.52
% Lipid extracted, liver [5]	12.20	11.86	11.67	9.76	12.03	11.72	12.97	13.42
Serum cholesterol, mg/100 ml	1586 ± 93	1083 ± 126	1439 ± 151	788 ± 95	993 ± 130	339 ± 20	757 ± 126	333 ± 27

[1] Menhaden oil added at 3% level at expense of equal amount of tallow.
[2] Ethoxyquin added at 0.05% level; − AO, no antioxidant added; + AO, antioxidant added.
[3] LM, low methionine diet; HM, high methionine diet.
[4] SE of mean.
[5] Samples were pooled by groups and used for fatty acid analysis.

treatment, indicating that the other 2 variables had no appreciable effect on weight gains. The feed-to-gain ratios further demonstrated that the diet was low in methionine, and that the HM groups utilized their feed much better. Antioxidant and fish oil supplements did not affect the feed-to-gain ratio within each methionine level. There were no conclusive differences in the proportions of extractable lipid from either the hearts or livers. Since these samples were pooled by groups, no statistical evaluation of the data can be made. Extractable lipid from liver tended to be more from the HM groups, but no difference was noted in the heart lipid.

Major differences in the serum cholesterol values were observed and are shown graphically in figure 1. Supplementation of the diet with methionine resulted in highly significant reductions ($P < 0.01$) in serum cholesterol values, both in the presence and absence of fish oil or antioxidant. That this effect was observed with a high protein diet excludes an effect of total protein per se. The addition of

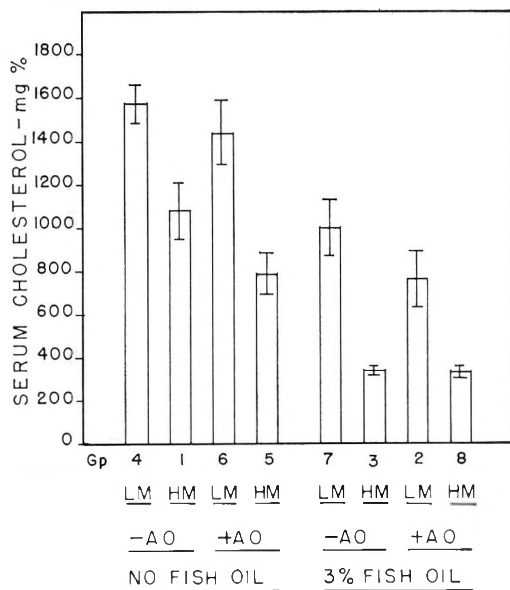

Fig. 1 Total serum cholesterol values (mg/100 ml) of chicks ± SE in 2 × 2 × 2 factorial experiment. LM, low methionine (0.68%); HM, high methionine (1.20%); − AO, no added antioxidant; + AO, + 0.05% ethoxyquin; no fish oil, basal ration without menhaden oil; + 3% fish oil, 3% menhaden oil replacing 3% tallow.

3% menhaden oil (+FO) also showed highly significant reductions ($P < 0.01$) in serum cholesterol in the absence or presence of the antioxidant or with either low or high methionine (LM and HM groups).

The effect of the added antioxidant was not as clear. In every comparison the ration with added ethoxyquin (+AO) caused lower cholesterol values than those groups without the added ethoxyquin (−AO), but the differences were not uniformly large (only 3 mg/100 ml for the groups fed high methionine and fish oil, but 147 to 295 mg/100 ml for the other 3 comparisons). Analysis of variance was calculated for the experiment (24). The F test values for the different treatments were: antioxidant effect, 5.63 ($P < 0.05$); methionine effect, 59.95 ($P < 0.01$); menhaden oil effect, 73.78 ($P < 0.01$). The F test for significance shows a significant overall difference in serum cholesterol values due to the presence of the added antioxidant ($P < 0.05$). The overall effect of the added antioxidant, always in the direction of lower serum cholesterol values, was opposite to the effect reported for rats by Nimni et al. (16).

The hypocholesterolemic effects of the fish oil supplement (+FO) and the increased methionine level (HM) were additive. The F values for the interaction terms in the analysis of variance were far from significant, indicating that there was no interaction between the 3 variables tested. The hypocholesterolemic effect of the fish oil was independent of growth.

TABLE 2

Total fatty acids in tissue lipids

Menhaden oil status [1]	No fish oil				3% fish oil			
Ethoxyquin status [2]	− AO		+ AO		− AO		+ AO	
Methionine status [3]	LM	HM	LM	HM	LM	HM	LM	HM
Group no.	4	1	6	5	7	3	2	8
	% of total fatty acids							
Liver								
Saturates	26.4	24.8	21.8	25.6	23.9	27.4	28.0	20.0
Monoenes	47.1	45.9	56.4	49.7	51.2	45.0	41.8	55.6
Total ω 3	5.3	4.9	3.4	5.2	10.1	11.7	10.1	11.3
Total ω 6	19.8	21.6	16.9	17.1	8.1	10.0	9.4	7.7
Total ω 9	1.2	1.6	0.7	1.5	5.8	5.5	5.2	4.8
Total PUFA	26.3	28.1	21.0	23.8	24.0	27.2	24.7	23.8
Heart								
Saturates	34.5	33.4	34.3	30.1	35.0	39.7	38.9	34.2
Monoenes	34.3	32.8	29.9	31.9	28.6	32.9	30.1	25.0
Total ω 3	1.7	2.0	1.8	2.4	7.9	5.7	6.9	5.2
Total ω 6	26.1	29.1	30.3	33.1	20.3	16.4	17.4	26.0
Total ω 9	1.6	2.1	2.1	2.2	7.2	3.7	5.0	8.8
Total PUFA	29.4	33.2	34.2	37.7	35.4	25.8	29.3	40.0
Muscle								
Saturates	31.6	36.0	30.5	32.6	35.5	35.0	35.0	31.0
Monoenes	35.8	31.8	30.2	30.5	33.3	30.1	29.8	30.3
Total ω 3	4.6	5.3	5.2	5.7	13.8	15.4	15.7	18.5
Total ω 6	23.6	22.4	28.6	26.8	10.2	11.1	11.4	11.4
Total ω 9	2.4	2.8	2.8	2.8	5.0	5.6	6.0	5.6
Total PUFA	30.6	30.5	36.6	35.3	29.0	32.1	33.1	35.5
Brain								
Saturates	38.2	41.2	48.1	43.1	40.9	41.3	42.5	42.4
Monoenes	22.6	21.2	17.7	17.3	19.7	19.8	18.2	20.5
Total ω 3	14.4	13.0	13.2	14.8	26.7	26.4	26.4	25.3
Total ω 6	20.7	19.6	18.6	21.2	8.7	8.6	9.1	8.1
Total ω 9	2.3	2.2	1.5	2.3	2.2	2.0	2.3	2.2
Total PUFA	37.4	34.8	33.3	38.3	37.6	37.0	37.8	35.6

[1] See table 1, footnote 1. [2] See table 1, footnote 2. [3] See table 1, footnote 3.

This was not true of the effect due to methionine supplementation because in every HM group the growth was better than in the LM groups.

The free radical concentrations of the individual liver samples were estimated. The mean values were variable and ranged from 4.0 to 8.1 microequivalents of DPPH-reactive material/g tissue, with no correlation with dietary treatment, although the four —AO groups averaged slightly higher (5.6) than the four +AO groups (4.8).

Fatty acid analyses of the tissue lipids were voluminous and are shown in condensed form in table 2. The fatty acid values were grouped in the following groups: saturates (14:0, 16:0, 17:0, 18.0 and 20:0), monoenes (16:1, 17:1 and 18:1), total $\omega 3$, linolenic acid family ($18{:}3\omega 3$, $20{:}5\omega 3$, $22{:}5\omega 3$ and $22{:}6\omega 3$), total $\omega 6$, linoleic acid family ($18{:}2\omega 6$, $20{:}3\omega 6$, $20{:}4\omega 6$, $22{:}3\omega 6$, $22{:}4\omega 6$ and $22{:}5\omega 6$), total $\omega 9$, oleic acid family ($20{:}3\omega 9$ and $22{:}3\omega 9$) and total PUFA.

Major differences arose from the inclusion of the 3% menhaden oil (+ fish oil) in the diet. In these groups the $\omega 3$ and $\omega 9$ PUFA were much higher than in the chicks not fed the fish oil. These differences were principally diminished levels of $18{:}2\omega 6$ and $20{:}4\omega 6$ with an increased level of $22{:}3\omega 9$, $22{:}5\omega 3$ and $22{:}6\omega 3$. These compositions reflected strongly the composition of the dietary lipid. The brain lipids were unique in that they contained very large amounts of $22{:}6\omega 3$, even in the absence of the fish oil supplement (12 to 13%), high levels of $20{:}4\omega 6$ (9 to 10% without dietary fish oil, 5 to 6% with dietary fish oil), and very low levels of $18{:}2\omega 6$ (linoleic acid, 0.3 to 0.8% in all groups).

The supplement of 0.05% ethoxyquin did not affect the fatty acid composition of the tissue lipids. This may be partially explained by the fact that the basal diet contained vitamin E (68 IU/kg), substantiated by the observation that none of the chicks showed signs of vitamin E deficiency. Furthermore, thiobarbituric acid analyses of liver homogenates from all groups showed no increased values after incubation at 37°, an indication that no lipid autoxidation took place.

Methionine supplements affected the tissue lipid composition most notably in the heart lipids. In the absence of fish oil, the HM groups had higher levels of PUFA than the LM groups, especially the $\omega 6$ acids. This was true also in the muscle lipids. When fed dietary oil and antioxidant, the HM groups had higher levels of $\omega 6$ and $\omega 9$ acids in the heart than in the absence of the antioxidant supplement. The differences in the liver and brain fatty acids in all groups were smaller and not consistent. The differences in fatty acid composition resulting from methionine supplementation were smaller than those resulting from menhaden oil supplementation.

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LITERATURE CITED

1. Barnes, R. H., E. Kwong, W. Pond, R. Lowry and J. K. Loosli 1959 Dietary fat and protein and serum cholesterol. II. Young swine. J. Nutrition, 69: 269.
2. Kokatnur, M., N. T. Rand, F. A. Kummerow and H. M. Scott 1958 Effect of dietary fat and protein on changes of serum cholesterol in mature birds. J. Nutrition, 64: 177.
3. Stamler, J., R. Pick and L. Katz 1958 Effects of dietary protein and carbohydrate level on cholesterolemia and atherogenesis in cockerels on a high-fat, high-cholesterol mash. Circulation Res., 6: 447.
4. Nishida, T., F. Takenada and F. Kummerow 1958 Effect of dietary protein and heated fat on serum cholesterol and beta-lipoprotein levels, and on the incidence of atherosclerosis in chicks. Circulation Res., 6: 194.
5. Leveille, G. A., and H. Fisher 1958 Plasma cholesterol in the growing chicken as influenced by dietary protein and fat. Proc. Soc. Exp. Biol. Med., 98: 630.
6. Leveille, G. A., A. S. Feigenbaum and H. Fisher 1960 The effect of dietary protein, fat and cholesterol on plasma cholesterol and serum components of the growing chick. Arch. Biochem. Biophys., 86: 67.
7. Moyer, A. W., D. Kritchevsky, J. B. Logan and H. R. Cox 1956 Dietary protein and serum cholesterol in rats. Proc. Soc. Exp. Biol. Med., 92: 736.
8. Leveille, G. A., and H. E. Sauberlich 1964 Plasma and liver lipids of mice as influenced by dietary protein and sulfur-containing amino acids. J. Nutrition, 84: 10.
9. Johnson, D., Jr., G. A. Leveille and H. Fisher 1958 Influence of amino acid deficiencies

and protein level on the plasma cholesterol level of the chick. J. Nutrition, 66: 367.
10. Seidel, J. C., and A. E. Harper 1962 Effects of ethionine and methionine on serum lipids and lipoproteins. Proc. Soc. Exp. Biol. Med., *111:* 579.
11. Leveille, G. A., J. W. Schockley and H. E. Sauberlich 1962 Influence of dietary protein level and amino acids on plasma cholesterol of the growing chick. J. Nutrition, *76:* 321.
12. Peifer, J. J., F. Janssen, P. Ahn, W. Cox and W. O. Lundberg 1960 Studies on the distribution of lipides in hypercholesterolemic rats. 1. The effect of feeding palmitate, oleate, linoleate, linolenate, menhaden, and tuna oils. Arch. Biochem. Biophys., *86:* 302.
13. Nicolaysen, R., and R. Ragård 1961 Effect of various oils and fats on serum cholesterol in experimental hypercholesterolemic rats. J. Nutrition, *73:* 299.
14. Peifer, J. J., F. Janssen, R. Muesing and W. O. Lundberg 1962 The lipid depressant activities of whole fish and their component oils. J. Am. Oil Chemists' Soc., *39:* 292.
15. Kahn, S. G., J. Vandeputte, S. Wind and H. Yacowitz 1963 A study of the hypocholesterolemic activity of the ethyl esters of the polyunsaturated fatty acids of cod liver oil in the chicken. J. Nutrition, *80:* 403.
16. Nimni, M. E., A. E. Mitta and M. Troparevsky 1961 Effect of short-term feeding of fish oils and of antioxidants on plasma and liver cholesterol in the rat. J. Nutrition, *73:* 243.
17. National Research Council, Committee on Animal Nutrition 1960 Nutrient requirements for poultry, pub. 827. National Academy of Sciences — National Research Council, Washington, D. C.
18. Reyntens, N., and G. deGroote 1962 The optimum levels of sulfur-containing amino acids in a high-energy diet for broiler chickens. Rev. Agr., *15:* 709.
19. Nelson, T. S., R. J. Young, R. B. Branfield, J. B. Anderson, L. C. Norris, F. W. Hill and M. L. Scott 1960 Studies on the sulfur amino acid requirement of the chick. Poultry Sci., *39:* 308.
20. Kahn, S. G. 1964 A study of the hypocholesterolemic activity of the ethyl esters of the polyunsaturated fatty acids of cod liver oil in the rat. J. Nutrition, *83:* 262.
21. Hofstetter, H. H., N. Sen and R. T. Holman 1965 Characterization of unsaturated fatty acids by gas-liquid chromatography. J. Am. Oil Chemists' Soc., 42: 537.
22. Abel, L. L., B. B. Levy, B. B. Brodie and F. E. Kendal 1952 A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. J. Biol. Chem., *195:* 357.
23. Mokadi, S., and P. Budowski 1963 Relationship between vitamin E and encephalomalacia in chicks. Brit. J. Nutrition, *17:* 347.
24. Snedecor, G. W. 1956 Analysis of variance of three-factor experiments. In: Statistical Methods in Agriculture and Biology, ed. 5 (chap. 12). Iowa State Press, Ames.

Tryptophan Utilization in a Threonine-induced Amino Acid Imbalance in Weanling Rats: Gain in Weight and Carcass Nitrogen with Two Feeding Methods [1]

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ABSTRACT The effect of a threonine-induced amino acid imbalance on tryptophan utilization in weanling rats was evaluated by determining weight gain and carcass nitrogen. Balanced and imbalanced niacin-free casein diets were either fed ad libitum or fed by a method in which the protein portion of the diet was given separately, twice daily, with the remainder of the diet available ad libitum. The depression in weight gain and food consumption, normally observed with ad libitum feeding of an imbalanced diet, was eliminated with separate feeding of the protein at levels equivalent to 0.4 to 0.8 g casein/day. Thus utilization of the protein did not appear to be impaired by the imbalance when the proteins were fed separately. When the percentage of the imbalanced mixture was increased in the diet of the ad libitum-fed animals, growth and food consumption were lower than those of the casein controls. Incorporation of niacin in the diet eliminated the adverse effects of the imbalance in the ad libitum-fed animals. Although there had been no depression in growth in the animals fed the proteins separately, the addition of niacin caused an increase in both weight gain and carcass nitrogen.

Minimal amino acid requirements have been determined for many species, but the effects of the changes in other nitrogenous components on these requirements have not been fully evaluated. A number of investigators have shown that the apparent requirement for tryptophan in the diets of rats is increased if various combinations of amino acids are added to a low protein diet. Most of the studies involved imbalanced diets in which tryptophan was the limiting amino acid, to which threonine or a protein low in tryptophan was added. Sauberlich and Salmon (1), using casein diets supplemented with corn or gelatin, attributed the increased tryptophan requirement to the increased percentage of ingested tryptophan which was excreted in the urine. They also suggested that the increase in tryptophan excretion could not account entirely for the decrease in growth, but that perhaps an increased rate of catabolism of amino acids had also occurred. Florentino and Pearson (2), using isotopically labeled tryptophan and a low casein diet, to which threonine was added, also showed that a higher percentage of ingested tryptophan was excreted in the urine. Wilson et al. (3), however, in a similar isotopic experiment, concluded that there was no increase in the rate of tryptophan excretion in the urine, no evidence of increased catabolism of tryptophan, and that the ingested tryptophan in the mixture was well utilized for protein synthesis.

In the series of experiments reported here, a quantitative study was made of the change in tryptophan requirement with changes in the amino acid pattern and in the level of nitrogen fed. Most of the previous experiments had been conducted with ad libitum-fed animals, in which total food intake varied with changes in the level of tryptophan in the diet. In the present studies all of the dietary ingredients were available ad libitum, except the protein moiety. Protein was fed separately, in controlled amounts, so that the effect of protein per se would be separated from the change in intake of all other dietary constituents.

EXPERIMENTAL PLAN

Male weanling rats of the Holtzman strain, weighing 45 to 55 g, were used in

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all experiments. For 2 to 3 days, animals were fed ad libitum an adequate pre-experimental diet containing 12% casein with sucrose and cornstarch as carbohydrates. In the experimental period 2 feeding methods were employed: 1) the entire diet was available ad libitum; and 2) the protein was fed separately in 2 weighed portions at 8:00 AM and 5:00 PM while other dietary components were available ad libitum at all times.

Each experimental group consisted of 5 animals and the average weight between groups did not vary by more than one gram. Weight gain and food consumption were measured individually 3 times weekly. Carcass nitrogen was determined at the end of the 2-week experimental period.

MATERIALS AND METHODS

Basal diets. The non-protein (basal) diet had the following percentage composition: sucrose, 87.6; salts B (4), 5.0; fat-soluble vitamins in corn oil, 5.0; choline chloride 0.15; niacin-free vitamin mix in sucrose (4), 0.25; and cellulose, 2.0; The fat-soluble vitamin mixture provided 400 IU vitamin A, 200 IU of vitamin D and 10.0 mg of α-tocopherol/100 g of diet.

Protein mixtures. Two protein mixtures were used in all the experiments. One mixture, which will be referred to as Cas, or balanced protein, contained vitamin-free casein and DL-methionine, and the other, referred to as Cas-threonine, or imbalanced protein, contained casein, DL-methionine and a defined amino acid mix. This mix, partially based on the amount and proportion of amino acids found in 12% gelatin, was known to depress growth in weanling rats when added to low casein diets (5). The proportions used in the 2 protein mixtures were as follows:

Casein (Cas):	
Vitamin-free casein	8.0
DL-Methionine	0.3
Total	8.3

Casein-threonine (Cas-threonine):	
Vitamin-free casein	8.000
DL-Methionine	0.300
DL-Threonine	0.360
DL-Phenylalanine	0.140
L-Lysine·HCl	0.162
L-Histidine·HCl	0.038
Total	9.000

The amount of Cas and of Cas-threonine mixture added to the diets was on the basis of the casein content. In ad libitum feeding, the percentage of casein in the diets was equal; in the separate feeding of protein, the actual amount of casein ingested was equal.

Food consumption. When the protein was fed separately, weighed portions were given to the animal twice daily, and the allotment was usually consumed within 5 to 10 minutes. The non-protein portion of the diet was fed ad libitum, and food consumption records were kept. Food spillage was collected daily for each group and total consumption figures corrected at the end of the experiment.

Carcass nitrogen. The animals were decapitated, the liver was removed for other determinations, and the remainder of the carcass was analyzed by a modification of Mickelsen's method (6). Carcass nitrogen was determined on individual animals or by experimental groups; when the latter method was used, each carcass was ground separately and homogenates were pooled before samples were taken.

RESULTS

Effect of graded levels of dietary tryptophan on weight gain and food consumption in rats fed ad libitum diets. In previous studies of tryptophan utilization with imbalanced diets, the level of the most limiting amino acid and the total protein content of the diet influenced the rate of growth (7, 8). In addition, a critical balance between tryptophan and threonine had been shown to exist when either casein or an amino acid diet was the nitrogen source (2, 9); in both studies a critical amount of tryptophan was needed to prevent the growth depression.

In the first experiment, the level of tryptophan necessary to overcome the growth depression with 9% of the Cas-threonine mixture fed ad libitum was studied. The results are shown in table 1. With 0.02% of tryptophan added to the Cas-threonine diet, the gain in weight was equivalent to that with the control Cas diet. The total food consumption of the imbalanced groups with added tryptophan increased, and the group with 0.02% tryptophan consumed an amount

TABLE 1

Effect of graded increments of tryptophan on weight gain and consumption of weanling rats fed a 9% Cas-threonine [1] diet, ad libitum

Group	Tryptophan added	Wt gain	Food intake
	%	*g/2 weeks*	*g/2 weeks*
1	0	10.0 ± 1.1 [2]	104
2	0.02	28.4 ± 5.0	127
3	0.03	49.8 ± 3.8	173
4	0.04	53.8 ± 1.1	180
5 [3] (control)	0	27.4 ± 2.9	136

[1] Cas-threonine contained vitamin-free casein, 8.0; DL-methionine, 0.3; DL-threonine, 0.36; L-Lysine·HCl, 0.16; DL-phenylalanine, 0.14; and L-histidine·HCl, 0.038.
[2] Averages ± SEM; 5 rats/group.
[3] Cas control contained vitamin-free casein 8.0; and DL-methionine 0.3, fed as 8.3% of the diet.

TABLE 2

Effect of graded levels of a Cas-threonine (imbalanced) mixture on weight gain and food consumption of weanling rats fed ad libitum

Group	Protein mixture [1]		Wt gain	Food consumption		mg Try [2]/g wt gain
	Cas	Cas-threonine		Total	Casein	
	%	%	*g/2 weeks*	*g/2 weeks*	*g/day*	
Without niacin						
1	8.3	—	35.7 ± 3.7 [3]	134	0.77	3.3
2		9.0	9.8 ± 1.9	80	0.46	7.2
3		10.5	21.8 ± 1.1	80	0.53	3.8
4		12.0	22.7 ± 3.6	80	0.61	4.2
5		13.5	34.6 ± 1.2	100	0.86	3.8
With niacin [4]						
6		9.0	48.8 ± 1.9	133	0.76	2.4
7		10.5	66.3 ± 2.4	155	1.03	2.4
8		12.0	63.8 ± 3.9	139	1.06	2.6
9		13.5	70.8 ± 5.2	143	1.23	2.7

[1] Cas contained vitamin-free casein, 8; and DL-methionine, 0.3. Cas-threonine contained vitamin-free casein, 8.0; DL-methionine, 0.3; DL-threonine, 0.36; L-lysine·HCl, 0.16; DL-phenylalanine, 0.14; and L-histidine·HCl, 0.038.
[2] Tryptophan content of Cas was 10.67 mg/g. Vitamin-free casein contained 1.3 g tryptophan/ 16 g N (20), 14.57% N (dry basis) and 6% moisture.
[3] Averages ± SEM; 5 rats/group.
[4] 2.5 mg nicotinic acid/100 g diet.

similar to that of the Cas control. Thus the increase in weight gain could be attributed to either a more favorable tryptophan-to-threonine ratio, or to an increase in tryptophan and total food intake.

Since no work had been carried out on growth depression when the ratio between casein and the "imbalanced mixture" was kept constant and the total nitrogen intake varied, the threonine-containing amino acid mixture was added to the intact protein and graded amounts of the entire mixture (Cas-threonine) added to the basal diet and fed ad libitum. In this way, the level of casein in the diet (and thus the tryptophan) could be varied, but the ratio of the casein or tryptophan to the threonine mixture was maintained. Results are shown in table 2. Weight gain declined from 35.7 g/rat/2 weeks (group 1) with the 8.3% Cas diet to 9.8 g with the 9% Cas-threonine diet (group 2), although the percentage of the casein in both diets was the same. Only at the highest level of Cas-threonine (group 5), equivalent to 12% Cas, did growth reach that of the 8.3% Cas control. Weight gain increased as the percentage of Cas-threonine mixture in the diet increased, but growth was not linear to actual casein intake. The depression in weight gain when Cas-threonine was fed ad libitum was accompanied by a decrease in the amount of food ingested. At all levels of Cas-threonine fed, food consumption was below that of the control group.

In these ad libitum studies, increasing the tryptophan-to-threonine ratio increased growth to that of the controls but maintaining the tryptophan-to-threonine ratio and increasing the total amount in the diet did not have as favorable a response. Apparently there was an increased requirement for tryptophan when these dietary mixtures were fed.

Effect of graded levels of Cas or Cas-threonine on growth, food consumption and tryptophan utilization. The previous results showed that Cas-threonine fed ad libitum caused a depression in weight gain and food consumption when compared with equivalent amounts of casein fed without the threonine mixture. With ad libitum feeding, the effect of the Cas-threonine per se could not be separated from the effect of an altered intake of both calories and protein, but separation would be possible if the amount of protein consumed could be controlled. The separate-feeding method described earlier was used. Five levels of the Cas-threonine were chosen to cover the range of protein intake of the animals fed this mixture ad libitum in the previous experiment. At each level, a corresponding group was fed an equal amount of Cas without the amino acid mixture (table 3). Groups 13 and 14 in which the protein was combined with the non-protein part of the diet and fed ad libitum served as controls.

With ad libitum feeding, weight gain declined from 35.5 g with Cas (group 13) to 15.2 g with Cas-threonine (group 14). However, no growth depression was observed when Cas-threonine was fed separately from the non-protein portion of the diet. In fact, weight gain was somewhat higher in animals fed the Cas-threonine mixture than in those fed Cas alone. With each protein source, gain in weight was linear to both the casein (fig. 1) and the nitrogen intake (table 3) over a wide range of intakes. In separate feeding, the level of intake of protein did not appear to influence the amount of the non-protein portion of the diet ingested (fig. 1); however, the particular protein mixture did have an effect. The animals fed Cas

TABLE 3

Weight gain, food consumption and tryptophan utilization of weanling rats fed controlled amounts of a Cas or Cas-threonine [1]

Diet no. [2]	Protein mixture [1]		Wt gain	Carcass nitrogen	Food intake		mg Try [3]/ g gain
	Cas	Cas-threonine			Protein mixture	Non-protein	
	g/day	*g/day*	*g/rat/2 weeks*	*g/rat*	*g/rat/2 weeks*		
			Fed by separate-feeding method				
1	0.84	—	34.2 ± 1.0 [4]	2.46	11.76	108	3.7
2	—	0.90	37.2 ± 2.1	2.63	12.60	105	3.3
3	0.72	—	26.6 ± 1.1	2.28	10.08	109	4.0
4	—	0.78	33.5 ± 0.7	2.58	10.92	96	3.2
5	0.60	—	21.4 ± 1.7	2.13	8.40	110	4.2
6	—	0.64	26.4 ± 1.6	2.37	8.96	97	3.3
7	0.48	—	17.8 ± 1.4	1.99	6.72	107	4.0
8	—	0.52	20.8 ± 1.0	2.16	7.28	92	3.4
9	0.36	—	11.0 ± 1.0	1.84	5.04	103	4.9
10	—	0.40	14.8 ± 1.6	1.94	5.60	99	3.7
11	0.48 [5]	—	15.0 ± 0.4	1.98	6.72	112	4.8
12	—	0.52 [5]	24.0 ± 1.4	2.23	7.28	109	3.0
			Fed ad libitum [6]				
13	0.80	—	35.5 ± 5.9	2.43	11.14	123	3.3
14	—	0.54	15.2 ± 2.0	1.96	7.54	76	4.9

[1] The percentage of the total food intake attributable to Cas in each of these diets is as follows: diets 1–10: 9.8, 10.0, 8.5, 9.4, 7.1, 7.9, 5.9, 6.8, 4.7, 4.8; diets 11, 12: 5.7, 5.8; and diets 13, 14: 8.3, 8.3.

[2] Cas contained vitamin-free casein, 8; and DL-methionine, 0.3. Cas-threonine contained vitamin-free casein, 8.0; DL-methionine, 0.3; DL-threonine, 0.36; L-lysine·HCl, 0.16; DL-phenylalanine, 0.14; and L-histidine·HCl, 0.038.

[3] Tryptophan content of Cas was 10.67 mg/g. Vitamin-free casein contained 1.3 g tryptophan/16 g N (20), 14.57% N (dry basis) and 6% moisture.

[4] Averages ± SEM; 5 rats/group.

[5] 2.5 mg nicotinic acid/100 g diet added.

[6] Protein mixture incorporated in diet and fed ad libitum.

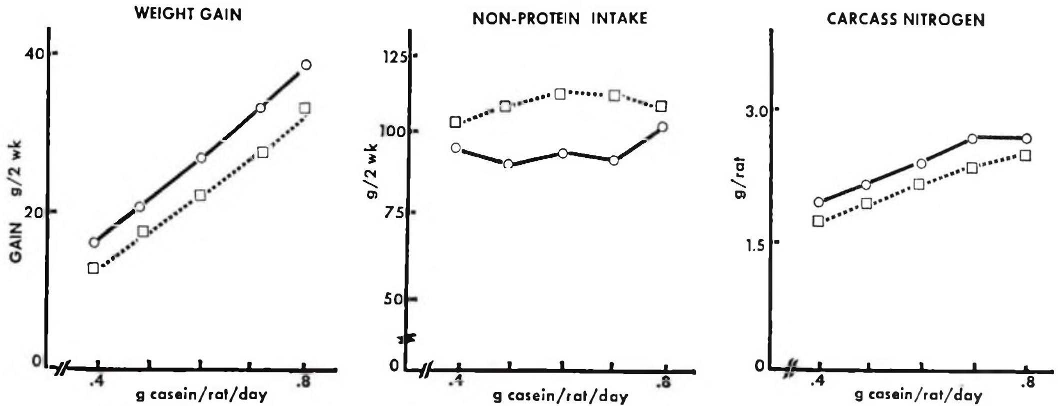

Fig. 1 Weight gain, non-protein food consumption and carcass nitrogen of rats fed graded amounts of Cas or Cas-threonine mixture by a separate-feeding method. Cas, □ – – – – □; Cas-threonine, ○ —— ○.

ate more non-protein diet than those fed Cas-threonine at any comparable level of protein intake. Therefore, although it appeared that there was a somewhat lower food consumption in the animals fed the Cas-threonine mixture, the gain in weight of the animals was equivalent to that of the controls fed Cas.

To determine whether weight gain was indicative of an increase in body protein, the total amount of nitrogen in the carcass was determined. With separate feeding, at each level of protein, animals fed Cas-threonine had more carcass nitrogen than those fed Cas (fig. 1). It appeared that the growing animal could incorporate nitrogen into the carcass from the Cas-threonine mixture as efficiently as from the Cas mixture alone. In these experiments, casein was the only source of tryptophan in the diet. With controlled protein intake, utilization of tryptophan for protein synthesis as measured by carcass nitrogen was greater in animals fed Cas-threonine than in those fed Cas.

Effect of addition of niacin to controlled amounts of Cas-threonine. The addition of niacin to imbalanced diets involving tryptophan has been shown to improve the growth rate (8). Most of the studies, however, were made with ad libitum feeding conditions, in which there was a concomitant increase in both total food intake and tryptophan intake. It was thus difficult to dissociate the effect of niacin itself from the increased intake of tryptophan.

The addition of niacin to the diet of rats fed Cas-threonine either ad libitum (table 2) or in separate feedings (table 3) increased both growth and food consumption. As niacin was used with only one nitrogen level with the separate feeding method in the second experiment, the last experiment was designed to extend the observations with separate feedings to other levels of protein intake with niacin added. Weanling rats were divided into 7 groups as shown in table 4 and fed the protein portion of the diet separately. Niacin (0.25 mg) was added to the protein portion of one group at each level. The amount of niacin added was based on an average daily food consumption of approximately 10 g of a diet containing 2.5 mg of nicotinic acid/100 g.

At all levels of Cas-threonine the addition of niacin increased growth (table 4), although no growth depression had been obtained when the imbalanced diet (Cas-threonine) was fed separately. The addition of niacin to the Cas control also increased growth, but the increase was not as great as that obtained when niacin was added to Cas-threonine. At all levels, animals fed Cas-threonine with niacin consumed more non-protein food than the corresponding group without niacin. There was also an increase in food intake when niacin was added to Cas, but this increase was not as great as when niacin was added to Cas-threonine.

TABLE 4

Effect of niacin on weight gain, food consumption and tryptophan utilization of rats fed controlled amounts of Cas or Cas-threonine [1] by separate-feeding method

Group	Protein mixture [1]		Wt gain	Carcass nitrogen	Food intake		mg Try [2]/ g gain
	Cas	Cas-threonine			Protein mixture	Non-protein	
	g/day	*g/day*	*g/2 weeks*	*g/rat*	*g/2 weeks*	*g/2 weeks*	
1	—	0.46	11.0 ± 2.2 [3]	1.85	6.44	82	5.8
2	—	0.46 [4]	23.2 ± 1.6	1.95	6.44	116	2.7
3	—	0.68 [4]	35.4 ± 1.4	2.32	9.32	127	2.6
4	—	0.90	32.6 ± 4.4	2.34	12.60	98	3.8
5	—	0.90 [4]	44.0 ± 1.9	2.60	12.60	130	2.8
6	0.80	—	31.2 ± 2.2	2.24	11.20	105	3.8
7	0.80 [4]	—	35.8 ± 0.7	2.19	11.20	120	3.3

[1] Cas contained vitamin-free casein, 8; and DL-methionine, 0.3. Cas-threonine contained vitamin-free casein, 8.0; DL-methionine, 0.3; DL-threonine, 0.36; L-lysine·HCl, 0.16; DL-phenlyalanine, 0.14; and L-histidine·HCl, 0.038.

[2] Trytophan content of Cas was 10.67 mg/g. Vitamin-free casein contained 1.3 g tryptophan/16 g N (20), 14.57% N (dry basis) and 6% moisture.

[3] Averages ± SEM; 5 rats/group.

[4] 0.25 mg nicotinic acid/day added to protein.

Since the addition of niacin to Cas-threonine increased both weight gain and non-protein food consumption, carcass nitrogen was determined to learn whether changes in weight were reflected in increased incorporation of nitrogen into body protein. The results (table 4) show that carcass nitrogen was greater when niacin was added to Cas-threonine than when niacin was omitted and the increase was greater at the higher level of protein intake. In contrast, when Cas was the protein source, carcass analysis showed that the increase in weight when niacin was added was not due to increased body nitrogen (table 4).

DISCUSSION

Amino acid imbalances, which have been characterized by a depression in growth and food consumption, result from an apparent increased requirement for the most limiting amino acid in the diet. Investigations with several proteins and amino acid combinations which cause growth depressions have been reviewed recently (10), i.e., fibrin and an amino acid mixture lacking histidine, casein and an amino acid mixture lacking threonine, and casein and an amino acid mixture lacking tryptophan. Of these combinations, only the imbalance which involves tryptophan is further complicated by the relationship with a vitamin. Thus in addition to the possible alteration of the requirement of tryptophan for protein synthesis, a further possible effect would be on the niacin requirement. Despite differences in mixtures used, all the amino acid imbalanced mixtures fed ad libitum have been characterized by reduced food consumption in the aminal.

Many studies have been made in an attempt to equalize the food consumption in the animals fed the imbalanced diets to that of animals fed the balanced or corrected diet. Kumta and Harper (11), using a fibrin-amino acid imbalance, increased food intake by injecting animals with insulin, and found that the animals fed the imbalanced protein diet had food intakes and growth rates similar to those of the controls. Klain et al. (12) placed the animals in a cold environment and found that with the fibrin-amino acid imbalance both food consumption and growth were restored to those of animals fed a fibrin control diet. Kumta (13), measuring nitrogen retention, reported that when balanced and imbalanced diets were pair-fed, the retention with the imbalanced diet was equal to or slightly greater than with the balanced diet.

The experimental conditions in the above experiments might have influenced the utilization of the imbalanced mixtures. In the studies in the present paper, however, protein intake was controlled without causing stress conditions or limiting the intake of any dietary component. The imbalanced protein was as well or better utilized than the balanced protein, and there was no indication of an increased requirement for tryptophan.

Although the imbalanced protein mixtures fed separately appeared to be well utilized, the same mixtures fed ad libitum in the diet caused a growth depression. In isotopic studies on the utilization of tryptophan from imbalanced diets by Florentino and Pearson (2) and Wilson et al. (3), two apparently opposing results have also been reported. Florentino, using labeled tryptophan incorporated in the diet and fed ad libitum on the last day of the experiment, showed that the animals fed the imbalanced diet excreted a greater percentage of the ingested tryptophan than the control animals. In contrast, Wilson, also using radioactive tryptophan, tube-fed a measured amount of the diet on the last day of the experiment, and showed neither an increased excretion of tryptophan, nor excessive catabolism of tryptophan. Thus when animals were fed ad libitum, as done by Salmon (7) and Florentino (2) and as shown in this paper, there appeared to be a decreased utilization of tryptophan, or an increased requirement per gram gain in weight. On the other hand, when a measured amount of protein was fed in a short period of time as in the force-feeding used by Wilson, or by the separate feeding method used in this paper, animals that were fed the imbalanced mixture utilized tryptophan as efficiently as controls. It is thus difficult to know whether the results in Wilson's paper, in which a change in feeding method occurred on the last day of the experiment, or the results in this paper with separate feeding, are a true measure of the fate of tryptophan from an imbalanced amino acid mixture fed ad libitum to the animal.

From the information available it is not known whether protein fed in a single meal is utilized differently from the same protein in an ad libitum situation nor whether the animal adapts to the imbalanced protein mixture. Studies which investigated these aspects will be reported in a subsequent paper.

In the separate-feeding method used here, the effect of niacin on the utilization of tryptophan was also investigated using conditions in which both niacin and tryptophan intakes were controlled. When niacin was added to the casein diets (i.e., balanced protein) little or no increase in weight gain was obtained, and there was no effect on the total carcass nitrogen. When niacin was added to the casein-threonine diet (i.e., imbalanced protein), in which no growth depression had occurred with the separate feeding technique, growth was increased, and this was reflected in an increase in the nitrogen in the carcass.

Thus, though there had been no growth depression with the imbalanced diet, the addition of niacin did cause a substantial increase in weight gain and carcass nitrogen. In the imbalanced mixture, extra threonine, which Harper (14) found to be either the second or third limiting amino acid in casein, was added. If tryptophan utilization was improved by adding niacin, and protein synthesis was no longer limited by threonine, an increase in carcass nitrogen with the addition of niacin to the casein-threonine diet might be expected. With the casein diet, which contained no additional threonine, threonine may have been the limiting amino acid. Therefore, the addition of niacin to this diet would not be expected to increase nitrogen incorporation into body protein.

Amino acid imbalances involving tryptophan in these and other studies have been alleviated in 3 ways: 1) by adding tryptophan to the diet of ad libitum-fed animals; 2) by adding niacin to the diet of ad libitum-fed animals, and 3) by feeding the protein portion of the diet separately with the other components ad libitum. Neither the cause of the imbalance nor the reason each of the treatments has been successful has been shown. Studies with animals fed similar imbalanced diets ad libitum led Morrison and associates (15) to suggest that the imbalances were not caused by a defect in the tryptophan-to-niacin pathway since no depression in the concentration of liver pyridine nucleotides (NAD) was observed. It had been assumed at that time that nicotinic acid was an intermediary metabolite in the pathway of tryptophan to NAD, and thus niacin would be formed prior to the formation of NAD. If niacin was then equally available for all parts of the body, liver NAD would directly reflect the availability of niacin. Recent work by Nishizuka (16)

indicates that niacin is not an intermediate in the pathway from tryptophan to NAD but enters at the nicotinic acid mononucleotide stage (NA MN). Thus nicotinic acid is not formed from tryptophan prior to the formation of NAD, but from the breakdown of NAD itself. Wagner (17) has reported that, in liver slices, tryptophan pyrrolase is inhibited by NA MN and NAD, but not by nicotinic acid itself. The relative effects of niacin and tryptophan in the intact animal on the formation of these intermediates with the subsequent inhibition of tryptophan pyrrolase is not known. In previous studies, the addition of a physiological level of niacin did not alter liver pyridine nucleotides (15), but did increase blood pyridine nucleotides (8) and in addition increased total food intake. Although part of the action of niacin, in increasing tryptophan utilization, may be in the formation of NAD in the liver and thus the inhibition of tryptophan pyrrolase, a significant action on the utilization may be at some other site in the body. Most of the studies on tryptophan-niacin interrelationships were made with ad libitum experiments which were complicated by the increase in tryptophan ingestion when niacin was added.

The alleviation of the imbalance when the protein portion of the diet was fed separately may have been due to a difference in the tryptophan metabolic pathways when the protein (or tryptophan) was ingested in this manner. Decreasing the amount of protein fed at each feeding in the separate-feeding method did not appear to decrease utilization; however, increasing the concentration of the protein in the diet of ad libitum-fed animals did not restore growth to that of the controls. The total food intake of the animals on these experiments was not controlled with either feeding method. There were wide variations of food intake both within and between groups, but the influence of the caloric intake on the utilization of the imbalanced diets has not yet been studied.

Since differences in the utilization of the protein appear to be attributable, at least in part, to the method of feeding, additional studies on the influence of feeding pattern on the utilization of amino acids seem necessary to aid in the interpretation of experimental animal studies to human nutrition problems. While there is some indication that amino acid imbalances may occur in humans (18, 19), imbalanced conditions have been difficult to show.

LITERATURE CITED

1. Sauberlich, H. E., and W. D. Salmon 1955 Amino acid imbalance related to tryptophan requirement of the rat. J. Biol. Chem., *214:* 463.
2. Florentino, R. F., and W. N. Pearson 1962 Effect of threonine-induced amino acid imbalance on excretion of tryptophan metabolites by the rat. J. Nutrition, *78:* 101.
3. Wilson, R. G., J. S. Wortham, D. A. Benton and L. M. Henderson 1962 Effect of threonine-induced amino acid imbalance on the distribution of isotope from DL-tryptophan-5-C^{14}. J. Nutrition, *77:* 142.
4. Harper, A. E. 1959 Amino acid balance and imbalance. I. Dietary level of protein and amino acid imbalance. J. Nutrition, *68:* 405.
5. Morrison, M. A., and A. E. Harper 1960 Amino acid balance and imbalance. IV. Specificity of threonine in producing an imbalance in diets deficient in niacin and tryptophan. J. Nutrition, *71:* 296.
6. Mickelsen, O., and A. A. Anderson 1957 A method for preparing intact animals for carcass analysis. J. Lab. Clin. Med., *53:* 282.
7. Salmon, W. D. 1954 The tryptophan requirement of the rat as affected by niacin and the level of dietary nitrogen. Arch. Biochem. Biophys., *51:* 30.
8. Morrison, M. A., M. S. Reynolds and A. E. Harper 1963 Effect of increments of tryptophan and niacin on growth and on concentrations of blood and liver pyridine nucleotides. J. Nutrition, *80:* 441.
9. Koeppe, O. J., and L. M. Henderson 1955 Niacin-tryptophan deficiency resulting from imbalances in amino acid diets. J. Nutrition, *55:* 23.
10. Harper, A. E. 1964 Amino acid toxicities and imbalances. In: Mammalian Protein Metabolism, vol. 2. Academic Press, New York, pp. 87–134.
11. Kumta, U. S., and A. E. Harper 1961 Amino acid balance and imbalance. VII. Effect of dietary additions of amino acids on food intake and blood urea concentrations of rats fed low-protein diets containing fibrin. J. Nutrition, *74:* 139.
12. Klain, G. J., D. A. Vaughan and L. N. Vaughan 1962 Interrelationships of cold exposure and amino acid imbalance. J. Nutrition, *78:* 359.
13. Kumta, U. S., A. E. Harper and C. A. Elvehjem 1958 Amino acid imbalance and nitrogen retention in adult rats. J. Biol. Chem., *233:* 1505.
14. Harper, A. E. 1959 Sequence in which the amino acids of casein become limiting for growth of the rat. J. Nutrition, *67:* 109.

15. Morrison, M. A., M. S. Reynolds and A. E. Harper 1960 Amino acid balance and imbalance. V. Effect of an amino acid imbalance involving niacin on liver pyridine nucleotide concentration in the rat. J. Nutrition, *72:* 302.
16. Nishizuka, Y., and O. Hayaishi 1963 Studies on the biosynthesis of nicotinamide adenine dinucleotide. I. Enzymic synthesis of niacin ribonucleotides from 3-hydroxyanthranilic acid in mammalian tissues. J. Biol. Chem., *238:* 3369.
17. Wagner, C. 1964 Regulation of the tryptophan-nicontinic acid-DPN pathway in the rat. Biochem. Biophys. Res. Comm., *17:* 668.
18. Hundley, J. M., H. R. Sandstead, A. G. Sampson and G. D. Whedon 1957 Lysine, threonine and other amino acids as supplements to rice diets in man: Amino acid imbalance. Am. J. Clin. Nutrition, *5:* 316.
19. Scrimshaw, N. S., R. Bressani, M. Behar and F. Viteri 1958 Supplementation of cereal proteins with amino acids. I. Effect of amino acid supplementation of corn masa at high levels of protein intake on the nitrogen retention of young children. J. Nutrition, *66:* 485.
20. Block, R. J. 1951 The Amino Acid Composition of Proteins and Foods. Charles C Thomas, Springfield, Illinois.

Nutritional Studies on Hysterectomy-obtained SPF Baby Pigs Fed Infant Formula Products

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ABSTRACT Three liquid formula products used for feeding human infants were fed to hysterectomy-obtained SPF (specific pathogen free) pigs from birth to 4 weeks of age to determine whether baby pigs could be raised with these low protein formulas and whether any differences in growth could be attributed to differences in formula composition. The baby pigs grew well but at a somewhat slower rate than those fed a simulated sow's milk formula. The lower weight gains, poorer caloric efficiencies and higher carcass fat levels observed with the infant formula products were related mainly to the low protein levels of the infant formulas. The lowest weight gain was noted with the infant formula which also contained levels of calcium and phosphorus below the requirements of the pig. Femur weights and ash content were also low in pigs fed this formula. Low hemoglobin levels in one group of pigs were apparently related to the low copper level of the formula fed. Some of the other differences in organ weights and composition may also be related to nutritional factors in these diets. The results indicate that the newborn baby pig may be a useful experimental animal for studying human infant formula products and for determining the effects of limiting levels of specific nutritional factors in the diet.

Nutritional studies on the growth of young laboratory animals fed liquid formulas or synthetic milks should, ideally, be carried out from birth. The rat has often been used in such studies on infant formula products, but usually only after weaning at 21 days of age; in addition, rats grow poorly when fed many of the high lactose formula products used for infants (1, 2). In studies with the baby pig on the nutritional value of infant formulas (3) and on growth and development with diets of varying protein content (4, 5) the pigs were usually a few days or weeks of age, and antibiotics were often included in the diets to minimize infection.

The recent development of SPF (specific pathogen free) pigs now provides newborn animals which may be used immediately, without nursing, and without inclusion of antibiotics in the diet, for studies of growth with formulas designed for feeding to human infants. We have previously described facilities and procedures which were developed for nutritional studies in these hysterectomy-obtained pigs (6).

The present report presents observations on the growth and development of SPF baby pigs fed 3 commercially available infant formula products. These formulas permitted moderate growth of the pigs from birth, even though they did not supply the levels of protein and minerals required by the baby pig for optimal growth. Some of the results may be correlated with differences in the composition of the various formula products.

MATERIALS AND METHODS

The special facilities and techniques which were developed to obtain the pigs and maintain them under pathogen-free conditions during the experiments were reported previously (6).

Animals. Male and female Hampshire-Yorkshire cross-bred pigs[1] were used. These were obtained by hysterectomy on the 112th day of gestation, 2 days prior to full term. Groups were selected on the basis of weight, sex, and litter, and the animals were placed on experiment within 5 hours after the hysterectomies. The pigs were maintained in individual, disposable cardboard incubators[2] for 7 days and then in individual metal cages for 22 days.

Two experiments were conducted; in experiment 1, 14 pigs from 2 litters were

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[1] Purchased from Pure-For-Sure, Flora, Indiana.
[2] Fort Dodge Container Corporation, Fort Dodge, Iowa.

distributed into 4 groups and fed formulas A, B, C and D, and in experiment 2, 12 pigs from 2 litters were distributed into 3 groups and fed formulas B, C and D.

Formulas. Formula A was a commercially available pig formula,[3] somewhat similar in composition to sow's milk, although containing only 840 kcal/liter (table 1), and was used as a control formula in this study. Formulas B, C, and D (also shown in table 1) were commercially available concentrated infant formula products providing 1350 kcal/liter. The infant formulas provided a much lower percentage of protein calories, slightly lower levels of fat calories and much higher percentages of carbohydrate calories than did pig formula A. The infant formulas differed in levels of minerals, some of which appeared to be slightly low for the baby pig, but only iron was added to formula D. Levels of vitamins (not shown) were assumed to be adequate on the basis of label claims and some analyses.

