THE JOURNAL OF NUTRITION

A COPYRIGHTED SCIENCE PERIODICAL PUBLISHED MONTHLY BY THE WISTAR INSTITUTE

Guide for Authors

(*Revised November 1962*)

New policy concerning page charge, abstracts and summaries, and numerical citation of literature

The Journal of Nutrition welcomes concise reports of original research bearing on the nutrition of any organism. Preliminary notes, or papers already published or in press elsewhere are not acceptable. Consideration will be given to the publication of supplements at the author's expense.

Manuscripts are to be typewritten on bond paper (8½ × 11 inches). Double spacing should be used throughout, including that on title page, tables, legends, footnotes and references. A margin of about 1¼ inches (or 3 cm) is needed at each side of the sheets.

An abstract of 200 words or less, typed on a separate sheet and double-spaced, should be included. When published, this will appear ahead of the introductory section of the text in lieu of a summary at the end. The abstract should present the scope of the work and the principal observations.

An original and a carbon copy of the manuscript and abstract should be sent *flat* by registered mail to

RICHARD H. BARNES, EDITOR
THE JOURNAL OF NUTRITION
GRADUATE SCHOOL OF NUTRITION
CORNELL UNIVERSITY, SAVAGE HALL
ITHACA, NEW YORK

The following information should be listed on page 1:

1. Complete title of paper (in upper and lower case letters).
2. Name or names of authors.
3. Institution or laboratory, with city and state.
4. Shortened form of title (not more than 48 letters and spaces).
5. Address and name of author to whom proof is to be sent.

(*All footnotes*, including those pertaining to the title page, should be placed on a separate sheet, typed double-spaced.)

The text should begin on page 2. The manuscript should be prepared in complete and finished form. Number all pages consecutively in the following order: title page, text, literature cited, footnotes, abstract, tables and figure legends. *Never divide or hyphenate a word at the end of a line,* and do not staple together the pages of the manuscript.

Start each of the following sections on a new page, typed double-spaced:

1. The abstract.
2. Tables, with appropriate heading (a separate sheet for each table).
3. Explanation of figures, numbered and listed consecutively (do not affix illustrations to these sheets).
4. Footnotes — including those referring to title, author, institution and text.
5. Literature cited, arranged numerically in the order of first citation in the text, as "Jones and Smith (1) and others (2) have reported . . . ."

LITERATURE CITED

1. Jones, K. Z., and X. Y. Smith 1972 Growth of rats when fed raw soybean rations. J. Nutrition, *95:* 102.
2. Brown, Q. R., V. A. Ham and I. V. Long 1971 Effects of dietary fat on cholesterol metabolism. J. Nutrition, *94:* 625.

The metric system is used for all units, and temperature is expressed in the centigrade scale. The words "et al.," "per se" and "ad libitum" are not underscored. The repeated use of a unit in a sentence may be avoided by stating the common unit used, as "the diet consisted of the following: (in grams) sucrose, 50; casein, 130; . . ." Or, in other instances, as "8, 12 and 14%." The expression "*fed* a diet" is preferred to "*on* a diet." Certain other preferred usage is described in the *Style Manual for Biological Journals*.[1] The use of laboratory jargon is to be avoided, as well as such comments as "it can be seen that," " it is interesting that," and "it can be noted that." The word "quite" is often misused. Use of the active voice in all writing is preferred to the passive voice.

Registered trade names. Registered trade names are not used in text, tables or figures, except when necessary to identify certain equipment. A trivial or descriptive name should be used in text and tables, with a footnote giving the registered trade name, manufacturer and address (city and state).

Footnotes. In the text, footnotes should be numbered consecutively, including any indicated on the title page. For tables, footnotes should be typed *double-spaced* directly beneath the table, and numbered 1, 2, 3, etc. Superscripts in the table should appear consecutively, starting at the top of the table and reading from left to right across each line. Footnotes to tables are independent of the other footnote numbers in the text. Symbols are not used as superscripts and subscripts.

Acknowledgments. Financial support should be listed as a footnote to the title. Credit for materials should be listed as a footnote in the text. Technical assistance and advice may be cited in a section headed Acknowledgments, which will appear at the end of the text.

Literature Cited. Accuracy and adequacy of the references are the responsibility of the author. Literature cited should be checked carefully with the original publication. References to abstracts of verbal reports and to other unedited material (as the abstracts in Federation Proceedings, theses, and industrial technical bulletins) should be treated as text footnotes. Reference to a paper in press may be included in Literature Cited. If such a paper would be useful in the evaluation of the manuscript under consideration, it is advisable to make a copy of it available to the Editor. When a manuscript is one of a series of papers, the preceding paper should be included in Literature Cited. Personal communications and unpublished experiments should be treated as footnotes to the text.

Numbers. Use Arabic numerals throughout, including those in tables and figures. If possible, avoid beginning a sentence with a numeral; when necessary, numbers so used should be spelled out as well as any units immediately *following*.

Abbreviations and symbols. Letters in abbreviations such as DPN or IU are not spaced and periods are omitted except when the abbreviation might be read as another word. Following is a list of the more common abbreviations and symbols used in the *Journal*:

average	avg (*in tables*)
centimeter(s)	cm
counts per minute	count/min
cubic centimeter(s)	cm^3
cubic millimeter	mm^3
degree(s)	°
degrees of freedom	df (*in tables*)
gram(s)	g
international unit(s)	IU (*to be used only when weight can not be given*)
kilogram(s)	**kg**
liter(s)	(spell out)
meter(s)	**m**
microgram(s)	μg (not γ)
micromicrogram(s)	μμg
microcurie(s)	μc
micron(s)	μ
micromicron(s)	μμ
micromolar (unit of concn)	μM
micromole (unit of mass)	μmole
milligram(s)	mg
milligrams %	(*never use*)
milliliter(s)	ml
millimeter(s)	mm
millimicrogram(s)	mμg
millimicron(s)	mμ
millimole(s)	mmole
molar (mole per liter)	M
parts per million	ppm
per cent	%
probability (in statistics)	P
square centimeter	cm^2

[1] Style Manual for Biological Journals 1960 American Institute of Biological Sciences, 2000 P street, N. W., Washington 6, D. C.

square meter	m^2
square millimeter	mm^2
standard deviation	SD
standard error	SE
t (Fisher's test)	*t*
weight (in tables)	wt

Other commonly accepted abbreviations may be found in the *Style Manual for Biological Journals.*[2] The isotope designation of a labeled compound should ordinarily appear *after* the name of the compound to which it applies. When following a symbol for a compound, it should be written as superscript (as, C^{14}); when the name of the compound is spelled out, the isotope designation should be written on the same line (as, carbon-14).

Tables and figures. Follow form in current issues for the use of upper and lower case letters and italics. Authors are urged to economize on space used for tables and figures. These should fit one