The pigs were fed fresh formula as the sole diet 3 times daily: at 7:00 and 11:30 AM and at 5:00 PM. The feeding was essentially ad libitum as the volume supplied lasted until almost the next feeding. Each animal's intake was recorded, and the amount provided each feeding gradually increased. After 10 days on the experiment, a small amount of water was also provided in a separate food cup.

On the 29th day, the pigs were fed half of the usual amount of diet early in the morning and killed a few hours later by exsanguination under CO_2 anesthesia. Blood was assayed for hemoglobin (7) and hematocrit (8), and plasma was separated for measurement of cholesterol (9), lipids (10), and proteins (11). The liver was excised, weighed and stored frozen for later determination of solids and fat (12), protein (13), ash (13), cholesterol (9), and glycogen (14). The right femur was removed, cleaned and stored frozen until analyzed for ash (13), calcium (15), and

[3] SPF-lac, The Borden Company, New York.

TABLE 1

Composition of formulas [1]

	Pig formula A	Infant formula products		
		B	C	D
Solids, g/liter	151	251	256	253
Protein, g/liter	52	33	38	32
Fat, g/liter	54	76	73	72
Linoleic acid,[2] %	12.0	25.2	33.2	16.9
Carbohydrate,[3] g/liter	37	136	137	144
Ash, g/liter	8.2	6.9	8.2	4.8
Ca, g/liter	1.2	1.3	1.5	0.7
P, g/liter	1.3	1.1	1.3	0.8
Fe, mg/liter	10.0	18.0	24.0	15.0 [4]
Mg, mg/liter	130	110	150	120
Na, g/liter	0.8	0.75	0.7	0.3
K, g/liter	1.4	1.6	2.3	1.4
Cl, g/liter	1.0	1.0	1.2	0.7
Zn, mg/liter	7.0	4.0	5.0	2.0
Cu, mg/liter	0.7	0.4	0.1	0.5
I, mg/liter	0.4	0.3	0.3	0.2
Kilocalories/liter	840	1360	1360	1350
	%	%	%	%
Distribution of calories				
Protein	25	10	11	10
Fat	58	50	49	48
Carbohydrate	17	40	40	42

[1] Average of 2 duplicate analyses.
[2] Per cent of total fatty acids.
[3] Estimated by difference.
[4] One drop Fer-In-Sol, (Mead Johnson & Company, Evansville, Indiana) added/100 ml of formula to give a total of 30 mg iron/liter.

phosphorus (13). Heart, kidneys, spleen and adrenals were also weighed, but were returned to the carcass for analysis; the gastrointestinal tract was removed and discarded. Each carcass was chopped into small pieces and stored frozen. It was then ground while frozen,[4] and aliquots were taken for analysis of solids and fat (12), protein (13), ash (13), and cholesterol (9).

RESULTS

The data from the 2 experiments are combined on the basis of weighted means (16) for comparison of the results with the 4 diets.

Weight gain and food efficiency. The pigs receiving formula A, somewhat similar to sow's milk, gained 5.5 kg during the 4-week period (table 2). In previous studies, pigs gained 5.8 kg with this formula and 7.4 kg with a more concentrated simulated sow's milk formula (6).

The 4-week weight gains of the pigs receiving the infant formulas B, C, and D were 4.5, 4.2, and 3.7 kg, respectively, or 82, 77, and 68% of the weight gain obtained with formula A. Although there were no statistically significant differences between the weight gains with the infant formulas, the gains of the pigs with formula D were consistently lowest in both experiments.

The average 4-week intake of the liquid formulas was 37.4 liters/pig with formula A (providing 840 kcal/liter), and varied from 25.6 to 21.4 liters with the infant formulas (providing 1350 kcal/liter). However, the caloric intake with the pig formula A, 31.3 megcal[5]/pig, was about the same as the average of the intakes on the infant formulas; namely, 34.8, 31.9, and 28.9 megcal/pig for formulas B, C, and D, respectively. The latter corresponded very closely to the respective weight gains with the infant formulas. Thus, the caloric efficiencies were quite similar—130, 130, and 128 g gain/megcal with formulas B, C, and D, respectively, but these were considerably lower than the 174 g/megcal with formula A. In contrast, the protein efficiency values with the infant formulas, which are low in protein, were considerably higher than the value with formula A, the high protein pig formula. The protein efficiency value with formula C, 4.7 g gain/g protein, was significantly less than the value of 5.4 g gain/g protein with formulas B and D, ($P < 0.05$).

Blood biochemistry. Hemoglobin, hematocrits and total plasma protein, lipid and cholesterol values are shown in table 3. Initial hemoglobin was approximately 5

[4] Frozen meat grinder, Model 4822, The Hobart Manufacturing Company, Troy, Ohio.
[5] Equivalent to 1000 kcal (17).

TABLE 2

Weight gains and food and water intakes of pigs receiving a pig formula and 3 infant formula products for 4 weeks

	Pig formula A	Infant formula products B	C	D
No. of animals	4	7	8	7
Initial wt, kg	1.2	1.1	1.1	1.0
Cumulative wt gain				
Week 1	0.6	0.4	0.4	0.4
Week 2	2.0	1.1	1.1	1.1
Week 3	3.3	2.4	2.3	2.1
Week 4	5.5 ± 1.1 [1]	4.5 ± 0.8	4.2 ± 1.5	3.7 ± 0.8
4-Week food, water consumption				
Formula, liters	37.4	25.6	23.5	21.4
Formula, solids, kg	5.6 ± 0.5	6.4 ± 0.9	6.0 ± 1.8	5.4 ± 0.9
Calories, megcal	31.3	34.8	31.9	28.9
Water, ml	1191	574	490	533
Grams gained/megcal	174 ± 30	130 ± 14	130 ± 11	128 ± 14
Grams gained/g protein	2.8 ± 0.5	5.4 ± 0.6	4.7 ± 0.4	5.4 ± 0.6

[1] SD.

TABLE 3

Hematocrit, hemoglobin and total plasma protein, cholesterol and lipids of pigs fed a pig formula and 3 infant formula products for 4 weeks

	Pig formula A	Infant formula products		
		B	C	D
No. of animals	4	7	8	7
Hematocrit,[1] %	—	37.5 ± 5.3	27.9 ± 4.4	36.7 ± 1.6
Hemoglobin, g/100 ml	10.6 ± 1.5[2]	10.2 ± 1.7	8.1 ± 2.1	10.3 ± 1.0
Plasma				
Total protein, g/100 ml	6.3 ± 0.6	4.5 ± 0.5	4.6 ± 0.7	4.3 ± 0.6
Total cholesterol, mg/100 ml	122 ± 15	118 ± 27	80 ± 19	110 ± 18
Total lipids, mg/100 ml	452 ± 42	497 ± 134	308 ± 95	470 ± 102

[1] Values from the second experiment only.
[2] SD.

g/100 ml. Final hemoglobin levels of pigs fed infant formulas B and D were quite similar to those for pigs receiving formula A. Pigs fed formula C had markedly and significantly lower hemoglobin levels than those receiving formulas A, B, and D, $P < 0.02$ and < 0.05, respectively. Hematocrit values were measured only in the second experiment and averaged 18% in the newborn pigs. After 4 weeks, they were significantly lower in pigs fed formula C than in other groups.

Plasma protein levels were low in animals receiving the infant formulas, but there were no differences between the 3 groups. The total plasma cholesterol level of 80 mg/100 ml with formula C was significantly lower than the values of 110 to 122 mg/100 ml observed with the other formulas. This may be related to the high level of linoleic acid in formula C (table 1). Total lipid values were also significantly lower in pigs receiving formula C.

Organ weights. The absolute and relative weights (on a body weight basis) of livers, spleen, heart, kidney and adrenals of the pigs fed the 4 diets are shown in table 4. The relative liver weights of the pigs receiving the infant formulas were somewhat greater than those of the pigs fed pig formula A. Differences such as these are probably related in great measure to the suboptimal level of protein in the formulas and the resultant smaller body weight gains in the pigs receiving these formulas. Livers were largest with formula D, but there was no statistically significant difference between values for formulas B, C and D.

Relative spleen weights were not markedly different with all 4 formulas. However, both heart and kidney weights of the pigs fed formula C were similar to those of the animals receiving the pig formula A and were significantly larger ($P < 0.02$) than those of the animals fed formulas B and D. The adrenals of the pigs fed both formulas C and D were significantly heavier ($P < 0.05$) than those of the animals fed infant formula B or pig formula A.

Liver composition. The livers of pigs receiving formula A had somewhat higher protein levels than those of the pigs fed the 3 low protein infant formulas B, C and D (table 5). This appeared to be related to the protein levels of the diets; however, liver protein levels in animals fed formula C were markedly higher than those in pigs receiving formulas B and D, even though there was little difference in the protein content of formulas. The levels of fat were similar in the groups given the infant formulas.

Further study of the data shows a reciprocal relationship between liver protein and liver glycogen levels in all 4 groups. Pigs fed formulas B and D, with lowest liver protein levels, had 5.8 and 6.8% liver glycogen, respectively, whereas those fed formulas A and C, with high liver protein, had liver glycogen levels of only 1.7 and 2.5%, respectively. This is difficult to explain since pigs in all groups were killed at similar times following the last feeding.

Carcass composition. The values for the carcass composition of the pigs fed pig formula A were quite similar to the values

TABLE 4

Organ weights of pigs fed a pig formula and 3 infant formula products for 4 weeks

	Pig formula A	Infant formula products		
		B	C	D
No. of animals	4	7	8	7
Body wt, kg	6.76	6.04	5.53	5.12
Liver, g	201	208	193	211
Liver, g/kg body wt	29.8 ± 2.2 [1]	34.7 ± 4.4	35.8 ± 6.7	40.7 ± 6.3
Spleen, g	11.54	12.85	11.41	8.60
Spleen, g/kg body wt	1.7 ± 0.4	2.2 ± 1.2	2.1 ± 0.8	1.7 ± 0.4
Heart, g	42.05	34.30	38.42	30.29
Heart, g/kg body wt	6.4 ± 1.4	5.7 ± 0.4	7.1 ± 1.3	5.9 ± 0.4
Kidneys, g	50.52	41.64	43.26	33.41
Kidneys, g/kg body wt	7.6 ± 1.4	6.9 ± 0.7	7.8 ± 0.5	6.5 ± 0.5
Adrenals, mg	605	601	682	648
Adrenals, mg/kg body wt	90.7 ± 23.2	99.6 ± 13.9	128.4 ± 28.9	126.0 ± 25.2

[1] SD.

TABLE 5

Composition of liver, carcass and femur of pigs fed a pig formula or 3 infant formula products for 4 weeks

	Pig formula A	Infant formula products		
		B	C	D
No. of animals	4	7	8	7
Liver composition				
Solids, %	27.2 ± 0.8 [1]	26.0 ± 0.7	24.9 ± 1.0	26.8 ± 0.5
Fat, %	4.0 ± 0.1	3.1 ± 0.3	3.6 ± 0.3	3.3 ± 0.6
Protein, %	19.5 ± 0.6	14.5 ± 1.5	17.3 ± 1.9	13.6 ± 0.9
Ash, %	1.6 ± 0.1	1.3 ± 0.2	1.4 ± 0.2	1.3 ± 0.2
Glycogen, %	1.7 ± 0.9	5.8 ± 1.7	2.5 ± 2.0	6.8 ± 1.0
Cholesterol, mg/g	3.1 ± 0.2	2.7 ± 0.3	2.8 ± 0.3	2.6 ± 0.2
Carcass composition				
Solids, %	35.3 ± 1.0	46.9 ± 1.8	43.0 ± 4.4	45.0 ± 1.4
Fat, %	17.3 ± 1.7	31.3 ± 1.5	26.6 ± 4.6	30.1 ± 1.8
Protein, %	14.9 ± 0.6	12.2 ± 0.5	13.0 ± 0.9	12.1 ± 0.9
Ash, %	2.7 ± 0.4	2.5 ± 0.3	2.8 ± 0.2	2.1 ± 0.3
Cholesterol, mg/g	1.2	1.1	1.1	1.2
Femur composition [2]				
Weight, g	26.4	19.3	19.3	14.6
g/kg body wt	3.9 ± 0.4	3.2 ± 0.3	3.5 ± 0.2	2.9 ± 0.2
Ash, %	14.6 ± 1.9	14.9 ± 1.9	14.8 ± 1.7	11.8 ± 1.6
Ca in ash, %	34.6 ± 0.5	35.6 ± 0.4	35.0 ± 0.6	34.4 ± 0.5
P in ash, %	17.9 ± 1.3	18.2 ± 1.2	18.9 ± 1.4	18.9 ± 1.3

[1] SD.
[2] Right femur.

reported by Brooks et al. (18) for 30-day-old pigs that were left with the sow and raised normally. The main effect of the low protein level of formulas B, C, and D on carcass composition was markedly higher carcass fat (26.6 to 31.3%) as compared with that observed in pigs fed the high protein formula A (17.3%, table 5). Increased levels of solids in the carcasses of the pigs fed the low protein infant formulas were also observed. Carcass protein levels were higher with the high protein formula, but when calculated on a fat-free basis, values were quite similar with all 4 formulas.

Femur composition. The femurs of the pigs fed formula A were relatively heavier than those of the animals receiving formulas B, C, and D, but the differences were not significant (table 5). The smallest

femurs and lowest ash content were observed in the pigs fed formula D, the formula lowest in calcium and phosphorus content. The levels of calcium and phosphorus in the ash of the femurs were similar in all 4 groups.

DISCUSSION

These experiments showed that hysterectomy-obtained SPF baby pigs could be raised from birth with infant formula products and that some differences in growth and body composition could be attributed to differences in composition of the formulas.

In 2 experiments, moderate rates of growth were observed with the 3 liquid infant formula products, but they appeared to be sufficient to permit some evaluation of nutritional differences in the formulas. Since these infant formulas contained much lower levels of protein and of some minerals than the baby pig requires for rapid growth, the observations in the pig may not apply directly to the human infant, but may help define effects of nutritional factors when present in the diet at limiting levels.

The low level of protein in the infant formulas appeared to be the main factor limiting growth. However, formula D gave consistently poorer growth than formulas B and C, although all three were essentially similar in protein levels. If the remainder of the composition of the formulas were precisely the same, it could be suggested that processing had affected the protein quality of formula D. However, examination of the mineral content showed that formula D contained only 0.3% calcium and 0.3% phosphorus on a dry-weight basis, levels slightly below the suggested requirements of 0.4% calcium and 0.5% phosphorus for the baby pig (19, 20). The additional observation that the pigs fed formula D had smaller femurs with lower ash content suggest that the calcium and phosphorus levels of this formula may have been the secondary growth-limiting factors.

The lower hemoglobin and hematocrit values observed with formula C appeared to be related to the lower copper content of formula C (21). The relatively larger heart and kidney weights in the pigs fed formula C cannot be readily explained at this time.

The significance of the high liver glycogen in pigs fed formulas B and D, is not clear and requires further study. Heard et al. (22) reported an increase in liver glycogen in older pigs fed diets very low in protein. However, in the present study, formula C was also low in protein but liver glycogen levels in these pigs were low rather than high. Although Filer et al. (5) did not measure liver glycogen, their data suggest that there may have been high liver glycogen levels in the pigs fed their low protein diets since analyses for moisture, protein, fat, and ash accounted for less of the total liver in the pigs fed the low protein diets than it did in those receiving the high protein diets.

Thus, a few of the differences noted between the pigs fed 3 different infant formula products can be attributed to specific differences in composition of the formulas. Dietary factors may be responsible for some of the other differences. These require further study. By feeding pigs well-defined formulas differing in levels of various nutritional factors it may be possible to delineate those effects which are related to inadequate or limiting levels of specific nutrients.

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LITERATURE CITED

1. Scott, M. L., and L. C. Norris 1949 Studies on the adequacy of human milk and artificial human milk for the rat. J. Nutrition, 37: 337.
2. Tomarelli, R. M., E. Linden, G. T. Durbin and F. W. Bernhart 1953 The effect of mucin on the growth of rats fed simulated human milk. J. Nutrition, 51: 251.
3. Diaz, F., V. C. Speer, P. G. Homeyer, V. W. Hays and D. V. Catron 1959 Comparative performances of baby pigs fed infant and baby pig diets. J. Nutrition, 68: 131.
4. Filer, L. J., Jr., L. S. Baur and H. Rezabek 1960 Influence of protein and fat content of diet on the body composition of piglets. Pediatrics, 25: 242.
5. Pond, W. G., R. H. Barnes, R. B. Bradfield, E. Kwong and L. Krook 1965 Effect of

dietary energy intake on protein deficiency symptoms and body composition of baby pigs fed equalized but suboptimal amounts of protein. J. Nutrition, *85:* 57.
6. Schneider, D. L., and H. P. Sarett 1966 Use of the hysterectomy-obtained SPF pig for nutritional studies of the neonate. J. Nutrition, *89:* 43.
7. Bell, C. H., J. W. Chambers and M. B. R. Waddell 1945 The routine estimation of haemoglobin as oxyhaemoglobin. Biochem. J., *39:* 60.
8. Van Allen, C. M. 1925 An hematocrit method. J. Lab. Clin. Med., *10:* 1027.
9. Abell, L. L., B. B. Levy, B. B. Brodie and F. E. Kendall 1952 A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. J. Biol. Chem., *195:* 357.
10. Sunderman, F. W., and F. W. Sunderman, Jr. 1960 Lipids and the Steroid Hormones in Clinical Medicine. J. B. Lippincott Company, New York, p. 7.
11. Kingsley, G. R. 1942 The direct biuret method for the determination of serum proteins as applied to photoelectric and visual colorimetry. J. Lab. Clin. Med., *27:* 840.
12. Sarett, H. P., and B. J. Jandorf 1947 Effects of chronic DDT intoxication in rats on lipids and other constituents of liver. J. Pharm. Exp. Therap., *91:* 340.
13. Association of Official Agricultural Chemists 1960 Official Methods of Analysis, ed. 9. Washington, D. C.
14. Scott, T. A., Jr., and E. H. Melvin 1953 Determination of dextran with anthrone. Analyt. Chem., *25:* 1656.
15. Scott, W. W. 1939 Standard Methods of Chemical Analysis, ed. 5. D Van Nostrand Company, New York, p. 211.
16. Snedecor, G. W. 1956 Statistical Methods, ed. 5. Iowa State College Press, Ames.
17. Harris, L. E. 1963 Symposium on feeds and meats terminology. III. A system for naming and describing feeds, energy terminology, and the use of such information in calculating diets. J. Animal Sci., *22:* 535.
18. Brooks, C. C., J. P. Fontenot, P. E. Vipperman, Jr., H. R. Thomas and P. P. Graham 1964 Chemical composition of the young pig carcass. J. Animal Sci., *23:* 1022.
19. Miller, E. R., D. E. Ullrey, C. L. Zutaut, B. V. Baltzer, D. A. Schmidt, J. A. Hoefer and R. W. Luecke 1962 Calcium requirement of the baby pig. J. Nutrition, *77:* 7.
20. Miller, E. R., D. E. Ullrey, C. L. Zutaut, J. A. Hoefer and R. W. Luecke 1964 Mineral balance studies with the baby pig: Effects of dietary phosphorus level upon calcium and phosphorus balance. J. Nutrition, *82:* 111.
21. Teague, H. S., and L. E. Carpenter 1951 The demonstration of a copper deficiency in young growing pigs. J. Nutrition, *43:* 389.
22. Heard, C. R. C., B. S. Platt and R. J. C. Steward 1958 The effects on pigs of a low-protein diet with and without additional carbohydrate. Proc. Nutrition Soc., *17:* xli.

Incorporation of Dietary Elaidic Acid in Tissues and Effects on Fatty Acid Distribution [1]

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ABSTRACT The incorporation of dietary elaidic acid into rat tissue and its effect on fatty acid distribution was studied with the use of infrared spectrophotometry and gas-liquid chromatography. Elaidic acid was incorporated into all of the 3 types of tissue analyzed, epididymal fat pad, liver mitochondria, and erythrocyte stroma. The patterns of tissue distribution of elaidic acid and other fatty acids were different in the 3 tissues; depot fat resembled the dietary lipid more closely than did erythrocyte or mitochondrial lipid. Traces of *trans* isomers of unsaturated fatty acids were present in tissue from rats receiving no detectable amount of these acids in the diet.

The *trans* isomers of unsaturated fatty acids,[3] which are part of the average diet (1), are incorporated into both structural and depot fat (2-4). A number of studies have been carried out which were largely concerned with the gross biological effects of these substances in the intact subject; for example, deposition in various tissues (5), effects upon weight gain (6), alleviation of essential fatty acid deficiency (7-9), and cholesteremia (10-13),[4] have been investigated. However, little is known about the effects of dietary *trans* fatty acids on cellular or subcellular functions. Since esterified fatty acids constitute a significant portion of all membranes and of mitochondria, and are known to play vital roles in the physiological processes of both, it appeared worthwhile to determine whether the introduction of a *trans* isomer of an unsaturated fatty acid in the diet would influence composition and biological function of these structures. In the first part of this study, reported here, some quantitative aspects of the incorporation of dietary *trans* fatty acids into red blood cell stroma, mitochondrial lipids, and into depot fat were investigated. In the second part,[5] the effects of incorporated *trans* fatty acids on a number of membrane functions will be reported.

EXPERIMENTAL PROCEDURES

Weanling male albino rats, Walter Reed strain, were fed the diets described below, a minimum of 12 rats/diet, with free access to water and food. They were weighed twice a week and observed as to their general well-being. The diets used in the study were formulated from a basal diet consisting of casein (15%), sucrose (60%), fat (8%), cod liver oil (2%), cellulose powder (10%), Fox-Briggs salt mixture (14) (4%), and a vitamin mixture (1%), supplying in mg/kg diet: thiamine·HCl, 10; riboflavin, 10; pyridoxine, 5; vitamin B_{12}, 1; nicotinic acid, 40; folic acid, 2; biotin, 0.3; *p*-aminobenzoic acid, 100; Ca pantothenate, 50; inositol, 300; choline chloride, 2,500; menadione, 2; vitamin A, 6; vitamin D, 55.5; and vitamin E, 400.

The fat in the experimental diet consisted of elaidinized olive oil containing 55% *trans* fatty acids; the control diet contained native olive oil. Olive oil was selected for these experiments because of its high content of oleic acid (78%). The latter can be easily converted to elaidic acid, the *trans* isomer which is the easiest to measure quantitatively by available methods. Elaidinized olive oil was prepared by the sulfur dioxide isomerization

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[3] Hereafter referred to as *trans* fatty acids.

[4] Tidwell, H. G., and P. Gifford 1965 Effect of ingestion of isomeric fatty acids on cholesterol and lipids of serum and liver. Federation Proc., 24: 192 (abstract).

[5] Decker, W. J., and W. Mertz, Effects of dietary elaidic acid on membrane functions, in preparation.

procedure described by Raulin (6). After a feeding period of 6 weeks, the rats were anesthetized by intraperitoneal injection of sodium pentobarbital, blood was withdrawn from the heart into a citric acid-sodium citrate-glucose solution (A.C.D. anticoagulant), and the liver and one epididymal fat pad were removed. The liver was immediately placed in ice-cold isotonic saline; the blood and fat pad were refrigerated.

Isolation of mitochondria. Liver mitochondria were prepared by a modification of the method of Schneider and Hogeboom (15), using a 10% liver homogenate obtained by grinding the tissue in an all-glass conical grinder with 0.32 M sucrose-0.001 M versene. The final wash, consisting of deionized 0.32 M sucrose solution, was decanted and the mitochondrial pellet blotted with filter paper.

Isolation of erythrocyte stromata. The blood was centrifuged for 10 minutes at 10,000 × *g*. Following removal of plasma and buffy coat by aspiration, the cells were washed 3 times with isotonic saline solution and were hemolyzed by the addition of 3 volumes of distilled water. After one-half hour, carbon dioxide-saturated water at a temperature of 4° was added, according to the method of Parpart (16). The mixture was centrifuged in the cold for 5 minutes at 10,000 × *g*, and the supernatant liquid decanted. The stromata were washed 3 times with cold carbon dioxide-saturated water; at this stage they were pale pink, indicating that most of the hemoglobin had been removed.

Extraction of lipids. Freshly prepared mitochondria and erythrocyte stromata were ground in an all-glass conical tissue grinder with 20 volumes of 3:1 ether-ethanol until all solids were in a state of fine suspension. The suspension was filtered and the residue was washed once with ether-ethanol and twice with ether. Solvent was removed by evaporation in a vacuum oven at 37°. Fresh epididymal fat pads were stirred with a glass rod in 20 volumes of 2:1 ether-ethanol. The supernatant mixture was filtered and processed in the same manner as were the extracts of mitochondria and stroma.

All extracts were stored under carbon dioxide at —15° until ready for use.

Assay for trans fatty acids. The *trans* fatty acids were measured quantitatively by infrared absorption spectrometry (17), after transesterification of fatty esters by a boron trifluoride methanol procedure (18). This step was found to be necessary in order to eliminate strong absorption peaks, probably due to phospholipids (19) which interfered with the absorption maxima of *trans* fatty acids.

Cholesterol, which also interfered with the absorption of the *trans* double bond, was precipitated with digitonin (20).

Ten to 15 mg of crude lipid extract were boiled with 3 ml of boron trifluoride-methanol reagent for 2 minutes (125 g BF_3 /liter of reagent-grade methanol), and then separated between water and petroleum ether. The filtered and washed petroleum ether layer was evaporated. The residue, dissolved in carbon tetrachloride[6] was used for quantitative determination of *trans* bonds in a Perkin-Elmer Model 21 Infrared Spectrophotometer. Elaidic acid subjected to the described procedures was recovered as the methyl ester with a yield of 97%. Infrared spectra showed no trace of an absorption peak at 10.36 μ after highly purified lecithin[7] had been transesterified with this procedure, indicating that the methanolysis of the phospholipid was essentially complete. Methyl elaidate, prepared by the BF_3-methanol technique, gave an absorption at 10.36 μ, quantitatively identical to that of elaidic acid, thereby indicating that the methylation technique has no deleterious effect on the absorption due to the *trans* double bond. Furthermore, samples of oleomargarine, a product known to contain mixtures of *trans* isomers of unsaturated fatty acids but no phospholipid, were transesterified and the peak at 10.36 μ was again unaffected. Transesterification was applied to ether-ethanol extracts of various biological materials, including yeasts, soybean protein, animal diets, rat depot fat, red blood cells, and liver. In each case where phospholipid was present, the procedure resulted in a depression of the absorption peak, either complete or partial. The absorption remaining after transesterification disap-

[6] Fisher Spectranalyzed.
[7] Sylvana Company, Millburn, New Jersey.

peared completely upon addition of bromine, demonstrating that this peak had been due to the *trans* configuration.[8]

Solutions of elaidic acid[9] of 0.005 M, 0.01 M, 0.02 M, and 0.04 M, were prepared in carbon tetrachloride. Two standard curves were obtained by plotting absorbance at 5.8 μ and 10.36 μ against molarity. By the use of these standard curves, and subtracting baselines at the two absorbance peaks, total *trans* isomer content and total fatty ester in a sample was determined, and the ratio of *trans* fatty ester to total fatty ester was calculated. The more rapid-scanning Beckman Model IR-5A infrared spectrophotometer was used to obtain standard curves and analyze the tissue extracts for both total *trans* isomer and fatty ester content.

Gas-liquid chromatography of methyl esters. A Barber-Colman Model 20 gas chromatograph fitted with a ^{90}Sr argon ionization detector was used. A 107 m (350-foot) stainless steel capillary column, i.d., 0.025 cm (0.01 in.), was coated with Apiezon L, using the method of Lipsky et al. (22). The column was operated at 205°, with injector and detector temperatures of 300° and 210°, respectively. Sample split was 100:1. Ionization voltage was 1250 volts; a scavenging flow of 60 ml/minute through the detector maintained an effective detector cell volume of a few milliliters. Flow through the column was 2 ml/minute at 60 psig argon. Under these conditions, methyl elaidate had a retention time of 50 minutes and a resolution of 10,200 theoretical plates. Pressure was programed in the following manner: the injection pressure of 20 psig was raised to 60 psig after the solvent had left the detector; finally it was raised to 80 psig after stearate had been eluted. Standards of gas chromatographically pure methyl esters of fatty acids[10] were used to determine relative retention times in order to identify individual fatty esters prepared from the lipid extracts. Gas chromatograms were quantitated by triangulation of peak (area = height $\times$ width at half-height); the concentration of an individual fatty ester was expressed in terms of per cent of total fatty ester.[11]

RESULTS

Growth of the experimental animals. Weight gain of the rats fed both experimental diets was approximately linear during the 6-week feeding period. The rates of weight gain were 18.8 and 20.9 g/week, respectively, for the *trans* fatty acid-supplemented group and for controls; the difference between rates was not significant ($P > 0.05$). Physical appearance of the rats in both dietary groups was identical; no gross pathologic lesions were evident when the animals were killed.

Distribution of fatty acids. The fatty acid composition of the dietary fats is listed in table 1. No *trans* fatty acid was detectable in either the native olive oil or the cod liver oil.

Fatty acid and aldehyde composition of extracts from epididymal fat pads, liver mitochondria, and erythrocyte stromata are shown in table 2. The major fatty acid components of the tissue lipids were palmitic, oleic, stearic, linoleic, arachidonic, and palmitoleic acids. That fatty aldehydes were found indicates the presence of plasmalogens. These compounds would be expected in extracts of membranes, but not in those of depot fat. A possible explanation is that despite the rather gentle extraction technique used for the isolation of depot fat, some lipid was extracted from fat-cell membranes. There was considerable incorporation of dietary elaidic acid into all of the 3 tissues studied; depot fat contained twice as much as the other 2 tissues. Tissue from animals that had not been fed *trans* fatty acids contained a small amount of elaidic acid (less than 0.1%). Elaidic acid (*trans*-octadec-9-enoic acid) was the only *trans* fatty acid identified in these tissues. It was unexpected that no palmitelaidic acid (*trans*-hexadec-9-enoic acid) was found, since the dietary elaidinized olive oil contained 0.4% of this isomer. Under the conditions of the gas-liquid chromatographic technique used,

[8] Morrison and Smith (21) have recently demonstrated that boiling with BF_3-methanol reagent for one hour does not affect *cis-trans* isomerism, even in the case of conjugated double bond systems.

[9] Hormel Foundation.

[10] See footnote 9.

[11] The animals were treated in accordance with the principles of laboratory animal care as promulgated by the National Society for Medical Research. The mentioning of trade names and manufacturers does not constitute an official endorsement of these products by the U. S. Government.

TABLE 1

Fatty acid composition of the dietary fats

Fatty acid	% of total fatty acids		
	Cod liver oil	Native olive oil	Elaidinized olive oil
Lauric	tr [1]	tr	tr
Myristic	2.9	tr	tr
Palmitoleic	11.3	0.8	0.2
Palmitelaidic	0.0 [2]	0.0	0.4
Palmitic	14.7	8.8	6.3
Octadecatetraenoic	2.1	0.0	0.0
Linolenic	1.1	tr	tr
Linoleic	2.8	8.8	0.4
Oleic	45.1	78.0	46.7 [3]
Elaidic	0.0	0.0	43.5
Stearic	4.3	2.5	2.2
Arachidonic	11.9	0.0	0.0
Docosahexaenoic	0.6	0.0	0.0

[1] Amounts of less than 0.1% are indicated as trace.
[2] The quantity "0.0" indicates a concentration of less than 0.005%, and the absence of any peak.
[3] This peak may contain some *trans* linoleic isomers.

TABLE 2

Fatty acid and aldehyde composition of tissue extracts

Component	% of total fatty acids					
	Fat pad		Mitochondria		Stromata	
	trans fatty acid-supplemented	Controls	*trans* fatty acid-supplemented	Controls	*trans* fatty acid-supplemented	Controls
Lauric	tr	tr	tr	tr	tr	tr
Myristoleic	tr	tr	tr	tr	tr	tr
Myristic	0.8	0.7	0.6	0.2	0.5	0.6
Pentadecenoic	tr	tr	tr	tr	0.3	0.2
Pentadecanoic	0.2	0.1	0.1	tr	0.4	0.1
Hexadecanal	0.1	tr	0.1	tr	0.4	0.6
Palmitoleic	7.3	6.6	7.3	3.7	2.9	1.5
Palmitic	17.6	23.7	37.8	30.9	36.4	37.2
Heptadecenoic	0.1	tr	0.2	0.1	0.8	0.2
Heptadecanoic	tr	0.2	0.1	0.1	0.5	0.9
Octadecanal	tr	tr	tr	tr	tr	0.4
Linoleic	tr	0.9	0.8	10.2	1.0	5.9
Oleic	43.6	62.9	27.7	24.8	29.6	30.9
Elaidic	24.8	tr	12.9	tr	13.9	tr
Stearic	1.2	2.3	11.0	23.4	9.8	14.4
Arachidonic	0.1	1.8	0.8	5.1	1.9	5.7
Docosahexaenoic	tr	tr	tr	1.0	0.9	0.7

trans isomers of polyunsaturated fatty acids could not be resolved into specific peaks; a very long retention time and more than one type of capillary column coating per analysis would have to be used (23).

In general, the relative concentrations of individual saturated fatty acids in *trans* fatty acid-supplemented tissues were lower than those in control tissues. A similar difference was noted in the case of polyunsaturated fatty acids (linoleic and arachidonic acids); this phenomenon may be an artifact by virtue of poor resolution of *trans* polyunsaturated fatty acids as described in the preceding paragraph. Although there was a considerable difference in the concentration of oleic acid between the 2 dietary groups in fat pad lipid, both the mitochondrial and the stromal lipid showed approximately the same oleic acid concentration with either diet. Other minor peaks, representing a total of less than

0.5% of total fatty acids, appeared on the chromatograms. Their retention time did not correspond to those of any available fatty acid standards. It is possible that they were breakdown products of polyunsaturated fatty acids despite the care tendered the lipid extracts to preclude significant air-induced oxidation and light-induced polymerization. A calculation of the ratio of oleic acid concentration in dietary native olive oil and that in elaidinized oil and its comparison with ratios of oleic acid in the tissues of control and *trans* acid-supplemented groups, demonstrated a difference between dietary fat and epididymal lipid in one instance (1:0.6 and 1:0.7, respectively) and mitochondria and red cell stromata (1:1.1 and 1:1, respectively) in the other. A similar difference was noted when ratios of elaidic to stearic acids were calculated for the dietary and tissue lipids which contained relatively large amounts of *trans* fatty acids. Both elaidinized olive oil and fat pad lipid had ratios of 22:1; those of mitochondrial and stromal lipids were 1:1 and 1.3:1, respectively.

Assay of tissues for total trans isomers. Erythrocyte stromata, liver mitochondria, and epididymal fat pads were extracted and processed as described. Concentrations of *trans* isomers were below the lower limit of quantification by infrared spectrophotometry in lipid extracts of tissues of rats fed the diet not containing *trans* fatty acid. A calculation of molar ratios between *trans* fatty acid and total fatty acid content in tissues from supplemented rats revealed interesting differences. The ratio was 1:0.55 in elaidinized olive oil, 1:0.27 in epididymal fat tissue, and only 1:0.18 and 1:0.15 in mitochondria and red blood cell stromata, suggesting different patterns of incorporation into depot and structural fat. A difference can be observed between the results for *trans* fatty acids obtained by infrared spectrophotometry and by gas-liquid chromatography (55% vs. 44% for elaidinized olive oil, 27% vs. 25% for fat pad, 18% vs. 13% for mitochondria, and 15% vs. 14% for stromata). An explanation for this apparent discrepancy is that the spectrophotometric method detects all *trans* isomers of fatty acids, whereas the chromatographic method does not quantitatively resolve any *trans* isomer other than elaidic acid. Thus, it appears that *trans* polyenoic fatty acids were present, and that above differences are an approximate measure of the concentration of these compounds.

DISCUSSION

These studies demonstrated by the use of 2 independent analytical techniques, that dietary elaidic acid is incorporated into lipids of rat mitochondria and erythrocyte stromata. They furthermore confirmed previous reports (2, 4, 6, 24) that dietary *trans* fatty acids are incorporated into depot fat, and are in good quantitative agreement with the observations of Loriette et al. (24). These authors fed *trans* fatty acids at 50 to 60% of total dietary fatty acids and measured the elaidic acid content of 22 to 23% of total fatty acids in epididymal fat pads, as compared with a 55% content in diet and 28% in the fat pads in the experiments reported here. The observation that the concentration of elaidic acid in mitochondrial and stromal lipid was approximately one-half that of depot fat, is in agreement with the conclusions of Collett and Favarger (4) that elaidic acid is incorporated to a greater extent into neutral lipid than into tissue phospholipid, since the majority of the fatty acids of membranes is contained in the phospholipids (25). It appears from this that the incorporation of elaidic acid into different tissues is subject to some sort of selective control mechanism. It is not unexpected, therefore, that the elaidic acid influenced the distribution of various other fatty acids to a different degree, depending on the tissue studied; for example, the concentrations of both palmitic and oleic acids were relatively depressed in the elaidic acid-supplemented fat pads; they remained nearly unchanged in red cell membranes, and relatively increased in mitochondria, when compared with control values. While this comparison shows a different pattern for each of the 3 tissues studied, a calculation of various ratios, as shown in the Results section, suggests a relatively similar pattern among the 2 structural lipids which is quite different from that of depot fat. The reasons for these differences are not clear. An interpretation of the data in a nutritional sense is complicated by the

unphysiologically high levels of elaidic acid which had to be fed. They were necessitated by the limitations of the analytical methods. It is possible that the relative incorporation into the 3 tissues is entirely different from that observed here, when lower, more nearly physiological amounts were used. Also, the possibility of a marginal deficiency of essential fatty acids, induced by the high amount of *trans* acid, cannot be ruled out entirely, even though cod liver oil was added to the diet and no gross symptoms of deficiency were observed. Of nutritional significance may be the observation that all tissues from rats presumably receiving no dietary *trans* fatty acids at all, contained a trace of elaidic acid. This trace was consistently present in the tissues but not in the dietary fat. Thus, if the latter did contain the *trans* acid at all, the tissues must have concentrated it to a considerably extent. Bacterial hydrogenation of *cis* polyenoic fatty acids in the gut with subsequent *trans* fatty acid production may be another source.[12] There is no experimental evidence for the third alternative, *cis-trans* isomerization within the mammalian organism.

LITERATURE CITED

1. Thomasson, H. J., and J. J. Gottenbos 1957 A comparison of the nutritional values of butter and margarine. Vlaamse Acad. Geneesk (Belgium), *19:* 369.
2. Sinclair, R. G. 1935 The metabolism of the phospholipids. VIII. The passage of elaidic acid into tissue phospholipids. Evidence of the intermediary role of liver phospholipid in fat metabolism. J. Biol. Chem., *111:* 515.
3. Sinclair, R. G., and O. Smith 1937 Turnover of phospholipids in intestinal mucosa. J. Biol. Chem., *121:* 361.
4. Collett, R. A., and P. Favarger 1949 Composition of canine plasma lipids following the absorption of trielaidin. Helv. Physiol., Pharmacol. Acta, *7:* C8.
5. Allen, R. R., A. A. Kiess, P. V. Johnston and F. A. Kummerow 1958 Metabolism of triglycerides containing *cis-* and *trans*-octadecenoic fatty acids. J. Am. Oil Chemists' Soc., *35:* 203.
6. Raulin, J. 1960 Influence of the stereochemical configuration of fatty acids on the nutritive value of dietary lipids. Ann. Nutr. (Paris) Mem., *14:* 201.
7. Holman, R. T. 1951 Metabolism of isomers of linoleic and linolenic acids. Proc. Soc. Exp. Biol. Med., *76:* 100.
8. Holman, R. T., and E. Aaes Jørgensen 1956 Effects of *trans* fatty acid isomers upon essential fatty acid deficiency in rats. Proc. Soc. Exp. Biol. Med., *93:* 175.
9. Mattson, F. H. 1960 An investigation of the essential fatty acid activity of some of the geometrical isomers of unsaturated fatty acids. J. Nutrition, *71:* 366.
10. Ahrens, E. H., Jr., W. Insul, Jr., R. Blomstrand, J. Hirsch, T. T. Tsaltas and M. L. Peterson 1957 The influence of dietary fats on serum lipid levels in man. Lancet., *1:* 943.
11. McOsker, D. E., F. H. Mattson, H. B. Sweringen and A. M. Kligman 1962 The influence of partially hydrogenated dietary fats on serum cholesterol levels. J.A.M.A., *180:* 380.
12. Beveridge, J. M., and W. F. Connell 1962 The effect of commercial margarines on plasma cholesterol levels in man. Am. J. Clin. Nutrition, *10:* 391.
13. Weigensberg, B. I., and G. C. McMillan 1964 Serum and aortic lipids in rabbtis fed cholesterol and linoleic acid stereoisomers. J. Nutrition, *83:* 314.
14. Fox, M. R. S., and G. M. Briggs 1960 Salt mixtures for purified-type diets. III. An improved salt mixture for chicks. J. Nutrition, *72:* 243.
15. Schneider, W. C., and G. H. Hogeboom 1950 Intracellular distribution of enzymes; further studies on distribution of cytochrome c in rat liver homogenates. J. Biol. Chem., *183:* 123.
16. Parpart, A. K. 1942 The preparation of red cell membranes. J. Cell. Comp. Physiol., *19:* 248.
17. Shreve, O. D., M. R. Heether, H. B. Knight and D. Swern 1950 Determination of *trans*-octadecenoic acids, esters and alcohols in mixtures. Analyt. Chem., *22:* 1261.
18. Metcalfe, L. D., and A. A. Schmitz 1961 The rapid preparation of fatty acid esters for gas chromatographic analysis. Analyt. Chem., *33:* 363.
19. Freeman, N. K. 1957 Infrared spectroscopy of serum lipids. Ann. N. Y. Acad. Sci., *69:* 131.
20. Natelson, S. 1961 In: Microtechniques of Clinical Chemistry. ed. 2. Charles C Thomas, Springfield, Illinois, p. 181.
21. Morrison, W. R., and L. M. Smith 1964 Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride-methanol. J. Lipid Res., *5:* 600.
22. Lipsky, S. R., R. A. Landowne and J. E. Lovelock 1959 Separation of lipides by gas-liquid chromatography. Analyt. Chem., *31:* 852.
23. Litchfield, C., A. F. Isbell and R. Reiser 1962 Analysis of the geometric isomers of methyl linoleate by gas chromatography. J. Am. Oil Chemists' Soc., *39:* 330.
24. Loriette, C., J. P. Carreau and J. Raulin 1963 Nutritional value of fats containing isomerized fatty acids. Ach. Sci. Physiol. (Paris), *17:* 273.
25. Spiro, M. J., and J. M. McKibbin 1956 Lipides of rat liver cell fractions. J. Biol. Chem., *219:* 643.

[12] Kepler, C. R., K. P. Hirons, J. J. McNeill and S. B. Tove 1965 Biohydrogenation of linoleic acid. Federation Proc., *24:* 290 (abstract).

Lysine-Arginine-Electrolyte Relationships in the Rat [1]

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ABSTRACT Lysine (1.5, 3, and 6% of diet) fed to weanling rats caused graded depressions in growth rates when added to an 18% casein diet and slight depressions when added to stock or isolated soy protein rations. Supplementation with 1% L-arginine eliminated the growth depression caused by 1.5 and 3% lysine and reduced that caused by 6% lysine. Changes in pancreatic trypsinogen or procarboxypeptidase B activities in animals fed lysine or ε-aminocaproic acid (in vitro enzyme inhibitors) could not be correlated with growth rates. After 14 days of supplementary lysine, cellular K in muscle decreased (not reversed by arginine) but cellular Na and bone composition were unchanged; concentrations of free lysine in plasma and muscle and of free arginine in muscle increased. These observations, data on plasma lysine and arginine concentrations after shorter test intervals, and data on urinary excretion of lysine and arginine indicate that competition for transport between lysine and arginine at the gut, tissue, or kidney is not the cause of the antagonism. Growth and food consumption curves of rats kept conventionally or at 4°, or previously protein-depleted, indicated that the growth depression was not due solely to decreased food consumption and that rats fed lysine were less efficient. It is suggested that lysine exerts its adverse effects by altering the intermediary metabolism of arginine.

The responses of organisms to variations in the amino acid composition of the diet depend upon a number of factors. In studies of these responses the levels of amino acids have been altered under a variety of experimental conditions, and some specific relationships have become apparent (these have been reviewed by Harper (1)). The work in this laboratory has been concerned with the relationship between lysine and arginine. This relationship has been described in the chick[2,3] (2) and guinea pig (3), and an inhibitory action of lysine on arginase (4), trypsin (5), and carboxypeptidase B (6) has been demonstrated.

A recent report (7) indicates that, under some conditions, the dietary requirement of the rat for arginine may be considerably in excess of the previous estimate, 0.3% of the diet. Thus, if it is assumed that the rat has a demonstrable arginine requirement, then a relationship among arginine requirement, lysine supplementation, and type of protein source may also exist in the rat as it does in the chick (2). Preliminary experiments indicated that, in rats fed excess lysine, different protein sources produced differences in response and that the depression of growth caused by excess dietary lysine could be reversed, in part, by additional dietary arginine.

Data obtained in young chicks (2) were interpreted as showing a decreased availability of dietary arginine as a result of high dietary intake of lysine because a portion of the ingested arginine (determined analytically) was not used by the animal.

An interrelationship between electrolytes (mainly potassium) and the dibasic amino acids has been described in many systems, including chick tissues (2), mammalian kidney (8, 9), tissue from potassium-deficient rats (10-12),[4] and isolated rat muscle (13). Although the decrease in cellular potassium in the young chick fed excess lysine reported previously (2) was not shown to be directly related to the symptoms observed in that species, it is difficult to understand why a similar decrease could not be observed in the rat (12).

In the present paper, efforts to define the cause and site of the antagonism be-

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[2] O'Dell, B. L., C. L. Limbaugh and J. E. Savage 1962 Arginine-lysine antagonism and deficiencies of casein for the chick. Federation Proc., *21:* 8 (abstract).

[3] Smith, G. H., and D. Lewis 1964 Agent and target in amino acid interactions. Proc. Nutrition Soc., *23:* 28 (abstract).

[4] Hall, P. W., and G. J. Gabuzda 1964 Effects of L-lysine feeding on muscle potassium and amino acid content. Am. J. Clin. Nutrition, *14:* 250 (abstract).

tween lysine and arginine will be described. Whether high lysine intake causes an increased need for arginine in the rat because of decreased digestion or absorption of arginine, or because of an altered metabolism of absorbed compounds, will be discussed. It also seemed worthwhile to try to relate cellular potassium content to the growth-depressing effects of excess dietary lysine. Therefore, the electrolyte distribution in tissues was studied under experimental conditions which would allow a clearer understanding of the relationship. These studies used procedures similar to those used in the chick study (2).

EXPERIMENTAL

Male weanling rats of the Sprague-Dawley strain, 40 to 55 g, were divided into groups of seven or more each; the rats were housed individually in screen-bottom cages in an air conditioned room and were given food and water ad libitum. When older animals or other variables were used, appropriate notations have been made in the text or tables. The basal diets contained: casein, 18%; salts IV (14), 4%; corn oil, 4%; L-cystine, 0.2%; choline chloride, 0.1%; and complete vitamins and sucrose to make 100%.[5] In some experiments, this diet was modified to contain 15.6% soy protein (nitrogen content similar to the casein basal ration), instead of casein, with 0.16% DL-methionine added. When added to the purified diets, amino acids[6] replaced sucrose. When a complete commercial stock diet[7] was used, added lysine replaced an equal amount of the total diet.

Methods of sampling and analysis were those reported previously (2) with the following additions: chloride was determined directly in plasma and in water extracts of tissue by the technique of Cotlove (15), and lipid extracted from muscle with $CHCl_3$: MeOH (3:1) was not included in calculating the electrolyte distribution because the lipid content (6.0% of wet weight) was not changed by the treatments. The femur and the gastrocnemius muscle are referred to in the text simply as bone and muscle.

When enzyme activities were to be determined, animals were decapitated, and the pancreases were removed quickly and homogenized in 20 parts (v/w) of cold 0.025 M tris buffer (pH 7.65) containing 0.1 M NaCl, 0.02 M $CoCl_2$, and 0.05 M $CaCl_2$. The homogenate was centrifuged at 18,000 × g for 20 minutes, and 5 ml of the resulting supernate was activated at 37° with 6 μg of trypsin for 2 or 2.5 hours for carboxypeptidase B or trypsin assays, respectively. Carboxypeptidase B was assayed with hippuryl-L-arginine as the substrate (16). The results are expressed as change in absorbancy at 254 mμ in a 1-cm cell/minute in a total volume of 3 ml. Trypsin was assayed with benzoyl-L-arginine *p*-nitroanilide as the substrate (17). The results are expressed as change in absorbancy at 410 mμ in a 1.9-cm cell/hour in a total volume of 6 ml. The activity of trypsin added to activate the proenzymes was determined and subtracted from the total tryptic activity of the homogenate. Particular effort was made to treat all specimens obtained at the same interval in exactly the same manner.

RESULTS AND DISCUSSION

Growth. Addition of 1.5, 3, and 6% L-lysine to casein diets inhibited rat growth from 9% to 50% (table 1, exps. 1 and 2). Arginine had little effect on growth when added to control diets but completely eliminated the inhibition caused by 3% L-lysine and significantly reduced that caused by 6% L-lysine. The only visible symptom other than decreased growth rate was increased irritability which was observed frequently in rats fed 6% L-lysine; this was eliminated by increased dietary arginine. To compare these observations with those previously found in the chick (2), lysine was added to diets of differing composition: a stock ration, one containing soy protein, and one containing dextrin.

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[5] This diet contains 0.6% potassium, 1.27% lysine, and 0.51% arginine. Vitamins added were, in mg/kg: riboflavin, 3.0; thiamine, 6.0; pyridoxine, 2.0; nicotinic acid, 25.0; Ca pantothenate, 20.0; menadione, 10.0; inositol, 100.0; biotin, 0.1; folic acid, 2.0; vitamin B_{12}, 0.02; α-tocopherol, 30.0; vitamin A acetate, 3.1, and calciferol, 0.045.

[6] L-Lysine and L-arginine were added as the monohydrochlorides. L-Lysine·HCl and crystalline B vitamins were kindly furnished by Merck and Company, Rahway, New Jersey; L-arginine·HCl was kindly furnished by General Mills, Minneapolis.

[7] Rockland Rat Diet (complete), A. E. Staley Manufacturing Company, Decatur, Illinois; contains 0.72% potassium, 1.15% lysine, and 1.39% arginine.

TABLE 1

Growth of rats fed diets supplemented with excess L-lysine or L-arginine

Exp. no. (days fed diet)	Protein source	Amino acid added [1]		Body wt gain [2]
		L-lysine	L-arginine	
		%	%	g
1 (30)	Casein	0	0	182 ± 2.4
		1.5	0	171 ± 5.5
		3	0	156 ± 8.7
		6	0	97 ± 3.3
		3	0.5	188 ± 5.9
		6	0.5	128 ± 7.4
	Crude diet	0	0	190 ± 6.0
		3	0	182 ± 4.5
		6	0	171 ± 4.3
2 (28)	Casein	0	0	158 ± 3.9
		0	1	152 ± 3.9
		0	2	159 ± 4.4
		0	4	151 ± 8.4
		6	0	78 ± 6.5
		6	1	123 ± 6.2
		6	2	126 ± 5.7
		6	4	114 ± 7.7
	Isolated soy protein	0	0	135 ± 8.8
		6	0	110 ± 9.7
3 (35) [3]	Casein-sucrose	0	0	200 ± 7.7
		6	0	90 ± 7.2
	Casein-dextrin	0	0	216 ± 3.1
		6	0	144 ± 5.2
4 (30)	Casein hydrolysate [4]	0	0	106 ± 4.6
		6	0	44 ± 1.7
		6	1	100 ± 8.4
		0	1	129 ± 5.5
	Casein	0	0	201 ± 6.0
		6	0	89 ± 3.8
5 (28) [5]	Casein	0	0	154 ± 2.8
and				179 ± 3.9
6 (30) [5]		6	0	81 ± 8.0
				87 ± 5.6
		0	ε-amino-caproic, 6	151 ± 6.1
				159 ± 6.1

[1] L-Lysine and L-arginine added as monohydrochlorides.
[2] Values are mean ± SE of 6 or 7 rats/group.
[3] Dextrin plus lysine different from sucrose plus lysine, $P \leq 0.01$. Lysine caused a significant depression in growth with either carbohydrate, $P \leq 0.01$.
[4] Enzymatic, casein hydrolysate, Nutritional Biochemicals Corporation, Cleveland.
[5] Results of 2 similar experiments are shown.

Addition of 3 and 6% L-lysine to a stock diet (24.3% protein) depressed growth 4 and 10%, respectively. Six per cent L-lysine added to an isolated soy protein diet depressed growth 19%. The data (table 1) indicate clearly that the rat, like the chick, is more susceptible to an excess of L-lysine when the protein of the ration is furnished by casein.

Since the use of dextrin in lieu of sucrose has been reported to decrease the requirement of the chick for arginine (18), sucrose was replaced by dextrin in one of our experiments. Although 6% L-lysine in a diet in which the carbohydrate was furnished by dextrin depressed growth significantly, the rats grew significantly more than did those receiving a comparable level of L-lysine added to the sucrose-containing diet (table 1, exp. 3). It is not readily apparent why lysine depresses growth less when fed with dextrin. The growth depression caused by lysine is considerably greater than the difference in growth between the sucrose and the dextrin controls. Therefore, it is unlikely that, as has been suggested under some experimental conditions, dextrin reduces the effect of high dietary lysine by allowing greater food consumption because of its physical

properties (19). It is possible that, in the presence of excess lysine, dextrin exerts its effect in a manner similar to that suggested by Romberg and Benton (20)—that is, in rats fed low protein diets, dextrin alters the mechanism of food intake control and also subsequent utilization and metabolism of ingested nutrients. Although other investigators have used different diets and conditions, it appears that the literature supports the conclusion that the adverse effects of excess lysine depend in part on the dietary protein and carbohydrate sources (see (21) for additional references).

Proteolytic enzymes. Earlier studies with chickens led to the hypothesis that high levels of dietary L-lysine could affect the utilization of dietary arginine by inhibiting the enzymes that hydrolyze the dietary proteins. The pancreatic enzymes trypsin and carboxypeptidase B were chosen for investigation because they are capable of hydrolyzing peptide bonds in which arginine furnishes the amino and carboxyl groups, respectively. Also, these enzymes isolated from other animals have been shown to be inhibited in vitro by lysine as well as by ε-aminocaproic acid, a compound that could be fed to rats (5, 6).

L-Lysine and ε-aminocaproic acid were added, at concentrations of 10^{-4} to 4×10^{-1} M, to activated pancreatic homogenates prepared as described above, and the trypsin and carboxypeptidase B activities were determined. When concentration of inhibitor was plotted against tryptic activity, similar smooth curves were obtained for both compounds, with inhibition values of 15% at 10^{-4} M and 40 to 50% at 4×10^{-1} M. L-Lysine at 10^{-4} to 4×10^{-1} M inhibited carboxypeptidase B activity 4 to 66%, whereas ε-aminocaproic acid inhibited this peptidase 23% at 10^{-4} M and 98% at 4×10^{-1} M.

On the assumption that the concentration of proenzymes in the pancreas would be directly related to the quantities secreted (see (22) for supporting evidence), an experiment was designed to test whether excess dietary lysine reduced the quantity of these 2 pancreatic proenzymes. Weanling rats were divided into groups, and the dietary treatments shown in table 2 were assigned. The rats were allowed free access to the diets. At the intervals shown, the rats were killed for proenzyme assay.

Addition of 6% ε-aminocaproic acid did not significantly affect the growth rate of the rat but did appear to affect the weight of the pancreas after the diet had been fed for 7 days. Carboxypeptidase B and trypsin activities were also depressed by this treatment at the same intervals. The effect of 6% L-lysine on pancreatic weights changed with time: they initially were higher than control values and then were lower. Initially, the enzyme activities were depressed, but they were increased at the last time interval. This may indicate an adaptation to the excess lysine.

The changes in weight of pancreas were not observed by Magee and Hong (23) with 1% L-lysine in a 7% casein diet, although they presented evidence that tryptic activity was depressed by this treatment. A considerable variation in the enzyme values within treatment groups prompted the plan of experiment B (table 2) in which animals were fasted for 10 hours prior to killing. Carboxypeptidase activity was depressed at 8 days after the start of lysine feeding but was not affected at 22 days. Tryptic activity increased at the 22-day interval in the control group but did not increase in rats fed the 6% lysine diet. It is apparent that the kind of treatment the animals receive prior to analysis for enzyme activity is important, and it may affect the results obtained. To determine the effect lysine and ε-aminocaproic acid actually have on the production of these enzymes would require a much more detailed study. However, the data indicate that the 2 compounds do have some effect on pancreatic proenzyme content but this effect cannot be correlated with the effect on growth rate.

Trypsin and carboxypeptidase B activities of the intestinal contents of rats fed control diets or diets with 6% L-lysine or 6% ε-aminocaproic acid were determined after the animals had been allowed access to the diets for 14 days. When expressed per unit body weight, the data indicate that neither compound significantly affected tryptic activity (not shown), whereas the carboxypeptidase activity was depressed 30% by lysine and appeared to

TABLE 2

Pancreatic trypsinogen and procarboxypeptidase B of rats receiving dietary L-lysine or ε-aminocaproic acid

Time fed diet	Treatment	Body wt	Pancreas wt	Pancreas wt	Carboxypeptidase B per g pancreas	Carboxypeptidase B per 100 g body wt	Trypsin per g pancreas	Trypsin per 100 g body wt
days		*g*	*g*	*% body wt*	*ΔOD/min*	*ΔOD/min*	*ΔOD/hr*	*ΔOD/hr*
				Experiment A [1]				
0	Control	50	0.221	0.440	25	12	84	39
2	Control	53	0.286	0.539 ± 0.014 [2]	42.3 ± 3.5	23	96.5 ± 14.4	52.0 ± 7.7
	+6% L-lysine	50	0.304	0.612 ± 0.035	32.1 ± 5.4	20	96	56
	+6% ε-aminocaproic acid	51	0.301	0.590	38	23	69.2 ± 12.6	42.0 ± 9.8
7	Control	84	0.675	0.813 ± 0.037	41.8 ± 3.8	34.6 ± 4.5	133.4 ± 20.0	110.2 ± 18.6
	+6% L-lysine	62	0.379	0.607 ± 0.025 [3]	26.3 ± 1.8 [3]	15.8 ± 0.6 [3]	73.5 ± 16.6 [4]	43.7 ± 9.3 [4]
	+6% ε-aminocaproic acid	78	0.392	0.503 ± 0.044 [3]	28.3 ± 3.2 [4]	14.2 ± 1.8 [3]	73.2 ± 21.6	40.0 ± 16.0 [4]
35	Control	279	1.414	0.504 ± 0.033	49.6 ± 2.5	24.9 ± 0.6	159.2 ± 1.8	80.8 ± 5.5
	+6% L-lysine	171	1.093	0.641 ± 0.028 [4]	37.2 ± 4.4 [4]	24.0	179.8 ± 32.7	109.2 ± 22.8
	+6% ε-aminocaproic acid	259	1.167	0.458 ± 0.010	29.4 ± 4.2 [3]	13.4 ± 2.3 [3]	131.4 ± 10.9 [4]	60.2 ± 6.2 [4]
				Experiment B [5]				
8	Control	83	0.764	0.92	22.4 ± 3.0	21.0 ± 3.4	50	46
	+6% L-lysine	61	0.538	0.89	14.4 ± 3.2	12.8 ± 1.2	43	41
22	Control	162	1.141	0.70	19	13	138.8 ± 11.4	93.0 ± 19.7
	+6% L-lysine	117	0.891	0.76	17	13	38.4 ± 2.6 [3]	29.4 ± 1.9 [4]

[1] Animals used in experiment A were allowed access to diets until they were killed.
[2] Mean ± SE of mean.
[3] Significantly different from control value, $P \leqq 0.01$.
[4] Significantly different from control value, $P \leqq 0.05$.
[5] Animals used in experiment B were fasted for 10 hours prior to being killed.

be increased by ε-aminocaproic acid (11.5 ± 1.9, 4.9 ± 1.4, and 25.5 ± 6.1 units/rat for control, 6% lysine, and 6% ε-aminocaproic acid groups, respectively). Since the enzymes were not separated from the dietary inhibitors nor were lysine or ε-aminocaproic acid concentrations in the intestinal contents determined, the decrease in peptidase activity may reflect residual inhibitor. It should be noted that little is known of the relative distribution of lysine and ε-aminocaproic acid in the gut and tissues of the rat. Despite all of the restrictions on the interpretation of these data imposed by the experimental plan, it may be concluded that lysine and ε-aminocaproic acid did not severely reduce the quantities of trypsin and carboxypeptidase B in the intestinal contents.

Another approach to elucidating the possible in vivo effect of lysine on the proteolytic enzymes was to determine the effect of excess dietary lysine in animals fed casein hydrolysate. Although less than optimal growth was observed in the control animals, 6% L-lysine severely depressed growth; the depression was eliminated by an additional supplement of 1% L-arginine (table 1).

Bone. The effect of different levels of dietary supplementation of lysine, arginine, or both on growth and bone composition was studied. The outline and data obtained are shown in table 3. The expected depression of growth by lysine and reversal by arginine were observed. In direct contrast with earlier observation in the chick (2), the lipid content of the bones was not significantly affected by the dietary treatment. The ash content was decreased by 6% L-lysine (when compared with controls) and was returned to normal by supplementation with arginine. The composition of the ash was relatively unaffected by the treatment, except for a possible increase in phosphorus with lysine supplementation. In the chick, L-lysine decreased the ash value (per cent of dry weight) to a much greater degree, whereas the composition of the ash was not affected by lysine or arginine. One interpretation of the differences in response of the 2 species is that in the chick the bone is directly affected by the decrease in circulating arginine, whereas in the rat the

TABLE 3

Growth and bone composition of rats fed diets supplemented with lysine, arginine, or both

Treatment	Diet [1]		Wt gain	Bones [2]					
							Ash		
	Lysine	Arginine		Dry wt	Lipid	Ash	Ca	Mg	P
	%	%	g	mg	% dry wt	% dry wt	%	%	%
1	0	0	156 ± 7.0 [2]	287 ± 12	6.4 ± 0.4	54.5 ± 0.5	35.3 ± 0.1	0.8 ± 0.09	18.8 ± 0.1
2	1.5	0	138 ± 9.0	—	—	—	—	—	—
3	3	0	130 ± 5.7	274	7.6	53.8 ± 0.9	36.8 ± 0.6 [3,6]	0.8 ± 0.2	19.1 ± 0.1
4	6	0	81 ± 2.9	217 ± 19 [3]	7.3 ± 0.8	51.8 ± 0.6 [3]	34.4 ± 0.4	1.8 ± 0.4	19.2 ± 0.1 [3]
5	0	0.5	157 ± 7.5	265	5.1 ± 0.8	55.8 ± 1.2 [6]	34.4 ± 0.4	1.3 ± 0.4	18.9 ± 0.1
6	0	1.0	151 ± 7.1	281	5.7 ± 0.4	55.1 ± 0.8 [6]	35.8 ± 0.5	0.9 ± 0.3	19.2 ± 0.1 [3]
7	3	0.5	149 ± 5.7	270	6.1	54.7 ± 0.9 [6]	35.0 ± 0.2	1.5 ± 0.45	19.2 ± 0.1 [5]
8	3	1.0	151 ± 6.1	257	5.7	55.2 ± 0.4 [5]	34.6 ± 0.8	1.2 ± 0.67	19.0 ± 0.1
9	6	0.5	103 ± 5.5	234 ± 8 [4]	7.4 ± 0.7	54.5 ± 0.5 [6]	35.3 ± 0.4	1.1 ± 0.48	19.3 ± 0.1 [3]
10	6	1.0	121 ± 5.5	231 ± 10 [4]	6.8 ± 0.4	55.2 ± 0.5 [5]	33.1 ± 1.2	0.7 ± 0.12 [6]	19.1 ± 0.1

[1] Five rats/group were fed the diets for 28 days.
[2] All values are mean ± SE of mean.
[3] Significantly different from treatment 1, $P < 0.05$.
[4] Significantly different from treatment 1, $P < 0.01$.
[5] Significantly different from treatment 4, $P < 0.01$.
[6] Significantly different from treatment 4, $P < 0.05$.

effect may be less specific, possibly reflecting a delayed maturation or abnormal metabolic state. The absence of any gross abnormalities in these rats is in agreement with the observations of Sidransky and Verney (24) who reported only increased liver glycogen and pancreatic trypsin as abnormalities in acute arginine deficiency in the rat.

Plasma and muscle amino acids. Two experiments were performed to determine the free amino acid concentrations in the plasma and muscle of rats fed various levels of lysine with and without arginine. The rats were allowed access to the diets until samples were obtained, at either 13 or 24 days.

In contrast with the chick (2), the concentrations of free amino acids in plasma and muscle of rats fed diets supplemented with L-lysine, L-arginine, or both, furnished little direct information as to the cause of the lysine-arginine relationship. Dietary supplementation with L-lysine resulted in increased plasma and muscle lysine concentrations and supplementation with arginine resulted in increased tissue arginine concentrations (table 4). Increased dietary lysine did increase muscle arginine concentrations, but the significance of the relatively small increase in plasma concentrations is questionable. Sauberlich (25) fed rats 6% casein rations supplemented singly at 5% with each of a number of different amino acids, including L-lysine, glycine, L- and DL-aspartic acid, and DL-tryptophan. He reported that of these, lysine increased and the others decreased plasma arginine concentration. The difference between his observations and those reported herein may be due to the time the animals were fed the diets, since it will be shown later in this report that plasma arginine concentration may be increased by dietary lysine after shorter times on the diets.

Nitrogen metabolism. The determination of concentrations of compounds, other than arginine, involved in the urea cycle—ornithine, citrulline, and urea—also yielded little information concerning the effect of lysine on general nitrogen metabolism. Muscle ornithine concentration was sharply increased by supplementary arginine regardless of lysine supplementation, indicating that probably there was an increased degradation of arginine detectable under these conditions; but it appeared that the rat had adapted to the increased intake of lysine, and it consumed only what it could metabolize so that additional end products of nitrogen metabolism did not accumulate. Possibly the tissues analyzed were not the ones which would show increased levels of these compounds.

Increased activities of the urea-cycle enzymes would indicate the potential for an increased synthesis of arginine. Schimke (26) has stated that 5% lysine added to a 15% casein diet did not affect urea excretion or the levels of urea-cycle enzymes in the liver of the rat. He reported that added arginine, 2.5 and 7.5%, did not reduce the activities of carbamylphosphate synthetase, ornithine transcarbamylase, argininosuccinate synthetase, argininosuccinase, or arginase but an arginine deficiency created by feeding an arginine-deficient amino acid mixture caused a decrease in arginase and an increase in the other enzymes. Thus, an arginine deficiency changes these enzymes in a direction favoring conservation of arginine, whereas they apparently were not changed by increased dietary lysine.

In Schimke's study (26) the concentration of the urea-cycle intermediates in liver were not altered by any of the conditions although the urea-cycle enzymes were altered. In the present studies, lysine and lysine plus arginine treatments significantly increased blood urea levels in rats sampled at 13 days: control, 466 μmoles/100 ml blood; 6% L-lysine, 633; and 6% L-lysine + 1% L-arginine, 583. However, the blood urea level was not significantly affected by increased dietary lysine alone at 28 and 30 days (see table 4 for data at 28 days).

There was a greater excretion of urea by rats that had been fed added lysine for 5 weeks, and there was an increase in urinary excretion of urea by rats when they were changed from an 18% casein diet to one with an added 6% L-lysine (table 5). Therefore, further investigation of these enzymes and the metabolism of arginine in animals fed lysine may be warranted.

TABLE 4

Free amino acids in young rats fed casein diets supplemented with lysine, arginine, or both [1]

	Plasma								Muscle								
Treatment no.	1		2	3		4	5	6	1		2	3		4	5	6	
L-Lysine added, %	0		3	6		3	6	0	0		3	6		3	6	0	
L-Arginine added, %	0		0	0		1	1	1	0		0	0		1	1	1	
Amino acid	μmoles/100 ml					μmoles/100 ml			μmoles/100 g					μmoles/100 g			
Taurine [2]	15		15	25		17	11	14	—		—	—		—	—	—	
Urea [2]	509		417	523		447	550	488	—		—	—		—	—	—	
Hydroxyproline	4		3	5		4	2	3	33			27			23	31	
Aspartic acid	2		1	2		2	1	2	21			28			23	39	
Threonine	54		41	25		39	31	36	159			108			124	160	
Serine	31		29	21		29	21	24	192			154			144	199	
Asparagine, Glutamine	67		59	48		31	40	26	437			471			227	448	
Proline	35		33	22		30	37	31	112			65			125	114	
Glutamic acid	17		13	16		19	11	17	122			130			171	163	
Citrulline	11		11	11		12	12	10	35			42			30	33	
Glycine	18		16	14		20	12	15	388			344			309	341	
Alanine	68		47	37		56	47	47	420			426			445	237	
Valine	22		21	15		13	19	21	30			19			24	29	
Half-cystine	4		7	2		5	4	3	—			—			—	—	
Methionine	9		7	6		5	6	5	10			12			11	13	
Isoleucine	13		9	7		9	8	9	11			8			10	13	
Leucine	18		14	11		14	13	15	17			12			14	18	
Tyrosine	19		10	5		13	11	13	26			9			20	26	
Phenylalanine	8		6	5		7	5	6	8			5			7	10	
Ornithine	6	7	5	3	4	5	9	10	9	27	12	7	12	41	25	26	76
Lysine	50	79	141	137	281	93	340	52	153	392	432	1513	984	1013	2050	176	397
Histidine	5	6	9	6	8	6	10	5	26	36	39	40	26	21	33	23	29
Anserine [3]	7		7	3		5	8	7	152	57	99	74	36	79	69	117	49
Carnosine	—		—	—		—	—	—	299	131	120	81	54	85	75	164	112
Arginine	7	9	11	8	9	13	17	15	20	27	62	57	51	130	97	74	95
Body wt, mean increase, g	156	58	130	81	30	150	122	151									62

[1] Values are means of 2 pooled samples, each from 3 to 4 rats, taken after the diets had been fed for 28 days, except for second columns under treatments 1, 3, and 6, which represent means of 2 pooled samples each from 5 rats, taken after the diets had been fed for 13 days.

[2] Muscle level not calculated.

[3] Identified in plasma by retention time only.

TABLE 5

Effect of dietary lysine on urinary excretion of sodium, potassium, and urea by the rat [1]

Treatment [2]	Day	Na	K	Urea
		mEq/rat/day		*mg/rat/day*
1 Casein basal [3]	1	0.72	1.15	164
	2	0.93	1.01	162
	3	0.83	0.92	163
	4	1.16	1.31	242
	5	1.33	1.34	265
	6	1.17	1.46	279
		1.02 ± 0.1 [4]	1.20 ± 0.1	212 ± 22.7
2 Treatment 1 to 6% L-lysine	1	0.94	2.08	347
	2	0.90	1.33	420
	3	0.45	0.47	296
	4	0.92	0.85	471
	5	0.46	0.49	484
		0.73 ± 0.4	1.04 ± 0.6	404 ± 36.0 [5]
3 Treatment 1 plus 6% L-lysine [3]	1	0.19	0.26	271
	2	0.53	0.45	264
	3	0.23	0.20	108
	4	0.57	0.57	437
	5	0.87	0.73	299
	6	0.45	0.40	250
		0.47 ± 0.1 [5]	0.44 ± 0.1 [5]	272 ± 42.9

[1] Mean values, 5 rats/group.
[2] Diets offered ad libitum.
[3] Rats had been maintained with this diet for 5 weeks previously.
[4] Mean ± SE.
[5] Different from casein basal, $P \leq 0.01$.

The distribution of histidine, another basic amino acid, did not parallel that of arginine, making it unlikely that the increased muscle arginine concentration was caused by a movement of arginine into the tissues as a cation. Because the muscle arginine concentration increased, it appears that, at the levels observed in the plasma of rats fed 6% L-lysine, lysine does not compete with arginine for transport into muscle. The increased muscle arginine concentration remains unexplained. Although lysine could conceivably alter the utilization of arginine by a variety of ways, the data from animals receiving a chronic load of lysine were of little help in determining the mechanism of the lysine-arginine relationship.

Other ninhydrin-positive substances detected on ion exchange chromatography were: β-alanine, which did not occur in plasma but was noted in the muscle samples; α-aminoadipic acid, a metabolic product of lysine, which was low in plasma but was increased when lysine was fed. However, the corresponding tissue levels could not be correlated with treatment; Tamura (27) noted an increased α-aminoadipic acid excretion in rats fed excess lysine. In addition, there was an unknown peak that appeared 30 ml after tyrosine and was correlated only with lysine supplementation.

Morrison and co-workers (28) reported an inverse relationship between dietary lysine and plasma threonine in the rat. In all of the present experiments in which plasma threonine was measured, it was decreased by increased dietary lysine (table 4). For these reasons, rats were fed diets in which various amounts of threonine (0.25 to 1.00%) were added to the basal casein ration and to a ration which also had 6% L-lysine added. The added threonine did not stimulate growth, either with or without added lysine; the rats fed added threonine grew 7 to 10 g less than did comparable controls in a 30-day period.

Food consumption. Alterations in the amino acid composition of a diet frequently affect the consumption of that diet when it is offered ad libitum. In many of these instances the animals appear to adapt to the diet after a short period and resume food consumption and growth at near normal rates. Thus, it is difficult to establish direct interactions between amino acids or

other factors unless food consumption or growth or both are equal on both treatments. In an attempt to separate effects of decreased food consumption from effects of lysine per se on depression of growth rate, the effect of 6% L-lysine on growth and food consumption was determined for rats maintained at 4 and at 27° and for rats previously depleted of protein.

The results with weanling rats conventionally housed at 27° are shown in figure 1; the food consumption and growth rate of rats were immediately depressed by diets containing 6% L-lysine. To determine whether the depression in growth was a reflection of the reduction in food consumption, the data at 17 days were analyzed by covariance analysis with adjustment of gain for differences in food intake. The 2 groups had a common slope of gain on food intake, and the test of adjusted means showed a significant difference ($P < 0.001$). Rats were maintained at 4° to increase their voluntary food intake, thereby forcing the use of amino acids as energy sources. This maneuver is interpreted as restriction of calories from nonprotein sources and probably is similar to adaptation of catabolic pathways to a high protein diet (Krauss and Mayer (29) accomplished this adaptation by placing rats on a very high protein intake).

Weanling rats were allowed to adjust to an ambient temperature of 4° for 6 days. Then one-half of the rats were fed the diet containing 6% L-lysine. The amount of food consumed per rat was very nearly equal in both groups for the first 6 days of the experiment (fig. 2). However, the growth rate of the rats given excess lysine was affected within the first day and remained depressed throughout the experimental period. The data obtained after the rats had been fed the experimental diets for 6 days were subjected to covariance analyses; the results were similar to those at 27°. The 2 groups had a common slope of gain on food intake, and the test of adjusted means showed a significant difference ($P < 0.001$). The food consumption of the rats maintained in a cold environment may depend on energy requirement only; however, although the rats adapted to use some protein for energy purposes, the addition of lysine still decreased the efficiency of diet utilization. This is in direct contrast with observations on leucine-isoleucine imbalance in which the growth-depressing effect of an imbalanced diet can be reduced by adapting the animals to a high protein diet prior to feeding them an imbalanced diet (29), by increased food consumption caused by a cold environment, or by insulin injection (see (1) for additional references). Since in both instances, at 4° and at 27°, the F values (covariance analyses) were highly significant, it is concluded that the quantity of food intake does not explain the

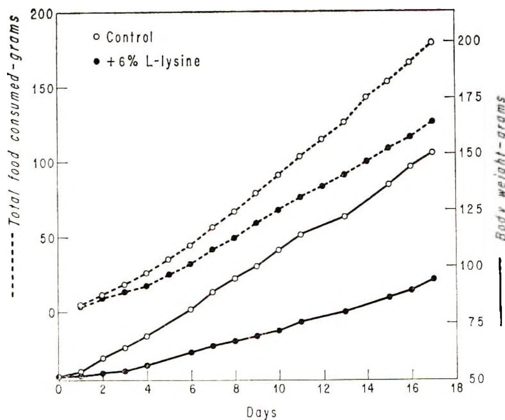

Fig. 1 Effect of 6% L-lysine on growth and food consumption of rats maintained under conventional conditions at 27°.

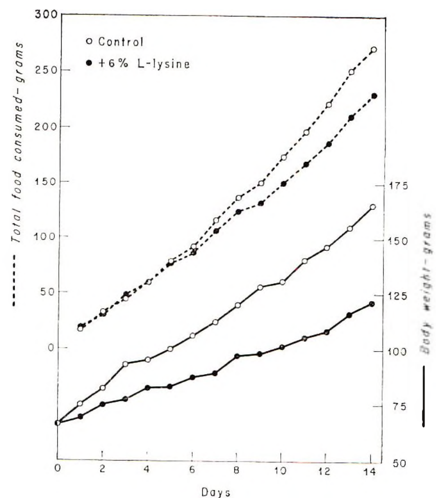

Fig. 2 Effect of 6% L-lysine on growth and food consumption of rats maintained at 4°.

differences in lot gains caused by lysine and that the diets consumed are not equally adapted to assimilation by the rat.

It has been shown that in protein-depleted animals the activities of the catabolic enzymes involved in nitrogen metabolism are decreased (26). Thus, if lysine has any effect on the enzymes involved in nitrogen metabolism the effect of excess lysine on metabolic processes, and therefore on the animal, should be measurable in such animals. To test this hypothesis, 35-day-old rats weighing about 120 g were depleted of protein for 10 days, by which time they had decreased in weight by an average of 30 g. They were then fed the control and lysine-containing diets under conventional conditions. The group receiving lysine showed a decrease in food consumption within 1 hour and definite reduction in growth within 20 hours (fig. 3). The greater gain in body weight during the first 8 hours in the animals receiving lysine can probably be attributed to retention of water caused by the consumption of lysine·HCl. These data, when compared with the 2 previous experiments, indicate that the protein-depleted rat initially is less able to handle excess dietary L-lysine.

Short-term changes. The data on free amino acid concentrations in tissues, presented in table 4, were obtained from animals which probably had adapted to a high, chronic consumption of lysine, indicated by a resumption of growth at a reduced rate after a short interval of severe growth depression. Therefore, data collected immediately after the animals received the diets might be of considerable value in determining the cause of the relationship, and such data would be influenced less by voluntary restriction of diet consumption caused by the addition of lysine to the diet. Postabsorption curves of plasma concentrations of amino acids have been used with success in assaying the biologic value of some selected proteins, and they appear to indicate whether an animal requires more of an amino acid than is furnished by the dietary protein (30). Therefore, this method should indicate whether the utilization of one amino acid (arginine) is altered prior to or during absorption by experimental treatment (high dietary lysine).

The basal casein diet and the casein diet supplemented with L-lysine were fed to rats, fasted for 10 hours, according to one of the following 3 treatments: 1) previously protein-depleted, 2) maintained in the conventional manner, and 3) adapted to 4°. The diet consumption (figs. 1, 2, and 3) of rats previously treated in a similar manner suggested that the intervals zero, 4, 10, 24, and 48 hours might be significant. Plasma lysine and arginine concentrations were determined microbiologically and the values are presented in table 6. The plasma lysine concentration of rats maintained conventionally

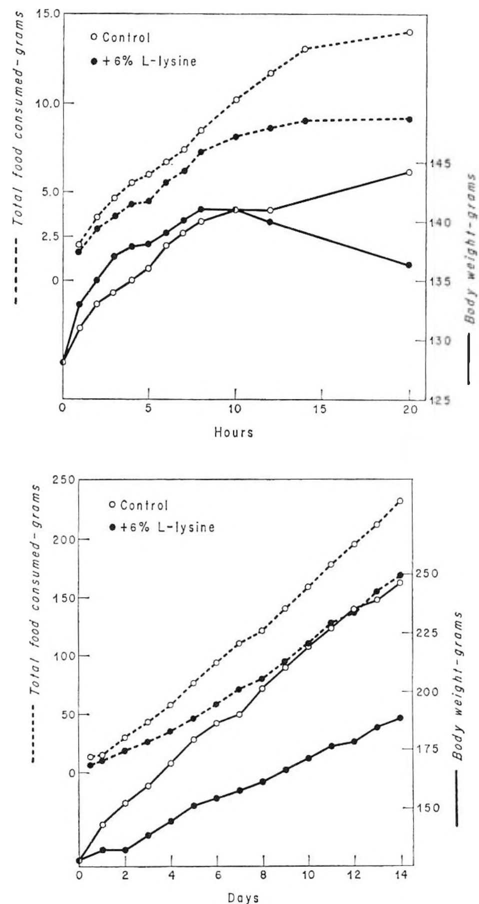

Fig. 3 Effect of 6% L-lysine on growth and food consumption on previously protein-depleted rats. *Upper*, first 20 hours. *Lower*, entire 14-day period.

TABLE 6

Plasma lysine and arginine concentrations in rats fed control or 6% L-lysine diets

Time after access to diets,[1] hours		0	4	8	10	13	24	48
Treatment [2,3]	Diet							
			Plasma lysine, μg/ml [4]					
27°	control	109	105		136		171	159
	+6% L-lysine		622 [5]		619 [5]		546 [5]	754 [5]
4°	control	99	147		177		107	135
	+6% L-lysine		642 [5]		935 [5]		502 [5]	732 [5]
Protein-depleted	control	18		111		87		
	+6% L-lysine			551 [5]		148		
			Plasma arginine, μg/ml					
27°	control	36	28		24		25	33
	+6% L-lysine		33 [5]		28		49 [5]	38
4°	control	33	40		40		29	44
	+6% L-lysine		32 [6]		37		46	49
Protein-depleted	control	28		20		14		
	+6% L-lysine			26 [6]		23 [6]		

[1] All rats were fasted for 10 hours prior to being allowed access to the diets.
[2] Weanling rats were allowed to adapt to temperature and basal diet 5 days prior to initiation of the experiment.
[3] Rats 35 days of age were depleted of protein for 10 days prior to initiation of the experiment.
[4] Mean of 4 samples of 2 animals/sample, values determined microbiologically.
[5] Different from respective control, $P \leqq 0.01$.
[6] Different from respective control, $P \leqq 0.05$

with the control diet reached a maximum at 24 hours after the rats had access to the diet, whereas in the animals maintained in the cold, it reached a maximum 14 hours earlier. When 6% L-lysine was added to the diets a high concentration of plasma lysine was reached by 4 hours in conventionally kept animals and by 10 hours in animals maintained at 4°. The concentration then decreased and subsequently increased again by 48 hours in both groups. The plasma arginine concentration in control, conventionally housed animals decreased by 4 hours and then increased to initial values by 48 hours, whereas in control rats maintained in the cold it increased by 4 hours and remained higher than in the conventionally housed animals during the remaining intervals. When 6% L-lysine was added to the diet the plasma arginine concentration in the conventional group, although fluctuating, was always higher than the control values. In the animals kept in the cold the 4- and 10-hour arginine values were lower than the respective cold controls but the 24- and 48-hour values were greater.

Thus the initial decrease in food consumption was probably not caused by a decreased plasma arginine concentration since, in animals receiving lysine in the cold, the arginine concentration was decreased but the animals continued to eat as well as the controls, whereas in the warm animals the controls had a greater decrease in plasma arginine concentration than did those receiving lysine. This conclusion is strengthened by the data, presented in table 6, obtained from previously protein-depleted rats. The metabolic processes involving nitrogen metabolism in these animals should have become adapted to minimal activity by the pretreatment—that is, the inverse of the cold-adapted rats. These animals were more sensitive to lysine supplementation (fig. 3) and their early refusal to eat the lysine-supplemented diet was reflected by the decrease in plasma lysine concentration in that group between 8 and 13 hours. As in the conventional animals, plasma arginine concentration decreased in the controls and was definitely increased above control values in the lysine-fed rats. It is not likely that the source of increased plasma arginine is tissue arginine because tissue arginine, although determined at a different interval, also appeared to be increased under such conditions (table 4). Hall and Gabuzda[8] reported an increased muscle arginine con-

[8] See footnote 4.

centration in the rat after giving 1 mmole of L-lysine/100 g of body weight daily for 3 days.

Although plasma arginine values indicated that this amino acid was possibly limiting in casein, as has been shown in dogs (29), the addition of lysine to the casein diet definitely did not appear to lower these values further. A large individual variation was noted in our postabsorption data and was probably due to the experimental plan—although a group of animals was used for each time interval, each animal was bled only once. To obtain a complete curve on both diets (with and without 6% lysine) from one animal, the procedure described by Longenecker and Hause (30) was performed with dogs. The results indicated that excess lysine does not depress postabsorption curves of arginine in dogs.[9] Injection of a high concentration of lysine into rats has been shown to inhibit arginase in vivo (4), which would tend to increase tissue concentrations of arginine even in spite of a possible decrease in intake or increased utilization via other pathways.

In using free amino acid concentrations of plasma for the evaluation of the amino acid status of an animal or for the evaluation of the quality of a dietary protein, certain assumptions are made, and some of them are open to criticism. One of these assumptions is that occurrence of a somewhat similar distribution of amino acids in plasma and in tissues means that, from a determination of amino acids in plasma and possibly another tissue such as muscle, it is possible to predict the overall status or distribution of these compounds. It is now apparent that the method used to prepare a tissue sample determines what one calls "free amino acids," that different tissues from the same animal have marked differences in amino acid patterns, and that not enough data have been published to allow conclusions without actually determining the distribution of amino acids. It may also be that not all tissues reflect abnormal concentrations of amino acids and metabolities. The data presented here indicate that prediction of the nutritional adequacy of a protein on the basis of plasma amino acid concentrations depends on the specific amino acid being studied and would be of most value in the evaluation of amino acids that are less active metabolically—an apparent arginine deficiency cannot be predicted from the plasma arginine concentration.

Electrolytes. A preliminary experiment was performed to determine whether dietary lysine or arginine or both would affect the cellular distribution of electrolytes. Addition of 6% L-lysine to the diet decreased muscle potassium by 27 mEq/kg wet weight when compared with controls, whereas sodium was not affected. When the cellular distribution was calculated on either a chloride or a sodium basis, 6% L-lysine decreased $[K]_c$ by 37 mEq/kg $(H_2O)_c$ or $[K]_c^{Na}$ by 34 mEq/kg $(H_2O)_c^{Na}$. The feeding of 1% arginine alone or with lysine had little effect on the distribution of potassium or sodium. These data were obtained from rats after the diets had been fed for 13 days and, although similar to those obtained in our laboratory in young chicks (2), were contrary to those reported by Eckel and co-workers (12) in the rat. This experiment was repeated because it contained only three or four observations per treatment and the plasma and muscle specimens were not obtained from the same animals.

Weanling rats were fed the diets for 14 days; the experimental outline and data obtained are shown in table 7. To avoid the error that could be introduced by analyzing a muscle specimen from an exsanguinated animal for electrolytes and also to allow a statistical evaluation of the data, tissue electrolytes were determined on animals that had been bled immediately before obtaining the muscle specimen and also on an equal number that had not been bled previously. The cellular distributions of sodium and potassium were then calculated for both groups of muscle on the basis of the plasma values obtained from the first group.

In this experiment, feeding L-lysine again severely decreased the growth rate, and arginine had a beneficial effect on growth only in the presence of excess lysine. Supplementation with either lysine or arginine increased the plasma chloride concentration. There was a significant

[9] Jones, J. D., and P. Burnett, unpublished data.

TABLE 7

Electrolyte distribution in the young rat fed lysine, arginine, or both

Treatment no.	1		2		3		4	
Supplemental L-lysine, %	0		6		0		6	
Supplemental L-arginine, %	0		0		1.5		1.5	
Gain in body wt, g [1]	72.4 ± 3.6		28.5 ± 2.8 [2]		76.4 ± 2.9		57.2 ± 3.3 [3]	
Composition of plasma [4]								
H_2O, g/liter	934.7 ± 0.05		—		—		—	
Na, mEq/liter	132.3 ± 1.9		135.8 ± 1.6		138.8 ± 0.7 [5]		137.0 ± 1.0	
K, mEq/liter	4.6 ± 0.3		4.4 ± 0.3		5.0 ± 0.4		4.2 ± 0.2	
Cl, mEq/liter	100.2 ± 1.5		109.4 ± 2.5 [5]		106.4 ± 1.5 [5]		109.2 ± 1.6 [5]	
Composition of muscle								
H_2O, g/kg wet wt	750 ± 0.8	745 ± 3.2 [6]	742 ± 6.1	740 ± 2.0	756 ± 2.1 [7]	747 ± 0.9	745 ± 3.8	741 ± 1.2
Na, mEq/kg wet wt	27.5 ± 0.8	26.4 ± 0.9	26.4 ± 0.8	26.7 ± 1.7	24.9 ± 0.8	26.8 ± 0.9	24.9 ± 0.8 [5]	25.4 ± 0.6
K, mEq/kg wet wt	103.4 ± 2.2	104.0 ± 1.1	85.4 ± 1.4 [2]	87.2 ± 2.4	107.8 ± 1.2 [3,8]	105.8 ± 1.4	89.0 ± 1.6 [2]	90.9 ± 1.4
Cl, mEq/kg wet wt	21.0 ± 0.6	20.5 ± 0.2	19.2 ± 0.2 [2]	20.2 ± 0.5	18.5 ± 0.6 [5]	23.2 ± 0.6	18.2 ± 0.2 [2]	20.9 ± 0.5
Data derived from results given above								
$(H_2O)_E^{Cl}$ [9], g/kg	192 ± 3.8	186	160 ± 4.4 [2]	168	159 ± 5.6 [2]	199	153 ± 2.4 [2]	175
$(H_2O)_c$, g/kg	558 ± 4.5	559	582 ± 7.2 [5]	572	597 ± 5.1 [5]	548	593 ± 2.9 [5]	566
$[Na]_c$, mEq/kg $(H_2O)_c$	3.5 ± 0.6	2.9	7.8 ± 1.5 [5]	6.5	6.4 ± 2.2	−1.8	6.2 ± 1.0	1.7
$[K]_c$, mEq/kg $(H_2O)_c$	194 ± 3.5	185	146 ± 4.1 [2]	151	180 ± 1.4 [3,8]	191	151 ± 2.8 [2]	159
$[K]_c^{Na}$, mEq/kg $(H_2O)_c^{Na}$	191 ± 5.9	189	154 ± 5.6 [2]	159	186 ± 3.9 [3,8]	189	158 ± 4.1 [2]	162

[1] Mean ± SE; n = 10 for weight gains after diet had been fed for 14 days; other values are mean ± SE, n = 5.

[2] Significantly different from treatment 1, $P \leq 0.01$.

[3] Significantly different from treatment 2, $P \leq 0.01$.

[4] A mean value of 934.7 ± 0.05 was used to calculate the derived data; the treatments did not alter this value.

[5] Significantly different from treatment 1, $P \leq 0.05$.

[6] These values are a mean of 5 rats that had not been bled prior to killing; the derived data below these numbers were obtained by using the mean plasma values that are shown.

[7] Significantly different from treatment 2, $P \leq 0.05$.

[8] Significantly different from treatment 4, $P \leq 0.01$.

[9] Symbols: $(H_2O)_E^{Cl}$ = extracellular water/kg tissue assuming all of Cl to be extracellular.
$(H_2O)_c$ = intracellular water/kg tissue.
$[Na]_c$ = Na/kg intracellular water.
$[K]_c$ = K/kg intracellular water.
$[K]_c^{Na}$ = K/kg intracellular water assuming all of Na to be extracellular.

increase in plasma sodium concentration only when arginine was fed without added lysine, although values for plasma sodium appeared to be higher when rats were supplemented with either lysine, arginine, or both. The observed increases in chloride may have been caused by the ingestion of the amino acids as monohydrochlorides rather than by a lysine-induced acid-base disturbance. The analysis of muscle confirmed the preliminary observations in that lysine caused a significant decrease in potassium in all animals and, although both lysine and arginine decreased the chloride in muscle from animals previously bled, they had no effect on muscle chloride in animals that were not previously bled. Addition of only arginine, which theoretically could exchange for potassium, did not cause an alteration in muscle potassium; this probably reflects the difference in the levels of lysine and arginine noted in the muscle (table 4). However, since arginine is readily metabolized by the rat the increase in plasmaariginine concentration in the arginine-supplemented animals was not comparable to that observed for lysine with lysine supplementation.

These data were used to derive cellular distribution of electrolytes, and in every instance the chloride changes were reflected in an increase in cellular water and a decrease in extracellular water. Cellular sodium calculated from chloride space tended to increase in rats fed lysine and arginine; lysine caused a highly significant decrease in potassium on a cellular basis when calculated from either chloride or sodium space.

On comparing the muscle values of animals not previously bled with those of animals that were bled for plasma determinations, very similar data were obtained with the exception of the group supplemented with 1.5% arginine; in this group the values for water were lower than would be predicted and for chloride were higher than would be predicted. These differences in chloride also affected the derived data. Since the relative values for cellular sodium depended on previous bleeding, it would be unwise to attempt an interpretation of those values at this time.

The data on the effect of lysine on the urinary excretion of electrolytes are shown in table 5. In this experiment, samples of urine from 8-week-old rats receiving the control diet were collected. The diet was then changed to contain 6% L-lysine. Urine was also collected from rats, of a comparable age, which had been receiving a diet containing 6% L-lysine for 5 weeks. Only a transient increase, similar to that observed by Eckel and co-workers (12), in urinary potassium was observed when the animals were fed the diet containing lysine. Whether this was caused by facilitation of tubular secretion rather than by inhibition of reabsorption in the kidney or reflected a release of potassium from the tissues was not determined. The lower urinary values for the animals maintained with 6% lysine probably reflected a decreased food intake. In our experiments in dogs[10] the plasma potassium concentration was not increased during the 6 hours immediately after feeding test meals containing 4% L-lysine, probably because the plasma lysine concentrations were not increased sufficiently.

These in vivo data confirm the conclusion of Levinsky and co-workers (13), drawn from in vitro experiments, that lysine competes with potassium in muscle. Thus, our data indicate that the displacement of muscle potassium by lysine, indicated by an increase in plasma potassium concentrations in the short-term experiment of Dickerman and Walker (8), is maintained for longer intervals in the rat by the increased plasma lysine levels caused by 6% L-lysine in the diet. Regardless of the mechanism (9) by which lysine increases potassium excretion, it has an initial effect which is most likely of a transient nature.

Eckel and co-workers (11), in a somewhat similar experiment, did not obtain data like those presented here. Although they fed the rats 10% L-lysine·HCl, they may not have produced plasma lysine concentrations high enough to compete with potassium for transport in muscle. This is likely because rats restrict their intake of a diet that contains a high supplement of lysine. The plasma lysine concentration appears to be a critical factor since

[10] See footnote 9.

the reabsorption of a competing ion by the kidney is not inhibited until a critical concentration is reached (8). Additional support for this statement is that in chicks the electrolyte distribution was altered more in those in which higher lysine levels were obtained (2).

That this electrolyte shift in muscle could be caused by the HCl accompanying the lysine is unlikely since the HCl accompanying arginine at 1.5% also increased the plasma chloride concentration but did not affect [K] calculated from either chloride or sodium space, and arginine·HCl added to diets containing lysine·HCl did not decrease cellular potassium by an additional increment.

The relationship of the electrolyte shifts to the adverse effects of excess lysine and the reduction by arginine of the effect of lysine on growth but not of that on electrolytes suggest that these 2 effects of lysine can be separated and studied independently. We have not determined the electrolytes in rats receiving other dietary levels of potassium. However, neutralization of a diet containing 6% lysine as the monohydrochloride with $KHCO_3$ did not significantly alter the growth depression caused by the lysine. It is possible that, when consumed in high concentrations, lysine affects the acid-base balance of the rat and this causes the reduced food consumption noted. However, if this were the case, then the chicks and the cold-adapted rats should be similarly affected soon after consuming such diets, but they were not. Unfortunately, we have no data on blood acid-base balance under any of these conditions.

Urinary excretion. Dietary lysine could also cause a loss of arginine via the urine by competition for reabsorption in the kidney. To test this possibility, urine was collected for 3-day periods from 4 rats while they were receiving the control diet and then while their diet was changed to contain 3% L-lysine and from 4 rats maintained with 3% L-lysine for 5 weeks. By microbiologic analysis, lysine excretion was 8, 22, and 15 mg/rat/day and arginine excretion was 4, 6, and 4 mg/rat/day for the respective groups. In a similar experiment, rats fed 6% L-lysine lost considerably more lysine in the urine and up to 9 mg/rat/day of arginine, determined by ion exchange, an insignificant portion of the arginine consumed. Therefore, it may be assumed that the apparent increased need for arginine is not caused by a loss of preformed arginine via the urine.

GENERAL COMMENT

Data presented here indicate that, in the rat, a lysine-arginine relationship can be demonstrated, which is quite similar to that described in the chick (2). However, the chick's inability to synthesize arginine introduces differences in the data obtained from the 2 species (a difference which may prove to be an asset in the eventual description of the mechanism of antagonism). In the chick a deficiency of arginine is readily reflected in a decreased plasma arginine concentration. This is not so in the rat and, therefore, our main evidence for a specific relationship is the elimination by arginine of the growth depression caused by lysine.

As in the chick, the mechanism of the relationship remains to be determined, but some of the possible explanations include interferences with digestion, absorption, excretion, or metabolism of arginine (degradation or use of arginine for synthetic purposes). Although the absorptions of lysine and arginine are competitive, our data would eliminate this as a cause of the antagonism for the following reasons: lysine has little effect when added to soy or stock rations; supplemental arginine relieves the effect of high lysine presumably after being absorbed; lysine did not cause a significant loss of arginine in the urine; and increased lysine concentrations in the muscle were accompanied by increased concentrations of arginine.

Although not completely eliminated as the site of antagonism, the effect of lysine on digestion appears less likely to be the site since 1) the alterations in the activities of the pancreatic enzymes thought to release arginine in the digestive processes could not be correlated with growth rates, and 2) lysine depressed growth when added to a diet containing enzymatically digested casein.

Results from those experiments suggest that a thorough assessment of the effect of excess lysine on digestive-absorptive proc-

esses would probably not yield the answer to the mechanism of the lysine-arginine antagonism. The absolute levels of lysine and arginine in the diets may explain the differences in response to excess lysine observed in animals receiving different dietary proteins. These results suggest that data from a study of feeding of varied levels of lysine added to crystalline amino acid diets would show a direct relationship between dietary lysine and arginine (this is currently under investigation).

It is possible that the metabolism of arginine is altered. Once absorbed, arginine is thought to be metabolized by 3 main processes: utilized for protein synthesis, used as an intermediate in urea excretion, and used in the synthesis of creatine.

It is unlikely that nitrogen—arginine specifically—is being used for increased protein synthesis because 1) the initial growth depression caused by excess L-lysine does not appear to be caused by decreased food intake since the animals maintained at low temperatures consumed a similar quantity of food as controls but grew less, and 2) lysine appeared to increase urea production which would also indicate a decreased efficiency of nitrogen utilization.

Although injection of a high concentration of lysine into rats has been shown to inhibit arginase in vivo (4) and thus would tend to increase tissue concentrations of arginine even in spite of a possible decrease in intake or increased utilization via other pathways, similar results, and conclusions, may not occur in animals fed lysine for a longer period of time.

It is also possible that enzymes concerned with the utilization of arginine for synthesis of essential metabolites—for example, creatine—are altered by lysine and that in the presence of excess lysine the feeding of arginine, by a process of mass action, can effect an adequate synthesis without altering the plasma arginine concentration significantly.

Therefore, the most probable interpretation of the data obtained to date in the rat is that excess lysine exerts its adverse effects on the rat via an effect(s) on the metabolism of arginine.

LITERATURE CITED

1. Harper, A. E. 1964 Amino acid toxicities and imbalances. In: Mammalian Protein Metabolism, vol. 2, eds., H. N. Munro and J. B. Allison. Academic Press, New York, pp. 87–131.
2. Jones, J. D. 1964 Lysine — arginine antagonism in the chick. J. Nutrition, *84:* 313.
3. O'Dell, B. L., and W. O. Regan 1963 Effect of lysine and glycine upon arginine requirement of guinea pigs. Proc. Soc. Exp. Biol. Med., *112:* 336.
4. Cittadini, D., C. Pietropaolo, D. de Cristofaro and M. d'Ayjello-Caracciolo 1964 *In vivo* effect of L-lysine on rat liver arginase. Nature, *203:* 643.
5. Chernikov, M. P. 1963 Effect of amino acids on the proteolytic activity of trypsin and chymotrypsin. Biokhimiia, *28:* 285.
6. Wolff, E. C., E. W. Schirmer and J. E. Folk 1962 The kinetics of carboxypeptidase B activity. I. Kinetic parameters. J. Biol. Chem., *237:* 3094.
7. Hepburn, F. N., and W. B. Bradley 1964 The glutamic acid and arginine requirement for high growth rate of rats fed amino acid diets. J. Nutrition, *84:* 305.
8. Dickerman, H. W., and W. G. Walker 1964 Effect of cationic amino acid infusion on potassium metabolism in vivo. Am. J. Physiol., *206:* 403.
9. Walker, W. G., H. Dickerman and L. J. Jost 1963 Mechanism of lysine-induced kaliuresis. Am. J. Physiol., *206:* 409.
10. Gershoff, S. N., E. Coutino-Abath, I. Antonowicz, A. L. Mayer, G. S. H. Shen and S. B. Andrus 1959 Some effects related to the potassium and lysine intake of rats. J. Nutrition, *67:* 29.
11. Eckel, R. E., J. E. C. Norris and C. E. Pope, II 1958 Basic amino acids as intracellular cations in K deficiency. Am. J. Physiol., *193:* 644.
12. Eckel, R. E., C. E. Pope, II and J. E. C. Norris 1958 Influence of lysine and NH_4Cl feeding on the electrolytes of normal and K-deficient rats. Am. J. Physiol., *193:* 653.
13. Levinsky, N. G., I. Tyson, R. B. Miller and A. S. Relman 1962 The relation between amino acids and potassium in isolated rat muscle. J. Clin. Invest., *41:* 480.
14. Phillips, P. H., and E. B. Hart 1935 The effect of organic dietary constituents upon chronic fluorine toxicosis in the rat. J. Biol. Chem., *109:* 657.
15. Cotlove, E. 1963 Determination of the true chloride content of biological fluids and tissues. II. Analysis by simple, nonisotopic methods. Analyt. Chem., *35:* 101.
16. Folk, J. E., K. A. Piez, W. R. Carroll and J. A. Gladner 1960 Carboxypeptidase B. IV. Purification and characterization of the porcine enzyme. J. Biol. Chem., *235:* 2272.
17. Erlanger, B. F., N. Kokowsky and W. Cohen 1961 The preparation and properties of two new chromogenic substrates of trypsin. Arch. Biochem., *95:* 271.

18. Monson, W. J., A. E. Harper, D. A. Benton and C. E. Elvehjem 1954 The effect of level of dietary protein on the growth of chicks fed purified diets containing sucrose or dextrin. J. Nutrition, *53:* 563.
19. Harper, A. E., and H. E. Spivey 1958 Relationship between food intake and osmotic effect of dietary carbohydrate. Am. J. Physiol., *193:* 483.
20. Romberg, B., and D. A. Benton 1965 Effect of type of carbohydrate on energy metabolism and body composition of rats fed low protein diets. J. Nutrition, *86:* 289.
21. Jansen, G. R. 1962 Lysine in human nutrition. J. Nutrition, 76(suppl 1): 1.
22. Abdeljlil, A. B., and P. Desnuelle 1964 Sur l'adaptation des enzymes exocrines du pancreas à la composition du regime. Biochim. Biophys. Acta, *81:* 136.
23. Magee, D. F., and S. S. Hong 1956 Changes in pancreatic enzymes brought about by amino acid additions to the diet. Am. J. Physiol., *184:* 449.
24. Sidransky, H., and E. Verney 1964 Chemical pathology of acute amino acid deficiencies. VII. Morphologic and biochemical changes in young rats force-fed arginine-, leucine-, isoleucine-, or phenylalanine-devoid diets. Arch. Pathol., *78:* 134.
25. Sauberlich, H. E. 1961 Studies on the toxicity and antagonism of amino acids for weanling rats. J. Nutrition, *75:* 61.
26. Schimke, R. T. 1963 Studies on factors affecting the levels of urea cycle enzymes in rat liver. J. Biol. Chem., *238:* 1012.
27. Tamura, A. 1962 Studies on amino acid supplementation and protein metabolism (6). Serum and liver transaminase activity and urinary amino acids in rats fed with excessive lysine (pt. 1). Eiyo to Shokuryo, *15:* 175.
28. Morrison, A. B., E. J. Middleton and J. M. McLaughlan 1961 Blood amino acid studies. II. Effects of dietary lysine concentration, sex, and growth rate on plasma free lysine and threonine levels in the rat. Canad. J. Biochem. Physiol., *39:* 1675.
29. Krauss, R. M., and J. Mayer 1964 Facilitatory effect of high protein intake on subsequent consumption of an amino-acid unbalanced diet. Nature, *202:* 600.
30. Longenecker, J. B., and N. L. Hause 1959 Relationship between plasma amino acids and composition of the ingested protein. Arch. Biochem., *84:* 46.

Acetate Metabolism in Ruminant Tissues [1]

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ABSTRACT Acetate-1-^{14}C was incubated with various tissue homogenates prepared from tissues of fed and 7-day fasted sheep in order to study the site and route of acetate metabolism in tissues of the ruminant. Acetate incorporation into CO_2, neutral lipids, free fatty acids, nondistillable organic acids, cations and proteins was followed. The extra hepatic tissues (adipose, muscle, heart) accounted for most of the acetate oxidation observed in intact animals. The decreasing order of acetate utilization by ruminant tissues was adipose tissue, kidney, muscle, heart, lung, liver and brain when expressed as incorporation per milligram of protein. A 7-day fast slightly decreased the overall acetate metabolism by the tissues, but $C^{14}O_2$ production from acetate was lowered only in liver and brain tissues. Inclusion of carnitine into the incubation medium with acetate increased acetate incorporation into free fatty acids and neutral lipids. The liver was the only organ to show an effect of carnitine upon acetate conversion to carbon dioxide. The largest stimulation occurred in incorporation of acetate into long-chain fatty acids where as much as a fivefold increase was observed.

It is well recognized that the volatile fatty acids (VFA), acetic, propionic and butyric, are major energy sources of ruminants. Acetate in particular contributes as much as 50% of the ruminant's energy supply and has a turnover time of 1.5 minutes (1). Several groups of investigators have reported that acetate oxidation by ruminant liver is very low (2-5), suggesting that the peripheral tissues must account for a substantial amount of the total acetate oxidation observed in the whole animal (1). With this in mind experiments were conducted using tissue homogenates from fed and 7-day fasted sheep to study the sites and routes of acetate metabolism in various ruminant tissues.

EXPERIMENTAL PROCEDURE

Four grade wether lambs weighing between 34.5 and 45.4 kg were used. All of these animals had been fed an all-roughage diet for at least 2 months before they were housed in individual metabolism cages. They were all kept in these cages 3 weeks and maintained with approximately one kilogram of chopped alfalfa hay per day before being killed. In experiments where fasted animals were used, the animal had no access to food for 7 days prior to time of killing, although water was available at all times. The animals used for the fed-animal experiments were trained to eat their total daily diet in 2 intervals during the day, 8 to 10 AM and 3:30 to 5 PM. At the time of the experiment the animals were killed 3 hours after being given their morning ration.

Acetate-1-^{14}C was incubated with homogenates prepared from liver (gastric area), adipose tissue (greater omentum), leg muscle (vastus lateralis), brain (cerebrum), kidney, heart and lung from fed and 7-day fasted sheep. Incorporation of radioactivity into carbon dioxide, neutral lipids, long-chain fatty acids, nondistillable organic acids, cationic fraction and protein was measured. The tissues were all homogenized within 20 minutes after the death of the animal. The homogenization was carried out in Waring blenders in 2 volumes of 0.25 M sucrose, 0.01 M nicotinamide and 0.001 M EDTA. The pH of the solution was immediately raised to 7.2-7.4 with potassium hydroxide. The

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supernatant from a 600 × *g* centrifugation was strained through one layer of cheesecloth before use for incubation.

Incubations were carried out in duplicate in Warburg flasks at 37° for one hour, using air as the gas phase. The shaking rate was 84 strokes/minute. The incubation media were as follows:[4] 140 μmoles potassium phosphate buffer, 30 μmoles $MgCl_2$, 20 μmoles $KHCO_3$, 15 μmoles ATP, 5 μmoles NAD, 0.15 μmoles cytochrome c, 150 μmoles KCl in presence of carnitine (20 μmoles) and 225 in its absence, 4 μmoles succinate and 50 μmoles of acetate/flask.

One milliliter of homogenate was used per flask. The acetate was added from the side arm after a 10-minute equilibration period. Two-tenths milliliter of 2 N NaOH plus a fluted filter paper (1.5 × 2 cm) were added to the center well. The total volume of the flask was 3 ml. At the end of the incubation period, 0.3 ml of 6 N H_2SO_4 was added to the media to stop the reaction and to assure liberation of all CO_2 from the media, and the flasks were left shaking for 3 hours.

At the end of the incubation, the paper wick and NaOH were removed from the center well and added to a volumetric flask. After washing the center well with water and adding the washings to the flask, the volume was made up to 2 ml. An aliquot of this was counted in a liquid scintillation counter using the scintillation mixture of Bray (6).

The acidified media in the main compartment of the Warburg flask were transferred into 50-ml flasks. The flasks were washed several times with chloroform-methanol (2:1) and the washings added to the acidic medium. The acidic medium containing 4 to 5 times its volume of chloroform-methanol was left in the cold for 12 hours. The protein was removed by centrifugation, washed several times and the washings combined with the original supernate. The chloroform-methanol extracts were extracted 4 times with equal volumes of 0.05 N aqueous NaOH. Such treatment of the chloroform-methanol extract should yield a relatively pure lipid fraction free of free fatty acids and amino acids (7). The NaOH washings were brought to a pH of 8.0 and run through a Dowex-50W, H^+ form, 20-50 mesh, column. The cationic fraction was eluted with 2 N NH_4OH, dried in vacuo, and counted in Bray's scintillation solution. The anions and neutral materials were brought to pH 10 and dried under vacuum at 55° to a volume of approximately 2 ml. The solution was acidified to pH 3 and steam-distilled using a modification of the Markham still. The volatile material was made basic, dried under vacuo and counted in Bray's solution. The nonvolatile material was made basic, dried under vacuo and added to a silicic acid column as described by Ramsey (8). The long-chain fatty acids were eluted with benzene, dried under vacuo and counted in toluene containing PPO (0.5%) and POPOP (0.01%). The nondistillable organic acids were eluted from the silicic acid with tertiary butanol-chloroform, dried in vacuo and counted in the toluene mixture described above. Two- to 4-mg samples of protein and the dried lipid fractions were dissolved in 1 ml of hyamine hydroxide and counted in the toluene scintillation counting solution.

Samples of the cation fraction from the Dowex-50W column were further extracted with picric acid (1%) and the traces of precipitated protein removed by centrifugation. The supernatant liquid was passed through a Dowex-2-10X column to remove the picric acid and anions. The column was washed with 0.02 N HCl and the eluate dried in vacuo to 3 ml. Aliquots were put on a Dowex-50 column of an amino acid analyzer and eluted with pH 3.25 and 4.25, 0.20 N Na-citrate buffers. The eluate was either run through a ninhydrin detector or collected for counting in a liquid scintillation counter.

Protein was estimated by the method of Lowry et al. (9).

RESULTS AND DISCUSSION

The distribution of radioactive acetate-carboxyl carbon into the various chemical classes are presented in tables 1 and 2 for 2 fed and 2 fasted sheep, respectively.

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[4] The ATP, NAD and cytochrome c were obtained from the Sigma Chemical Company, St. Louis; carnitine from K and K Laboratories, Inc., Jamaica, New York; and the acetate from Volk Radiochemical Company, Chicago.

TABLE 1

Acetate incorporation into various metabolites by tissues from fed sheep [1]

Tissue	Carnitine [2]	CO_2	Lipids	Long-chain fatty acids	Nondistillable organic acids	Cations
				mμ atoms acetate-carboxyl carbon/mg protein/hr		
Liver	–	10.1 (11.5, 8.6) [3]	0.049 (0.060, 0.037)	0.08 (0.07, 0.09)	13.4 (12.2, 14.6)	18.5 (15.3, 21.7)
	+	17.8 (21.4, 14.2)	0.078 (0.093, 0.062)	0.46 (0.38, 0.54)	21.7 (16.5, 26.8)	27.2 (18.3, 36.1)
Heart	–	39.5 (43.6, 35.4)	0.082 (0.031, 0.133)	0.31 (0.42, 0.20)	24.4 (26.1, 22.7)	37.8 (29.6, 46.0)
	+	38.3 (38.3, 38.3)	0.069 (0.062, 0.076)	1.69 (0.70, 2.68)	28.4 (23.3, 33.4)	47.6 (38.3, 56.9)
Lung	–	21.7 (22.5, 20.8)	0.071 (0.101, 0.040)	0.14 (0.16, 0.11)	9.7 (12.1, 7.2)	23.7 (20.5, 26.9)
	+	21.2 (22.9, 19.5)	0.052 (0.077, 0.026)	0.31 (0.22, 0.39)	9.8 (11.6, 8.0)	39.7 (23.6, 55.8)
Muscle	–	77.9 (85.5, 70.2)	0.076 (0.096, 0.056)	0.10 (0.13, 0.07)	22.3 (13.2, 31.3)	52.0 (75.1, 28.8)
	+	85.0 (94.7, 75.2)	0.081 (0.058, 0.104)	0.35 (0.14, 0.55)	20.1 (17.5, 22.6)	95.6 (146.6, 44.6)
Adipose	–	274.5 (320.3, 228.6)	0.521 (0.472, 0.569)	0.88 (0.82, 0.93)	28.0 (30.2, 25.7)	238.2 (227.4, 249.0)
	+	274.7 (320.3, 229.0)	1.030 (0.916, 1.144)	2.76 (1.69, 3.83)	36.5 (41.1, 31.8)	306.4 (307.8, 305.0)
Kidney	–	133.2 (120.0, 146.4)	0.088 (0.142, 0.034)	0.30 (0.48, 0.12)	29.8 (41.5, 18.0)	28.9 (39.6, 18.2)
	+	129.2 (115.7, 142.7)	0.096 (0.140, 0.051)	0.60 (1.03, 0.17)	33.7 (43.1, 24.2)	46.1 (42.3, 49.8)
Brain	–	11.3 (10.9, 11.7)	0.111 (0.141, 0.081)	0.19 (0.33, 0.06)	13.0 (14.6, 11.3)	28.5 (35.9, 21.0)
	+	13.5 (13.2, 13.8)	0.080 (0.101, 0.058)	0.23 (0.40, 0.05)	16.3 (15.8, 16.7)	48.5 (39.2, 57.7)

[1] Homogenates from 2 sheep were incubated for 1 hour and contained: liver 91.5 and 49 mg, heart 68 and 16 mg, lung 56 and 23 mg, muscle 34 and 20.5 mg; adipose 5.5 and 1.7 mg, kidney 56 and 36 mg, and brain 47 and 26 mg protein/flask for sheep 1 and 2, respectively. Each vessel contained 50 μmoles acetate (1 μc) and 4 μmoles succinate. The individual values for sheep 1 (37.2 kg) and 2 (45.4 kg) are in parentheses.
[2] Carnitine was added at the level of 20 μmoles/flask when used.
[3] Individual values for sheep 1 and 2, respectively.

The results are presented in terms of millimicron atoms acetate-carboxyl carbon incorporated per milligram of protein per hour. When the same set of data was expressed on a day-matter basis, the comparative order of incorporation of label was essentially the same with the exception of adipose tissue which contains smaller amounts of protein than the other tissues considered.

Acetate metabolism by various tissues in the fed sheep. The adipose tissue, kidney, muscle and heart tissues were those which oxidized acetate at the highest rates. Holdsworth et al. (10) and, Busch and Baltrush (11) with sheep and rat tissue homogenates, reported that the heart and adipose tissues were the most active sites of acetate oxidation. The present data support the view that most of the acetate is oxidized in extra hepatic tissues. Quantitatively the main sites of acetate oxidation appear to be the muscle and adipose tissues.

Acetate incorporation into the long-chain free fatty acids and neutral lipids took place at the greatest rate in the adipose tissue. These results corroborate those of Favarger and Gerlach (12) which pointed out that the liver does not play a major role in lipogenesis. These authors indicated that liver fat synthesis accounts

TABLE 2

Acetate incorporation in various metabolites by tissues from 7-day fasted sheep [1]

Tissue	Carnitine [2]	CO_2	Lipids	Long-chain fatty acids	Nondistillable organic acids	Cations
		mμ atoms acetate-carboxyl carbon/mg protein/hr				
Liver	−	5.9 (9.5, 2.2) [3]	0.015 (0.019, 0.011)	0.03 (0.03, 0.03)	4.3 (6.0, 2.5)	11.6 (17.6, 5.5)
	+	9.0 (14.4, 3.5)	0.016 (0.018, 0.014)	0.05 (0.05, 0.05)	4.6 (5.3, 3.9)	17.4 (23.1, 11.7)
Heart	−	33.5 (33.1, 33.9)	0.071 (0.051, 0.090)	0.24 (0.27, 0.21)	9.6 (12.2, 6.9)	32.3 (30.0, 34.5)
	+	33.5 (33.1, 33.9)	0.064 (0.048, 0.080)	0.47 [4] (0.47, —)	9.4 (12.1, 6.6)	22.7 (11.4, 33.9)
Lung	−	19.3 (12.8, 25.7)	0.039 (0.044, 0.033)	0.11 (0.13, 0.09)	5.6 (4.8, 6.4)	9.8 [4] (9.8, —)
	+	21.0 (10.4, 31.6)	0.042 (0.041, 0.042)	0.23 [4] (0.23, —)	4.7 (3.8, 5.6)	39.0 (13.9, 64.1)
Muscle	−	75.7 (84.3, 67.0)	0.021 (0.016, 0.025)	0.16 (0.22, 0.10)	8.7 (6.8, 10.5)	30.4 (37.7, 23.0)
	+	70.3 (91.2, 49.4)	0.022 (0.016, 0.028)	0.27 (0.33, 0.20)	9.3 (7.1, 11.5)	32.7 (44.7, 20.7)
Adipose	−	317.8 (199.6, 435.9)	0.206 (0.221, 0.190)	0.40 (0.48, 0.32)	19.5 (15.9, 23.0)	196.0 (288.1, 103.8)
	+	253.3 (177.9, 328.7)	0.234 (0.248, 0.220)	0.64 (0.73, 0.54)	53.1 (71.7, 34.4)	232.2 (162.8, 301.6)
Kidney	−	149.5 (205.2, 93.7)	0.014 (0.017, 0.010)	0.20 (0.19, 0.21)	22.4 (21.7, 23.0)	19.2 (23.1, 15.3)
	+	81.5 [4] (—, 81.5)	0.019 [4] (—, 0.019)	0.36 (0.41, 0.30)	20.7 (10.3, 31.0)	22.7 (32.8, 12.5)
Brain	−	1.3 (1.8, 0.8)	0.022 (0.019, 0.024)	0.13 (0.13, 0.13)	7.6 (12.9, 2.2)	18.9 (9.1, 28.7)
	+	1.5 (1.6, 1.3)	0.020 (0.013, 0.027)	0.28 (0.31, 0.25)	4.8 (5.3, 4.2)	23.3 (23.8, 22.7)

[1] Homogenates from 2 sheep were incubated for 1 hour and contained: liver 35 and 94 mg, heart 16 and 31 mg, lung 20 and 43 mg, muscle 14 and 31 mg, adipose 1.2 and 4 mg, kidney 21 and 79 mg and brain 21 and 41 mg protein/flask for sheep 3 and 4, respectively. Each vessel contained 50 μmoles acetate (1 μc) and 4 μmoles succinate. The individual values for sheep 3 (34.5 kg) and 4 (44.5 kg) are in parentheses.
[2] Carnitine was added at the level of 20 μmoles/flask when used.
[3] Individual values for sheep 3 and 4, respectively.
[4] One sample lost.

for only 4% of the body lipogenesis of the mouse.

The incorporation of the acetate carboxylcarbon into the nondistillable organic acids and the cations (see Methods for definition of this fraction) paralleled the extent of acetate oxidation to carbon dioxide. For all tissue studies the cation fraction was the most labeled one (tables 3 and 4). The radioactivities in the neutral and acidic amino acids of the cation fraction were determined in a pooled sample from the liver homogenates of sheep 1 and 2. Glutamic and aspartic acid accounted for 51% and 10%, respectively, of the cation activity (table 5). The radioactivity in the neutral and acidic amino acids represented 88% of the cation activity. The high labeling of glutamate may possibly explain the results observed by Sabine and Johnson (1). These authors reported that a constant specific activity of blood acetate is reached rapidly during continuous infusion of ^{14}C-acetate, whereas the specific activity of expired carbon dioxide does not reach equilibrium for 4 hours. This indicates that acetate is rapidly converted to other metabolites which are more slowly oxidized. On the basis of the present in vitro experiments

TABLE 3

Percentage of recovered radioactivity in various metabolic products from tissues of fed sheep

Tissue	Carnitine	Total activity recovered	CO_2	Lipids	Long-chain fatty acids	Nondistillable organic acids	Cations [1]	VFA [2]
		% dose	*% recovered activity*					
Liver	−	87.3 (92.3, 82.2) [3]	1.65 (2.28, 1.02)	0.008 (0.012, 0.005)	0.013 (0.014, 0.011)	2.09 (2.42, 1.75)	2.81 (3.02, 2.59)	93.43 (93.07, 94.65)
	+	85.9 (94.0, 77.8)	2.99 (4.19, 1.79)	0.013 (0.018, 0.008)	0.071 (0.074, 0.068)	3.30 (3.21, 3.38)	4.06 (3.56, 4.55)	89.57 (88.94, 90.23)
Heart	−	86.3 (85.6, 87.0)	4.12 (6.93, 1.30)	0.005 (0.005, 0.005)	0.036 (0.066, 0.007)	2.50 (4.15, 0.84)	3.20 (4.70, 1.69)	90.14 (84.11, 96.11)
	+	93.3 (94.1, 92.4)	3.43 (5.53, 1.33)	0.006 (0.009, 0.003)	0.097 (0.101, 0.093)	2.26 (3.36, 1.16)	3.75 (5.53, 1.97)	90.46 (85.35, 95.67)
Lung	−	80.9 (78.0, 83.8)	2.18 (3.23, 1.14)	0.009 (0.015, 0.002)	0.015 (0.023, 0.006)	1.04 (1.74, 0.399)	2.21 (2.94, 1.48)	94.55 (91.80, 96.90)
	+	78.9 (70.8, 87.0)	2.33 (3.62, 1.03)	0.007 (0.012, 0.001)	0.028 (0.035, 0.021)	1.13 (1.84, 0.423)	3.34 (3.73, 2.95)	93.16 (90.68, 95.40)
Muscle	−	85.5 (77.7, 93.4)	5.28 (7.47, 3 08)	0.005 (0.008, 0.002)	0.025 (0.046, 0.003)	1.27 (1.15, 1.38)	3.91 (6.56, 1.26)	89.51 (84.58, 94.43)
	+	90.7 (88.3, 93.0)	5.31 (7.29, 3.32)	0.005 (0.005, 0.005)	0.042 (0.060, 0.024)	1.18 (1.35, 1.00)	6.63 (11.28, 1.97)	86.83 (79.86, 93.76)
Adipose	−	79.4 (73.4, 85.4)	2.29 (3.71, 0.86)	0.005 (0.007, 0.002)	0.008 (0.012, 0.004)	0.28 (0.45, 0.10)	2.17 (3.41, 0.93)	95.25 (92.10, 98.10)
	+	78.2 (74.6, 81.8)	2.28 (3.65, 0.90)	0.010 (0.014, 0.005)	0.020 (0.025, 0.015)	0.37 (0.61, 0.12)	2.87 (4.54, 1.19)	94.45 (91.15, 97.56)
Kidney	−	87.7 (87.0, 88.4)	13.69 (15.45, 11.92)	0.010 (0.018, 0.003)	0.036 (0.061, 0.010)	3.41 (5.34, 1.47)	3.29 (5.10, 1.48)	79.56 (74.25, 84.84)
	+	91.7 (91.4, 92.0)	12.68 (14.18, 11.17)	0.010 (0.017, 0.004)	0.072 (0.128, 0.013)	3.54 (5.18, 1.90)	4.59 (5.28, 3.89)	79.11 (75.27, 83.04)
Brain	−	82.9 (94.2, 71.6)	0.97 (1.09, 0.85)	0.010 (0.014, 0.006)	0.019 (0.033, 0.004)	1.14 (1.46, 0.82)	2.56 (3.59, 1.53)	95.30 (93.84, 96.65)
	+	89.1 (89.4, 88.8)	1.10 (1.39, 0.81)	0.007 (0.011, 0.003)	0.023 (0.042, 0.003)	1.32 (1.66, 0.98)	3.75 (4.12, 3.38)	93.80 (93.07, 94.78)

[1] See experimental procedures for definition of cation fraction.

[2] Volatile fatty acids.

[3] Individual values for sheep 1 and 2, respectively.

TABLE 4

Percentage of recovered radioactivity in various metabolic products from tissues of fasted sheep

Tissue	Carnitine	Total activity recovered	CO_2	Lipids	Long-chain fatty acids	Nondistillable organic acids	Cations [1]	VFA [2]
		% dose	*% recovered activity*					
Liver	−	74.4 (62.6, 86.2) [3]	0.78 (1.06, 0.49)	0.002 (0.002, 0.002)	0.005 (0.003, 0.007)	0.61 (0.67, 0.54)	1.59 (1.97, 1.21)	97.01 (96.49, 97.91)
	+	75.3 (71.6, 79.0)	1.13 (1.41, 0.84)	0.003 (0.002, 0.003)	0.009 (0.005, 0.012)	0.55 (0.51, 0.59)	2.53 (2.26, 2.80)	95.78 (95.81, 97.78)
Heart	−	74.6 (59.4, 89.8)	2.08 (2.02, 2.14)	0.004 (0.003, 0.005)	0.014 (0.014, 0.014)	0.57 (0.65, 0.48)	1.98 (1.60, 2.36)	95.35 (95.62, 94.88)
	+	77.3 (65.2, 89.4)	1.97 (1.61, 2.33)	0.004 (0.002, 0.005)	0.023 (0.023, —) [4]	0.53 (0.59, 0.46)	1.45 (0.56, 2.33)	96.02 (97.24, 95.29)
Lung	−	76.6 (63.4, 89.8)	1.63 (0.81, 2.44)	0.003 (0.003, 0.003)	0.007 (0.005, 0.009)	0.46 (0.31, 0.61)	0.62 (0.62, —) [4]	97.28 (98.11, 96.88)
	+	71.2 (61.4, 81.0)	2.00 (0.67, 3.33)	0.004 (0.003, 0.004)	0.015 (0.015, —) [4]	0.42 (0.25, 0.58)	3.83 (0.90, 6.76)	93.73 (98.06, 89.38)
Muscle	−	76.1 (71.4, 80.8)	4.16 (3.21, 5.11)	0.002 (0.001, 0.002)	0.008 (0.008, 0.008)	0.54 (0.26, 0.81)	1.59 (1.43, 1.75)	93.70 (94.96, 92.33)
	+	77.0 (65.8, 88.2)	3.57 (3.69, 3.45)	0.002 (0.001, 0.002)	0.014 (0.014, 0.014)	0.55 (0.29, 0.81)	1.65 (1.85, 1.45)	94.21 (94.22, 94.33)
Adipose	−	77.1 (64.6, 89.6)	2.32 (0.74, 3.89)	0.002 (0.001, 0.002)	0.003 (0.002, 0.003)	0.04 (0.06, 0.02)	1.00 (1.07, 0.93)	96.63 (98.14, 95.09)
	+	78.3 (68.2, 88.4)	1.81 (0.63, 2.98)	0.002 (0.001, 0.002)	0.004 (0.003, 0.005)	0.14 (0.25, 0.03)	1.65 (0.57, 2.73)	96.39 (98.53, 94.12)
Kidney	−	80.0 (74.0, 86.0)	13.1 (8.99, 17.21)	0.002 (0.001, 0.002)	0.025 (0.011, 0.039)	2.81 (1.38, 4.23)	2.06 (1.32, 2.80)	82.00 (88.38, 75.80)
	+	87.0 (77.6, 96.4)	13.4 (—, 13.36) [4]	0.003 (—, 0.003) [4]	0.036 (0.022, 0.049)	2.57 (0.06, 5.08)	1.92 (1.78, 2.05)	82.07 (97.68, 79.46)
Brain	−	78.9 (68.0, 89.8)	0.1 (0.11, 0.08)	0.002 (0.001, 0.002)	0.010 (0.008, 0.012)	0.50 (0.80, 0.20)	1.59 (0.56, 2.61)	97.80 (98.53, 97.11)
	+	79.0 (70.4, 87.6)	0.1 (0.09, 0.12)	0.002 (0.001, 0.003)	0.022 (0.019, 0.024)	0.36 (0.31, 0.40)	1.74 (1.35, 2.13)	97.78 (93.50, 97.49)

[1] See experimental procedures for definition of cation fraction.
[2] Volatile fatty acids.
[3] Individual values or sheep 3 and 4, respectively.
[4] One sample lost.

TABLE 5

Specific activities of cations isolated from liver homogenates incubated with acetate-1-^{14}C

Compound [1]	Total radioactivity	Specific activity
	dpm	*dpm/mμmole*
Glycerophosphoethanolamine and phosphoethanolamine	549	— [2]
Aspartic acid	1042	3.07
Serine	869	2.29
Asparagine and glutamine	624	— [2]
Glutamic acid	5150	28.60
Glycine	615	0.56

[1] The following amino acids were also isolated but did not contain any significant counts: threonine, alanine, α-amino-*n*-butyric acid, valine, cysteine, isoleucine, leucine, tyrosine, and phenylalanine.
[2] Quantitative estimations of these 2 fractions were not obtained.

glutamate and aspartate appear to be quantitatively important intermediates in acetate oxidation.

Effects of fasting. With the exception of brain homogenates, fasting did not decrease the oxidation rates of acetate (tables 1 and 2). This is at variance with results obtained with liver slices of fed and fasted sheep (2). Fasting in the present experiment caused a tenfold decrease in oxidation of labeled acetate. This marked decrease in acetate oxidation in the brain is noteworthy since the most commonly accepted view is that the brain has an absolute requirement for glucose as an energy source (13). In contrast with this view, it has been reported that the brain can maintain its excitability and oxygen consumption for over an hour if perfused with a fluid free of glucose (14). In vivo (15) and in vitro (16-18) experiments have shown that fatty acid-activating enzymes are present in the brain and that fatty acids can be oxidized to carbon dioxide in brain preparations. These experiments suggest that a significant portion of the carbon dioxide production by the brain is derived from the oxidation of substrates other than glucose, possibly lipids. Fasting decreased consistently acetate incorporation into the nondistillable organic acids and cation fractions in all tissue considered. Since most of the cation radioactivity was present as glutamate, it is possible that a more active transamination of α-ketoglutarate prevails in tissues of fed animals where levels of ammonia are higher than in fasted sheep. Glutamate formation would deplete the tricarboxylic acid cycle of its intermediates. Repletion could be accomplished by carbon dioxide fixation involving pyruvate and the malic enzyme. This hypothesis would also have the merit of explaining the failure to observe an increase in the apparent rate of acetate oxidation with feeding. The analytical procedure used to evaluate the labeling of CO_2 is only a measure of the $^{14}CO_2$ in the NaOH at the end of the incubation period. The $^{14}CO_2$ radioactivity does not reflect the rate of release of CO_2 from acetate or the total amount of acetate-^{14}C oxidized.

Effect of carnitine on acetate metabolism. The presence of carnitine in the incubation media increased acetate oxidation to CO_2 only in liver tissue homogenates. This absence of stimulation in other tissues is in agreement with previously published work (5, 19). Carnitine increased acetate oxidation approximately 1.6-fold in both fed and fasted sheep liver.

The largest stimulation of acetate incorporation by carnitine occurred in long-chain fatty acid synthesis. In fasted animals this was not as pronounced as in the fed animals where fatty acid synthesis was more active. In tissues where fatty acid synthesis was high, heart and adipose tissues, carnitine increased acetate incorporation four- to fivefold, whereas in other tissues a threefold increase was observed. Bressler and Katz (20) have reported that carnitine increased acetate, pyruvate and glucose incorporation into fatty acids in guinea pig liver and adipose tissue both in vivo and in vitro, approximately threefold. Presumably this increase in fatty acid synthesis occurred by shuttling of intramitochondrially formed acetyl-CoA to the extramitochondrial sites of fatty acid synthesis via acetylcarnitine.

Carnitine did not generally increase acetate conversion into nondistillable organic acids except in the liver where also an increase in $^{14}CO_2$ was observed. Incorporation of acetate into acetyl carnitine probably accounts for the observed increased labeling of the cation fraction.

As indicated by Block et al. (21), the metabolic fate of acetate depends on the tissue which is metabolizing the compound. This study indicates that adipose and muscle tissues are the main quantitatively important sites of acetate oxidation.

LITERATURE CITED

1. Sabine, J. R., and B. C. Johnson 1964 Acetate metabolism in the ruminant. J. Biol. Chem., *239:* 89.
2. Leng, R. A., and E. F. Annison 1963 Metabolism of acetate, propionate and butyrate by sheep liver slices. Biochem. J., *86:* 319.
3. Gallagher, C. H., and S. H. Buttery 1959 Biochemistry of sheep tissues. Enzyme systems of liver, brain and kidney. Biochem. J., *72:* 575.
4. McCarthy, R. D., J. C. Shaw and S. Lakshmanan 1958 Metabolism of volatile fatty acids by the perfused goat liver. Proc. Soc. Exp. Biol. Med., *99:* 560.
5. Mayfield, E. D., J. L. Smith and B. C. Johnson 1965 Metabolism of acetate by sheep liver homogenates. J. Dairy Sci., *48:* 93.
6. Bray, G. A. 1960 A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Analyt. Biochem., *1:* 279.
7. Folch, J., M. Lees and G. H. S. Stanley 1957 A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem., *226:* 497.
8. Ramsey, H. A. 1963 Separation of organic acids in blood by partition chromatography. J. Dairy Sci., *46:* 480.
9. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall 1951 Protein measurement with the folin phenol reagent. J. Biol. Chem., *193:* 265.
10. Holdsworth, E. S., E. Neville, C. Nader, I. G. Jarrett and O. H. Filsell 1964 The metabolism of acetate in the sheep. Biochim. Biophys. Acta, *86:* 240.
11. Busch, H., and H. A. Baltrush 1954 Rates of metabolism of acetate-1-C^{14} in tissues *in vivo.* Cancer Res., *14:* 448.
12. Favarger, P., and J. Gerlach 1955 Recherches sur la synthese des graisses a partir d'acetate ou de glucose. Helv. Physiol. Pharmacol. Acta, *13:* 96.
13. Kety, S. S. 1957 General metabolism of the brain *in vivo.* Metabolism of the nervous system, ed., D. Richter. Pergamon Press, New York.
14. Geiger, A. 1958 Correlation of brain metabolism and function by the use of a brain perfusion method *in situ.* Physiol. Rev., *38:* 1.
15. Sacks, N. 1957 Cerebral metabolism of isotopic glucose in normal human subjects. J. Appl. Physiol., *10:* 37.
16. Geyer, R. P., L. W. Matthews and F. J. Stare 1949 Metabolism of emulsified trilaurin and octanoic acid by rat tissue slices. J. Biol. Chem., *180:* 1037.
17. Vignais, P. M., C. H. Gallagher and I. Zabin 1958 Activation and oxidation of long chain fatty acids by rat brain. J. Neurochem., *2:* 283.
18. DiPietro, D., and S. Weinhouse 1959 Glucose oxidation in rat brain slices and homogenates. Arch. Biochem. Biophys., *80:* 268.
19. Bremer, J. 1962 Carnitine in intermediary metabolism. Reversible acetylation of carnitine by mitochondria. J. Biol. Chem., *237:* 2228.
20. Bressler, R., and R. I. Katz 1965 The effect of carnitine on the rate of incorporation of precursors into fatty acids. J. Biol. Chem., *240:* 622.
21. Black, A. L., M. Kleiber and A. M. Brown 1961 Butyrate metabolism in the lactating cow. J. Biol. Chem., *236:* 2399.

Action of Amprolium on the Thiamine Content of Rat Tissues

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ABSTRACT A study was made of the action of Amprolium on rats raised with a thiamine-deficient diet. The animals were given supplements of thiamine alone or together with Amprolium, by the oral or parenteral route. The administration of thiamine alone resulted in an adequate thiamine level in the tissues. The simultaneous administration of thiamine and Amprolium prevented the establishment of normal tissue levels of thiamine. This effect of Amprolium was obtained only when it was given per os, showing that its antagonism to thiamine was exerted only during absorption from the intestinal tract.

Amprolium,[2] the coccidiostat recently synthesized by Rogers et al. (1), exerts an anti-thiamine action, which is manifest in fowls at doses much higher than anti-parasitic doses.[3]

The symptoms it induces are typical of avian beriberi: anorexia, weight loss, polyneuritis and death. They may be prevented by giving an adequate dose of thiamine.

This anti-vitaminic activity is, however, rather weak, since fowls tolerate up to 1000 ppm of Amprolium in the diet, mice up to 5000 ppm and pigs up to 8000 ppm, without any symptoms of thiamine deficiency.[4] Moreover, the so-called anti-vitaminic index, i.e., milligrams of anti-vitamin inhibiting the effect of 1 mg of thiamine mononitrate on chicken growth, is 500 for Amprolium, and 15 and 80, respectively, for the classical thiamine antagonists, pyrithiamine and oxythiamine,[5] which for that reason are much more efficient anti-metabolites.

The mode of action of Amprolium apparently differs from that of the classical thiamine antagonists: the latter operate essentially at the enzymatic level (2), whereas Amprolium appears to compete with thiamine only at the intestinal level, an excess of Amprolium notably reducing the absorption of thiamine and vice versa.[6, 7]

In view of the peculiar mechanism of the anti-thiamine action of Amprolium, we felt it would be worthwhile to study the properties of the substance more fully, particularly to ascertain whether it has a direct action on the cellular metabolism of thiamine. In the present investigation we therefore compared the effect of oral and subcutaneous administration of the compound on the total thiamine content of some organs of the rat under various experimental conditions.

The determination of the thiamine content of tissues is, of course, one of the most direct means of evaluating anti-vitaminic action and has been used previously in the study of pyrithiamine and oxythiamine (3).

A preliminary note on this subject has been published (4).

EXPERIMENTAL

Male albino rats weighing 65 to 75 g were kept in individual cages at a temperature of 22° and fed a thiamine-deficient diet consisting of: (in per cent) casein, washed and defatted, 18; cornstarch, 65;

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[2] Coccidiostat obtainable from Merck and Company, Inc., Rahway, New Jersey.

[3] Ott, W. H., A. M. Dickinson and A. Van Iderstine 1960 Amprolium. 3. Tolerance studies in chickens. Poultry Sci., *39:* 1280 (abstract).

[4] Polin, D., personal communication.

[5] Ott, W. H., A. M. Dickinson and A Van Iderstine 1962 Amprolium. 8. Comparison with oxythiamine and pyrithiamine as antagonists of thiamine in chicks. Poultry Sci., *41:* 1672 (abstract).

[6] Polin, D., E. R. Wynosky and C. C. Porter 1962 Amprolium. 9. Studies on the absorption of Amprolium and its competition with thiamine for absorption in the chick. Poultry Sci., *41:* 1673 (abstract).

[7] Polin, D., E. R. Wynosky and C. C. Porter 1962 Amprolium. On thiamine absorption in vivo from ligated segments of digestive tract in chicks. Federation Proc., *21:* 261 (abstract).

olive oil, 10; cod liver oil, 2; Osborne and Mendel salt mixture (5), 5.

The diet was supplemented with the following amounts of vitamin B complex, excluding thiamine, given by mouth on alternate days: (in micrograms) riboflavin, 80; pyridoxine, 40; nicotinamide, 800; Ca pantothenate, 40; *p*-aminobenzoic acid, 20; inositol, 40; and choline, 850. In addition, every week 1 mg of *α-dl*-tocopherol was administered per os.

The animals were maintained with this diet for periods varying from 24 to 34 days, and were weighed on alternate days.

The substances used were: thiamine chloride hydrochloride,[8] and Amprolium (1 - (2 - *n* - propyl - 4 - amino - pyrimidyl-methyl)-2-picoline chloride hydrochloride).[9]

Determination of total thiamine in tissue

Extraction of thiamine from the tissues, enzymatic dephosphorylation, Decalso[10] chromatography of the extract and elution with acidic KCl were carried out according to the procedure described in Methods of Vitamin Assay (6).

The oxidation of thiamine to thiochrome was effected on 3 ml of eluate with 1 ml of 40% NaOH containing 1.2 mg of potassium ferricyanide; the excess was eliminated with 0.1 ml of buffered Perhydrol,[11] freshly prepared (7). The thiocrome was extracted with 5 ml of isobutanol. A blank was prepared in a similar way, except that 40% NaOH only was added. Simultaneously, the whole procedure starting with the absorption of Decalso was carried out using a standard solution of thiamine (0.1 μg/ml).

The fluorescence was read on 1 ml of isobutanol in a Farrand microfluorimeter, model A[12] (primary filter, no. 5860; secondary, nos. 4308 and 3389), calibrated with a solution of quinine sulfate (0.3 μg/ml in 0.1 N H_2SO_4).

Since the alkaline oxidation of Amprolium produces a fluorescent substance, extractable with isobutanol (8), which might interfere with the determination of thiamine, the above procedure was tested on solutions of Amprolium and of thiamine plus Amprolium. The results of several experiments indicated that Amprolium, although fixed by Decalso and eluted by acidic KCl in practically the same way as

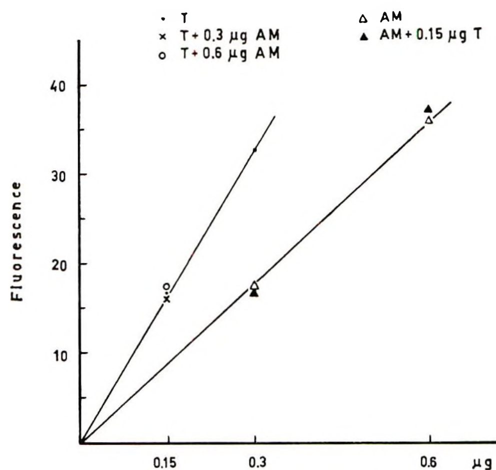

Fig. 1 Fluorescence of solutions of thiamine (T), Amprolium (AM) and mixtures of thiamine plus Amprolium, after oxidation with ferricyanide, measured as indicated in the text.

thiamine (in 5 experiments the range of the percentage recoveries of 0.3 or 0.6 μg of Amprolium was 102 to 110), did not interfere with the determination of thiamine under the above conditions (fig. 1). In particular, 0.5 μg of thiamine in the presence of 0.3 and 0.6 μg of Amprolium was recovered, respectively, for 98.2% (88.2 to 113.3; 7 determinations) and 100% (86.1 to 114.2; 8 determinations).

Design of experiments

The rats were treated as described below. At the end of each treatment the total thiamine content of liver, heart, brain and kidneys was determined.

Experiment 1. Four groups of 7 rats were maintained for 24 days with a thiamine-deficient diet and were given the following substances, dissolved in 0.2 ml of H_2O, daily, from day 21 to day 24, per os: group A (controls), 0.2 ml of H_2O; group B, 20 μg of thiamine; group C, 20 μg of thiamine plus 8 mg of Amprolium; and group D, 8 mg of Amprolium.

Experiment 3. Two groups of 7 rats each were maintained with the thiamine-

[8] Roche, Milan.
[9] Kindly supplied by Merck and Company, Rahway, New Jersey.
[10] Sodium alumino-silicate; The Permutit Company, New York.
[11] 35% hydrogen peroxide as hydrogen peroxide carbamide.
[12] We wish to express our thanks to the Research Corporation, New York, for the generous gift of a model A, Farrand fluorimeter.

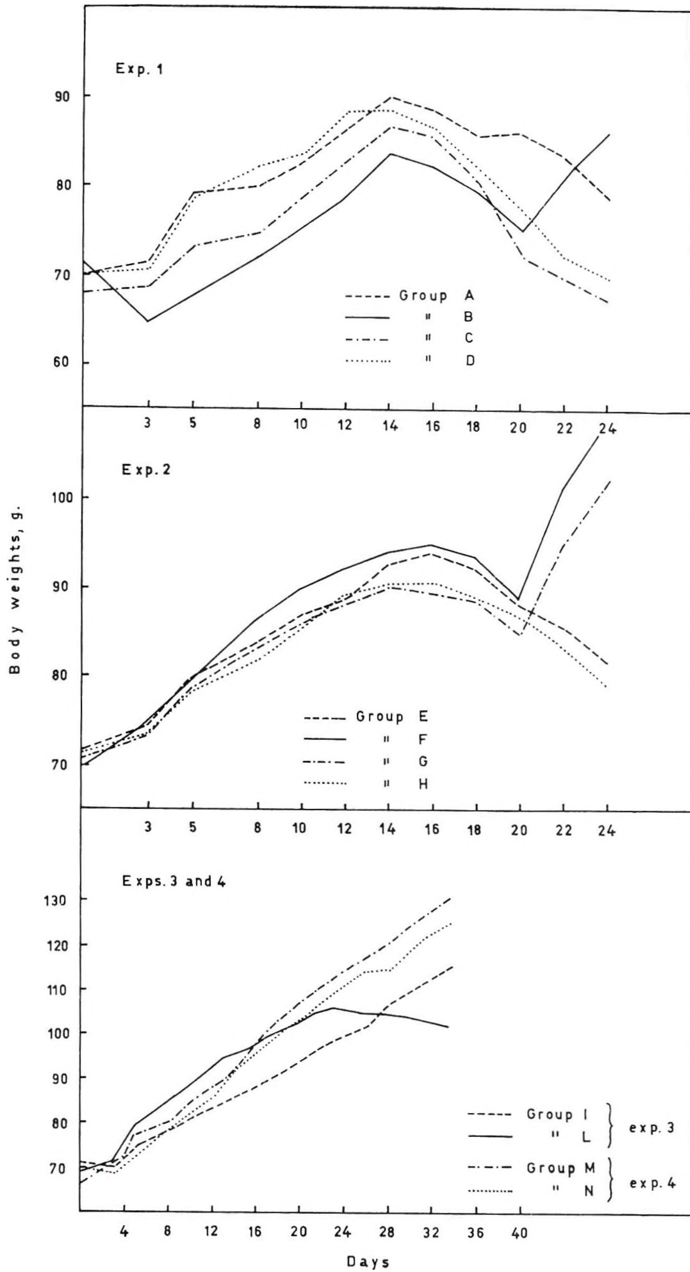

Fig. 2 Body weight of rats maintained with a thiamine-deficient diet and receiving:

Exp. 1. Group A, H_2O; group B, 20 μg of thiamine; group C, 20 μg of thiamine + 8 mg of Amprolium; per os, from day 21 to day 24.

Exp. 2. Group E, H_2O; group F, 20 μg of thiamine; group G, 20μg of thiamine + 8 mg of Amprolium; group H, 8 mg of Amprolium; by subcutaneous injection, from day 21 to day 24.

Exp. 3. Group I, 10 μg of thiamine; group L, 10 μg of thiamine + 2 mg of Amprolium; per os, from day 1 to day 34.

Exp. 4. Group M, 10 μg of thiamine; group N, 10 μg of thiamine + 2 mg of Amprolium; by subcutaneous injection, from day 1 to day 34.

deficient diet for 34 days. Every day each animal received, per os, one of the following substances dissolved in 0.2 ml of H_2O: group I, 10 μg of thiamine; and group L, 10 μg of thiamine plus 2 mg of Amprolium.

Experiments 2 and 4. The experimental conditions were as for experiments 1 and 3, respectively, except that the substances were injected subcutaneously (groups E, F, G and H, exp. 2; groups M and N, exp. 4).

RESULTS

The results obtained are recorded in tables 1 and 2.

Experiment 1 (table 1). The thiamine content, lowered by the vitamin B_1-deficient diet, increased significantly after oral administration of thiamine for 4 days, although it did not reach the values usually noted in animals fed an adequate diet (9). However, when Amprolium was administered together with thiamine, this increase did not occur and the deficiency levels remained practically unchanged after oral administration of Amprolium alone.

Experiment 2 (table 1). Subcutaneous injection of thiamine, with or without Amprolium, to thiamine-deficient rats in the same amounts as in experiment 1 induced a rapid increase in the thiamine content of the tissues in 4 days. No increase was observed on injection of Amprolium alone.

The trend of body weight (fig. 1) reflected fairly closely the trend of the thiamine levels of the tissues. In fact, in experiment 1, the body weight increased only after the administration per os of thiamine alone; neither Amprolium, nor thiamine given together with Amprolium, had any effect. In experiment 2 the subcutaneous injection of thiamine, alone or with Amprolium, caused an increase in body weight, whereas Amprolium alone had no effect.

Experiment 3 (table 2). The daily oral dose of 10 μg of thiamine for 34 days to rats maintained with the thiamine-deficient diet ensured the maintenance of a tissue level of thiamine which the simultaneous administration of 200 times the quantity of Amprolium did not. In fact, when Amprolium was administered with vitamin B_1 (group L, table 2), the thia-

TABLE 1

Total thiamine content in organs of rats maintained with a thiamine-deficient diet for 24 days and treated from day 21 to day 24 with thiamine and Amprolium[1] *(see text)*

Group	Treatment	Route	No. rats	Thiamine content			
				Liver	Brain	Kidney	Heart
				µg/g fresh tissue	*µg/g fresh tissue*	*µg/g fresh tissue*	*µg/g fresh tissue*
			Experiment 1				
A	Control[2]	per os	7	0.58 ± 0.03[3]	1.09 ± 0.03	0.40 ± 0.04	0.62 ± 0.025
B	20 µg thiamine	per os	7	1.29 ± 0.06	1.72 ± 0.09	0.89 ± 0.09	1.26 ± 0.15
C	20 µg thiamine + 8 mg Amprolium	per os	7	0.89 ± 0.11	1.07 ± 0.07	0.56 ± 0.08	0.64 ± 0.10
D	8 mg Amprolium	per os	7	0.58 ± 0.04	1.05 ± 0.12	0.38 ± 0.12	0.66 ± 0.04
			Experiment 2				
E	Control[2]	sc	6	0.45 ± 0.04	0.70 ± 0.02	0.26 ± 0.02	0.43 ± 0.02
F	20 µg thiamine	sc	6	1.36 ± 0.09	1.61 ± 0.05	1.09 ± 0.11	1.51 ± 0.22
G	20 µg thiamine + 8 mg Amprolium	sc	6	1.31 ± 0.06	1.41 ± 0.07	0.95 ± 0.09	1.33 ± 0.12
H	8 mg Amprolium	sc	6	0.46 ± 0.03	0.70 ± 0.03	0.39 ± 0.07	0.60 ± 0.10

[1] Amprolium (coccidiostat), Merck and Company, Inc., Rahway, New Jersey.
[2] Fed the thiamine-deficient diet ad libitum.
[3] Mean ± SE.

TABLE 2

Total thiamine content in organs of rats maintained with a thiamine-deficient diet for 34 days and treated daily with thiamine and Amprolium [1] *(see text)*

Group	Treatment	Route	No. rats	Thiamine content: Liver	Brain	Kidney	Heart
				µg/g wet tissue	*µg/g wet tissue*	*µg/g wet tissue*	*µg/g wet tissue*
			Experiment 3				
I	10 µg thiamine	per os	7	1.52 ± 0.14 [2]	2.07 ± 0.09	1.46 ± 0.06	1.97 ± 0.06
L	10 µg thiamine + 2 mg Amprolium	per os	7	0.68 ± 0.03	1.11 ± 0.06	0.40 ± 0.04	0.55 ± 0.08
			Experiment 4				
M	10 µg thiamine	sc	7	2.20 ± 0.11	2.11 ± 0.04	2.02 ± 0.11	2.24 ± 0.18
N	10 µg thiamine + 2 mg Amprolium	sc	7	2.10 ± 0.10	2.18 ± 0.06	1.98 ± 0.12	2.07 ± 0.20

[1] Amprolium (coccidiostat), Merck and Company, Inc., Rahway, New Jersey.
[2] Mean ± SE.

mine content of the tissues did not differ greatly from that of the controls of experiment 1 (group A, table 1).

The body weight reflected these facts, as in experiments 1 and 2; only when thiamine was given together with Amprolium (group L) did it tend to decrease, especially in the last 10 days.

Experiment 4 (table 2). Both thiamine alone (10 µg) and thiamine plus Amprolium, injected subcutaneously daily for 34 days into thiamine-deficient rats, yielded an approximately equal thiamine level in the organs.

In both cases the trend of body weight was practically analogous, tending constantly to increase (groups M and N).

DISCUSSION

The results of the experiments (exps. 1 and 2), in which the substances were administered only on the last 4 days of thiamine deficiency, indicate clearly that the thiamine-Amprolium antagonism only exists at the level of the intestinal epithelium. The penetration of thiamine into the cells of other tissues does not appear to be affected by the simultaneous administration of Amprolium. In fact, when the 2 substances were administered together by subcutaneous injection, thus by-passing the intestine, the increase in thiamine was evident in all the tissues and it was quantitatively similar to that obtained with the vitamin alone.

In these experimental conditions the administration of Amprolium per se did not appear to affect the thiamine content of the organs, including the brain (4).

The results of the experiments (exps. 3 and 4), in which the substances were given for 34 days, substantiate the existence of the thiamine-Amprolium antagonism at the intestinal level only. In fact, only when Amprolium was injected subcutaneously with thiamine (exp. 4), did it fail to prevent deposition of the vitamin in the organs, whose levels reached practically those obtained by the injection of thiamine alone.

These results show that the presence of Amprolium in the diet prevented the absorption of thiamine from the intestine, thus causing a thiamine deficiency. The thiamine content of the brain, however,

did not reach such a point in rats as to induce the neuromuscular symptoms of beriberi, which, as is well-known, result from a much lower content (3).

Our results are in agreement with those of Brin (10), who observed decreased transketolase activity in all tissues of rats receiving thiamine and Amprolium in suitable proportions, except in the brain.

The increase in body weight appears to be related to the attainment of an adequate level of thiamine in the tissues; the experimental conditions which allowed the thiamine content of the organs to increase above a deficiency level were also those which allowed an increase in body weight. This is evident in experiments 1 and 2, as well as in experiments 3 and 4, even though in the latter experiments the thiamine-to-Amprolium ratio was lower.

Sharma and Quastel (11) showed recently that Amprolium blocks the active transport mechanism whereby thiamine penetrates the cells of rat brain slices. This blockade, however, does not appear to be substantiated by the results of our experiments in vivo; in fact, when Amprolium was injected together with thiamine, it did not prevent the latter from penetrating the cells of cerebral tissue, as is evident from the increase in thiamine we observed in the brain (exp. 2, group G.). Furthermore, according to Brin (10), the brain is the only organ in which transketolase is not affected by oral administration of Amprolium; this suggests that its thiamine diphosphate, and hence thiamine content, is not lowered, as otherwise the enzyme would be affected, given its marked sensitivity to thiamine deficiency (12).

LITERATURE CITED

1. Rogers, E. F., R. L. Clark, A. A. Pessolano, H. J. Becker, W. J. Leanza, L. H. Sarett, A. C. Cukler, E. McManus, M. Garzillo, C. Malanga, W. H. Ott, A. M. Dickinson and A. Van Iderstine 1960 Antiparasitic drugs. III. Thiamine — reversible coccidiostats. J. Am. Chem. Soc., *82:* 2974.
2. Rindi, G. 1963 Recenti acquisizioni sul meccanismo di azione di alcuni analoghi strutturali della tiamina. Boll. Chim. Farm., *102:* 363.
3. De Caro, L., G. Rindi, V. Perri and G. Ferrari 1954 The in vivo effects of pyrithiamine and oxythiamine in the rat. Internat. Ztschr. Vitaminforsch., *26:* 343.
4. Rindi, G., G. Ferrari and U. Ventura 1963 Influenza dell'Amprolium sul contenuto in tiamina dei tessuti di ratto. Il Farmaco, *18:* 1001.
5. Osborne, T. B., and L. B. Mendel 1912 Feeding experiments with fat-free mixtures. J. Biol. Chem., *12:* 81.
6. Association of Vitamin Chemists, Inc. 1951 Methods of Vitamin Assay, ed. 2. Interscience Publishers, New York, p. 111.
7. Burch, H. B., O. A. Bessey, R. H. Love and O. H. Lowry 1952 The determination of thiamine and thiamine phosphates in small quantities in blood cells. J. Biol. Chem., *198:* 477.
8. Polin, D., E. R. Wynosky and C. C. Porter 1962 Amprolium. V. Studies on thiamine deficiency in laying chickens and their eggs. J. Nutrition, *76:* 59.
9. De Caro, L., G. Rindi and L. de Giuseppe 1961 Content in rat tissues of thiamine and its phosphates during dietary thiamine deficiency. Internat. Ztschr. Vitaminforsch., *31:* 333.
10. Brin, M. 1964 The antithiamine effects of Amprolium in rats on tissue transketolase activity. Toxicol. Appl. Pharmacol., *6:* 454.
11. Sharma, S. K., and J. H. Quastel 1965 Transport and metabolism of thiamine in rat brain cortex in vitro. Biochem. J., *94:* 790.
12. Brin, M. 1962 Effects of thiamine and of oxythiamine on rat tissue transketolase. J. Nutrition, *78:* 179.

Effect of Hormones on the Testicular Lipids of Vitamin A-deficient Rats [1]

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ABSTRACT The phospholipids of the testes from vitamin A-deficient rats and from vitamin A-deficient rats receiving injections of follicle-stimulaitng hormone (FSH), luteinizing hormone (LH), testosterone and various combinations of these hormones were determined quantitatively by silicic acid chromatography and thin-layer chromatography. A vitamin A deficiency decreased cardiolipin, phosphatidyl ethanolamine and phosphatidyl choline and increased phosphoinositides and sphingomyelin. Gonadotropins or testosterone, or both, altered the phospholipids in the testis of the vitamin A-deficient rat. FSH increased cardiolipins; FSH + LH restored phosphatidyl ethanolamine and phosphoinositides to normal; FSH + testosterone or FSH + LH restored phosphatidyl choline and testosterone and LH restored sphingomyelin to normal. The alterations in the testicular lipids accompanying sterility induced by a deficiency of vitamin A suggests a functional role of phospholipids in spermatogenesis and a hormonal control of testicular lipids via the pituitary gland. Vitamin A may be involved in the synthesis or secretion of gonadotropins by the pituitary gland.

Although vitamin A is required for spermatogenesis by the male rat, very little is known about the function of vitamin A in spermatogenesis. Mayer and Truant (1) attributed the histological changes in the testis from vitamin A-deficient rats to a decrease in circulating androgen and later suggested the gonadotropin secretion or synthesis by the pituitary gland was decreased (2). Beaver (3) reported that vitamin A was required to maintain the size of the pituitary gland. Howell et al. (4) reported that the basic lesion of a vitamin A deficiency in spermatogenesis was at a stage in spermatocytogenesis before the meiotic division of the spermatocytes to form spermatids.

Pituitary hormones can alter testicular lipids in many species. Lofts (5) reported that hypophysectomy caused an accumulation of lipids in the testes of frogs and arrested spermatogenesis. Gonadotropins reversed this effect and restored spermatogenesis. Hypophyseal hormones were reported by Ellefson and Mason (6) to affect the phospholipids and fatty acid metabolism in the rat liver. Borell et al. (7) also reported that hypophyseal hormones affected the phosphorus metabolism of the rat testis. Posaloky et al. (8) used histochemical techniques and reported alterations in testicular lipids as spermatogenesis progressed and suggested that lipids, possibly phospholipids may be related to functional activity of the testis.

The purpose of the present work was to determine the changes in the phospholipids of the rat testis that accompanied sterility produced by a chronic vitamin A deficiency and to determine whether either gonadotropins or testosterone, or both, could affect the phospholipids of the testis in vitamin A-deficient rats.

METHODS

Weanling male rats of the Sprague Dawley strain were housed in individual cages and fed ad libitum a normal diet or a vitamin A-deficient diet. The percentage composition of the normal diet was as follows:[3] dextrose, 70; vitamin-free casein, 20; salts, 4;[4] powdered cellulose, 2; molec-

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[3] The dextrose, vitamin-free casein, powdered cellulose, α-tocopherol powder, and vitamin A powder were obtained from General Biochemicals Inc., Chagrin Falls, Ohio.

[4] McCollum salt mixture no. 185, Hawk, P. B., B. L. Oser and W. H. Summerson 1951 Practical Physiological Chemistry, Blakiston Company, Philadelphia, p. 1274; obtained from General Biochemicals Corporation.

ularly distilled lard, 4.[5] The vitamins were added in the following amounts in milligrams per kilogram of feed: thiamine · HCl, 10; riboflavin, 12; pyridoxine, 8; Ca pantothenate, 60; nicotinic acid, 30; *p*-aminobenzoic acid, 12; biotin, 2; inositol, 900; folic acid, 3; vitamin B_{12} (0.1%), 2; choline, 1500; menadione, 50; α-tocopherol powder, 135 IU; calciferol, 0.25; vitamin A powder, 4000 IU. The vitamin A-deficient diet did not have any added vitamin A. The B vitamins were added as a premix with dextrose and the powdered cellulose carrier and was prepared monthly. The diets and premix were stored at 0°.

The rats were maintained with the diet for 11 weeks and during the last 3 weeks of this period the rats were injected intramuscularly with the following hormones in 0.2 ml saline: group 1, saline; group 2, testosterone (0.25 mg); group 3, follicle-stimulating hormone (FSH, 1 mg or 1.18 Armour units[6]); group 4, luteinizing hormone (LH, 0.5 mg or 2.2 Armour units[6]); group 5, FSH + testosterone (0.5 + 0.25 mg); group 6, FSH + LH (0.5 + 0.25 mg). Group 7 received oral supplements of 20 IU vitamin A added to 0.5 ml corn oil daily and group 8 was fed a normal diet for 11 weeks and injected with 0.2 ml saline. Each group contained 8 rats and all groups except group 7 received 5 injections during the last 3 weeks but only groups 7 and 8 received any added vitamin A during this treatment.

The experiment was terminated at the end of 11 weeks. The rats were weighed, killed in ether and the testes removed, blotted and weighed. Approximately one-third of a testicle was fixed in Zenker's solution, sectioned, stained with hematoxylin-eosin and examined for pathological changes. The remainder of the testes was used for lipid analysis.

The procedures for the isolation of the testicular lipids and the chemical determination of lipid phosphorus were previously described by Harris and Gambal (9). The total lipid in the testes was determined gravimetrically and the percentage lipid was calculated using the wet weight of the testes. The percentage of phospholipids was calculated from the chemical determination of the total lipid phosphorus.

Silicic acid[7] was prepared according to the procedure of Barron and Hanahan (10), stored in a closed container in a desiccator and activated at 130° for 24 hours prior to use. A chloroform solution of the total lipids from each rat was chromatographed under nitrogen pressure o 3.5 g silicic acid (10 × 180 mm column with a 125-ml reservoir) and the non-phosphatide lipids were eluted with 65 ml of chloroform. The phospholipids were eluted with the following solvents: 40 ml of 20% methanol in chloroform (v/v) eluted noncholine-containing phospholipids; 40 ml of 40% methanol in chloroform eluted a lecithin-rich fraction; 40 ml of methanol eluted a sphingomyelin-lecithin-rich fraction. The phospholipid eluates were evaporated in vacuo and made to 10 ml with chloroform.

Aliquots of the noncholine-containing phospholipids from each rat were combined to give an average sample for each hormonal treatment. The sample was rechromatographed on 3 g silicic acid and the phospholipids were fractionated by increasing the methanol concentration in chloroform from 5, 10, 15, to 20% (v/v). Six to 18 tubes containing 3-ml fractions were collected for each eluting solvent. The lecithin-rich and sphingomyelin-lecithin-rich fractions were treated similarly except that the eluting solvents were 22, 30, 35 and 40% methanol in chloroform for the lecithin-rich fractions and 45, 65, 80 and 100% methanol in chloroform for the sphingomyelin-lecithin-rich fraction. The lipid phosphorus was determined on an aliquot from each tube; the recovery of lipid phosphorus from the columns was quantitative, usually ranging between 97 to 102%. Peak tubes were combined and the phospholipids identified by chromatography on silicic acid-impregnated paper and thin-layer chromatography (TLC) according to the procedures described by Marinetti (11) and Skipski et al. (12), respectively. The phospholipids were also qualitatively identified by the reagents described by Skidmore and Entenman (13). Phosphatidyl choline and sphingomyelin in

[5] Distillation Products, Rochester, New York.

[6] Armour Pharmaceutical Company, Kankakee, Illinois.

[7] Silicic Acid, 100 mesh, suitable for chromatography; Mallinckrodt Chemical Company, St. Louis.

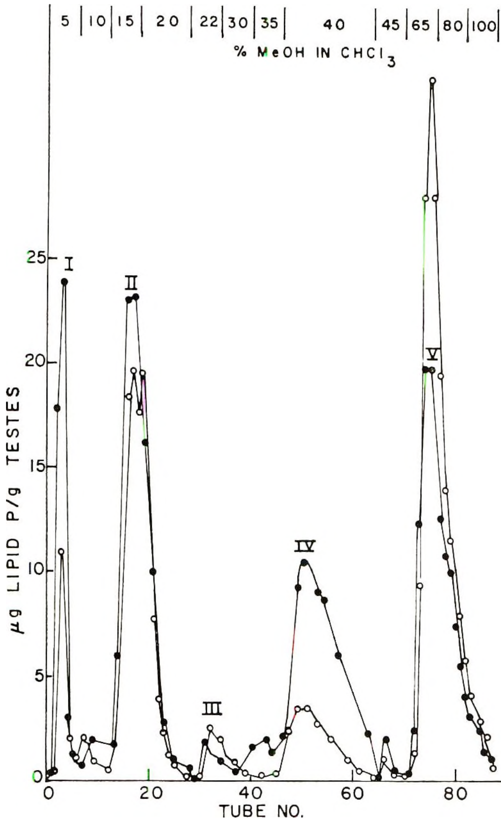

Fig. 1 Fractionation of testicular phospholipids by silicic acid chromatography: (●) normal control; (○) vitamin A-deficient. Numerals refer to peaks described in text. Numbers within lines refer to percentage of methanol in chloroform and tube at which eluting solvent was changed.

peak V (fig. 1) were separated by TLC and the phosphorus in each spot was determined. The phosphatidyl choline phosphorus in peak V was added to the phosphatidyl choline in peak IV to obtain the total phosphatidyl choline phosphorus in the testes for fig. 2.

RESULTS

At the end of the 11-week period, all rats receiving the vitamin A-deficient diet had a rough hair coat, slight incoordination, xerophthalmia and retarded growth. The diet contained some vitamin A and the results are for a chronic deficiency of vitamin A. Pathological changes in the testes were similar to those described and attributed to a deficiency of vitamin A by Follis (14). A deficiency of vitamin A decreased the body weight of the rat and the weight of the testes (table 1). Refeeding vitamin A for 3 weeks increased the body weight from 72 to 87% and the testes weight from 41 to 83% of the control values. Only LH increased the body weight but only FSH + testosterone increased the weight of the testes. However, FSH + testosterone, FSH + LH or FSH increased the relative weight of the testes.

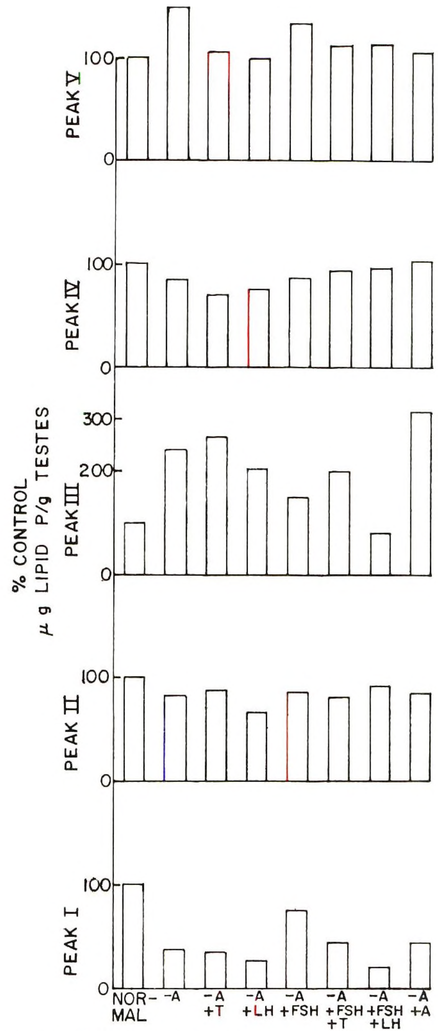

Fig. 2 Effects of hormones on the testicular phospholipids in vitamin A-deficient rats: vitamin A-deficient, (−A); testosterone, (T); luteinizing hormone, (LH); follicle-stimulating hormone, (FSH); vitamin A, (+A).

A deficiency of vitamin A or the administration of hormones to vitamin A-defi-

TABLE 1

Effect of hormones on the testicular lipids of vitamin A-deficient rats

Treatment [1]	Body wt	Testes wt	Relative wt of testes	Lipid	Lipid phosphorus in testes	PL [2] in total lipid	Lipid phosphorus in eluates/testis		
							Non-choline	PC-rich	Sph-PC rich
	g	mg	g/100 g	% wet wt	µg/g	%	µg/g	µg/g	µg/g
One-day	302 ± 13 [3]	3150 ± 98	1.046 ± 0.041	1.92 ± 0.04	482 ± 7	61.9 ± 1.6	208 ± 5	140 ± 2	129 ± 4
−A + saline	220 ± 13 [4]	1306 ± 95 [4]	0.590 ± 0.018 [4]	2.09 ± 0.14	414 ± 13 [4]	50.5 ± 2.3 [4]	159 ± 4 [4]	57 ± 3 [4]	201 ± 7 [4]
+ T	236 ± 14	1575 ± 187	0.671 ± 0.068	1.98 ± 0.08	359 ± 19 [5]	46.2 ± 3.1	139 ± 6 [4]	70 ± 10	140 ± 4 [4]
+ FSH + T	244 ± 7	1936 ± 212 [4]	0.795 ± 0.086 [4]	2.33 ± 0.07	449 ± 23 [5]	48.8 ± 3.1	171 ± 12	92 ± 11 [4]	159 ± 2 [4]
+ LH	247 ± 7 [5]	1355 ± 58	0.551 ± 0.030	1.96 ± 0.09	377 ± 34	50.2 ± 2.5	137 ± 7 [4]	82 ± 8 [4]	137 ± 4 [4]
+ FSH + LH	225 ± 16	1756 ± 330	0.778 ± 0.026 [4]	2.06 ± 0.45	369 ± 21	45.5 ± 2.1	142 ± 11	82 ± 11 [5]	146 ± 8 [4]
+ FSH	238 ± 23	1638 ± 212	0.691 ± 0.062 [5]	2.32 ± 0.20	427 ± 34	46.5 ± 0.9	158 ± 16	70 ± 17	179 ± 8
+ A	265 ± 2 [4]	2642 ± 163 [4]	0.994 ± 0.059 [4]	1.89 ± 0.04	458 ± 8 [4]	61.0 ± 1.4 [4]	171 ± 5	126 ± 4 [4]	142 ± 3 [4]

[1] −A indicates vitamin A deficient; T, testosterone; + A, added vitamin A.
[2] PL indicates phospholipid; PC, phosphatidyl choline; Sph, sphingomyelin.
[3] Averages ± SE of mean.
[4] $P < 0.01$. Saline group compared with 1-day hypophysectomized rat; hormone-treated compared with saline group.
[5] $P < 0.05$.

cient rats did not affect the total lipid concentration in the testis (table 1) although the lipid content of the testis decreased. The total lipid phosphorus concentration was decreased in vitamin A-deficient rats (table 1) and the lipid phosphorus content was decreased. Testosterone reduced the total lipid phosphorus concentration but FSH + testosterone increased it. Refeeding vitamin A for 3 weeks increased the lipid phosphorus concentration of the testis, but did not return it to normal. Under these conditions the hormones did not return the lipid phosphorus concentration to normal, but FSH + testosterone increased this value. Similarly the percentage of phospholipids in the total lipids of the testis was decreased by a deficiency of vitamin A and could be restored to normal by refeeding vitamin A, but not by the hormones under these conditions (table 1).

A vitamin A deficiency decreased the concentration of the noncholine-containing phosphatides and the lecithin-rich fraction, but increased the sphingomyelin-lecithin-rich fraction in the rat testis (table 1). Testosterone and LH produced a further reduction in the concentration of noncholine-containing phosphatides in the vitamin A-deficient rat and were the only hormones that had any effect on this group of phospholipids. FSH + testosterone, LH, FSH + LH or refeeding vitamin A increased the concentration of the lecithin-rich fraction in the vitamin A-deficient testis, but only vitamin A returned this group of phospholipids to a normal value. Testosterone, FSH + testosterone, LH, FSH + LH or refeeding vitamin A decreased the concentration of the sphingomyelin-lecithin-rich fraction to a nearly normal value.

Cardiolipin (peak I) and phosphatidyl ethanolamine (peak II) were obtained by rechromatographing the noncholine-containing phospholipids on silicic acid columns (fig. 1). The lecithin-rich and the sphingomyelin-lecithin-rich fractions yielded phosphoinositides (peak III), phosphatidyl choline (peak IV) and sphingomyelin (Peak V) (fig. 1). All peak tubes were comparatively homogeneous on TLC (12) except peaks II and V. The contaminants in peak II were estimated to be approximately 5%. A fast-moving com-

ponent was cardiolipin and the phosphatidyl ethanolamine spot also contained a phosphatidal ethanolamine. Peak V contained sphingomyelin, phosphatidyl and phosphatidal choline and traces of lysophosphatidyl choline. Phosphatidyl and phosphatidal choline were 65% of the lipid phosphorus in peak V. The sphingomyelin spot also gave a positive test with 2,4-dinitrophenylhydrazine. A minor component between peaks III and IV was tentatively identified as phosphatidyl serine, but positive identification could not be made because of the small amount of material.

A deficiency of vitamin A decreased the concentration of cardiolipins (figs. 1 and 2) and FSH (fig. 2) increased the concentration and content of lipid phosphorus in this peak. Testosterone and LH had a tendency to decrease cardiolipins and when administered with FSH, they counteracted the beneficial effects of FSH (fig. 2). Refeeding vitamin A increased cardiolipins slightly (fig. 2).

The concentration of phosphatidyl ethanolamine was decreased slightly by a vitamin A deficiency and was increased by testosterone or FSH + LH and possibly by refeeding vitamin A (fig. 2). LH decreased the concentration of phosphatidyl ethanolamine significantly, and this effect could be counteracted by FSH (fig. 2). Phosphoinositides were a minor component in the testicular phospholipids and the concentration was increased by a vitamin A deficiency (fig. 2).

LH, FSH, FSH + testosterone and FSH + LH decreased the concentration of the phosphoinositides, but FSH + LH was the most effective (fig. 2). The concentration of the minor component between phosphoinositides and phosphatidyl choline was restored by FSH or refeeding vitamin A (fig. 1). The concentration of phosphatidyl choline was reduced by a deficiency of vitamin A and FSH, FSH + testosterone or FSH + LH increased the concentration of phosphatidyl choline (fig. 2) but testosterone or LH decreased the concentration of phosphatidyl choline. The concentration of sphingomyelin was increased by a vitamin A deficiency and testosterone, LH or refeeding vitamin A restored the value to normal (fig. 2). FSH + testosterone and FSH + LH decreased the concentration of sphingomyelin, but did not return it to normal.

DISCUSSION

Xerophthalmia, the pathological changes in the testis and the alleviation of these symptoms by feeding vitamin A emphasize that the rats were deficient in vitamin A. Mason (15) has reported that atrophy of the germinal epithelium occurred more rapidly with a vitamin A-deficient diet than similar morphological alterations resulting from inanition. At the termination of this experiment, the deficient rats ate approximately two-thirds the amount of feed as the controls and inanition could not explain these results.

The weight of the testes was affected more rapidly by a vitamin A deficiency than the body weight since the percentage decrease in the weight of the testes was greater than the decrease in the weight of the body (table 1). After feeding vitamin A for 3 weeks, the weight of the testes was restored faster than the body weight. FSH + testosterone increased the weight of the testes and this could not be merely a change in water balance, since histological examination of the testes indicated the size of the seminiferous tubules had increased. Similarly the percentage of lipid in the wet weight of the testes was not affected by the hormones (table 1) and if the increase in testicular weight was due to an increase in water content and not other constituents, then the hormones should have decreased the percentage of lipid in the wet weight of the testes.

Since the percentage of lipids in the testes was not changed by a vitamin A deficiency but the total lipid phosphorus content of the testes and the concentration of phospholipids in the total lipids were decreased, the neutral lipids must have increased. Since the phospholipid content of the testes decreased and the neutral lipids increased, vitamin A may decrease the conversion of α, β-diglycerides to phospholipids as described by Weiss et al. (16) and increase the conversion of the diglycerides to triglycerides.

The concentration of phosphatidyl choline, sphingomyelin, phosphoinositides and phosphatidyl ethanolamine was restored

to normal and cardiolipin was partially restored to normal by hormones (fig. 2). Gonadotropin secretion or synthesis by the pituitary gland was decreased (4). The changes in the concentration of testicular lipids may be an indirect effect of a vitamin A deficiency and may be more directly related to the abnormal synthesis or secretion of the gonadotropic hormones by the pituitary gland. Lofts (5) also reported that gonadotropins decreased the accumulation of lipids in the seminiferous tubules of the testes from hypophysectomized frogs and lipids accumulated in the tubules of frogs testes during the nonbreeding season and disappeared during the breeding season. Similarly Posaloky et al. (8) suggested that lipids may have a functional role in spermatogenesis. The changes in phospholipid concentrations in the rat testis accompanying sterility with a vitamin A-deficient diet support the contention that lipids may have a functional role in spermatogenesis.

The concentration of sphingomyelin and phosphatidyl choline appeared to vary inversely with sphingomyelin during a vitamin A deficiency. FSH + testosterone or FSH + LH increased the phosphatidyl choline to near normal and also decreased the sphingomyelin to normal. Similar changes in sphingomyelin and lecithin content were reported by Balint et al. (17) in the liver from patients with Niemann-Pick disease. In a vitamin A deficiency the phosphorylcholine moiety of CDP-choline[8] may be diverted into sphingomyelin via phosphoryl choline-ceremide transferase as described by Scribney and Kennedy (18) rather than into lecithin via phosphoryl choline glyceride transferase as described by Weiss et al. (16) and these metabolic pathways may be controlled by hormones. Strickland and Rossiter (19) diverted diglycerides into lecithin rather than triglycerides in vitro by the addition of CDP-choline.

Testosterone or LH, either alone or in combination with FSH, produced similar qualitative but not quantitative results. The major difference between the response to LH and testosterone was that the concentration of phosphatidyl ethanolamine and phosphoinositides were increased by testosterone and decreased by LH (fig. 2). Similarly FSH + testosterone was more effective in increasing the concentration of cardiolipin than FSH + LH but not as effective in increasing phosphatidyl ethanolamine or decreasing phosphoinositides. Hall and Eik-Nes (20, 21) reported that LH stimulated the production of androgen by the testis. Since FSH + LH is generally more effective than FSH + testosterone, the utilization of LH or the production of androgen may be decreased.

Similarly, one-half of the vitamin A-deficient rats treated with FSH + testosterone had testes that were histologically normal, but none of the testes from rats receiving FSH + LH were normal. Panos et al. (22) reported that hormones improved the histology of the testes from essential fatty acid-deficient rats. Refeeding vitamin A restored the histology and the concentration of sphingomyelin and phosphatidyl choline to normal, but cardiolipin, phosphatidyl ethanolamine and phosphoinositides were not returned to normal.

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LITERATURE CITED

1. Mayer, J., and A. P. Truant 1949 Effects of administration of testosterone on vitamin A-deficient rats. Proc. Soc. Exp. Biol. Med., 72: 436.
2. Mayer, J., and J. W. Goddard 1951 Effects of administration of gonadotropic hormone on vitamin A-deficient rats. Proc. Soc. Exp. Biol. Med., 76: 149.
3. Beaver, D. L. 1961 Vitamin A deficiency in the germ-free rat. Am. J. Pathol., 38: 335.
4. Howell, J. M., J. N. Thompson and G. A. Pitt 1963 Histology of the lesions produced in the reproductive tract of animals fed a diet deficient in vitamin A alcohol but containing vitamin A acid. J. Reprod. Fertil., 5: 159.
5. Lofts, B. 1961 The effects of follicle-stimulating hormone and luteinizing hormone on the testis of hypophysectomized frogs, (Rana Temporaria). Gen. Comp. Endocrinol., 1: 179.
6. Ellefson, R. D., and H. L. Mason 1963 Effects of L-thyroxine on lipids in the hypophysectomized rats. Endocrinology, 72: 131.

[8] Cytidine diphosphorylcholine.

7. Borell, U., E. DiczFalusy and A. Westman 1952 Effect of chorionic gonadotropin on turnover of phosphorus in testicles of hypophysectomized rats. Acta Endocr., *9:* 59.
8. Posaloky, Z., A. Gyevai and B. Bukulya 1961 Data on mechanism of spermatogenesis based on studies with acid-fast dyes on lipid and succinic dehydrogenase activity. Acta Morphol. Acad. Sci. Hung., *10:* 137.
9. Harris, R. A., and D. Gambal 1963 Fluorometric determination of total phospholipids in rat tissues. Analyt. Biochem., *5:* 479.
10. Barron, E. J., and D. J. Hanahan 1958 Observations on the silicic acid chromatography of the neutral lipids of rat liver, beef liver and yeast. J. Biol. Chem., *231:* 493.
11. Marinetti, G. V. 1962 Chromatographic separation, identification and analysis of phosphatides. J. Lipid Res., *3:* 1.
12. Skipski, U. P., R. F. Peterson and M. Barclay 1962 Separation of phosphatidyl ethanolamine, phosphatidyl serine and other phospholipids by thin-layer chromatography. J. Lipid Res., *3:* 467.
13. Skidmore, W. D., and C. Enteman 1962 Two-dimensional thin-layer chromatography of rat liver phosphatides. J. Lipid Res., *3:* 471.
14. Follis, R. J., Jr. 1948 Pathology of nutritional disease. Charles C Thomas, Springfield, Illinois.
15. Mason, K. E. 1935 Foetal death, prolonged gestation and difficult parturition in rats as a result of vitamin A deficiency. Am. J. Anat., *57:* 303.
16. Weiss, S. B., S. W. Smith and E. P. Kennedy 1959 The enzymatic formation of lecithin from cytidine diphosphate choline and D-1,2-diglyceride. J. Biol. Chem., *231:* 53.
17. Balint, J. A., W. L. Nyhan, P. Lietman and D. A. Turner 1961 Lipid patterns in Niemann-Pick disease. J. Lab. Clin. Med., *58:* 548.
18. Scribney, M., and E. P. Kennedy 1958 The enzymatic synthesis of sphingomyelin. J. Biol. Chem., *233:* 1315.
19. Strickland, K. P., and R. J. Rossiter 1962 Lipid synthesis in rat liver particles. Diversion of synthesis from triglyceride to lecithin by the in vitro addition of cytidine diphosphate choline. Canad. J. Biochem. Physiol., *40:* 1051.
20. Hall, P. F., and K. B. Eik-Nes 1962 The influence of gonadotropins in vivo upon the biosynthesis of androgens by homogenates of rat testis. Biochim. Biophys. Acta, *71:* 438.
21. Hall, P. F., and K. B. Eik-Nes 1962 The action of gonadotropic hormones upon rabbit testis in vitro. Biochim. Biophys. Acta, *63:* 411.
22. Panos, T. C., G. F. Klein and J. C. Finerty 1959 Effects of fat deficiency on pituitary-gonad relationships. J. Nutrition, *68:* 509.

Effect of Pyridoxine Deficiency on the Metabolism of Linoleic Acid in the Rat [1,2]

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ABSTRACT The influence of pyridoxine upon the metabolism of linoleic acid was studied in rats fed a pyridoxine-free diet for several weeks and then supplemented for 1 or 6 days with zero, 10 or 50 μg pyridoxine hydrochloride and given an oral tracer dose of methyl linoleate-1-^{14}C. Supplementation with 50 μg for 6 days reduced the amount of radioactivity expired as $^{14}CO_2$. Supplementation also resulted in decreased amount of ^{14}C in lipids of liver and of testes. In both organs most of the ^{14}C was in the phosphatide lipid. Extensive catabolism of linoleate-1-^{14}C occurred in all groups with re-incorporation of ^{14}C into saturated and unsaturated shorter chain fatty acids. In testicular phosphatides the amount of ^{14}C in linoleate was significantly reduced and the amount in shorter chain fatty acids and in other polyunsaturated acids increased by pyridoxine supplementation. There was no effect on ^{14}C distribution in hepatic fatty acids. Pyridoxine supplementation for 6 days resulted in the following changes in fatty acid composition: slightly reduced concentrations of linoleate and slightly increased concentrations of arachidonate in liver phosphatides; slight increase in concentration of stearic acid in liver triglycerides; increase in concentration of arachidonate, slight increase in linoleate, and an increase in concentration of docosapentaenoate in testicular phosphatides; and an increase in concentration of docosapentaenoate in testicular triglycerides.

The possible role of pyridoxine in the metabolic conversions of essential fatty acids has been of interest since the report by Witten and Holman (1) that tissue concentrations of arachidonic acid were lower in rats maintained with a diet deficient in pyridoxine and essential fatty acids and given linoleate than in those maintained with a similar diet and given pyridoxine and linoleate. The relationship between pyridoxine and essential fatty acid metabolism is still to be clarified despite numerous investigations in this area during recent years (2-7). Recently, Scheier and Williams (8) reported increased arachidonate levels in phospholipids of livers of rats maintained with a diet deficient in pyridoxine and fat and supplemented with pyridoxine when compared with nonsupplemented animals. The greatest effect was obtained during the first few days of supplementation. In a previous paper from this laboratory, Kirschman and Coniglio (5) reported that a significant decrease in tetraenoate concentrations in liver and carcass of pyridoxine-deficient rats compared with pair-fed, pyridoxine-supplemented controls did not occur. In view of the results of Swell et al. (3) that arachidonate content of liver phospholipids and triglycerides is affected by pyridoxine deficiency and of Scheier and Williams (8) during the first few days of supplementation with pyridoxine, a more detailed study of the role of pyridoxine in the metabolism of linoleic acid was deemed necessary. Analyses of fatty acid content of tissues were supplemented with results of ^{14}C-linoleate catabolism and conversion to other polyunsaturated fatty acids, including arachidonic acid.

MATERIALS AND METHODS

Male, weanling rats of the Sprague-Dawley strain were fed a purified diet free of pyridoxine and containing 20% fat as vegetable shortening [3] as described previously (5). The animals were maintained with this diet until growth stopped, morphological symptoms of pyridoxine deficiency began to appear, and serum transaminase levels dropped (8 to 10 weeks

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[2] Presented in part at annual meeting of the American Institute of Nutrition, Atlantic City, New Jersey, April, 1966.
[3] Crisco, containing added polyunsaturates, Procter and Gamble Company, Cincinnati.

after start of pyridoxine-free diet). At this time the rats were divided into groups which received the following supplements of pyridoxine hydrochloride: 1, none; 2A, 10 μg/day for 1 day; 2B, 10 μg/day for 6 days; 3A, 50 μg/day for 1 day; 3B, 50 μg/day for 6 days. Rats supplemented with pyridoxine for 6 days were pair-fed to rats in the non-supplemented group and all rats were continued with the pyridoxine-deficient diet. Controls of rats supplemented for only one day were allowed to eat ad libitum. At the end of the supplementation period, each rat was given by stomach tube 10 μc (0.2 ml) of methyl linoleate-1-^{14}C (radiopurity, 98% after purification by preparative thin-layer chromatography using $AgNO_3$-impregnated silica gel) and placed in a metabolic chamber for 6 hours for collection of expired air. After decapitation, liver and testes were removed quickly, weighed, and kept at —20° until analyzed.

The ^{14}C activity of expired air was measured as $Ba^{14}CO_3$ using a thixotropic gel[4] and liquid scintillation counting. Total lipids were extracted from each organ by the procedure of Folch et al. (9) and separated into classes by preparative thin-layer chromatography on plates of 1 mm silica gel[5] in an atmosphere of nitrogen. Multiple development (10) with the solvent system hexane-diethyl ether-acetic acid (73:25:2) was used. The plates were dried in an atmosphere of nitrogen and the individual zones scraped off, following the recommendations of Privett et al. (11). The solvent system ethanol:chloroform:water:acetic acid (100:30:20:2)[6] was used for extraction of ^{14}C from the silica gel. The extract was washed by agitation and the layers separated by centrifugation at room temperature. Separated lipids were saponified with 10% ethanolic KOH on a steam bath in an atmosphere of nitrogen for 60 minutes. After removal of non-saponifiable material by extracting with petroleum ether, the soap solution was acidified and the free fatty acids were extracted with petroleum ether (b.p. 40-60°). An aliquot was used for ^{14}C determination by liquid scintillation counting and the rest of the fatty acids methylated according to the method of Metcalfe and Schmitz (12).

Gas-liquid chromatographic analyses of methyl esters were made using an argon ionization detector calibrated with standards obtained from the Hormel Foundation, National Institutes of Health, and Applied Science Corporation. The liquid phase was diethylene glycol succinate polyester (12%) supported on Chromosorb W (110-120 mesh) in a glass column, 183 cm (6 ft.) by 4 mm. Column temperature was 190° and carrier gas flow rate 125 to 150 ml/minute. For gas-liquid radiochromatography an 244 cm (8 ft.) by 4 mm column containing 15% diethylene glycol succinate polyester (110-120 mesh) was used. Other conditions were identical to those used in the analytical determinations. This instrument was fitted with a stream splitter which allowed 15% of the column effluent to go to the detector and 85% to a manual collector. Individual methyl esters were collected in cartridges packed with *p*-terphenyl.[7] The contents of each cartridge were then dissolved in 10 ml of toluene containing diphenyloxazole and 1,4-bis-2-(5-phenyloxazolyl)-benzene, and ^{14}C determination made in the liquid scintillation spectrometer.

RESULTS

Pyridoxine deficiency in these rats resulted in high mortality, a slight but significant hypertrophy of the liver and a significant decrease in testicular weight. Most of the animals of all groups were also cryptorchids although fewer of these were noted in the groups supplemented for 6 days. Morphologically all the deficient animals had rough skin, nasal edema and scaling of the tail. Administration for one day of 10 or 50 μg of pyridoxine hydrochloride did not have any significant effect on the morphological changes. The 6-day-long administration of 10 μg of pyridoxine hydrochloride/day slightly reversed the morphological changes and led to increased food consumption and slightly im-

[4] Cab-O-Sil (trademark of Godfrey L. Cabot, Inc.), obtained from Packard Instrument Company, LaGrange, Illinois.

[5] Silica gel HF 254 (product of E. Merck, W. Germany), obtained from Brinkmann Instruments, Westbury, New York.

[6] Biezenski, J. J. 1964 Quantitation and preparation of phospholipids by elution following improved thin layer chromatography separation. Federation Proc., 23: 503 (abstract).

[7] Obtained from Packard Instrument Company, LaGrange, Illinois.

proved growth curves. Administration of 50 μg of pyridoxine hydrochloride/day for 6 days resulted in almost complete reversal of the morphological symptoms and in normal growth. Weights of livers and of testes of the rats are shown in table 1.

Results of ^{14}C analyses of respiratory CO_2 and of ^{14}C incorporated from linoleate-1-^{14}C into total lipids of livers and testes are also shown in table 1. Deficient animals incorporated more radioactivity into hepatic and testicular lipids and oxidized more of the linoleate-^{14}C to $^{14}CO_2$. Individual figures were widely variable but each deficient animal had more ^{14}C in respiratory CO_2 and hepatic or testicular lipids than its pair-fed control. Supplementation of 10 μg/day for one day had no apparent effect on the results. A decreased incorporation of radioactivity into total lipids was apparent even with a supplementation of 10 μg pyridoxine/day for one day ($P < 0.001$). However, with respect to the expired $^{14}CO_2$ data, only the difference between groups 1 and 3B was statistically significant ($P < 0.005$).

Aside from the effect on total incorporation, pyridoxine deficiency did not have significant effect on the distribution of radioactivity among individual lipid classes of the liver. Results are shown in table 2 for groups 1 and 3B only, as the differences were generally the largest between these 2 groups. The greatest amount of ^{14}C activity was in the phosphatides and the smallest in the cholesterol ester fraction. An apparent decrease in amount of ^{14}C in triglycerides was not significant ($P < 0.2$) but there was an increase in incorporation into sterol esters in the supplemented groups ($P < 0.02$).

In testes the greatest incorporation was also in the phosphatide fraction. In table 2 are shown data for groups 1 and 3B. An increase in ^{14}C activity with pyridoxine supplementation ($P < 0.02$) and a lower incorporation into triglyceride ($P < 0.01$) occurred. Incorporation into sterol esters was not different between the groups ($P < 0.3$). Results obtained with group 2B (supplementation of 10 μg/day for 6 days) were similar to those of group 3B, whereas values obtained with groups 2A and 3A (one day supplementations) were not significantly different than group 1.

TABLE 1

Incorporation of ^{14}C from linoleate-1-^{14}C into expired $^{14}CO_2$ and into hepatic and testicular lipids

Group	Pyridoxine supplementation	$^{14}CO_2$[1]	Weight of organ		^{14}C Incorporation[2]	
			Liver	Testes	Liver	Testes
		% of administered dose	*g*	*g*	*% administered dose/g tissue*	
1	None	33.7 ± 2.6[3]	5.14 ± 0.21	1.33 ± 0.03	2.41 ± 0.11	0.082 ± 0.006
2A	10 μg, one day	31.2 ± 2.1	5.36 ± 0.19	1.39 ± 0.06	1.42 ± 0.09	0.030 ± 0.002
2B	10 μg/day, 6 days	28.8 ± 1.7	4.87 ± 0.16	1.77 ± 0.03	1.69 ± 0.13	0.039 ± 0.005
3A	50 μg, one day	26.5 ± 2.8	4.51 ± 0.09	1.48 ± 0.05	1.19 ± 0.12	0.025 ± 0.007
3B	50 μg/day, 6 days	17.9 ± 1.9	3.73 ± 0.11	2.01 ± 0.08	1.84 ± 0.16	0.024 ± 0.009

[1] Three animals for each of groups 1 and 3B; 2 animals for each of other groups.
[2] Six animals/group.
[3] All results are expressed as average ± SE of mean.

TABLE 2

Distribution of ^{14}C activity among lipid components

Lipid class	Liver [1]		Testes [1]	
	Group 1	Group 3B	Group 1	Group 3B
	% of total activity		*% of total activity*	
Phosphatides	88.3 ± 5.8 [2]	86.6 ± 7.6	63.3 ± 3.9	82.5 ± 6.2
Triglycerides	13.8 ± 2.7	8.9 ± 1.2	25.3 ± 3.1	12.6 ± 1.4
Sterol esters	1.1 ± 0.1	4.2 ± 1.2	5.5 ± 0.71	3.8 ± 0.77

[1] Six animals/group.
[2] Average ± SE of mean.

Extensive degradation of linoleic acid-1-^{14}C to 2-carbon fragments occurred as evidenced by incorporation of ^{14}C into fatty acids of chain lengths varying from about ten carbons to eighteen. Most of the activity in such fatty acids of liver was found in triglycerides and phosphatides, and supplementation with pyridoxine tended to lower this amount (table 3). The labeled shorter chain acids (< 16:0)[8] occurred predominantly in the triglycerides.

The major amount of ^{14}C in hepatic triglycerides and phosphatides was in the linoleic acid fraction, but in sterol esters ^{14}C activity was more evenly distributed in various fatty acids. Fatty acids containing the major portion of the ^{14}C incorporated are shown in table 3. Labeled arachidonic acid was found mostly in phosphatides and cholesterol esters. The slight increase in ^{14}C activity in arachidonate with supplementation is only of borderline significance ($0.05 < P < 0.10$). Considerable incorporation of ^{14}C also occurred in the eicosatrienoic acid fraction of all 3 lipid classes and in the docosatetraenoic acid fractions of cholesterol esters.

Most of the ^{14}C activity in testicular phosphatides was in linoleic acid, but in triglycerides a more general distribution was observed (table 3). The amount of ^{14}C in linoleic acid of phosphatides was significantly reduced by pyridoxine supplementation and increased both in fatty acids more saturated or of shorter chain length than 18:2 and more unsaturated than 18:2. Incorporation into phosphatide fatty acids of retention time greater than that of linoleate were 10.5 ± 2.1, group 1; 10.3 ± 1.9, group 2A; 19.3 ± 2.2, group 2B; 11.8 ± 3.2, group 3A; and 29.0 ± 4.4, group 3B. The difference between groups 1 and 2B and between group 1 and 3B are significant ($P < 0.02$ and $P < 0.01$, respectively). The large incorporation into 20:3 of cholesterol esters was not affected by pyridoxine supplementation.

Concentrations of most of the fatty acids of liver and testicular lipids were not affected by pyridoxine supplementation. Therefore, these are shown in table 4 only for group 1, the non-supplemented rats. In table 5 are summarized the concentrations of fatty acids which were affected by pyridoxine supplementation. A slightly lower concentration of linoleic and slightly higher concentration of arachidonic acid were found in liver phosphatides of rats supplemented for 6 days. An increase in concentration of stearic acid was observed in liver triglycerides of supplemented rats. In testicular phosphatides an increase in concentration of arachidonic and slight decrease in linoleic acid also occurred. In addition, the concentration of docosapentaenoic acid was significantly higher in most of the supplemented groups. The concentration of docosapentaenoic acid also was higher in testicular triglycerides of rats supplemented for 6 days than it was in those supplemented for one day or not supplemented. Triglyceride linoleic acid concentrations were generally constant in testicular triglycerides.

DISCUSSION

The data presented in this paper indicate that linoleic acid is catabolized to a greater degree by pyridoxine-deficient rats than by supplemented, pair-fed controls. This is in agreement with data reported previously by Kirschman and Coniglio (5). The increased incorporation of radioactivity by livers and testes of pyridoxine-deficient rats may represent a preliminary step for the oxidation. Incomplete oxida-

[8] Number of carbons in molecule:number of double bonds in molecule.

TABLE 3

^{14}C Incorporation from linoleic-1-^{14}C into tissue fatty acids

	< 18:2			18:2			20:3			20:4			22:4		
	P [1]	T [1]	CE [1]	P	T	CE	P	T	CE	P	T	CE	P	T	CE
							% of total counts incorporated								
Liver [2]															
1	21.4	24.3	19.6	47.2	58.4	7.7	5.1	9.0	10.1	10.4	tr	11.6	4.7	1.1	20.2
2A	20.5	23.8	16.1	45.2	59.1	11.2	5.5	8.0	11.2	6.9	0.9	12.3	3.2	1.9	18.0
2B	20.6	13.1	8.6	48.0	68.3	15.6	7.2	4.2	7.5	9.6	2.2	15.2	3.8	1.0	20.2
3A	10.7	18.6	16.0	57.1	50.1	16.7	6.9	3.6	8.1	17.0	1.8	11.9	3.2	1.1	21.1
3B	1.4	9.7	15.9	70.5	80.0	10.5	4.5	5.6	8.6	14.8	1.2	16.8	8.9	4.2	21.4
													22:5 [3]		
Testes [2]															
1	13.0	31.4	14.8	69.4	16.1	43.5	2.5	11.6	18.3	3.6	3.9	13.9	0.4	7.5	3.7
2A	—	28.1	9.6	73.6	11.2	39.6	1.6	8.9	14.7	4.0	3.9	10.1	0.3	7.5	4.2
2B	14.5	34.6	15.1	59.0	9.6	34.5	9.6	9.5	16.4	7.2	5.3	17.5	2.4	5.2	4.8
3A	19.0	25.3	9.6	60.8	17.2	41.2	2.6	10.6	17.7	4.3	1.2	15.0	tr	5.1	3.9
3B	25.4	34.2	15.6	46.4	11.7	33.3	7.2	3.2	18.1	7.2	3.2	18.1	7.7	3.2	6.7

[1] P = phosphatides; T = triglycerides; CE = cholesterol esters.
[2] Six animals/group.
[3] ^{14}C incorporation in 22:4 in testes was significant only in triglycerides; therefore, data for 22:5 are given in place of 22:4.

tion of unsaturated fatty acids with production of water-soluble, small molecules may indicate inefficient use of substrates by the deficient rats. This was observed in pyridoxine-deficient rats given linoleate-1-^{14}C by Kirschman,[9] in fat-deficient rats given arachidonate-1-^{14}C by Coniglio et al. (13) and in pyridoxine-deficient rats given arachidonate-1-^{14}C.[10] In the latter two experiments the compound containing the ^{14}C activity has been identified as acetoacetate.

In the present experiments linoleate was furnished at 10% of total calories, an amount which would by the criteria of Caster et al. (14) be in excess of the nutritional requirement for essential fatty acids and would be available for storage in depot fat. The pyridoxine-deficient rat appears to metabolize unsaturated fat available for storage in a different manner than the supplemented, pair-fed control. This may in part account for the decrease fat formation in pyridoxine-deficient animals observed by other workers (15).

Results of our ^{14}C experiments lend no support for a specific effect of pyridoxine supplementation on conversion of linoleate to arachidonate in liver and these are in agreement with results obtained previously by Kirschman and Coniglio (5). The slight increase in ^{14}C activity in arachidonate in livers of supplemented rats could be due to decreased oxidation, increased storage, esterification or mobilization as supported by Leitch and Hepburn (16) rather than to actual synthesis.

Incorporation of ^{14}C into polyunsaturated fatty acids of testicular lipids was affected more by pyridoxine supplementation than was incorporation into those of liver. The effect may be an indirect one as in about 70% of the animals descent of testis to scrotum had not occurred, and abdominal testes may have a different pattern of lipid metabolism than scrotal testes. Whether the cryptorchidism is a sequel to the deficiency-induced arrested growth or to a decreased synthesis of pituitary gonadotrophins (17) is not known. However, it is clear that in interpreting results ob-

[9] Kirschman, J. C. 1961 A study on the effects of age, sex, and pyridoxine deficiency on the metabolism of polyunsaturated fatty acids in the rat. Ph.D. Thesis, Vanderbilt University, Nashville, Tennessee.
[10] Unpublished observations.

TABLE 4

Composition of major fatty acids of lipids of liver and of testes of pyridoxine-deficient, non-supplemented rats [1]

Fatty acid	% of total fatty acids					
	Phosphatides		Triglycerides		Cholesterol esters	
	Liver	Testes	Liver	Testes	Liver	Testes
16:0	15.8 ± 1.3	38.7 ± 3.5	34.5 ± 4.0	44.8 ± 4.4	22.8 ± 2.4	28.6 ± 3.0
16:1	1.6 ± 0.1	2.7 ± 0.2	7.4 ± 0.4	—	9.1 ± 1.1	4.7 ± 0.7
18:0	19.8 ± 2.2	9.7 ± 0.9	2.1 ± 0.2	9.5 ± 1.2	12.9 ± 1.8	14.3 ± 1.8
18:1	18.0 ± 2.3	19.3 ± 2.9	27.5 ± 3.2	17.5 ± 2.5	36.3 ± 3.0	19.0 ± 1.8
18:2	19.2 ± 4.2	8.8 ± 1.1	18.3 ± 4.6	10.9 ± 2.3	11.1 ± 1.9	9.5 ± 1.0
20:4	23.7 ± 3.3	11.1 ± 1.3	< 0.5	2.8 ± 0.3	8.1 ± 1.1	2.2 ± 0.3
22:5	tr	16.8 ± 1.2	—	9.6 ± 1.2	—	23.9 ± 2.5
22:6	4.0 ± 0.7	—	—	—	—	—

[1] Six rats/group.

TABLE 5

Effect of pyridoxine supplementation on concentration of selected fatty acids of hepatic and testicular lipids

Lipid fraction	Fatty acid	% of total fatty acids [1]				
		1	2A	2B	3A	3B
Liver phosphatides	18:2	19.2 ± 4.2	19.5 ± 1.8	11.8 ± 1.1	15.2 ± 2.3	13.3 ± 1.2
	20:4	23.7 ± 3.3	21.5 ± 3.2	27.7 ± 4.2	22.4 ± 4.9	30.6 ± 4.8
Liver triglycerides	18:0	2.1 ± 0.2	3.0 ± 0.4	5.3 ± 0.9	3.9 ± 0.6	6.2 ± 0.8
Testicular phosphatides	18:2	8.8 ± 1.1	6.8 ± 0.9	3.2 ± 0.8	7.3 ± 0.9	4.2 ± 1.0
	20:4	11.1 ± 1.3	9.9 ± 1.1	17.6 ± 3.2	12.1 ± 1.2	17.9 ± 2.4
	22:5	16.8 ± 1.2	17.8 ± 1.0	26.5 ± 1.7	23.1 ± 1.4	30.0 ± 2.0
Testicular triglycerides	18:2	10.9 ± 2.3	11.1 ± 2.1	5.2 ± 1.1	8.4 ± 1.8	8.1 ± 2.0
	22:5	9.6 ± 1.2	7.9 ± 1.2	16.5 ± 2.8	11.8 ± 2.2	18.4 ± 3.0

[1] Six rats/group.

tained from in vivo studies on chronic pyridoxine-deficient rats care must be exercised to consider the involvement of pyridoxine in other aspects of metabolism.

In experiments reported by Kirschman and Coniglio (5) no differences were observed in linoleate and arachidonate concentrations in liver total fatty acids between rats maintained for several weeks with a pyridoxine-free diet and pair-fed rats fed the same diet but supplemented with pyridoxine. Scheier and Williams (8) reported higher concentrations of arachidonate in phospholipids of liver and heart of rats given linoleate plus pyridoxine for 6 days compared with rats given linoleate alone. Our present experiments were conducted more similarly to theirs than to those reported by Kirschman and Coniglio (5). In the present experiments slightly higher concentrations of arachidonate in liver phospholipids were observed. Arachidonate and docosapentaenoate concentrations in testicular phospholipids were also increased in the pyridoxine-supplemented animals. Another interesting result was accumulation of some ^{14}C activity into eicosatrienoic acid which was only slightly decreased by pyridoxine supplemenation. This presumably arose from radioactive 2-carbon units resulting from degradation of linoleate-1-^{14}C. Brenner and José (18) postulated some involvement of pyridoxine in conversion of oleic to eicosatrienoic acid. Our present data do not support this, but in vitro studies in progress indicate that incubation of ^{14}C-acetyl CoA with liver microsomes from pyridoxine-deficient rats fed 20% fat results in accumulation of $\Delta^{6,9}$ octadecadienoic acid, a precursor of $\Delta^{5,8,11}$ eicosatrienoic acid. Whether this is an effect of pyridoxine deficiency per se or of the level and nature of the dietary fat is yet to be determined.

Pertinent to possible effects of pyridoxine on the concentration of unsaturated

fatty acids is the observation by Haskell and Snell (19) that lipids of yeast grown in a pyridoxine-deficient medium contain smaller amounts of palmitoleic acid (16:1) than yeast grown in a medium containing pyridoxine. At present there is no known role of pyridoxine in desaturation.

In testes, as in liver, incorporation into phosphatides was affected more than incorporation into triglycerides and sterol esters. Thus, phospholipid formation may be influenced by pyridoxine deficiency, especially with respect to sphingosine—and ethanolamine-containing phosphatides. The recent report of Haskell and Snell (19) of decreased concentration of phytosphingosine in yeast grown on a pyridoxine-deficient medium is pertinent. The authors point out that the structure of phytosphingosine suggests that it, like sphingosine, may be formed through a pyridoxal phosphate-dependent condensation of L-serine with a fatty acyl derivative.

LITERATURE CITED

1. Witten, P. W., and R. T. Holman 1952 Polyethenoid fatty acid metabolism. VI. Effect of pyridoxine on essential fatty acid conversions. Arch. Biochem. Biophys., *41:* 266.
2. Goswami, A., and D. P. Sadhu 1961 Distribution of unsaturated fatty acids in pyridoxine-deficient hypercholesterolemia. Biochem. J., *78:* 732.
3. Swell, L., M. D. Law, P. E. Schools, Jr. and C. R. Treadwell 1961 Tissue lipid fatty acid composition in pyridoxine-deficient rats. J. Nutrition, *74:* 148.
4. Greenberg, L. D., and H. D. Moon 1961 Alterations in the blood fatty acids in single and combined deficiencies of essential fatty acids and vitamin B_6 in monkeys. Arch. Biochem. Biophys., *94:* 405.
5. Kirschman, J. C., and J. G. Coniglio 1961 The role of pyridoxine in the metabolism of polyunsaturated fatty acids in rats. J. Biol. Chem., *236:* 2200.
6. Söderhjelm, L. 1962 Influence of pyridoxine and dietary fat on the distribution of serum fatty acids in dogs. J. Nutrition, *78:* 438.
7. Mueller, J. F., and J. M. Iacono 1963 Effect of desoxypyridoxine-induced vitamin B_6 deficiency on polyunsaturated fatty acid metabolism in human beings. Am. J. Clin. Nutrition, *12:* 358.
8. Scheier, G. E., and M. A. Williams 1964 Sequential changes in liver and heart lipids after giving linoleate or linoleate plus pyridoxine to rats depleted of fat and pyridoxine. Biochem. J., *92:* 422.
9. Folch, J., M. Lees and G. H. Sloane-Stanley 1957 A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem., *226:* 497.
10. Halpaap, H. 1963 Preparative layer chromatography. Chemie Ingenieur-Technik, *35:* 488 (reprinted in Shandon Instrument Applications Monograph, no. 4, March, 1964).
11. Privett, O. S., M. L. Blank, D. W. Codding and E. C. Nickell 1965 Lipid analysis by quantitative thin-layer chromatography. J. Am. Oil Chemists' Soc., *42:* 381.
12. Metcalfe, L. D., and A. A. Schmitz 1961 The rapid preparation of fatty acid esters for gas chromatographic analysis. Analyt. Chem., *33:* 363.
13. Coniglio, J. G., J. T. Davis and S. Aylward 1964 Metabolism of ethyl arachidonate-1-C^{14} in rats fed complete or fat-free diets. J. Nutrition, *84:* 265.
14. Caster, W. O., P. Ahn, E. G. Hill, H. Mohrhauer and R. T. Holman 1962 Determination of linoleate requirement of swine by a new method of estimating nutritional requirement. J. Nutrition, *78:* 147.
15. Beaton, G. H., A. M. Haufschild and E. W. McHenry 1956 Prevention of inanition in vitamin B_6-deprived rats by insulin treatment. J. Nutrition, *60:* 455.
16. Leitch, I., and A. Hepburn 1961 Pyridoxine: metabolism and requirement. Nutrition Abstr. Rev., *31:* 389.
17. Hsu, J. M. 1963 Interrelations between vitamin B_6 and hormones. Vitamins Hormones, *21:* 113.
18. Brenner, R. R., and P. José 1965 Action of linolenic and docosahexaenoic acids upon the eicosatrienoic acid level in rat lipids. J. Nutrition, *85:* 196.
19. Haskell, B. E., and E. E. Snell 1965 Effect of vitamin B_6 deficiency on the composition of yeast lipids. Arch. Biochem. Biophys., *112:* 494.

Effects of Twelve Common Fatty Acids in the Diet upon the Composition of Liver Lipid in the Rat [1]

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ABSTRACT To test whether effects and interactions of fatty acids when fed singly also apply in complex dietary mixtures, a study was undertaken using natural fats and mixtures. Twenty-one dietary fatty acid ester mixtures were prepared from common fats and oils plus small amounts of purified fatty acid esters. The compositions of these mixtures were adjusted to allow independent but simultaneous study of twelve of the dietary fatty acids. The lipid mixtures were fed as 10% of the dietary calories to groups of weanling male rats for 66 days. The rats were killed and the liver lipids extracted and analyzed for fatty acid content by gas chromatography. Metabolic interactions and conversions were indicated by intercorrelations calculated with the aid of a digital computer. Equations for estimating the amounts of 4 dietary fatty acids from analysis of tissue, and of 7 tissue fatty acids from an analysis of diet are presented. The linolenate requirement of the rat is estimated from these latter equations to be 0.14% of caloric intake. There are high, positive diet-tissue correlations between acids of the linoleate family and between acids of the linolenate family. The amounts of certain saturated and monoenoic fatty acids in the diet had marked effects upon these correlations.

The effect upon tissue lipid composition of feeding controlled amounts of certain fatty acids has been described in quantitative terms for only the essential fatty acids (1). The present experiment attempts to extend this information to describe the dietary effects of most of the fatty acids present in substantial amount in the common edible fats and oils. Excluded from consideration are the polyunsaturated fatty acids with four or more double bonds and any individual consideration of short-chain fatty acids found characteristically in coconut oil and butterfat.

To be meaningful, such an experiment requires that each of the fatty acids studied should be varied over as wide a range of intakes as is possible in practical diets, and that each fatty acid be varied both independently and in combination with all of the others. When all of these goals are rigorously satisfied and more than 12 fatty acids are to be considered, experimental designs result which require unrealistically large numbers of animals and amounts of purified fatty acids. The present experimental design approximates these goals within defined limits. It has the advantages of using a reasonable number of animals and using common fats and oils as the major source of fatty acids in the diets.

PROCEDURES

Notation. The shorthand notation used here indicates chain length, number of methylene-interrupted double bonds and location of the double bond nearest the methyl group, counting its position from the terminal (ω) methyl group. Thus linoleic acid is $18{:}2\omega6$. This terminology has been chosen because fatty acids related metabolically have the same structure at the methyl end of the molecules. Thus fatty acids with the same number following the *omega* are metabolically related.

Experimental design. Initially, a group of natural fats and oils, listed in the upper portion of table 1, was assembled having as widely different compositions as feasible. The analyzed fatty acid composition of these materials obtained by gas-liquid chromatography (GLC) was tabulated and the intercorrelations between these fatty acids were computed with the aid of digital

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TABLE 1

Percentage fatty acid composition of dietary fats

Dietary oil or fat	6:0–12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0	22:1
Soybean	0	0	10.9		3.7	28.6	50.9	5.9					
Safflower	0	0.5	17.4		1.8	10.0	67.9	2.4					
Cottonseed	0	0.9	23.1	0.6	2.1	18.3	55.0	0.3					
Linseed	0	0	5.3		3.4	20.2	14.6	56.6					
Corn	0	0.01	11.8	0.3	1.8	29.6	54.6	0.8	0.5	0.3			
Olive	0		9.6	0.6	2.3	74.9	5.1	0.8					
Rapeseed	0		2.8		0.9	29.2	18.4	9.4		9.2	0.1		30.0
Macademia	0.1	0.7	12.0	18.8	1.4	59.3	1.8		2.3	2.4		1.1	
Hydrogenated coconut	55.0	19.7	10.9		12.2	2.7							
Hydrogenated whale oil	0.1	12.6	20.9	13.7	6.4	35.0		0.7	2.6	2.4	0.6	1.8	2.3
Lard	0.2	1.4	23.0	2.7	14.5	44.6	11.0	0.2			1.9	0.1	0.1
Tallow	0.2	3.7	21.7	2.9	28.9	40.3	1.9	0.2		0.05	0.1		
50% Corn + 50% rapeseed	0		7.55	0.15	1.19	29.26	40.87	4.91		4.58		0.26	10.91
75% Palmitate + 25% erucate	0		81.00										19.00
60% Corn + 40% rapeseed	0		8.30	0.18	1.26	30.03	42.55	4.41		3.93		0.13	9.03
60% Hydrogenated coconut + 40% safflower	36.8	10.14	9.37		9.44	7.08	26.89	0.34					
50% Macademia + 50% soybean	0	0.35	9.66	7.68	2.88	51.44	23.44	3.46		0.80		0.28	
30% Macademia + 70% lard	0	0.88	22.44	6.27	8.10	54.02	6.30	0.75		0.92		0.16	
50% Linoleate + 50% oleate	0					50.09	49.91						
50% Linoleate + 50% hydrogenated whale oil	0	5.10	8.93	6.82	3.31	15.85	55.15	0.27		1.69	1.4	0.08	0.69
50% Linoleate + 50% tallow	0	1.32	12.36	1.09	10.34	19.31	55.30	0.13		0.13			

computers. Where high correlations between 2 fatty acids existed, additional lipid mixtures were devised which were calculated to reduce the intercorrelations to a minimum. Some of these make use of purified fatty acid esters as components. Each of the last 9 lipid mixtures listed in table 1 was designed to serve 2 purposes: (a) to minimize certain correlations, and (b) to assure that each fatty acid appeared in the different diets at a number of different levels. In most cases these levels approximate a geometric progression so as to provide equal spacing when dietary data are plotted on a logarithmic scale (2). In the 21 lipid mixtures used, the relative amounts of 14:0, 16:0, 16:1, 18:0, 18:1, 18:2, and 18:3 in the fat mixtures fed (see table 1) varied from zero to 20, 81, 19, 29, 75, 68, and 57%, respectively, of the total fatty acid fed. For each of these acids there are at least 6 dietary intake levels, differing from each other by roughly twofold, represented within the experiment. These 7 acids are dietary fatty acids of major nutritional importance and are major components of natural fats and oils. The correlation between any pair of these acids (see table 2) in no case exceeds 0.42, and in most cases is less than 0.23 (for which $P = 0.01$ with the 126 rats used in this experiment). Also listed as experimental variables in tables 1 and 2 are 5 other dietary fatty acids (20:0, 20:1, 20:2, 22:0, and 22:1) and the short chain group (6:0 to 12:0). These are less prominent dietary fatty acids. In many cases their intakes were varied over smaller ranges and with fewer distinct levels being represented. In most cases the correlations between each of these and all other fatty acids are small. Of the 81 correlations in table 2, 94% are smaller than 0.50, and 60% are smaller than 0.23.

The above experimental design could be further improved by adding lipid mixtures with progessively more complex compositions, and thereby reducing the correlations in table 2 to nearly any desired level. Practical limitations are provided, however, by factors of cost and experimental error, and by judgments concerning the statistical and nutritional significance of expected results. An examination of the correlation coefficients in table 2 which are larger than $r = 0.50$ indicates that the diets fail to distinguish clearly between arachidic (20:0), behenic (22:0) and palmitoleic (16:1) acids. They fail to distinguish between the monoenoic acids 20:1 and 22:1. They provide little differentiation (hydrogenated whale oil vs. coconut oil) between myristic acid (14:0) and all of the shorter chain saturated fatty acids (6:0 through 12:0). Most of these failures were deemed of too little nutritional or biochemical interest to justify the cost of a further extension of the experimental design. Most of the dietary fatty acids which are critical in later discussions are clearly independent. It was on the basis of these calculations and considerations that the fatty acid mixtures of table 1 where chosen.

The major portion of the statistical work, required both in planning the experiment and in analyzing the resultant data,

TABLE 2

Degree of independence of the dietary variables as expressed in terms of the correlation between the amount of each fatty acid in the diets and the amounts of all other fatty acids in the diets in this experiment

Acid	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0	22:1
6:0–12:0	**0.78**	−0.08	−0.16	0.24	−0.40	−0.26	−0.10	−0.10	−0.16	−0.11	−0.11	−0.12
14:0		−0.03	0.13	0.37	−0.42	−0.34	−0.19	0.25	−0.14	0.12	0.29	−0.21
16:0			0.01	0.08	−0.28	−0.34	−0.21	0.03	−0.25	0.06	0.03	0.23
16:1				−0.01	0.41	−0.37	−0.19	**0.84**	0.09	0.21	**0.83**	−0.22
18:0					−0.03	0.36	−0.15	−0.10	−0.31	0.23	−0.10	−0.32
18:1						−0.25	−0.14	0.25	0.04	0.02	0.25	−0.22
18:2							−0.08	−0.34	−0.02	−0.03	−0.36	−0.11
18:3								−0.12	0.03	−0.13	−0.12	0.04
20:0									0.14	0.06	**0.95**	−0.11
20:1										−0.03	0.21	**0.80**
20:2											0.08	−0.11
22:0												−0.07

was carried out on the Control Data 1604 or IBM 7094 digital computers, using the biomedical computer programs of Dixon (3) or programs described previously by Caster et al. (1, 2, 4, 5). Unless otherwise specified, each correlation coefficient, r, is a Pearson product-moment correlation coefficient.

Laboratory methods and procedures. One-hundred-twenty-six male weanling rats of the Sprague-Dawley strain were divided into 21 groups of six. Each group was fed the basal fat-free diet of Mohrhauer and Holman (6) plus the ethyl esters of the supplemental oil in an amount equivalent to 10% of the total calories. The composition of these 21 ester supplements is listed in table 1. Esters were mixed with the basal diet in batches sufficient for one week's food supply and these diets were refrigerated at −20° until used. Sufficient fresh diet was given each pair of rats daily to provide ad libitum feeding. After the animals had been fed these diets for 66 days, the livers of the 120 survivors were removed under ether anesthesia, and were stored in saline solution at −20° until they were analyzed.

The fat supplements were derived mainly from naturally occurring fats and oils. These were interesterified using 2% sulfuric acid in absolute ethanol. The ethyl esters were analyzed by GLC using a Barber-Coleman Model 10 Gas Chromatograph with a 210 cm × 5 mm id column packed with 20% ethylene glycol succinate on Gaschrom P, 80-100 mesh. The flow rate was 60 ml of argon/minute at 16 psi. Inlet heater was kept at 270° and the detector cell at 250°. Esters with retention times shorter than 18:3 were chromatographed at 180°. Approximately 5 μliters of a 10% solution of the methyl esters in light petroleum ether were injected. The long-chain esters were analyzed separately at 200° by injecting approximately 30 to 50 μliters of sample. Quantitation was by triangulation, and results are expressed in terms of area per cent. Individual fatty acids where identified using equivalent chain length values derived from authentic substances. All dietary ethyl ester mixtures were protected from autoxidation by addition of 0.025% of ethoxyquin.[3] The tocopherol and vitamin A content of the diets was incorporated in the fatty ester supplements.

The liver tissues were homogenized and extracted with chloroform-methanol (2:1) according to the method of Folch et al. (7). The lipids were transesterified by refluxing with 30 volumes of a 5% solution of HCl in methanol. All operations were conducted under nitrogen. The methyl esters were analyzed by GLC as described above.

RESULTS

Table 3 lists the correlations between the concentration of different fatty acids in liver lipid and the amounts of individual fatty acids in the diet. No tissue fatty acid had a statistically significant correlation ($r = 0.23$) with dietary 20:2, therefore this dietary variable was deleted. The variables in table 3 are arranged to place metabolically related acids (saturated, ω7, ω9, ω6, and ω3) together so as to demonstrate certain general effects. Feeding saturated and ω7 fatty acids tends to increase all ω7 and ω9 acids and most saturated acids in liver lipid, whereas it decreased 18:0 and all ω6 and ω3 components. Dietary 18:2 increases all tissue ω6 components, decreases most saturated and ω7 and ω9 acids, but has little effect upon ω3 acids. Feeding 18:3 has the effect of increasing ω3 acids in liver lipid, but has little effect (negative, if any) upon other acids. An examination of table 2 demonstrates that these general effects cannot be explained in terms of similar correlations between the different fatty acids within the diets.

Estimation equations. A series of least squares equations which allow the estimation of the amount of certain fatty acids in the diet from a knowledge of liver lipid composition have been calculated and are given as equations 1 to 4 in table 4. Each equation is derived from tissue analysis and dietary data from 120 rats. The letters *D* and *T* are used to distinguish between dietary and tissue fatty acid components in these equations. Hence, D18:1 represents the amount of oleic acid in the diet expressed as a percentage of the total caloric intake, whereas T18:1 represents the concentration of oleic acid in the liver

[3] Santoquin, Monsanto Chemical Company, St. Louis.

TABLE 3

Correlation between the amount of each fatty acid in the diet and the concentration of each fatty acid in liver lipid

Dietary acid	6:0–12:0	14:0	16:0	18:0	20:0	22:0	16:1ω7	18:1ω9	20:1ω9	22:1ω9	18:2ω6	18:3ω3
Tissue												
14:0	**0.79**	**0.79**	0.06	0.20	0.05	0.05	0.02	−0.38	0.01	0.03	−0.35	−0.23
16:0	0.14	−0.03	0.07	0.02	−0.11	−0.12	−0.21	−0.01	0.11	0.26	−0.24	0.16
18:0	−0.32	−0.41	−0.33	−0.27	−0.25	−0.27	−0.16	0.03	0.07	−0.07	**0.65**	−0.05
16:1ω7	0.25	0.43	0.36	0.35	0.37	0.41	0.34	0.06	−0.03	0.06	**−0.67**	−0.22
18:1ω9	0.30	0.43	0.45	0.43	0.43	0.45	0.43	0.21	−0.08	0.01	**−0.83**	−0.31
20:1ω9	−0.10	0.05	0.37	−0.29	0.37	0.41	0.23	−0.07	0.29	0.39	−0.33	0.12
20:3ω9	0.26	0.39	**0.50**	0.32	**0.55**	**0.51**	**0.53**	0.14	−0.11	−0.01	**−0.78**	−0.26
18:2ω6	−0.27	−0.29	−0.35	−0.35	−0.33	−0.32	−0.25	−0.22	0.06	−0.09	**0.86**	0.01
20:3ω6	−0.14	−0.20	−0.34	−0.30	−0.29	−0.32	−0.31	−0.14	0.04	0.01	**0.61**	0.04
20:4ω6	−0.26	−0.31	−0.35	−0.27	−0.37	−0.38	−0.31	−0.07	−0.11	−0.23	**0.88**	−0.24
22:5ω6	−0.06	0.02	−0.04	−0.03	−0.12	−0.20	−0.12	−0.17	−0.38	−0.38	**0.51**	−0.32
18:3ω3	−0.08	−0.03	−0.13	−0.10	0.09	0.10	−0.04	−0.13	−0.04	−0.05	−0.14	**0.86**
20:5ω3	−0.09	−0.18	−0.21	−0.14	−0.11	−0.11	−0.17	−0.12	0.07	0.08	−0.12	**0.99**
22:5ω3	−0.10	−0.20	−0.22	−0.16	−0.13	−0.13	−0.21	−0.16	0.11	0.12	−0.07	**0.98**
22:6ω3	−0.20	−0.38	−0.33	−0.23	−0.24	−0.17	−0.25	0.09	0.45	0.38	−0.03	**0.71**

lipid expressed as a percentage of the total fatty acid in the extracted sample. The linolenic acid intake (D18:3) of the rat can be computed from equation 1, in which error of estimation is $\sigma = 0.14\%$ of calories, and the multiple correlation coefficient is $R^2 = 0.994$. In this experiment the dietary intake of linolenic acid ranged from zero to 5.6% of calories. If linolenic acid is not the major source of the $\omega 3$ components in the diet, such as may be the case if fish oils are fed, it may be desirable to use equation 2 which is independent of the higher molecular weight $\omega 3$ components.

The amount of saturated fatty acid in the diet can be estimated within $\sigma = 1.67\%$ of calories by equation 3 which depends upon the facts that all dietary saturated fatty acids tend to increase the concentration of the $\omega 7$ and $\omega 9$ components in liver, and the feeding of the saturated fatty acids of short-chain length increases the amount of myristic acid in the liver lipid. Probably the most consistent effect of all dietary saturated fatty acids is the increase of oleic acid concentration in liver lipid.

The amount of linoleic acid in the diet has previously been estimated by Caster and Holman (1) on the basis of the tissue lipid concentrations of linoleate and other polyunsaturated fatty acids (PUFA). These estimation equations were deduced from studies in which linoleate was the only fatty acid in the diet. It now appears (table 3) that dietary saturated fatty acids also have an important effect upon the linoleate content of tissue. In the case of normal diets, containing a mixture of fatty acids, a better estimate of linoleate can be obtained using equation 4 in which a factor involving tissue oleate concentration is used to correct for changes in saturated fatty acid intake.

Equations which allow the estimation of fatty acid concentrations in liver lipid from a knowledge of dietary intake are listed as equations 5 to 11 in table 4. The equations for estimating tissue levels of the different $\omega 6$ components were omitted because they do not differ appreciably from the equations previously presented by Caster and Holman (1). Again these diet-tissue relationships were curvilinear and

TABLE 4

Estimation equations which express the quantitative relationship between the amounts of certain fatty acids in the diet (D) and the concentration of fatty acids in tissue (T) [1,2]

No.	Equation	Statistic
(1)	$D18{:}3\omega3 = -0.022 + 0.369T18{:}3 + 0.638T20{:}5\omega3 + 0.002T22{:}5\omega3 + 0.031T22{:}6\omega3$	$R^2 = 0.994$
(2)	$D18{:}3 = 0.122 + 1.833T18{:}3$	$r = 0.859$
(3)	$DSat. = -2.59 + 3.49T14{:}0 + 0.068T16{:}1 + 0.04T18{:}1 + 0.24T20{:}3\omega9$	$R^2 = 0.773$
(4)	$D18{:}2\omega6 = 2.47 - 0.094T18{:}1 + 0.138T18{:}2\omega6 + 0.176T20{:}4\omega6$	$R^2 = 0.924$
(5)	$T14{:}0 = 0.881 + 0.129D(6\text{–}12) + 0.262D14{:}0 - 0.017D18{:}1 - 0.022D18{:}2$	$R^2 = 0.844$
(6)	$T18{:}1 = 32.02 + 1.22D14{:}0 + 0.54D16{:}0 + 0.59D18{:}0 - 2.26D18{:}2 - 1.69D18{:}3$	$R^2 = 0.927$
(7)	$T20{:}3\omega9 = 1.78 + 0.500D(6\text{–}12) + 0.034D14{:}0 + 0.708D16{:}0 + 2.343D16{:}1 + 0.404D18{:}0 - 0.546D18{:}2$	$R^2 = 0.901$
(8)	$T18{:}3 = -0.024 + 0.402D18{:}3$	$r = 0.859$
(9)	$T20{:}5\omega3 = -0.33 + 1.26D18{:}3$	$r = 0.988$
(10)	$T22{:}5\omega3 = -0.03 + 0.87D18{:}3$	$r = 0.979$
(11)	$T22{:}6\omega3 = 5.45 - 3.14e^{(-8.64D18:3)} - 2.10e^{(-0.37D18:3)}$	$R^2 = 0.961$
(12)	$T22{:}5\omega6 = 0.58 + 0.058T18{:}2 + 0.072T20{:}4\omega6 - 0.295T22{:}6\omega3$	$R^2 = 0.822$

[1] Dietary intake data (D) are expressed as a percentage of the total caloric intake.
[2] Tissue fatty acid concentrations (T) are expressed as a percentage of the total fatty acid in the tissue.

equations were best expressed in logarithmic or exponential form.

Equations 8 to 11 allow the estimation of $\omega3$ acids in liver lipid from knowledge of the dietary intake of linolenate. All but the equation for $22{:}6\omega3$ were essentially linear in the range studied. This confirms the results of a parallel study in this laboratory in which linolenate was the only variable. Figure 1 shows a plot of four of the diet-tissue relationships in the $\omega3$ series. Only in the case of $22{:}6\omega3$ was the relationship clearly curvilinear. For this reason the equation for $22{:}6\omega3$ given above is expressed in exponential form. Exponents in such equations have been interpreted in terms of nutritional requirements by Caster et al. (2). On this basis, the nutritional requirement for linolenic acid is estimated as 0.14% of calories ($I_{1/2} = 0.08\%$ of calories). This leads us to suggest that if a need for linolenic acid

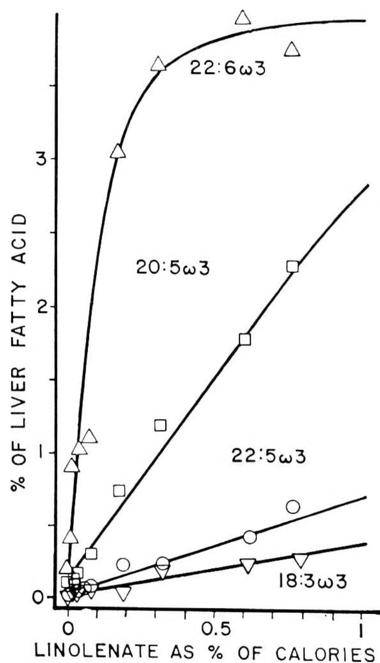

Figure 1

Fig. 1 Relationship between dietary intake of linolenate and the concentration of ω3 acids in liver lipid.

is established in the rat, this requirement will be satisfied by linolenic intakes of about 0.14% of calories. Furthermore, this need will be related to 22:6ω3 metabolism. The other diet-tissue relationships for linolenic acid, being substantially linear, perhaps represent storage and show no indication of a metabolic requirement within the range studied here.

Interaction between tissue fatty acids. Table 5 shows the correlations between the concentrations of different fatty acids in liver lipid. Of the 105 possible correlations in the data of table 5, 68 are statistically significant. This is despite the fact that the dietary intakes of these same fatty acids were substantially uncorrelated (table 2). In table 5, high positive correlations are observed between fatty acids which share common metabolic pathways, and negative correlations are observed wherever metabolic competition is known to exist. The overall pattern of these relationships can be seen more easily by carrying out a factor analysis (4) on the data for tissue fatty acid concentrations and by expressing the results graphically as shown in figure 2. In this figure all of those points which are close together represent tissue fatty acids which have high positive correlations with each other. Those on opposite sides of the origin have negative correlations.

All ω3 components are highly correlated and represent a discrete metabolic group. Stearic acid (18:0) and most of the ω6 components are highly correlated with each other. This confirms previous reports (8-10). The association of stearic acid with the ω6 fatty acids is seen as a consequence of its occurrence in the α-position together with polyunsaturated fatty acids in the β-position of lecithin which is the major lipid in liver. The ω6 acids have strong negative correlations with 20:3ω9 and other ω9 and ω7 components, and have essentially zero correlation with the ω3 components. The ω6 group does not form as tight a cluster (as highly intercorrelated a group) as in the case of the ω3 components. Liver arachidonate (20:4ω6) and 22:5ω6 are slightly displaced from the ω6 group because of their stronger metabolic interaction (negative correlation) with the ω3 components. In par-

TABLE 5

Correlation between each fatty acid in liver lipid and the concentration of all other fatty acids in the same lipid samples

Acid	16:1	18:0	18:1	18:2	20:1	20:3ω9	20:3ω6	20:4ω6	20:5ω3	22:5ω6	22:5ω3	22:6ω3
14:0	0.45	**−0.52**	0.43	−0.29		0.35		−0.38				−0.29
16:0	0.36	−0.46		**−0.50**	−0.23		−0.29	−0.45		**−0.51**		0.30
16:1		**−0.76**	**0.74**	**−0.75**		**0.63**	**−0.61**	**−0.70**		−0.36	−0.25	−0.26
18:0			**−0.71**	**0.64**	−0.23	**−0.55**	**0.54**	**0.79**		0.39		
18:1				**−0.81**	0.31	**0.87**	**−0.63**	**−0.77**	−0.28	−0.33	−0.32	−0.37
18:2						**−0.72**	**0.63**	**0.83**		**0.51**		
18:3ω3					0.24			−0.29	**0.81**	−0.24	**0.83**	**0.51**
20:1						0.37		−0.37				
20:3ω9							**−0.61**	**−0.74**	−0.23	−0.27	−0.27	−0.36
20:3ω6								**0.66**		0.38		
20:4									−0.27	**0.65**	−0.23	
20:5ω3										−0.32	**0.98**	**0.70**
22:5ω6											−0.35	**−0.59**
22:5ω3												**0.75**

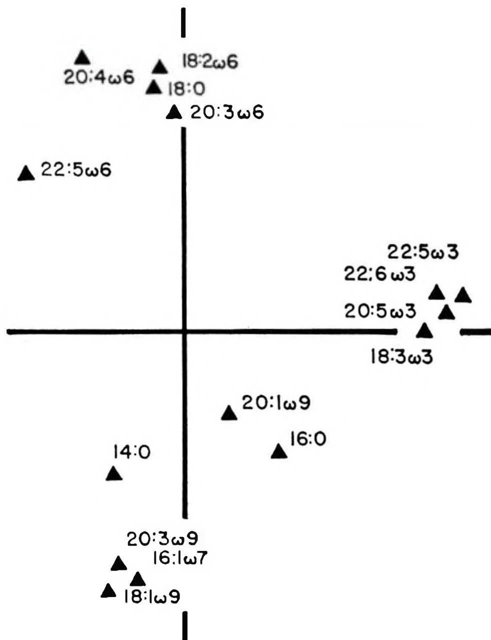

Fig. 2 Factor analysis representation of the data in table 5. Tissue fatty acid variables are plotted in a 2-dimensional correlation space. The higher the correlation between any 2 variables, the closer together will be the points representing them. The first factor loadings are plotted as ordinates and generally represent the ω6 acid metabolism in the positive direction, which is in opposition to the ω7, ω9 and saturated acid group. The second factor loadings are plotted as abscissas and are related to ω3 acid metabolism.

ticular, the content of 22:5ω6 in liver is strongly related to the content of other metabolically related liver fatty acids. The concentration of 22:5ω6 in liver lipid can be estimated within $\sigma = 0.62\%$ from equation 12 which requires only a knowledge of the concentrations of 20:4ω6 and 22:6ω3 in this lipid.

DISCUSSION

Heretofore the interrelationships between dietary fatty acids and tissue fatty acids have been deduced from experiments in which single fatty acids were fed and their influence upon tissue fatty acids assessed either by measurement of radioactivity or by measurement of changes in steady state concentrations. In such experiments, involving one fatty acid at a time, it is possible to overlook important interactions of one fatty acid upon another. This report presents results obtained when 12 acids were varied simultaneously and independently and when fat deficiencies are not involved. In most cases the results of the present experiment tend to confirm results obtained in experiments with more simple design. A contrary example may be found in the metabolic interaction between the saturated fatty acids and the ω6 fatty acids.

It is commonly thought that an increase in the amount of any fatty acid in the diet will result in a corresponding increase of this fatty acid in tissue lipid. In the cases of 14:0, 16:1, 18:2, 18:3 and 20:1 this was true. There were significant positive correlations between each of these fatty acids in the diet and the concentration of that fatty acid in liver lipid. However, there were zero and negative correlations between dietary and tissue contents of 16:0, 18:0 and 18:1. Dietary oleic acid shows little positive correlation with the concentration of any of the liver fatty acids. The concentration of oleic acid in liver lipid is controlled by a number of factors. T18:1 is directly proportional to the amount of saturated fatty acid in the diet and is inversely proportional to the ω6 and ω3 polyunsaturated acids in the diet. All saturated fatty acids in the diet tend to increase the ω7 and ω9 acids in liver lipid. The short-chain saturated acids 6:0 to 14:0 are distinguished from the other saturated acids in that they selectively increase liver myristate. Feeding linolenic acid also tends to suppress ω7, ω9 and part of the ω6 components in liver lipid, but its major effect is to increase in the tissue concentration of ω3 components.

Taken together, the data of the present experiment suggest that there are 4 independent dietary fatty acid variables: the short-chain saturated fatty acids (6:0-14:0), the long-chain saturated fatty acids (16:0-22:0), linoleate and linolenate. This concept may be useful both for the planning of nutrition experiments and in deciding which fatty acid data need to appear in nutritionally useful tables of food composition. Dietary oleic acid had very little effect upon tissue fatty acid composition (table 3). It thus appears that oleic acid may prove useful as a source of fatty acid calories which will have minimal

influences upon fatty acid metabolism. The dietary effects of palmitoleate need further study. Most of the correlations with this dietary variable in table 3 resemble those of the dietary saturated fatty acids, but this may be the indirect effect of the high correlations (table 2) between 16:1 and both 20:0 and 22:0 in these diets. The very highly unsaturated acids (20:4 and above), which were not included as dietary variables in this experiment, are presently under study in our laboratories.

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LITERATURE CITED

1. Caster, W. O., and R. T. Holman 1961 Statistical study of the relationship between dietary linoleate and the fatty acids of heart and blood lipids. J. Nutrition, *73:* 337.
2. Caster, W. O., P. Ahn, E. G. Hill, H. Mohrhauer and R. T. Holman 1962 Determination of linoleate requirement of swine by a new method of estimating nutritional requirement. J. Nutrition, *78:* 147.
3. Dixon, W. J. 1964 Biomedical Computer Programs. Health Sciences Computing Facility, University of California, Los Angeles.
4. Caster, W. O. 1962 Use of a digital computer in the study of eating habit patterns. Am. J. Clin. Nutrition, *10:* 98.
5. Caster, W. O., E. G. Hill and R. T. Holman 1963 Estimation of essential fatty acid intake in swine. J. Animal Sci., *22:* 389.
6. Mohrhauer, H., and R. T. Holman 1963 The effect of dose level of essential fatty acids upon fatty acid composition of rat liver. J. Lipid Res., *4:* 151.
7. Folch, J., M. Lees and G. H. Sloane-Stanley 1957 A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem., *226:* 497.
8. Peifer, J. J., and R. T. Holman 1959 Effect of saturated fat upon essential fatty acid metabolism of the rat. J. Nutrition, *68:* 155.
9. Tove, S. B., and F. H. Smith 1959 Kinetics of the depletion of linoleic acid in mice. Arch. Biochem. Biophys., *85:* 352.
10. Dhopeshwarkar, G. A., and J. F. Mead 1961 Role of oleic acid in the metabolism of essential fatty acids. J. Am. Oil Chemists' Soc., *38:* 297.

Dietary and Defaunation Effects upon Plasma Amino Acid Concentrations in Sheep [1]

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ABSTRACT When 2 rations were each fed to a group of 3 sheep fed twice daily, plasma amino acid concentrations were greater than pre-feeding concentrations with an 8.1% protein ration, whereas the converse was true for a 12.5% protein ration. In a second experiment, substitution of a concentrate ration for a roughage ration on the sample day only, resulted in lower plasma amino acid concentrations. In a third experiment, the infusion of a starch-glucose mixture into the rumen at the usual feeding time resulted in reduced plasma amino acid concentrations. Infusion at 1.5% of the metabolic weight ($B.W.^{0.75}$) gave a greater reduction than did infusion at 1.2% of the metabolic body weight ($B.W.^{0.75}$). Defaunated lambs showed higher concentrations of plasma amino acids and a greater decrease in concentration following starch glucose infusion than did comparable faunated animals.

While a considerable number of publications concerning various aspects of amino acid nutrition and metabolism of simple-stomach animals is available, the literature with respect to ruminant animals is relatively restricted. Both Downes (1) and Black et al. (2) using ^{14}C-labeled acetate have indicated that the amino acids, threonine, valine, methionine, isoleucine, leucine, phenylalanine, lysine, histidine, and tryptophan are essential for the ruminant. An influence of the dietary protein source upon abomosal ingesta hydrolysate and plasma free amino acid patterns in sheep has been reported,[2] despite the modifying influence of the rumen microbial population. Changes in plasma acid concentrations in the bovine with fasting have been reported by Brown et al. (3). These results are essentially similar to those reported by Hill and Olsen (4) for chickens.

The present work was undertaken to investigate daily patterns in plasma amino acid concentrations, the influence of various modifying factors, and the feasibility of using modified plasma amino acid ratios to indicate limiting amino acids in ruminants.

MATERIALS AND METHODS

Experiment 1. Rations which contained protein at levels of either 8.1% or 12.5% were each fed to 3 lambs (rations A and B, table 1) twice daily at a level of 6% of their metabolic body weight ($B.W.^{0.75}$) per day. Rumen content and carotid blood samples were collected 3, 6, 9, 12, 16, 20, and 24 hours after the last feeding. The animals were not fed during the sampling period. Throughout these experiments the lambs weighted 36 to 45 kg.

Experiment 2. A ration with a high content of roughage material (ration D, table 1) was fed to 2 lambs twice daily for 14 days at a level of 6% of their metabolic body weight ($B.W.^{0.75}$) per day. On the seventh and fourteenth days one of the two lambs received a concentrate ration (ration C, table 1) at the morning feed. Carotid blood and rumen content samples were collected prior to feeding and 1, 4, and 8 hours after feeding on the seventh and fourteenth days.

Experiment 3. Eight lambs were fed the 12.5% protein ration (ration B) twice daily at a level of 6% of their metabolic body weight ($B.W.^{0.75}$) per day for 7 days. Four of the lambs contained protozoa and four were protozoa-free; the animals were defaunated using the method of Eadie and Oxford (5). Blood and rumen samples were collected prior to feeding on the

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[2] Poley, G. E., and A. H. Trenkle. J. Animal Sci., 22: 1139, 1963 (abstract).

TABLE 1
Ration compositions

	Ration			
	A	B	C	D
	%	%	%	%
Alfalfa meal	—	38.0	31.5	25.8
Ground corn cobs	31.0	7.8	14.8	37.9
Ground shelled corn	50.0	47.0	39.0	4.8
Molasses	—	—	1.9	1.6
Glucose [1]	3.0	4.8	7.8	—
Starch	7.0	—	—	—
Cellulose [2]	5.0	—	—	—
Wheat straw	—	—	—	25.8
Urea	0.6	—	1.0	0.8
Mineral [3]	3.0	2.4	3.3	2.7
Na_2SO_4	0.45	—	—	—
Vitamins [4]	0.25	0.25	0.8	0.6

[1] Included as Cerelose, Corn Products Company, Argo, Illinois.
[2] Included as Solka Floc, Brown Company, Berlin, New Hampshire.
[3] Minerals consisted of trace mineralized salt, Cura-phos (14% P rock phosphate, M. J. Baker and Brother, New York) and limestone (3:2:1). Trace mineralized salt contained: (in %) Nacl, 99; Mn, 0.200; Fe, 0.160; Cu, 0.033; Co, 0.010; I, 0.007; and Zn, 0.005.
[4] Included in proportions recommended by the National Research Council (19) for finishing pigs including those listed as not necessarily required.

seventh day and then instead of feeding, a 2:1 (w/w) mixture of starch and glucose in 500 ml of water was infused into the rumen. This was given to 2 lambs of each group at a level of 1.2% (dry weight) of the metabolic body weight ($B.W.^{0.75}$); the other 2 lambs in each group received the starch and glucose at a level of 1.5% of the metabolic body weight ($B.W.^{0.75}$). Carotid blood and rumen samples were taken 4 hours later.

Blood samples. Each of the animals had had carotid loops established (6) and blood was drawn from these. One milliliter of blood to be used for glucose analysis was treated immediately with sodium fluoride, the glucose concentrations were determined enzymatically by the method of Huggett and Nixon (7) using a commercial enzyme preparation.[3] Plasma urea concentrations were determined by the method of Conway (8). Tungstic acid filtrates (9) were used for plasma free amino acid determinations using a Technicon automatic analyzer.

Rumen samples. Volatile fatty acids (VFA) were determined quantitatively by gas-liquid chromatography as described by Erwin et al. (10). Rumen ammonia was determined by the method of Conway (11).

RESULTS

Experiment 1. Pre-feeding[4] plasma free amino acid concentrations for the 2 rations are shown in table 2. Ration B (12.5% protein) generally supported higher levels of plasma amino acids at this time. Amino acid concentrations throughout the 24-hour period (figs. 1–4) are expressed as a ratio of the pre-feeding concentrations to facilitate comparison of the feeding to feeding variations. A distinct pattern was apparent, particularly with the essential amino acids. Also the patterns for the 2 rations were different. With ration A the general pattern was one of an elevated plasma amino acid concentration following feeding followed by a subsequent return to the pre-feeding level. This pattern was repeated in the second 12 hours despite the fact that the animals were deprived of their feed at this time. However, the levels were slightly higher in the second 12 hours and at 24 hours most of the amino acid concentrations were elevated to some degree. On the other hand, with ration B, the general pattern was one of a decrease in concentration following feeding, with a subsequent return to the pre-feeding level. Again despite feed deprivation, this pattern was repeated in the second 12 hours.

Rumen ammonia (fig. 5) concentrations were greater with ration B than A and both tended to increase with the onset of

[3] Worthington Biochemical Corporation, Freehold, New Jersey.
[4] Pre-feeding is used hereafter to refer to samples taken immediately prior to feeding.

TABLE 2

Pre-feeding concentration of plasma free amino acids in sheep fed two different rations

Essential amino acids			Nonessential amino acids		
Amino acid [1]	Ration A	Ration B	Amino acid	Ration A	Ration B
	mg/100 ml			*mg/100 ml*	
Threonine	1.65	1.96	aspartic	0.50	0.59
Valine	1.35	1.75	glutamic	1.28	1.39
Methionine	0.22	0.27	citrulline	1.83	3.22
Isoleucine	0.75	1.06	glycine	5.50	8.08
Leucine	1.32	1.33	alanine	2.14	2.23
Phenylalanine	0.88	0.73	cystine	0.35	0.29
Lysine	0.58	0.95	tyrosine	1.77	1.16
Histidine	1.36	1.58	ornithine	0.64	0.86
			arginine	0.75	1.37

[1] Tryptophan not determined.
[2] Pre-feeding refers to samples taken immediately prior to feeding.

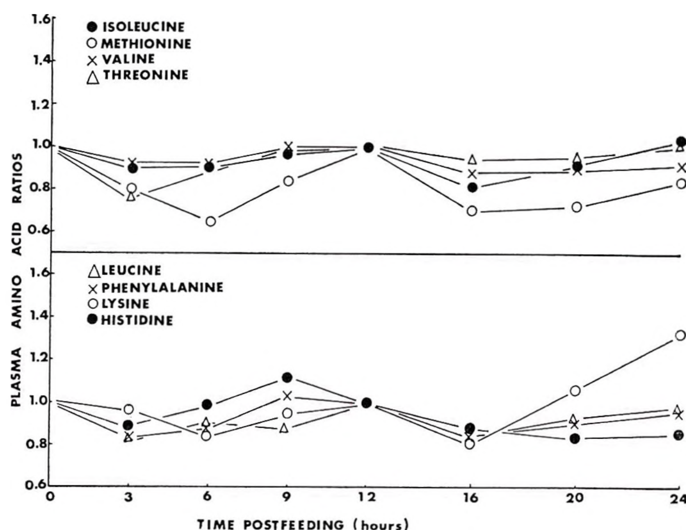

Fig. 1 Essential plasma amino acid concentrations in lambs fed ration A (8.1% protein) expressed as a ratio of the pre-feeding concentration. The animals were deprived the one-half daily ration normally fed 12 hours after the previous feed. Each value is the mean of the results from 3 animals.

fasting. Blood urea (fig. 5) concentrations reflected the rumen ammonia values. Total rumen VFA concentrations were greater with ration B than with A (fig. 6) following feeding, but the converse was true 12 to 24 hours post-feeding, whereas plasma glucose concentrations reflect the onset of fasting in showing a decrease 20 and 24 hours post-feeding.

Experiment 2. Pre-feeding plasma amino acid concentrations and the ratios at the various times after feeding are shown in table 3. The substitution of ration C for D on the sampling day resulted in generally lower amino acid concentrations, particularly at 4 hours post-feeding. At this time the mean percentage ratio was 61 for ration C and 78 for ration D.

VFA concentrations expressed as a percentage of the pre-feeding value are shown in figure 7. The substitution of ration C for D on the sampling day resulted in elevated VFA concentrations in the rumen; this effect was most marked one hour after feeding. Molar proportions of the individual acids were not influenced by this treatment.

Experiment 3. The plasma amino acid concentrations for the faunated and de-

faunated sheep both before and 4 hours after the starch-glucose administration are shown in table 4. Plasma amino acid concentrations were generally lower in faunated lambs than in defaunated lambs and the amino acid concentrations were reduced in both groups following the administration of the starch-glucose mixture.

In table 5 essential amino acid values from experiment 3 are given as percentage ratios of the pre-feeding amino acid concentrations. Ratios for individual animals in experiment 3 indicate that the higher level of the starch-glucose mixture (1.5%) resulted generally in lower ratios. These results also show that on the average, ratios were lower for defaunated than for faunated lambs, these were 56 and 82, respectively. These values may be compared with the ratios shown in figure 3,

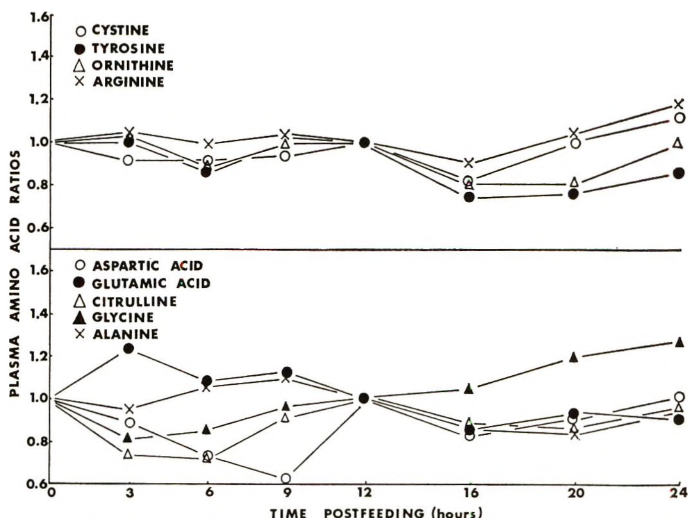

Fig. 2 Nonessential plasma amino acid concentrations in lambs fed ration A (8.1% protein). Conditions were as described for figure 1.

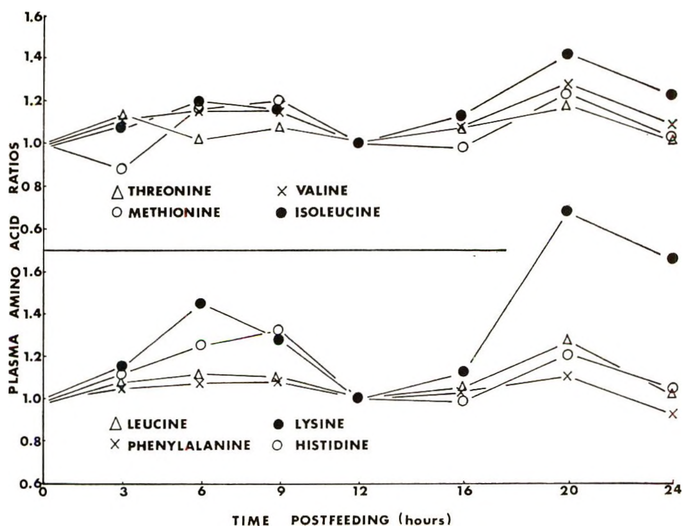

Fig. 3 Essential plasma amino acid concentrations in lambs fed ration B (12.5% protein). Conditions were as described for figure 1.

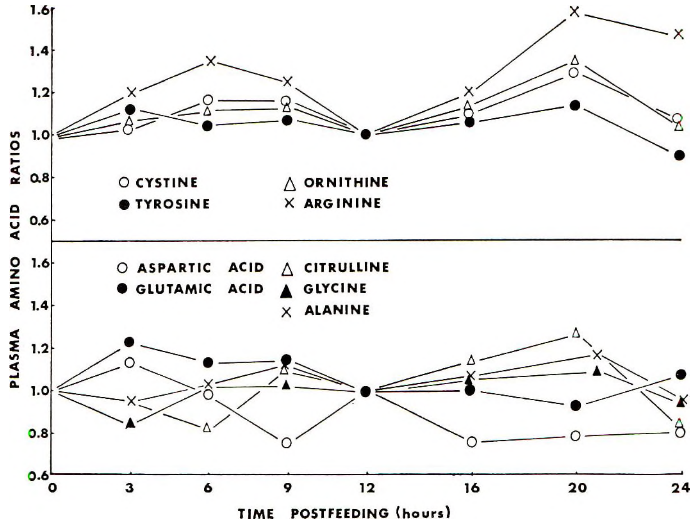

Fig. 4 Nonessential plasma amino acid concentrations in lambs fed ration B (12.5% protein. Conditions were as described for figure 1.

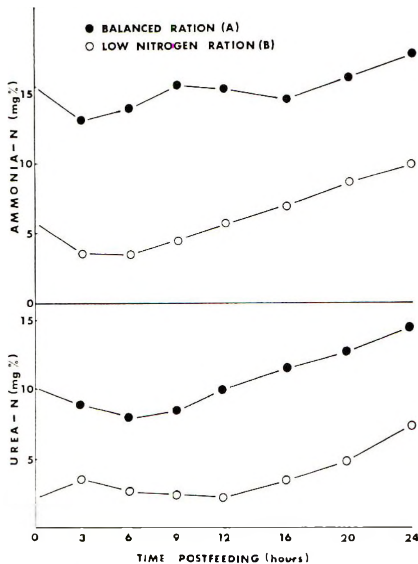

Fig. 5 Rumen ammonia nitrogen and blood urea nitrogen concentrations in lambs fed ration A (8.1% protein) and ration B (12.5% protein) in experiment 1. Each value is the mean of results from 3 animals.

when the animals received a normal feed, and it is apparent that the starch-glucose infusion resulted in lower average ratios.

DISCUSSION

The many factors influencing plasma amino acid concentrations have been well-documented (11, 12) and factors influencing amino acid imbalances have been discussed by Harper (13). In the present experiments it was not possible to distinguish between the various contributing factors. However, the establishment of different amino acid patterns following the feeding of 2 different rations in experiment 1 was probably due to variations in the relative magnitude of amino acid supply or energy availability, or both. Thus, the relative decrease in plasma amino acid concentrations following feeding ration B compared with the relative increase in plasma amino acid concentrations with ration A, was probably due to a greater relative availability of energy at this time. That the different patterns were due to quantitatively more amino acids being available with ration A is unlikely since this ration had a lower N content. Two explanations for this relative energy effect are possible. First, the relative increase following feeding ration A may have been due to less energy being available for protein synthesis, or second, maximal amino acid absorption from the alimentary tract may have occurred at a different time with ration A than with ration B, being in optimal combination with energy availability with ration B.

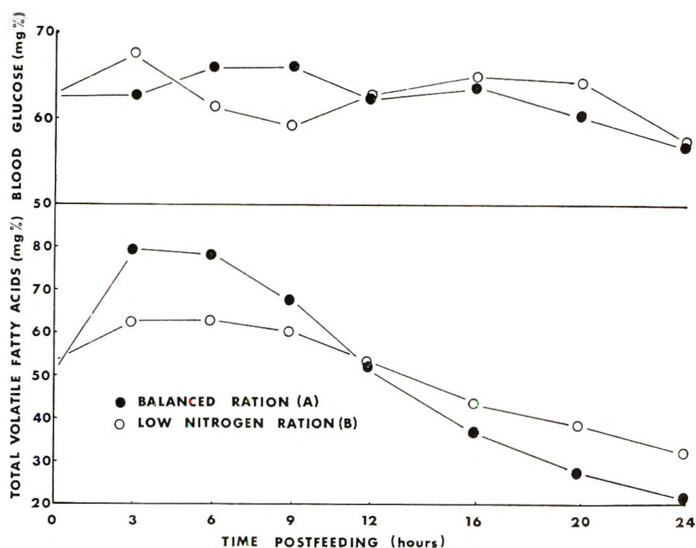

Fig. 6 Blood glucose and rumen VFA concentrations in lambs fed ration A (8.1% protein) and ration B (12.5% protein) in experiment 1. Each value is the mean of results from 3 animals.

TABLE 3

Pre-feeding plasma concentration of essential amino acids in sheep and the ratio (%) at various times after feeding

Amino acid [1]	Pre-feeding conc		Ratio of Pre-feeding conc (time post-feeding) [3]					
			1		4		8	
	Ration C	Ration D	Ration C	Ration D	Ration C	Ration D	Ration C	Ration D
	mg/100 ml		*mg/100 ml*		*mg/100 ml*		*mg/100 ml*	
Valine	3.08 [2]	2.89	88	93	69	83	84	88
Methionine	0.20	0.15	83	105	60	91	50	85
Isoleucine	1.18	1.00	81	92	69	76	89	85
Leucine	1.16	1.06	77	69	55	43	89	87
Phenylalanine	0.80	0.71	67	88	67	89	85	89
Lysine	2.04	2.19	67	69	32	42	45	45
Histidine	1.56	1.42	70	103	77	124	73	98
Mean			76	88	61	78	74	82

[1] Threonine and tryptophan were not determined.
[2] Each value is the mean of results from 2 sheep.
[3] Ratios are the values obtained at the respective times expressed as a percentage of the prefeeding value.

Additional evidence supporting the conclusion that absorption of amino acids from the alimentary tract occurred at different times for the 2 rations is provided by the fact that the plasma amino acid concentrations showed a similar pattern in the second 12 hours to that exhibited in the first 12 hours, even though at this time the total rumen volatile fatty acid concentrations with ration B were greater than with ration A and with both had reached a relatively low level (fig. 6). Plasma glucose concentrations also tended to decrease at 24 hours. That energy was not available for promoting amino acid utilization in this latter part of the sampling period is confirmed by the increasing rumen ammonia and blood urea levels (fig. 5). Miller and Payne (14) have discounted the practical importance of the timing of nutrients in large animals such as man, but in view of the nature of the

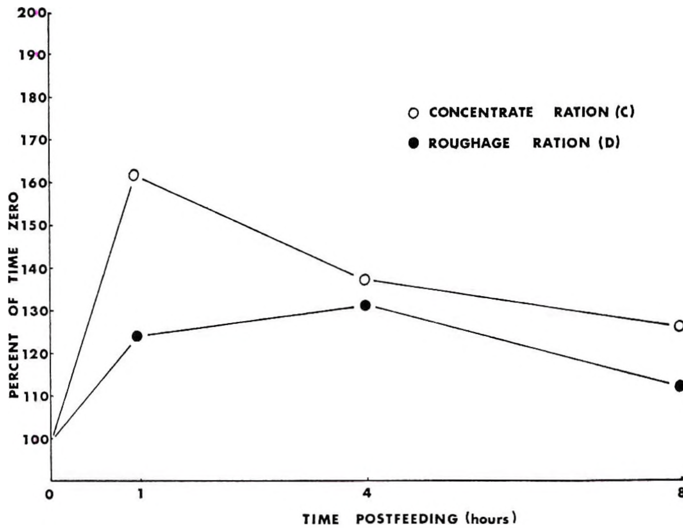

Fig. 7 Rumen VFA concentrations in lambs fed ration C and D (ration C on the day of sampling only), expressed as a percentage of the pre-feeding concentration. Each value is the mean of results from 2 animals.

TABLE 4

Plasma concentration of essential amino acids in faunated and defaunated sheep before and after the addition of starch and glucose to the rumen [1]

Amino acid	Before starch-glucose addition [2]		After starch-glucose addition (4 hours) [3]	
	Faunated	Defaunated	Faunated	Defaunated
	mg/100 ml		*mg/100 ml*	
Threonine	1.28 ± 0.59 [4]	3.70 ± 1.02	1.07 ± 0.83	2.12 ± 0.35
Valine	2.60 ± 0.47	6.41 ± 1.23	2.03 ± 0.26	3.31 ± 0.11
Methionine	0.12 ± 0.03	0.30 ± 0.09	0.10 ± 0.01	0.15 ± 0.04
Isoleucine	0.81 ± 0.09	1.61 ± 0.34	0.77 ± 0.04	0.72 ± 0.09
Leucine	1.27 ± 0.16	2.44 ± 0.88	1.01 ± 0.12	1.28 ± 0.28
Phenylalanine	0.62 ± 0.12	1.23 ± 0.38	0.68 ± 0.32	0.80 ± 0.07
Lysine	1.43 ± 0.24	2.69 ± 0.89	0.97 ± 0.34	0.87 ± 0.23
Histidine	0.87 ± 0.12	1.59 ± 0.31	0.67 ± 0.27	1.02 ± 0.07
Glutamic	0.88 ± 0.14	1.34 ± 0.23	1.11 ± 0.08	1.09 ± 0.11
Citrulline	2.56 ± 0.45	3.08 ± 0.40	2.68 ± 0.70	1.81 ± 0.42
Glycine	5.78 ± 1.14	5.98 ± 0.54	5.15 ± 2.81	4.58 ± 0.24
Alanine	1.16 ± 0.24	1.75 ± 0.40	1.23 ± 0.30	1.00 ± 0.13
Cystine	0.23 ± 0.08	0.64 ± 0.18	0.16 ± 0.04	0.43 ± 0.14
Tyrosine	0.83 ± 0.17	2.10 ± 0.38	0.69 ± 0.14	1.01 ± 0.29
Ornithine	0.92 ± 0.10	1.61 ± 0.21	0.76 ± 0.13	0.87 ± 0.09
Arginine	1.33 ± 0.22	2.87 ± 0.53	1.22 ± 0.19	0.91 ± 0.13

[1] Starch and glucose (2:1 w/w) infused into the rumen at either 1.2 or 1.5% of the metabolic body weight.
[2] Each value is the mean of results from 4 animals.
[3] Each value is the mean of values from 4 animals; two received starch and glucose at the 1.2% level and two at the 1.5% level.
[4] SE.

ruminant digestive system and the above results such an effect seems worthy of further investigation.

The second experiment was carried out in an attempt to distinguish more clearly between the effect of energy per se and amino acid absorption differences upon the plasma amino acid concentrations. Thus, since the animals had been fed identical rations until the sampling period, the composition of material passing from the rumen into the lower alimentary tract

TABLE 5

Plasma amino acid ratios and the order of the limiting amino acids in faunated and defaunated sheep [1,2]

Amino acid	Faunated sheep no.					Defaunated sheep no.				
	1 [3]	3 [3]	2 [4]	4 [4]	Mean	5 [3]	7 [3]	6 [4]	8 [4]	Mean
Threonine	66 [3]	100	100	28 [1]	69 [2]	70	89	40 [2]	47	62
Valine	81	86 [3]	89	62	77 [3]	67	62	42 [3]	43	54 [3]
Methionine	100	90	77 [1]	65	83	87	45 [3]	59	26 [2]	54 [3]
Isoleucine	101	113	86 [3]	87	97	62 [1]	52	44	29 [3]	47 [2]
Leucine	82	77 [2]	80 [2]	78	79	62 [1]	48	44	67	53
Phenylalanine	94	92	145	90	105	67	41 [2]	70	35	73
Lysine	53 [2]	67 [1]	99	52 [2]	68	64 [3]	36 [1]	28 [1]	18 [1]	37 [1]
Histidine	43 [1]	105	107	56 [3]	78 [1]	82	63	72	49	67
Mean	78	91	98	65	82	70	55	50	39	56

[1] Ratios are the values obtained 4 hours after the starch-glucose administration expressed as a percentage of the initial value.

[2] Limiting order of the amino acids determined by that amino acid showing the smallest plasma amino acid ratio. These are identified in the table by superscripts 1, 2, and 3, representing the first, second, and third limiting amino acid, respectively.

[3] Starch and glucose (2:1 w/w) infused into the rumen at a level of 1.2% of the metabolic body weight.

[4] Starch and glucose (2:1 w/w) infused into the rumen at a level of 1.5% of the metabolic body weight.

should have been similar. Hence, the principal effect of the concentrate ration substitution was one of greater volatile fatty acid concentration in the rumen (fig. 7), presumably giving rise to greater availability of energy for amino acid utilization. Molar proportions of the acids were not different and hence any differences should have been due to quantitative differences in the energy supply. It seems probable, therefore, that in this experiment decreased plasma amino acid concentrations resulted from greater energy availability for amino acid utilization. Munro and Thompson (15) have shown that glucose, but not fat, caused a decrease in plasma amino acid concentrations in humans, and Crofford (16) has also shown decreases in plasma amino acid concentrations following glucose or glucose plus insulin administration.

The third experiment was carried out to investigate the possibility of using the above information in the development of a technique capable of delineating the sequence of limiting amino acids in ruminants under specific dietary conditions. Such a technique would be extremely valuable in view of the impossibility of using feeding trials or supplementation experiments for such determinations. The virtual impossibility of obtaining fasting values, uncomplicated with starvation effects, from ruminants is a major obstacle in applying either the plasma amino acid ratio technique of Longnecker and Hause (17) or the plasma amino acid score technique of McLaughlin (18). Furthermore, daily essential amino acid requirements of ruminants are unknown, a necessary factor in the method of Longnecker and Hause (17).

The expression of the amino acid concentrations, 4 hours after starch-glucose administration, as a percentage ratio of the pre-feeding value may be analagous to the plasma amino acid score used by McLaughlan (18) to delineate limiting amino acids. The superscripts 1, 2, and 3 in table 5 indicate the first, second, and third limiting amino acid using such a system. This suggests that in three out of the four defaunated lambs, lysine was the limiting amino acid, whereas in the faunated lambs, no single amino acid was limiting in more than one animal. This aspect will be discussed in detail in a paper specifically concerned with the role of protozoa in ruminant metabolism.

The validity of using these ratios to indicate limiting amino acids is dependent upon a major assumption, namely, that the decrease in plasma amino acid concentration following starch-glucose administration was proportional to the daily essential amino acid requirement of the animal. This assumption is supported by the work of Munro and Thompson (14) who showed the change in plasma amino

acid concentration in humans following glucose administration to be proportional to the daily requirement of essential amino acids. Certainly, considerable work is required to substantiate the validity of the suggested method.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. Downes, A. M. 1961 On the amino acids essential for the tissues of the sheep. Australian J. Biol. Sci., *14:* 254.
2. Black, A. L., M. Kleiber, H. M. Smith and D. N. Stewart 1957 Acetate as a precursor of amino acids of casein in the intact dairy cow. Biophys. Acta, 23: 54.
3. Brown, H. E., H. O. Kunkel and J. M. Prescott 1962 Fasting amino acid patterns in plasma of normal and dwarf cattle. J. Animal Sci., *21:* 970.
4. Hill, D. C., and E. M. Olsen 1963 Effect of starvation and a nonprotein diet on blood plasma amino acids, and observations on the detection of amino acids limiting growth of chickens fed purified diets. J. Nutrition, *79:* 303.
5. Eadie, J. M., and A. E. Oxford 1957 A simple and safe procedure for the removal of holotrich ciliates from the rumen of an adult fistulated sheep. Nature, *179:* 485.
6. Bone, J. F., J. Metcalfe and J. T. Parer 1962 Surgical preparation of a coratid loop in sheep. Am. J. Vet. Res., *23:* 1113.
7. Huggett, A. St. G., and D. A. Nixon 1957 Use of glucose oxidase, perioxidase and o-dianisiodine in determination of blood and urinary glucose. Lancet, *2:* 368.
8. Conway, E. I. 1950 Microdiffusion Analysis and Volumetric Error. Crosby Lockwood and Son Ltd., London.
9. Hawk, P. B., B. L. Oser and W. H. Summerson 1947 Practical Physiological Chemistry, Churchill Ltd., London.
10. Erwin, E. S., G. J. Marco and E. M. Emery 1961 Volatile fatty acid analysis of blood and rumen fluid by gas chromatography. J. Dairy Sci., *44:* 768.
11. Munro, H. N. 1964 Regulation of protein metabolism. In: Mammalian Protein Metabolism, vol. 1, eds., H. N. Munro and J. B. Allison. Academic Press, New York.
12. Holden, J. T. 1962 Amino Acid Pools. Elsevier Company, New York.
13. Harper, A. E. 1964 Amino acid toxicities and imbalances. In: Mammalian Protein Metabolism, vol. 2, eds., H. N. Munro and J. B. Allison. Academic Press, New York.
14. Miller, D. S., and P. R. Payne 1964 Dietary factors influencing nitrogen balance. Proc. Nutrition Soc., *23:* 11.
15. Munro, H. N., and N. S. T. Thompson 1953 Influence of glucose on amino acid metabolism. Metabol. Clin. Exp., *2:* 354.
16. Crofford, O. B., P. W. Felts and W. W. Lacy 1964 Effect of glucose infusion on the individual plasma free amino acids in man. Proc. Soc. Exp. Biol. Med., *117:* 11.
17. Longenecker, J. B., and N. L. Hause 1959 Relationship between plasma amino acids and composition of the ingested protein. Arch. Biochem. Biophys., *84:* 46.
18. McLaughlan, J. M. 1964 Blood amino acid studies. V. Determination of the limiting amino acids in diets. Canad. J. Biochem., *42:* 1353.
19. National Research Council, Committee on Animal Nutrition 1964 Nutrient requirements of swine, pub. 1192. National Academy of Sciences — National Research Council, Washington, D. C.

In vivo Intestinal Absorption of L-Alanine in Irradiated Rabbits [1]

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ABSTRACT The effects of ionizing radiation (^{60}Co) on intestinal absorption of L-alanine were studied by means of an in vivo technique permitting measurement of radioactivity in a large number of blood samples taken from the portal and femoral veins of albino rabbits following duodenal injection of 20 μCi of L-alanine-^{14}C. The experiments were carried out 4 days after irradiation. The curves so obtained show a very rapid rise and fall of activity in the portal vein under all three experimental conditions (control, 300 r, and 600 r). Absorption was practically completed 50 minutes after the injection of this amino acid. Absorption trends were similar and no significant difference was observed between those of irradiated and unirradiated animals. At the doses used, histological examination 4 days after irradiation showed slight effects on the absorbing cells of the intestine.

The effect of ionizing radiations on intestinal absorption has been studied for a number of food constituents including calcium and strontium, plutonium, sodium, and water (1-5), sugars (6-8), fat (9), and proteins (10). With respect to amino acids, we are not aware of any research other than that of Shishova (11) who studied their absorption in irradiated, adrenalectomized rats.

We therefore considered it worthwhile to supplement the information available about unirradiated animals (12, 13) with some results concerning the effect of ionizing radiations on the intestinal absorption of amino acids.

Although in vitro methods have advantages from the standpoint of experimental convenience, we chose an in vivo technique in order to maintain physiological conditions as normal as possible. The technique used also made it possible to draw a large number of samples from the same animal and thus to plot a curve for absorption in relation to time.

The present work concerns a comparison of the intestinal absorption of L-alanine in control rabbits and in rabbits exposed to 300 and 600 roentgens. The absorption studies were made 4 days after irradiation. In this work, the term absorption refers to the amount of alanine transferred from the intestine into the blood during the experimental period.

MATERIAL AND METHODS

Albino rabbits weighing between 1.8 and 2.2 kg and fasted for 24 hours were anesthetized with an intravenous injection of pentobarbital sodium[2] at the rate of 30 to 50 mg/kg. An opening 8 cm long was made in the right flank through which the portal vein and the posterior vena cava could be reached by slightly displacing the intestines. A catheter was then inserted into each of these 2 veins to permit blood samples to be taken periodically. The point of the catheter in the portal vein was inserted as far as the liver, and that in the vena cava as far as the junction of the femoral veins.

By this method it was possible to follow the results of intestinal absorption in the blood of the 2 veins without harming or disturbing the normal intestinal functions of the anethetized animals. Rabbits so prepared have proved to be excellent blood donors for a fairly long period (about 8 hours).

Experience has shown that sampling over a period of 2 hours is quite sufficient for studying intestinal absorption of L-alanine. However, we continued our observations up to 3 hours. In order to satisfy

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[1] This work was made possible by a research grant from the National Research Council of Canada and a study bursary from the Canadian Cancer Institute.

[2] Nembutal, Abbott Laboratories, North Chicago, Illinois.

ourselves as to the good physiological condition of the animals during the experiment, we took regular measurements of the hematocrit and the respiratory rate and also made electrocardiograms.

Following the duodenal injection of 2 ml of water containing 20 μCi of uniformly labeled L-alanine-^{14}C (specific activity: 120 mCi/mmole)[3] 7 cm from the pylorus, about 40 minutes after anesthesia, the opening in the flank was closed quickly. By means of tuberculin syringes, 0.5-ml samples of blood were taken at intervals from both catheters.

Altogether, 40 to 45 samples were taken in this way from the 2 catheters in each animal. Before every such sampling, care was taken to withdraw the residual blood (0.17 ml) from the catheter. Syringes were used only once in order to avoid contamination. Blood samples were then deproteinized by adding 2.5 ml of 7% trichloroacetic acid. Following centrifugation the supernatant fluid was transferred directly to counting bottles and evaporated under a vacuum. The residue remaining after evaporation was taken up in 0.5 ml of ethyl alcohol and then the scintillation solution, toluene-PPO-dimethyl POPOP at standard concentration, was added to it.

The protein separated from the serum by centrifugation was washed 3 times to recover amino acids remaining in it. Each washing resulted in the recovery of an amount of radioactivity equal to 9% of that contained in the supernatant fluid of the previous washing. In this way about 90% of the total activity present in each sample was recovered. The efficiency of counting of the system ranged from 79 to 81%.

The animals were irradiated by means of a cobalt-60 source delivering 5.6 roentgens/minute at a distance of 45 cm (from the source to the middle of the animal).

RESULTS

Physiological tests. Injection of pentobarbital sodium into the test animals had a pronounced effect on the rate of respiration, greatly slowing it in relation to the depth of anesthesia, but 20 to 30 minutes after the injection, the initial physiological state was restored and the respiratory rhythm remained normal throughout the actual experimental period.

Variations in the hematocritic values during the 30 minutes following the operation were negligible. On the average, this value diminished from 40 to 34% over a period of 180 minutes. Up to 30 ml of blood can be drawn from an animal, i.e., about 20% of the blood in a rabbit weighing 2 kg.

Histological observations. Histological examination, 4 days after irradiation, of the intestinal tract of 10 animals (four irradiated at 300 r and six at 600 r) revealed only the complete disappearance of lymph nodules and the presence of large pale nuclei in a great many epithelial cells.

Radioactivity in blood from the portal vein. The 2 curves in figure 1 show typical variations of radioactivity in the portal and in the femoral blood of an unirradiated animal.

Curves of a different shape were obtained for some animals but were disregarded because their departure from the mode was thought to be due to the presence of food remnants in the small intestine of these fasted animals. The shapes of the curves in figure 1 are considered to be typical for the absorption of small quantities of L-alanine in our irradiated and unirradiated rabbits. It should be pointed out, however, that although the curves obtained in this series of experiments follow the same general trend, there is a considerable range in the magnitude of activity in individual animals during the first hour. Apparently certain biological conditions, including individual differences in intestinal motility and rate of flow may easily lead to large variations in rate of absorption.

The typical curve indicates a rapid increase in the radioactivity shown by the deproteinized serum of blood taken from the portal vein after duodenal injection of L-alanine-^{14}C, attainment of a maximum after about 7 minutes, and then an almost equally rapid decrease.

The first 15 minutes following the introduction of alanine are of special interest and 10 to 12 samples were therefore taken from the portal vein during that time.

[3] Obtained from the New England Nuclear Corporation, Boston.

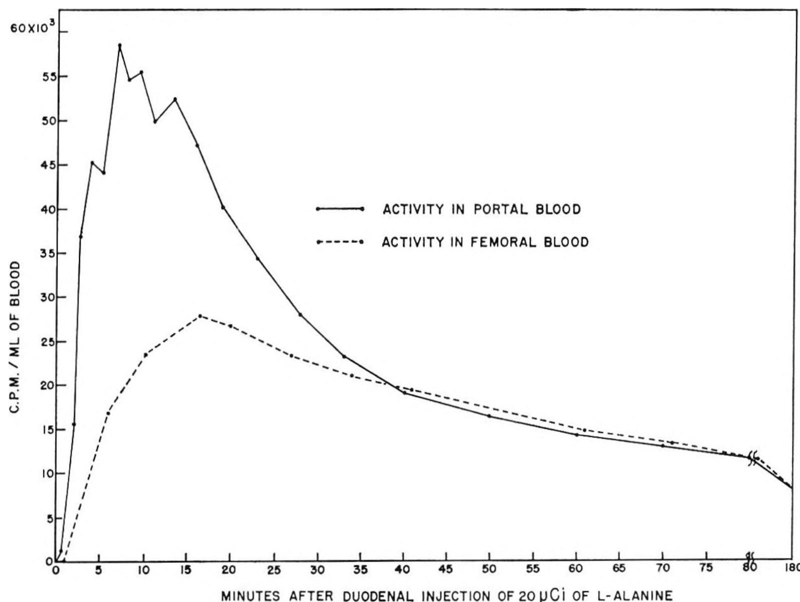

Fig. 1 Typical variation of radioactivity in the portal and in the femoral blood of an animal.

After 38 minutes, the 2 curves (representing activity in the portal and in the femoral blood) cross each other, the femoral curve maintaining a slightly higher level.

The curves in figure 2 are based, respectively, on mean values obtained from 12 control (unirradiated) rabbits, ten rabbits exposed to 300 r, and ten to 600 r. The variations encountered for each of these mean values are not shown on the graphs because they would overlap. However, the variations for the entire area under each portal curve, from zero to 50 minutes after duodenal injection of L-alanine, are shown in table 1.

It is necessary to describe how these curves were obtained because, in practice, it was impossible to take the blood samples at precisely identical times from every animal. In order to calculate the mean activity corresponding to a given time, the activity for that time was found on the curve plotted for each of the ten or, as the case may be, 12 animals (by interpolation when necessary). The arithmetic mean of these individual values was then plotted and in this way an average curve was obtained for activity in the portal blood and, similarly, for that of the femoral blood. An estimate of net portal activity and, by inference, of intestinal absorption of alanine during a given period was obtained by subtracting the area corresponding to that period under the femoral curve on the graph from the total area for the same period under the portal curve.

The main characteristics of curves showing mean activity in the portal blood may be summarized as follows:

1. Very rapid appearance of activity in the blood (within a minute after duodenal injection of alanine), increasing to a maximum between the seventh and eighth minute and then falling, thereby indicating a decrease in absorption. This decrease in activity after the peak can be explained as being due to less material being available for intestinal absorption.

2. Apparently almost identical rates of absorption during the increasing phase for all 3 experimental conditions (control, 300 r, and 600 r), the similarity persisting longer in the case of the irradiated animals.

3. Virtual termination of absorption after about 50 minutes, the activity in the portal blood by that time closely approaching that of the femoral blood. In all cases, the decline in activity in the portal blood was slow. The same applies to decline in activity in the femoral blood.

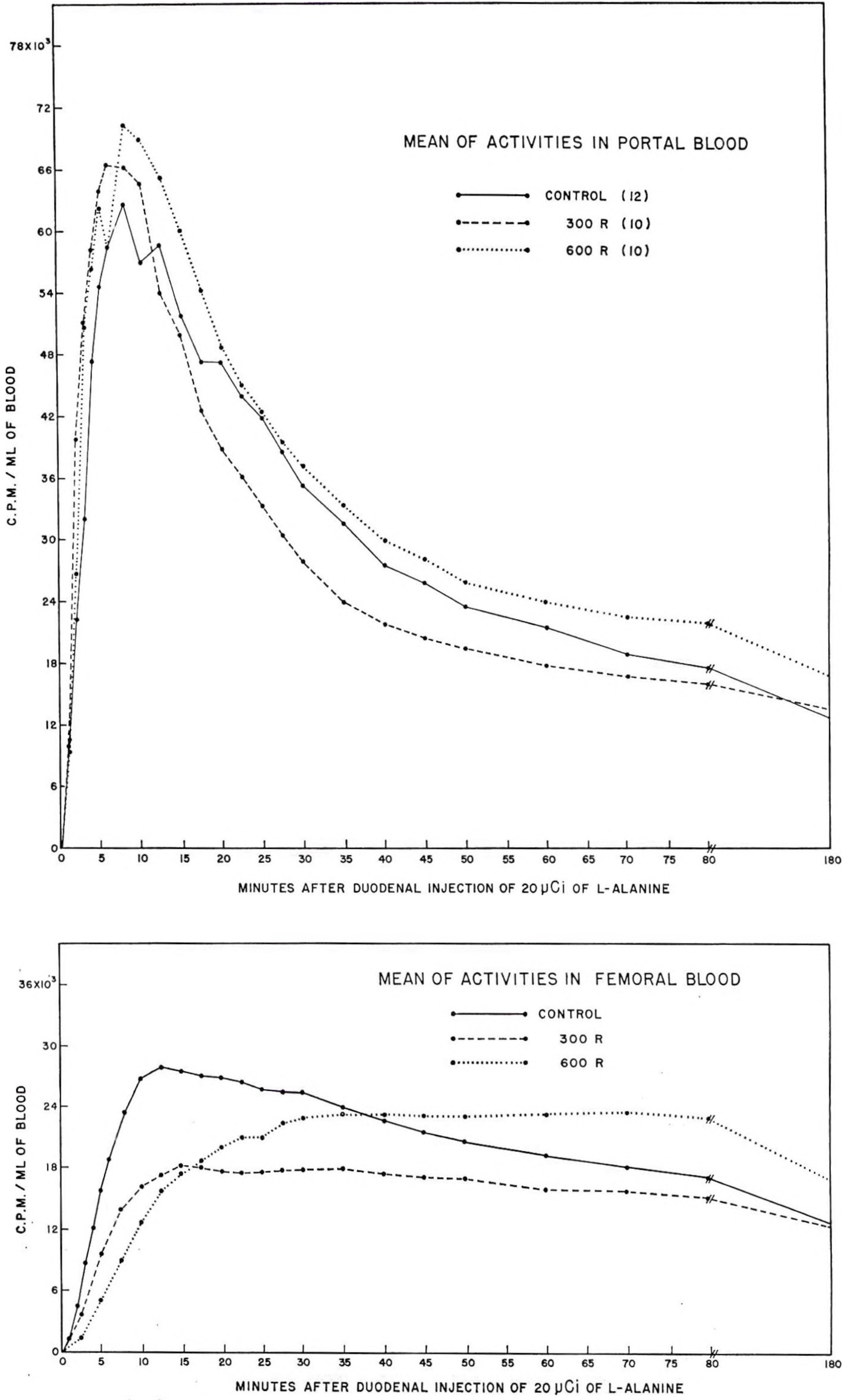

Fig. 2 Mean of activities in portal and femoral blood in control and irradiated rabbits.

TABLE 1

Areas under portal blood activity curves between zero and 50 minutes (arbitrary units)

Controls			300 r			600 r		
No.	Area	n − n̄	No.	Area	n − n̄	No.	Area	n − n̄
1	5.279	−0.951	1	8.950	3.369	1	6.207	−0.483
2	3.273	−2.959	2	5.056	−0.525	2	7.150	0.460
3	4.840	−1.392	3	9.150	3.569	3	4.348	−2.342
4	9.357	3.125	4	3.441	−2.140	4	4.380	−2.310
5	4.470	−1.762	5	5.050	−0.531	5	7.088	0.398
6	5.925	−0.307	6	3.131	−2.450	6	3.760	−2.930
7	7.060	0.828	7	6.200	0.619	7	11.375	4.685
8	4.944	−1.288	8	5.450	−0.131	8	10.925	4.235
9	6.392	0.160	9	3.855	−1.726	9	5.515	−1.175
10	7.472	1.240	10	5.526	−0.055	10	6.150	−0.540
11	9.070	2.838						
12	6.708	0.476						
n̄ = 6.23		σ = 1.75	n̄ = 5.58		σ = 2.1	n̄ = 6.69		σ = 2.5

Controls–300 r: $t = 0.77$
Controls–600 r: $t = 0.49$

Assuming that the net areas under the portal curves in figure 2 are proportional to the amount of alanine absorbed, our results suggest increased absorption in the irradiated animals (see table 2). This effect may be due to a reduction in intestinal motility under the influence of radiation, as postulated by Lengemann (1) in order to explain greater absorption of calcium and strontium in irradiated rats. But in view of the magnitude of the standard deviation σ and the small value for t obtained in a statistical comparison of the areas under the curves for the first 50 minutes, we cannot consider this increase to be significant (see table 1). Comparisons of the areas under the curves for the first 30 minutes and for the first 80 minutes gave similar results.

Activity in the femoral blood. About 50 minutes after the start of the experiment, the activity in the femoral blood approximated that in the portal blood; in a number of animals it slightly exceeded it, suggesting the uptake of alanine by intestinal tissue. Nevertheless, the difference in activity in the portal and the femoral blood at any given time after completion of absorption was always very small. It is interesting to compare the trends of curves showing activity in the peripheral blood of unirradiated and irradiated rabbits. In the former the activity increased rapidly, reaching a maximum in approximately 12 minutes, and then steadily and very gradually decreased. In rabbits receiving 300 r, activity in the peripheral blood increased much more slowly and, after about 15 minutes, reached its maximum, which was lower than in the case of the control animals. Decline in this activity was negligible within the experimental period.

In rabbits receiving 600 r, activity in the peripheral blood increased at less than half the speed at which it increased in the unirradiated animals. After about 35 minutes it reached a plateau and remained constant for an hour. Assuming that the activity in the peripheral blood reflects the metabolism of alanine, it appears that radiation may have a marked effect on the fate of this amino acid.

TABLE 2

Mean area after deduction for activity in femoral blood between zero and 50 minutes (arbitrary units)

Controls	300 r	600 r
n̄ = 2.96	n̄ = 3.10	n̄ = 3.86

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We wish to thank Dr. A. Gagnon for the interest he has shown in our experimental work.

LITERATURE CITED

1. Lengemann, F. W. 1960 The effect of whole body x-irradiation on the absorption of calcium-45 and strontium-85 from the intestinal tract of the rat. Radiat. Res., 13: 892.

2. Foreman, H., J. Post and C. Finnegan 1957 The effect of x-irradiation on the absorption of plutonium in the gastrointestinal tract. Radiat. Res., *7:* 267.
3. Curran, P. F., E. W. Webster and J. E. Hovsepian 1960 The effect of x-irradiation on sodium and water transport in rat ileum. Radiat. Res., *13:* 369.
4. Lengemann, F. W., and C. L. Comar 1961 The effect of whole-body x-irradiation on the passage and absorption of strontium and calcium in the gastrointestinal tract of the rat. Radiat. Res., *14:* 662.
5. Vaughan, B. E., and E. L. Alpen 1961 Moderate level x-ray effect on active transport mechanism in dog intestine. Internat. J. Radiat. Biol., *3:* 265.
6. Detrick, L. E., H. C. Upham, D. Highby, V. Debley and T. J. Haley 1955 Influence of x-irradiation on glucose transport in the rat intestine. Radiat. Res., *2:* 483.
7. Farrar, J. T., M. D. Small, D. Bullard and F. J. Ingelfinler 1956 Effect of total body irradiation on absorption of sugars from the small intestine. Am. J. Physiol., *186:* 549.
8. Lourau, M. 1961 Cinétique de l'absorption d'un dose unique de glucose par l'intestin de cobayes irradiés totalement par les rayons X. Arch. Sci. Physiol., *15:* 309.
9. Mead, J. F., A. B. Decker and L. R. Bennett 1951 The effect of x-irradiation upon fat absorption in the mouse. J. Nutrition, *43:* 485.
10. Bennett, L. R., S. M. Chastain, A. B. Decker and J. F. Mead 1951 Effect of Roentgen irradiation upon protein absorption in the mouse. Proc. Soc. Exp. Biol. Med., *77:* 715.
11. Shishova, O. A. 1959 The effect of ionizing radiation, adrenalectomy and cortisone treatment on the intestinal absorption of amino acids. Biokhimiya, *25:* 812 (cited in Biol. Abst., *35:* no. 52466, 1960).
12. Wilson, T. H. 1962 Intestinal Absorption. W. B. Saunders Company, Philadelphia.
13. Wiseman, G. 1964 Absorption from the Intestine. Academic Press, New York.

Post-ruminal Degradation and Absorption of Carbohydrate by the Mature Ruminant

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ABSTRACT Sheep that had been fed a high carbohydrate ration were killed 3, 6 or 24 hours post-feeding to determine the relative amount and form of carbohydrate reaching the abomasum. The carbohydrate concentrations in abomasum contents, 3 hours post-feeding, was equal to 55% of the ration concentration. More total carbohydrate, due to more starch, was observed in the abomasum 3 hours post-feeding than 6 or 24 hours post-feeding. Starch and maltose were hydrolyzed and absorbed as glucose from the small intestine. The rate of hydrolysis of maltose and absorption of glucose was appreciably faster than the rate of blood glucose clearance, whereas the rate of starch hydrolysis was only slightly greater than the rate of blood glucose clearance. The latter difference was detectable only when a large amount of soluble starch was administered into the abomasum of sheep fed a high concentrate diet.

The information concerning the post-ruminal utilization of carbohydrate by the ruminant is incomplete and conflicting. Most reports have indicated that little starch or glucose survived rumen microbial degradation (1), and that oral administration of glucose to sheep or cattle failed to increase the venous blood glucose concentration (2, 3). However, other experiments suggest that orally administered glucose increased blood sugar in functioning ruminants (4) and that appreciable amounts of glucose were absorbed by cattle fed a corn-alfalfa ration (5).

Weller and Gray (6), using the lignin ratio technique, reported that only 5 to 10% of the dietary starch reached the abomasum when low carbohydrate rations were fed to sheep. The degree of enzymatic degradation of starch within the small intestine was not determined. Dollar and Porter (7) observed that only glucose and lactose would increase blood reducing sugars when given orally to calves younger than 4 weeks of age. In their studies, maltose, sucrose, dextrin and soluble starch were not utilized. The lactase activity in the intestinal mucosa was high in the very young calf but maltase was low. Pancreatic amylase and intestinal maltase activity increased with age, whereas lactase activity decreased. Apparent quantitative post-ruminal degradation of maltose and absorption of glucose has been reported by Larsen et al. (8). However, these same investigators failed to demonstrate any apparent digestion of starch in the small intestine of 9-month-old calves.

Evaluation of the degree to which ruminants can utilize carbohydrates has been complicated by conflicting results of other studies that involved enzyme additions to ruminant diets (9).

The following studies were designed to determine the relative amount of carbohydrate, from a high concentrate ration, that reached the abomasum. A further objective was to determine the degree of post-ruminal carbohydrate digestion in sheep.

TABLE 1
Composition of basal diet

	%
Cracked corn	64.0
Chopped alfalfa	15.0
Soybean oil meal	9.1
Molasses	5.0
Corn cobs	5.0
Minerals [1]	1.9

[1] Contained: (in per cent) calcium carbonate, 40; defluorinated phosphate, 30; and iodized salt, 30.

EXPERIMENTAL

Twelve, 35-kg, wether lambs were individually fed the basal ration (table 1) ad libitum for a 21-day period. Approximately one-half of the total daily feed intake was consumed within the first 2 to 3 hours after fresh diet was offered each morning. On day 21, four sheep were killed

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3 hours after feeding, 4 sheep 6 hours after feeding, and 4 sheep 24 hours after feeding and following a 16-hour fast. Contents of the abomasum and of 3 equal-length segments of the entire small intestine were collected. One gram of NaF was added/100 ml ingesta and the samples were frozen with dry ice.

Thawed aliquots were homogenized in conical glass homogenizers, jacketed with ice. One milliliter of the homogenate was dried at 105° for dry matter determination. Another aliquot representing approximately 200 mg dry matter was dried at 70° and subjected to a 2-step acid hydrolysis (10). Total reducing sugars were determined with dinitrosalicylic acid (11), following lead acetate-sodium phosphate precipitation to remove noncarbohydrate reducing substances (12). Glucose was determined with glucose oxidase.[1] Recoveries of glucose, maltose or starch ranged from 96 to 111%.

Starch and soluble carbohydrates were separated by a modification of the procedure described by Weller and Gray (6). Ten milliliters of homogenate and 3 volumes of ethanol (95%) were allowed to stand overnight for the complete precipitation of starch. After centrifugation the precipitate was suspended and washed with three 15-ml portions of ethanol, then dried at 70°. The starch in the precipitate was rendered water-soluble by refluxing for 30 minutes with 25 ml 1% concentrated HCl in ethanol. After centrifugation, while still hot, the starch-containing precipitate was washed with ethanol, and dried at 70°. The supernatants from each of these steps were combined and the ethanol evaporated prior to glucose or reducing sugar determination. Recovery of added glucose or maltose following the precipitation of noncarbohydrate-reducing substances (12) was quantitative.

Thirty milliliters of water were added to the dried starch-containing precipitate, the mixture was refluxed for 30 minutes then built to 50 ml. The resulting solution, following centrifugation, was used for enzymatic starch hydrolysis (12). One milliliter of 0.5 M sodium acetate buffer (pH 6), 1 ml 0.25 N NaCl, and 1 ml aqueous solution containing 300 units bacterial α-amylase[2] were added to 10 ml of the starch solution. The reaction was stopped after 2 hours at 38.5° and noncarbohydrate reducing substances precipitated by the addition of 0.5 ml saturated lead acetate followed in 5 minutes by 2 ml saturated Na_2HPO_4, the final volume was adjusted to 25 ml. Starch content of the filtrate was measured using dinitrosalicylic acid (11). A standard curve was prepared by α-amylase hydrolysis of known amounts of cornstarch under identical conditions. Sample blanks were prepared by performing the lead acetate-sodium phosphate precipitation on an aliquot of the soluble starch solution prior to dinitrosalicylic acid addition. Starch recoveries from simulated gastric or intestinal fluid ranged from 101 to 112%.

Experiments were conducted to determine the relative rates of hydrolysis of maltose and starch and the rate of glucose absorption. Six 32-kg wether lambs surgically fitted with abomasal fistulas[3] were fed the basal ration (table 1) for 4 weeks prior to and during the experimental period. Reagent grade glucose or maltose was administered into the abomasum in a 70% w/v aqueous solution; reagent grade soluble starch was given as a 35% w/v aqueous suspension. Dosing was begun approximately 2 hours after feeding. Jugular vein blood was obtained at 30- or 60-minute intervals following single doses of 2 or 4 g/kg body weight or 4-hourly doses of 0.5 or 1 g/kg body weight. Blood glucose and total reducing sugars were determined by methods described above.

RESULTS AND DISCUSSION

The carbohydrate concentration in abomasal contents (table 2), 3-hours post-feeding, was equal to 55% of the ration concentration. The relative amount of dietary starch as well as the total amount of reducing sugars reaching the abomasum in the present study was several-fold greater than that reported by Weller and Gray (6). In their experiments, sheep were fed 150 g, or less, of starch/day in 800 g total ration. In the present experi-

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[1] Worthington Biochemical Corporation 1963 Technical Bulletin, Freehold, New Jersey.

[2] α-Amylase from *Bacillus subtilus*, type II; Sigma Chemical Company, St. Louis.

[3] Bardex 860, 10 French catheter.

TABLE 2

Carbohydrate concentration in the abomasum of sheep 3, 6 and 24 hours after feeding

Hours post-feeding	Acid hydrolysis: Total reducing sugars	Acid hydrolysis: Glucose	Amylase hydrolysis: Starch equivalent	Ethanol-soluble: Total reducing sugars
	% of dry matter	*% of dry matter*	*% of dry matter*	*% of dry matter*
3	41.3 ± 7.5 [1]	35.8 ± 7.0	29.4 ± 12.1	0.5 ± 0.3
6	27.9 ± 3.3	20.9 ± 2.0	15.6 ± 6.6	0.8 ± 0.2
24 [2]	23.7 ± 3.9	16.3 ± 2.4	6.6 ± 2.3	0.4 ± 0.1

[1] Mean ± SE of mean.
[2] Fasted the last 16 hours.

ment, 800 to 850 g starch in 1500 to 1600 g ration were consumed/sheep/day. It is apparent that starch or total nutrient intake can influence the extent of carbohydrate degradation in the rumen. The data also suggest that appreciable amounts of carbohydrate reach the abomasum of sheep fed a high carbohydrate ration ad libitum. No attempt was made to separate bacterial or protozoal carbohydrate from dietary carbohydrate.

The glucose contribution from non-starch carbohydrate, such as cellulose, was calculated by subtracting the starch equivalent determined by α-amylase hydrolysis from the total glucose produced by acid hydrolysis. The difference between total reducing sugar concentration and glucose concentration was used as an estimate of polysaccharides containing sugars other than glucose.

In samples collected 3-hours post-feeding, α-amylase labile starch accounted for 71% of the total reducing sugars. Glucose arising from the acid hydrolysis of cellulose or mixed hexosans was equivalent to 16% of the total reducing sugars. The remaining 13% of the total reducing sugars are considered to have been produced by acid hydrolysis of pectins, hemicellulose, pentosans, or fructans. None of the abomasal samples contained significant amounts of ethanol-soluble carbohydrates and all were practically devoid of free glucose. Dye and Orsini[4] found no free glucose in abomasal contents from a 6-month-old calf, but appreciable quantities of fermentable and non-fermentable reducing substances were present after acid hydrolysis of the abomasal contents.

Abomasal carbohydrate was reduced 6-hours post-feeding. This reduction may have resulted from increased carbohydrate degradation by rumen microorganisms; other workers (6, 13) have reported that starch is not degraded in the abomasum. Six hours post-feeding, the contribution of glucose from starch to the total abomasal carbohydrate decreased, whereas the contribution from cellulose and other polysaccharides remained essentially unchanged. This suggests that the longer ingesta remain in the rumen the greater the extent of starch degradation, or that the rate at which starch-containing material leaves the rumen differs from that of other material. The variation in abomasum starch concentration among animals killed 3 or 6 hours after feeding indicated that 3 hours approached the minimal time after feeding at which ingesta began to leave the rumen.

Following a 16-hour fast and 24-hours after the last feeding, α-amylase labile starch accounted for only 6% of the abomasal dry matter, and contributed only 28% of the total carbohydrate content. The contribution from cellulose was calculated to be 40% of the total reducing sugars or approximately twice the amount it contributed at 3 or 6 hours post-feeding. The absolute contribution from other carbohydrates remained relatively constant at each sampling period.

Intestinal contents (table 3), handled in the same manner as abomasal contents, contained very little α-amylase labile starch regardless of time after feeding or segment of the intestine sampled. This suggests very rapid hydrolysis of starch within the intestine. The small, constant,

[4] Dye, J. A., and D. Orsini 1952 Glycemic levels and rumen development in calves. Federation Proc., *11:* 39 (abstract).

TABLE 3

Carbohydrate concentration in the small intestine [1] *of sheep 3, 6 or 24 hours after feeding*

Hours post-feeding	Acid hydrolysis		Amylase hydrolysis	Ethanol-soluble
	Total reducing sugars	Glucose	Starch equivalent	Total reducing sugars
	% of dry matter	*% of dry matter*	*% of dry matter*	*% of dry matter*
3	6.9 ± 0.2 [2]	2.5 ± 0.4	0.7 ± 0.2	1.6 ± 0.8
6	8.0 ± 0.8	2.9 ± 0.1	1.2 ± 0.5	0.8 ± 0.4
24 [3]	6.4 ± 1.1	1.7 ± 0.5	1.0 ± 0.5	0.7 ± 0.2

[1] Contents of first one-third of small intestine.
[2] Mean ± SE of mean.
[3] Fasted the last 16 hours.

residual starch value may represent a material attacked by bacterial α-amylase but not by ovine amylase. The intestinal content of ethanol-soluble dextrins was higher than comparable abomasal levels, suggesting the presence of starch hydrolysis intermediates of which approximately 10% was free glucose. The contribution to total reducing sugars from hemicellulose, pectins, etc. was relatively much greater than in the abomasum, demonstrating the inability of intestinal enzymes to hydrolyze these substances. These data demonstrate complete digestion of starch in the intestine of sheep and are not in agreement with those reported for the calf (8) showing no intestinal digestion of starch. Differences in type of diet, method of feeding or sampling may be involved. In agreement with the present results are the data of Foster (14) and Bergman et al. (15) which demonstrate amylolytic activity within the small intestine of ruminents. The present data also suggest that the apparent digestion of starch by steers[5] was not a result of microbial degradation of starch in the cecum or colon.

Following abomasal administration, glucose was rapidly absorbed from the post-ruminal gastrointestinal tract (fig. 1). Peak jugular vein blood levels were observed 1.5 to 3 hours post-dosing. During this period the rate of absorption was in equilibrium with rate of clearance. The quantitative absorption of glucose from the small intestine has been reported by Larsen et al. (8) who observed that the most rapid absorption occurred from the jejunum. Based upon the estimated blood volume and rate of blood glucose clearance, only approximately 0.1 g glucose/kg body weight would have been required to produce the observed increase in blood glucose. Thus, extremely rapid turnover of plasma glucose is apparent. Armstrong (1) reported that the turnover rate of glucose per unit surface area was as rapid in the cow and sheep as in the rat or dog.

If an equal amount of glucose was given in fractional doses at hourly intervals a less pronounced increase in blood glucose was observed (fig. 1). Blood glucose increased with each successive dose of 1 g/kg body weight and reached a peak one hour after the last dose.

Reducing the amounts of glucose given decreased the magnitude of blood glucose increase. The pattern observed (fig. 1) was similar to that from larger glucose doses. The highest blood glucose level from the fractionated dose of 0.5 g/kg body weight/hour occurred one hour after the second dose. No further increases were observed following the third or fourth doses. This suggests that these sheep could utilize 0.5 g/kg body weight/hour, or 350 g glucose/day without showing an increase in blood glucose.

Maltose infusion resulted in less pronounced increases in blood glucose concentrations (fig. 1). The increase in blood glucose following maltose administration was much less than that following glucose administration. This indicates that the rate of hydrolysis of maltose was slower than the rate of glucose absorption. There was no detectable maltose in the blood at the times peak blood glucose was reached.

A small but significant increase in glucose was observed between 3 and 4 hours following the single dose (4 g/kg body

[5] Karr, M. R., C. O. Little, G. E. Mitchell, Jr. 1965 Ruminal and post-ruminal digestion of starch by steers. J. Animal Sci., 24: 890 (abstract).

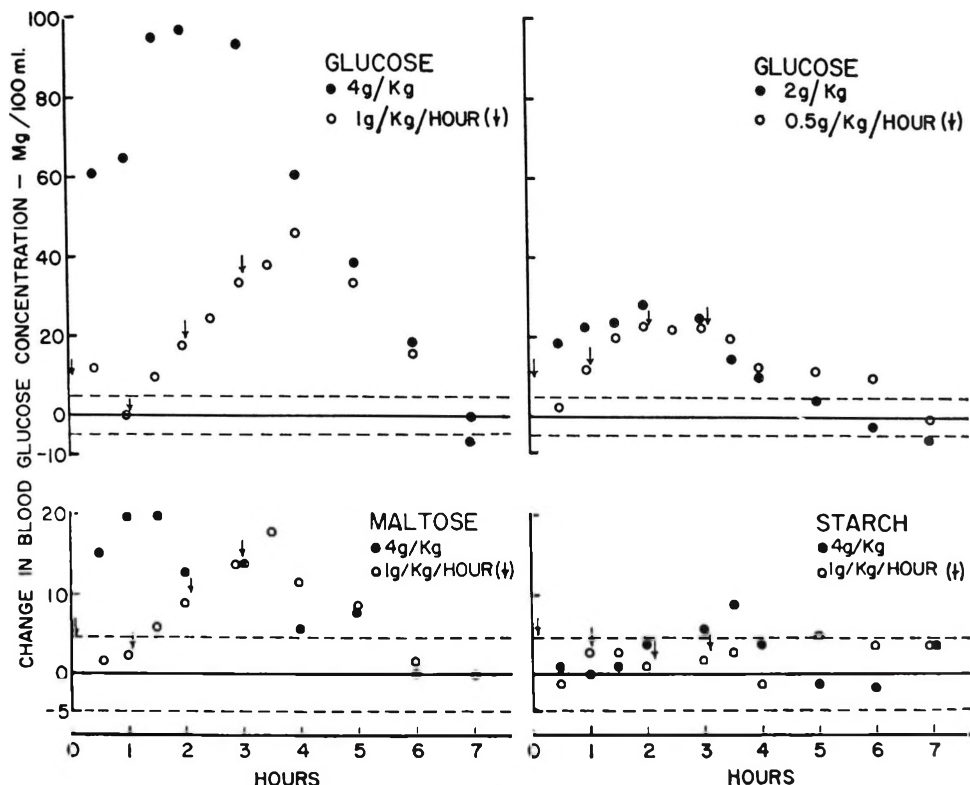

Fig. 1 Changes in blood glucose concentrations following single or multiple doses of glucose, maltose or starch into the abomasum of fistulated wethers. Broken lines represent 99% confidence limits for sham-dosed animals.

weight) of soluble starch (fig. 1). This increase in blood glucose following starch administration was much smaller than that obtained from equal amounts of either glucose or maltose. However, in the preceding study starch was shown to be almost completely digested and absorbed from the intestine, even though relatively high amounts were present in abomasal contents. These results indicate that the rate of hydrolysis of starch was only slightly faster than the rate of tissue utilization of glucose. Therefore, an increase in blood glucose following starch administration could only be observed when a large amount of soluble starch had been infused into the abomasum of sheep that had been fed a high carbohydrate ration.

The data from the present investigations demonstrate that the ruminant possesses an adequate capacity to digest carbohydrates post-ruminally and that glucose absorbed as such is important in supplying the energy requirement of the ruminant.

LITERATURE CITED

1. Armstrong, D. G. 1965 Carbohydrate metabolism in ruminants and energy supply. Second International Symposium on the Physiology of Digestion in the Ruminant. Butterworth's, Washington, D. C.
2. Schambye, P. 1951 Volatile acids and glucose in portal blood of sheep. 3. The influence of orally administered glucose. Nord. Vet. Med., 3: 1003.
3. Bell, F. R., and E. R. Jones 1945 Glucose tolerance in the bovine. J. Comp. Pathol. Therap., 55: 117.
4. Dougherty, R. W., C. S. Klavano, W. M. Dickson and P. A. Kalavano 1956 Blood sugar levels in cattle and sheep fed various amounts of glucose. Cornell Vet., 46: 397.
5. Fries, G. F., and G. H. Conner 1960 Observations on the absorption of glucose into the portal vein. J. Dairy Sci., 43: 1851.
6. Weller, R. A., and F. V. Gray 1954 The passage of starch through the stomach of the sheep. J. Exp. Biol., 31: 40.
7. Dollar, A. M., and J. W. G. Porter 1957 Utilization of carbohydrates by the young calf. Nature, 179: 1299.

8. Larsen, H. J., G. E. Stoddard, N. L. Jacobson and R. S. Allen 1956 Digestion and absorption of various carbohydrates posterior to the rumino reticular area of the young bovine. J. Dairy Sci., *15:* 473.
9. Rust, J. W., N. L. Jacobson and A. D. McGilliard 1963 Supplementation of dairy calf diets with enzymes. I. Effect on rate of growth. J. Animal Sci., *22:* 1104.
10. Saeman, J. F., W. E. Moore and M. A. Millett 1963 Sugar units present. Hydrolysis and quantitative paper chromatography. Meth. Carbohyd. Chem., *3:* 54.
11. Sumner, J. B. 1925 The estimation of sugar in diabetic urine using dinitrosalicylic acid. J. Biol. Chem., *62:* 287.
12. Hassid, W. Z., R. M. McCready and R. S. Rosenfels 1940 Determination of starch in plants. Ind. Eng. Chem. (Anal. Ed.), *12:* 142.
13. Heald, P. J. 1951 The assessment of glucose-containing substances in rumen microorganisms during a digestion cycle in sheep. Brit. J. Nutrition, *5:* 84.
14. Foster, M. 1867 Notes on amylolytic ferments. J. Anat. Physiol., *1:* 107.
15. Bergman, H. D., H. H. Dukes and J. H. Yarborough 1924 A study of the enzymic action of extracts of the duodenal gland regions of the domestic animals. J. Am. Vet. Med. A., *65:* 313.

Effect of Vitamin E, Arginine and Methionine on Free Amino Acids and Lipids in Selected Rat Tissues [1]

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ABSTRACT The effect of dietary arginine, methionine and vitamin E on selected free amino acids in plasma and on lipids in liver was investigated. Weanling male rats were fed a vitamin E-deficient ration supplemented with methionine, arginine or methionine plus arginine with and without vitamin E. Supplementary feeding of arginine, methionine or a combination of these amino acids increased in plasma the concentration of the supplementary amino acid. The effect of vitamin E deficiency on growth had become apparent only in animals fed added arginine or methionine. Vitamin E had no effect on the concentration of arginine, methionine or tyrosine in plasma. Feeding of supplementary methionine or tocopherol decreased the concentration of cholesterol in liver. Vitamin E decreased the amount of total lipids in liver of all animals. The data suggest that a marked change in the proportion of arginine and methionine in plasma may accelerate the development of vitamin E deficiency in the rat.

Recent observations have suggested interrelationships among vitamin E, arginine and sulfur-containing amino acids. Machlin and Shalkop (1) reported that methionine or cystine prevented syndromes similar to those of muscular dystrophy which occurred in chicks fed vitamin E-deficient diets containing 15% of casein and 10% of gelatin. Nesheim et al. (2) reported that degenerative changes in muscle tissue were not observed in chicks fed diets deficient in vitamin E, sulfur amino acids and arginine. Later work by Scott and Calvert (3) indicated that the proportion of arginine to methionine was important in prevention of the myopathic condition in chicks and the authors suggested that the adverse effect of added arginine was due to decreased availability of methionine for biosynthesis of cystine.

Since information with respect to the concentration of free amino acids in blood may provide valuable indexes of metabolic interrelationships (4), a study of the effect of vitamin E, arginine and methionine on the concentration of selected amino acids in plasma seemed appropriate. Analyses of liver for cholesterol and total lipids were also made.

EXPERIMENTAL

Male rats of the Sprague-Dawley strain, weighing 40 to 60 g, were assigned to 4 groups of 16 rats each according to body weight and litter. The experimental diets consisted of a basal vitamin E-deficient ration and this ration supplemented with 1% of L-methionine, 1% of L-arginine·HCl or 1% of L-methionine and 1% of L-arginine·HCl. Eight rats of each group received orally 9 mg of *dl-α*-tocopherol/week. The animals were housed in individual wire-bottom cages. Water and fresh food were provided daily ad libitum during the 12-week experimental period. The rations were prepared twice weekly and stored in the refrigerator.

The percentage composition of the basal diet was: sucrose, 75; vitamin-free casein,[2] 12; salt mixture,[3] 4; cellulose,[4] 4; and antioxidant-free lard, 5. The following were provided in mg/kg of ration: thia-

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[1] Scientific series paper no. 898, published with the approval of the Director of the Colorado Agricultural Experiment Station. Supported in part by funds from Regional Project W-57 of the Western Agricultural Experiment Stations.
[2] General Biochemicals, Inc., Chagrin Falls, Ohio.
[3] U. S. Pharmacopoeia XIV, 1950.
[4] Solka Floc, Brown Company, San Francisco.

mine·HCl, 5.0; pyridoxine·HCl, 2.5; riboflavin, 8.0; Ca pantothenate, 20.0; niacin, 10.0; biotin, 0.1; folacin, 2.0; vitamin B_{12}, 0.02; inositiol, 100.0; *p*-aminobenzoic acid, 100.0; choline chloride, 1300.0; and menadione, 0.5. The fat-soluble vitamin mixture provided per kilogram of diet: vitamin A, 4000 IU; and vitamin D, 2000 IU. During the last 6 weeks of the study 300 μg of an aqueous suspension of natural vitamin A were given orally to each rat once a week. The amino acids were added to the diet at the expense of sucrose.

Blood from decapitated rats was collected in centrifuge tubes which contained 0.2 mg of sodium heparinate/ml of blood (5). Equal volumes of plasma from 4 rats in each dietary group were pooled. Protein-free filtrates were preparted (6) and assayed microbiologically for arginine, methionine and tyrosine (7).

Liver from each rat was analyzed for cholesterol (8) and total lipids of liver were measured by the method of Bloor (9) as modified by Okey and Lyman (10).

RESULTS

Free amino acids in plasma. The amounts of free arginine, methionine and tyrosine in the plasma are shown in table 1. Supplementation of the basal ration with arginine or methionine individually or in combination increased in plasma the concentration of the supplemental amino acids ($P < 0.05$). The concentration of tyrosine in plasma was not affected by the addition of arginine or methionine to the diet. A significant interrelationship between the supplemental amino acids as determined by the level of the amino acids in plasma was not observed. Feeding of the diet deficient in vitamin E had no significant influence on the concentration of arginine, methionine or tyrosine in plasma under the conditions of this experiment.

Liver cholesterol. The concentrations of cholesterol in liver of animals fed supplemental methionine or methionine combined with arginine were lower ($P < 0.05$) than those of animals fed the basal diet or the basal diet plus arginine (table 2). Arginine alone did not influence the level of hepatic cholesterol. The concentration of cholesterol in liver of animals fed supplemental vitamin E was less than that of animals fed the vitamin E-deficient diet ($P < 0.05$).

Total lipids. The concentration of total lipids in liver was not affected significantly by supplementation of the basal ration with arginine or methionine alone or together (table 2). Supplemental feeding of vitamin E decreased the amount of total lipids in all groups of animals ($P < 0.01$).

Growth. Supplementation of the basal ration with methionine, arginine or a combination of the 2 amino acids with or without vitamin E increased ($P < 0.01$) the weight gain (table 2). Addition of vitamin E to diets containing 12% of casein supplemented with arginine or methionine increased growth of the animals approximately 8 and 9%, respectively ($P < 0.05$). Differences in weight gain attributed to vitamin E intake were not observed in animals fed the basal ration or the basal

TABLE 1

Effect of arginine and methionine supplementation on free amino acids in plasma of rats fed a diet containing 12% of casein with and without vitamin E

Supplementation			Free amino acids					
			Arginine		Methionine		Tyrosine	
L-Arginine·HCl	L-Methionine	*dl*-a-tocopherol	Group values	Mean	Group values	Mean	Group values	Mean
%	%	mg	μg/ml		μg/ml		μg/ml	
—	—	—	24.8 26.5	25.6	7.9 9.6	8.8	24.3 31.1	27.7
—	—	9	18.1 25.7	21.9	10.2 10.4	10.3	31.5 32.4	32.0
1	—	—	35.2 38.3	36.8	8.6 9.6	9.1	21.1 33.3	27.2
1	—	9	27.5 36.9	32.2	7.0 8.4	7.7	21.0 24.4	22.7
—	1	—	15.1 20.0	17.6	38.4 43.8	41.1	23.0 25.0	24.0
—	1	9	15.5 18.6	17.0	36.7 43.5	40.1	24.5 28.4	26.4
1	1	—	38.8 39.1	39.0	34.1 44.4	39.2	22.1 29.8	26.0
1	1	9	33.0 38.4	35.7	40.0 41.0	40.5	23.5 24.6	24.0

TABLE 2

Effect of arginine and methionine supplementation on weight gain, cholesterol and total lipids in liver of rats fed a diet containing 12% of casein with or without vitamin E

Supplementation			Weight gain	Total cholesterol	Total lipids
L-Arginine·HCl	L-Methionine	*dl-a*-tocopherol			
%	%	*mg*	*g*	*mg/g*	*mg/g*
—	—	—	203 ± 5.8 [1]	2.23 ± 0.07	41.6 ± 1.4
—	—	9	208 ± 5.2	2.10 ± 0.08	38.3 ± 1.5
1	—	—	238 ± 6.9	2.29 ± 0.10	42.4 ± 1.5
1	—	9	258 ± 11.6	2.13 ± 0.13	37.9 ± 1.4
—	1	—	234 ± 5.9	2.00 ± 0.04	39.8 ± 1.5
—	1	9	258 ± 5.5	1.91 ± 0.06	35.2 ± 1.0
1	1	—	255 ± 8.6	2.04 ± 0.05	40.3 ± 1.9
1	1	9	257 ± 5.3	1.94 ± 0.07	37.8 ± 1.9

[1] Mean ± SE.

ration supplemented with the combination of arginine and methionine.

DISCUSSION

The lower levels of cholesterol observed in liver of animals fed supplemental methionine or vitamin E are in agreement with other reports. Experiments with animals have indicated that supplementation of low protein diets with methionine decreases tissue cholesterol (10-13). Other investigations have shown that the concentration of lipids in skeletal muscle and serum increased in vitamin E-deficient animals (14-17). It is possible that the effect of vitamin E on liver lipids observed in this study would be enhanced by increased intake of dietary fat. A previous study (18) showed that a vitamin E-deficient diet containing 10% of fat produced a more pronounced effect on the concentration of cholesterol and total lipids in liver than the ration used in this experiment which contained 5% of fat. Witting and Horwitt (19) reported that the vitamin E requirement of the rat increased as the dietary fat was increased from 1 to 7.5%.

The increase in concentration of methionine in plasma which resulted from feeding supplemental methionine was greater than the increase in arginine produced by the addition of arginine to the diet. Decreased serum cholesterol levels were observed in animals with the lowest plasma arginine-methionine ratios. Kokatnur and Kummerow (20) reported that the addition of 0.5% of arginine and 0.3% of methionine produced a marked increase in serum cholesterol of chicks. These observations raise the question whether the effect of dietary methionine on blood cholesterol is related to dietary arginine. Similar studies of the relation of arginine, methionine and vitamin E in the rat have not been reported previously.

Since pigmentation of tissues has been reported in tissues of vitamin E-deficient rats (21), the concentration of tyrosine in plasma was determined. Tyrosine concentration in plasma was not affected by tocopherol supplementation or by supplementary amino acids used in this study.

The data show that the supplementary feeding of arginine or methionine increased in plasma the concentration of the supplementary amino acid. Since the feeding of either amino acid did not produce a corresponding increase in plasma of the amino acid which was not supplemented, the arginine-methionine ratio in plasma varied considerably. The effect of vitamin E on weight gain had become apparent only in animals fed supplementary methionine alone, which resulted in the lowest arginine-methionine ratio in plasma, or arginine which produced the highest ratio. The deleterious effect of the vitamin E-deficient diet on growth of the rat was accompanied by increased amounts of plasma arginine or methionine. Since supplementation of the basal ration with either amino acid increased the rate of growth, it appears that the decreased weight gain in animals fed the vitamin E-deficient diet was not due to additional stress imposed by the supplemental amino acids. A recent report by Jenkins et al. (22) indicated that the effect of dietary

arginine on the development of muscular lesions described by Scott and Calvert (3) was not specific. Jenkins and co-workers (22) reported that muscular lesions were observed in chicks fed diets supplemented with selected essential amino acids when "the sulfur amino acids became first limiting for growth."

The implications of data reported in this study are that a marked change in the proportion of arginine and methionine in plasma may accelerate the decline in growth rate of animals deprived of vitamin E. The data support the work of Scott and Calvert (3) which indicated a relationship between arginine and methionine in prevention of symptoms characteristic of animals fed vitamin E-deficient diets.

LITERATURE CITED

1. Machlin, L. J., and W. T. Shalkop 1956 Muscular degeneration in chickens fed diets low in vitamin E and sulfur. J. Nutrition, 60: 87.
2. Nesheim, M. C., C. C. Calvert and M. L. Scott 1960 Effect of arginine deficiency on nutritional muscular dystrophy in the chick. Proc. Soc. Exp. Biol. Med., *104:* 783.
3. Scott, M. L., and C. C. Calvert 1962 Evidence of a specific effect of cystine in the prevention of nutritional muscular dystrophy in vitamin E-deficient chicks. J. Nutrition, 77: 105.
4. Richardson, L. R., L. G. Blaylock and C. M. Lyman 1953 Influence of the level of vitamins in the diet on the concentration of free amino acids in the plasma of chicks. J. Nutrition, *49:* 21.
5. Interdepartmental Committee on Nutrition for National Defense, National Institutes of Health 1957 Manual for Nutrition Surveys. U. S. Government Printing Office, Washington, D. C., p. 55.
6. Hier, S. W., and O. Bergeim 1945 Microbiological determination of free leucine, isoleucine, valine, and threonine in dog plasma. J. Biol. Chem., *161:* 717.
7. Henderson, L. M., W. L. Brickson and E. E. Snell 1948 A micromethod for the microbiological determination of amino acids. J. Biol. Chem., *172:* 31.
8. Sperry, W. M., and M. Webb 1950 A revision of the Schoenheimer-Sperry method for cholesterol determination. J. Biol. Chem., *187:* 97.
9. Bloor, W. R. 1928 The determination of small amounts of lipid in blood plasma. J. Biol. Chem., 77: 53.
10. Okey, R., and M. M. Lyman 1954 Dietary constituents which may influence the use of food cholesterol. II. Protein, L-cystine and DL-methionine intake in adolescent rats. J. Nutrition, *53:* 601.
11. Nishida, T., F. Takenaka and F. A. Kummerow 1958 Effect of dietary protein and heated fat on serum cholesterol and beta-lipoprotein levels, and on the incidence of atherosclerosis in chicks. Circulation Res., 6: 194.
12. Seidel, J. C., N. Nath and A. E. Harper 1960 Diet and cholesterolemia. V. Effects of sulfur-containing amino acids and protein. J. Lipid Res., *1:* 474.
13. Leveille, G. A., J. W. Shockley and H. E. Sauberlich 1962 Influence of dietary protein level and amino acids on plasma cholesterol of the growing chick. J. Nutrition, 76: 321.
14. Morgulis, S., V. M. Wilder, H. C. Spencer and S. H. Eppstein 1938 Studies on the lipid content of normal and dystrophic rabbits. J. Biol. Chem., *124:* 755.
15. Heinrich, M. R., and H. A. Mattill 1943 Lipids of muscle and brain in rats deprived of tocopherol. Proc. Soc. Exp. Biol. Med., 52: 344.
16. Oppenheimer, H., S. Shulman, S. Roberts and A. T. Milhorat 1958 Serum proteins, lipoproteins and glycoproteins. In muscular dystrophy of vitamin E deficiency. Proc. Soc. Exp. Biol. Med., 97: 882.
17. Alfin-Slater, R. B. 1960 Relation of vitamin E to lipid metabolism. Am. J. Clin. Nutrition, 8: 445.
18. Harrill, I., G. Minarik and E. D. Gifford 1965 Effect of vitamins A and E on lipids in selected rat tissues. J. Nutrition, 87: 424.
19. Witting, L. A., and M. K. Horwitt 1964 Effects of dietary selenium, methionine, fat level and tocopherol on rat growth. J. Nutrition, *84:* 351.
20. Kokatnur, M. G., and F. A. Kummerow 1961 Amino acid imbalance and cholesterol levels in chicks. J. Nutrition, 75: 319.
21. Tappel, A. L. 1955 Studies of the mechanism of vitamin E action. III. In vitro copolymerization of oxidized fats with protein. Arch. Biochem. Biophys., *54:* 266.
22. Jenkins, K. J., D. C. Hill and L. M. Hutcheson 1964 Nonspecific role of arginine in the promotion of muscular dystrophy in the chick. J. Nutrition, 82: 415.

An Abnormality of Circulating Erythrocytes in Untreated and in Coenzyme Q_{10}-treated Vitamin E-deficient Monkeys [1]

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ABSTRACT In a study of the anemia of vitamin E deficiency and its inadequate response to oral coenzyme Q_{10} treatment, survival of ^{51}Cr-labeled erythrocytes was measured. Half-times for erythrocyte survival were: 14, 14.5 and 16 days in 3 control monkeys; 21 and 23 days in two previously vitamin E-deficient monkeys treated with vitamin E; and 8, 10, 10.5 and 12 days in 4 vitamin E-deficient monkeys. Erythrocytes from vitamin E-deficient monkeys exhibited increased susceptibility to hemolysis by hydrogen peroxide, but a cause and effect relationship between a low environmental capacity to protect erythrocytes from oxidative destruction and shortened erythrocyte survival was not demonstrated. In fact, in experiments in which labeled erythrocytes were transfused into recipients other than the donor, erythrocytes from vitamin E-deficient monkeys had no improvement in survival when transfused into the vitamin E-replete environment of control monkeys, and erythrocytes from a control monkey survived normally when transfused into a vitamin E-deficient monkey. These results indicate that there is an intrinsic defect in the erythrocyte of the vitamin E-deficient monkey. Exogenous coenzyme Q_{10} did not improve erythrocyte survival; 3 coenzyme Q_{10}-treated, vitamin E-deficient monkeys had erythrocyte survival half-times of 5, 6 and 6 days. This persistence of shortened erythrocyte survival explains the reticulocytosis without an increase in hemoglobin concentration which occurred in these monkeys. Inadequate intracellular accumulation of the intact coenzyme Q_{10} molecule is proposed to explain the inadequate response of vitamin E-deficient monkeys to exogenous coenzyme Q_{10}.

Although the anemia of vitamin E deficiency can be attributed primarily to abnormal erythropoiesis (1), the circulating erythrocyte has a shortened survival (2) and this might contribute significantly to the anemia under certain conditions. An example of one such condition is the treatment of vitamin E-deficient monkeys with coenzyme Q_{10}. Exogenous coenzyme Q_{10} causes reticulocytosis without otherwise appreciably affecting the anemia, whereas treatment with vitamin E or with hexahydrocoenzyme Q_4 induces a complete hematologic remission (3).

A shortened erythrocyte survival must be the result either of an intrinsic abnormality in the erythrocyte or of an abnormality in the circulating erythrocyte's environment. In the vitamin E-deficient monkey, neither mechanism can be excluded on the basis of previously available information. On the contrary, it has been necessary to suspect both mechanisms since an intrinsic abnormality might develop during abnormal erythropoiesis and since the environmental lack of enough vitamin E to protect against oxidative hemolysis might contribute to a shortened erythrocyte survival.

The present report describes studies of erythrocyte survival which provide new information on the circulating erythrocyte in vitamin E-deficient monkeys and on the inadequate response of vitamin E deficiency anemia to exogenous coenzyme Q_{10}.

METHODS

Young Rhesus monkeys (*Macaca mulata*) of both sexes were fed a purified vitamin E-deficient diet containing by weight 18% of isolated soybean protein, 11% of fat, and 66% of carbohydrate plus minerals and vitamins with the exception of vitamin E. This diet was fed alone or with supplements either of choline or of choline,

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methionine, and cystine. Control monkeys received oral supplements of 80 mg of *dl-α*-tocopheryl acetate dissolved in 0.5 ml of ethanol 3 times per week. As in previous experiments (4), even the vitamin E-deficient monkeys ate well until they were moribund. After 1 to 2 years the monkeys receiving the diet without the α-tocopherol supplement developed the typical vitamin E-deficiency syndrome of anemia (table 1) and muscular dystrophy, and supplementing the diet either with choline or with choline, methionine and cystine did not affect the time of appearance or the characteristics of the syndrome (3). Details of the diet for each of the monkeys including those used for the present erythrocyte survival studies, the general experimental procedures, and a description of the deficiency syndrome which developed have been published (3, 5). Only monkeys that were maintaining a relatively stable clinical course were used to study erythrocyte survival (see table 1 for weight and hemoglobin values). Since choline, methionine and cystine supplements did not affect the deficiency syndrome, the vitamin E-deficient monkeys are considered to represent a single group in the present report.

To measure erythrocyte survival, the cells from 10 ml of blood were first labeled with 60 μCi of ^{51}Cr (specific activity approximately 270 μCi/μg) (6); then, after washing and resuspending in 0.9% NaCl solution, they were injected intravenously into the donor or into a selected recipient. Half-times were estimated from the disappearance of radioactivity from whole blood. The duration of these studies varied from 5 to 18 days depending on the half-times. When the half-time was short, blood was obtained at short intervals so that the survival curve could be based on several points. Prior to injection of the labeled erythrocytes into a recipient other than the donor, it was always ascertained that serum from the recipient would not cause clumping of cells from the donor after incubation for 30 minutes both at room temperature and at 37°.

In vitro hemolysis of erythrocytes by hydrogen peroxide was measured by the method of Gordon et al. (7).

RESULTS

The half-times for erythrocyte survival, weights, hemoglobin concentrations, and reticulocyte counts are recorded in table 1. From this table both the severity of the anemia of vitamin E deficiency and the magnitude of the reticulocyte response to coenzyme Q_{10} treatment are evident. Little published data are available on erythrocyte survival in the Rhesus monkey, but the values for the control animals in the present experiments are comparable to the values reported by Marvin et al. (2), and are considered to be normal. In confirmation of the earlier report (2) erythrocyte survival decreased in the vitamin E-deficient monkeys. An even further reduction in erythrocyte survival was observed in vitamin E-deficient monkeys being treated with coenzyme Q_{10}. However, in previously vitamin E-deficient monkeys treated with vitamin E, erythrocyte survival was somewhat longer than normal, as would be expected with the increased proportion of young erythrocytes in these recently treated animals.

The half-times for survival of erythrocytes transfused into monkeys other than the donor also are shown in table 1. Erythrocytes from the control monkey survived normally after transfusion into a vitamin E-deficient monkey; erythrocytes from a vitamin E-deficient monkey had the same half-time after transfusion into a control monkey as they did in the donor; and, erythrocytes from a vitamin E-deficient monkey treated with coenzyme Q_{10} had the same short half-time after transfusion into a control monkey as they did in the donor. Antibodies to the erythrocytes of the vitamin E-deficient monkeys were demonstrated in the sera of their respective recipients several weeks after the studies were completed. Such antibodies were not present intially and it is not known whether they developed soon enough to affect the early part of the survival curves, but this possibility is considered unlikely. In this respect, the normal survival of control erythrocytes in a vitamin E-deficient monkey cannot be questioned.

The erythrocyte survival and reticulocyte counts of a coenzyme Q_{10}-treated, control monkey are compared with similar

TABLE 1

Effect of vitamin E deficiency and coenzyme Q_{10} treatment on erythrocyte survival

Monkey no.		Survival study begun	Weight		Hemoglobin conc		Initial [1] reticulocyte count	Survival $T_{1/2}$
			Initial [1]	Final	Initial [1]	Final		
		day	*kg*	*kg*	*g/100 ml*		*%*	*days*
231	Control	610	3.5	3.6	12.5	12.6	1.5	14.5
233	Control	636	3.1	3.1	12.7	13.2	1.5	16
245	Control	671	3.5	3.5	11.8	12.2	1.7	14
235	Vitamin E-deficient	699	2.5	2.5	8.6	8.5	4.8	12
239	Vitamin E-deficient	671	3.2	3.2	8.2	8.7	4.7	10.5
234 [2]	Vitamin E-deficient	627	2.8	2.6	4.7	4.5	6.1	8
234	Vitamin E-deficient treated with coenzyme Q_{10}	656	2.5	2.4	3.7	6.8	34.0	5
234	Treated with vitamin E	705	3.8	4.1	13.3	14.5	1.9	23
246 [3]	Vitamin E-deficient	655	2.5	2.4	6.7	7.3	5.8	10
246	Vitamin E-deficient treated with coenzyme Q_{10}	678	2.5	2.6	6.8	6.5	10.6	6
246	Treated with vitamin E	732	3.1	3.3	13.8	12.4	0.3	21
246	Vitamin E-deficient-coenzyme Q_{10}-treated cells into control (233)	678	3.2 [4]	3.3 [4]	11.9 [4]	12.7 [4]	1.8 [4]	6
235	Vitamin E-deficient cells into control (245)	699	3.5 [4]	3.6 [4]	13.7 [4]	12.7 [4]	1.4 [4]	12
245	Control cells into vitamin E-deficient (239)	699	3.2 [4]	2.6 [4]	7.5 [4]	7.0 [4]	5.1 [4]	17

[1] The terms, initial and final, refer to the beginning and end of the survival study.

[2] Monkey 234 was given 100 mg of coenzyme Q_{10} without a suspending vehicle orally each day from day 643 through day 660. From day 658 through 667, 100 mg of *dl-α*-tocopheryl acetate dissolved in ethanol were given orally each day and thereafter 80 mg of *dl-α*-tocopheryl aceate dissolved in ethanol was given orally 3 times each week.

[3] Monkey 246 was given 100 mg of coenzyme Q_{10} without a suspending vehicle orally each day from day 676 through 686; 100 mg of *dl-α*-tocopheryl acetate dissolved in ethanol were given orally each day from day 692 through 701, and thereafter 80 mg of *dl-α*-tocopheryl acetate dissolved in ethanol were given orally 3 times each week.

[4] Values for the monkey receiving the transfusion are shown.

studies in a coenzyme Q_{10}-treated, vitamin E-deficient monkey in figure 1. In the control monkey, coenzyme Q_{10} treatment was associated with a questionable increase in reticulocyte count but the erythrocyte survival half-time of 14 days is comparable to an earlier value of 14.5 days for the same control monkey, number 231 (table 1). Thus, there is no evidence that coenzyme Q_{10} increases peripheral destruction of normal erythrocytes.

The results of the tests of erythrocyte susceptibility to hemolysis by hydrogen peroxide are shown in table 2. The susceptibility to hydrogen peroxide hemolysis was greater in erythrocytes from vitamin E-deficient monkeys than in erythrocytes from control or from previously vitamin E-deficient monkeys treated with vitamin E.

TABLE 2

In vitro hemolysis of erythrocytes by hydrogen peroxide

Monkev no.	Conditions	Hemolysis
		%
231	Control	0
233	Control	0
245	Control	16
234	Vitamin E-treated	19
246	Vitamin E-treated	12
235	Vitamin E-deficient	89
239	Vitamin E-deficient	90
236	Vitamin E-deficient	63

DISCUSSION

It is well-established that erythrocytes from a variety of animals, now including the monkey, exhibit increased susceptibility to hemolysis by oxidizing agents, such as hydrogen peroxide, if the animal is deprived of vitamin E (7-9). Normal plasma concentrations of tocopherol somehow protect the erythrocyte from hemolysis and this protection can be demonstrated by the addition of vitamin E in vitro to erythrocytes from vitamin E-deficient animals (8). The concomitant occurrence of increased susceptibility to hemolysis by hydrogen peroxide and a shortened erythrocyte survival makes it desirable to consider the relationship between the 2 phenomena.

The present data and certain observations by others (9-12) indicate that the increased susceptibility to oxidative hemolysis is not causally related to the shortened erythrocyte survival. It is true that erythrocytes from vitamin E-deficient monkeys have an increased susceptibility to oxidative destruction by hydrogen peroxide in vitro (table 2), but their survival was not improved by transfusing them into a control, vitamin E-replete monkey. Conversely, the exposure of normal erythrocytes to potential oxidative damage in vivo by transfusing them into the low vitamin E environment of a vitamin E-deficient monkey did not shorten their survival.

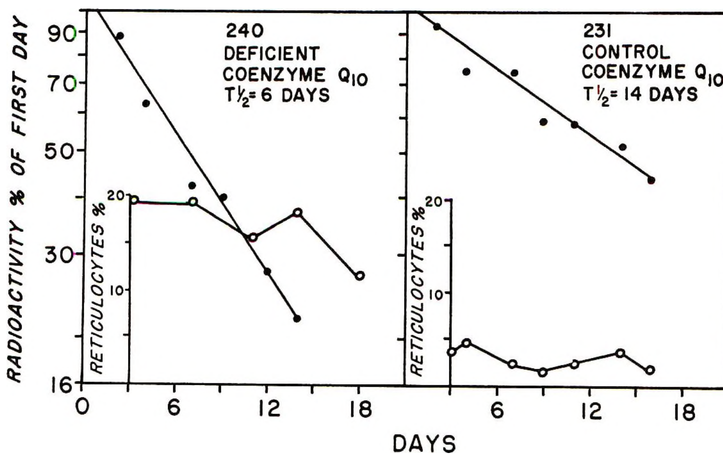

Fig. 1 Erythrocyte survival curves and reticulocyte counts of a control and of a vitamin E-deficient monkey during coenzyme Q_{10} treatment. Each monkey received 100 mg of coenzyme Q_{10} orally without a suspending vehicle each day during the survival study and for 5 days (monkey 231) or 8 days (monkey 240) preceding the survival study.

Moreover, man (9-11) and rats (12) receiving diets deficient in vitamin E show increased erythrocyte susceptibility to hydrogen peroxide in vitro with little or no shortening of the erythrocyte survival. These observations are most compatible with the view that the shortened erythrocyte survival in the vitamin E-deficient monkey is due to an intrinsic abnormality in the erythrocyte, probably occurring during its development.

During coenzyme Q_{10} treatment of vitamin E-deficient monkeys, the erythrocyte survival was unusually short; and this, undoubtedly, accounts for the failure of the anemia to improve despite a sustained reticulocytosis. Since other studies have shown that a coenzyme Q of lower molecular weight and with an almost completely saturated side chain—hexahydrocoenzyme Q_4—produces not just a retculocytosis but a complete hematologic remission (3), we suggest that the inadequate response to exogenous coenzyme Q_{10} results from failure either to achieve or to maintain effective intracellular concentrations of the coenzyme.

If the exogenous coenzyme Q_{10} which does enter the cell causes reticulocytosis by functioning in one of the metabolic roles usually served by endogenous coenzyme Q_{10}, it could be argued that coenzyme Q activity in the vitamin E-deficient monkey is too limited to meet metabolic needs. Tentatively, then, vitamin E could be assigned a role in maintaining coenzyme Q activity. In fact, changes in coenzyme Q activity in monkeys receiving vitamin E-deficient diets could explain all of the presently known interrelationships between exogenous coenzyme Q, vitamin E, a structurally unrelated antioxidant (N,N'-dipheny-*p*-phenylenediamine), and dietary unsaturated fatty acids (3, 4). At present, there is no reason to propose that vitamin E is either a precursor or an essential factor in coenzyme Q biosynthesis. However, coenzyme Q_{10} may require antioxidant protection in vivo since it has a long side chain containing 10 double bonds; and vitamin E, a lipid-soluble antioxidant which the mammalian body apparently retains in a special way (3, 13), would be the naturally occurring compound most likely to provide this protection.

The importance of these considerations to our understanding of the metabolic role of vitamin E emphasizes the need for further studies both to determine whether tissue concentrations of coenzyme Q_{10} are low in vitamin E-deficient monkeys and to determine whether the incomplete response to exogenous coenzyme Q_{10} is due to inadequate intracellular accumulation of the intact molecule.

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LITERATURE CITED

1. Porter, F. S., C. D. Fitch and J. S. Dinning 1962 Vitamin E deficiency in the monkey. IV. Further studies of the anemia with emphasis on bone marrow morphology. Blood, *20:* 471.
2. Marvin, H. N., J. S. Dinning and P. L. Day 1960 Erythrocyte survival in vitamin E-deficient monkeys. Proc. Soc. Exp. Biol. Med., *105:* 473.
3. Fitch, C. D., J. S. Dinning, F. S. Porter, K. Folkers, H. W. Moore and J. L. Smith 1965 Vitamin E deficiency in the monkey. VII. The role of vitamin E and the vitamin-like activity of coenzyme Q. Arch. Biochem. Biophys., *112:* 488.
4. Fitch, C. D., and J. S. Dinning 1963 Vitamin E deficiency in the monkey. V. Estimated requirements and the influence of fat deficiency and antioxidants on the syndrome. J. Nutrition, *79:* 69.
5. Fitch, C. D., W. E. Harville, J. S. Dinning and F. S. Porter 1964 Iron deficiency in monkeys fed diets containing soybean protein. Proc. Soc. Exp. Biol. Med., *116:* 130.
6. Wintrobe, M. M. 1961 Clinical Hematology. Lea and Febiger, ed. 5. Philadelphia, p. 164.
7. Gordon, H. H., H. M. Nitowsky and M. Cornblath 1955 Studies of tccopherol deficiency in infants and children. I. Hemolysis of erythrocytes in hydrogen peroxide. Am. J. Dis. Child., *90:* 669.
8. Rose, C. S., and P. György 1952 Specificity of hemolytic reactions in vitamin E-deficient erythrocytes. Am. J. Physiol., *168:* 414.
9. Horwitt, M. K., B. Century and A. A. Zemen 1963 Erythrocyte survival time and reticulocyte levels after tocopherol depletion in man. Am. J. Clin. Nutrition, *12:* 99.
10. Goldbloom, R. B. 1960 Investigations of tocopherol deficiency in infancy and child-

hood. Studies of serum tocopherol levels and of erythrocyte survival. Canad. Med. Assoc. J., *82:* 1114.
11. Asfour, R. Y., and S. Firzli 1965 Hematologic studies in undernourished children with low serum vitamin E levels. Am. J. Clin. Nutrition, *17:* 158.
12. Marvin, H. N. 1963 Erythrocyte survival of rats deficient in vitamin E or vitamin B_6. J. Nutrition, *80:* 185.
13. Fitch, C. D., and J. F. Diehl 1965 Metabolism of *l*-alpha-tocopherol by the vitamin E-deficient rabbit. Proc. Soc. Exp. Biol. Med., *119:* 553.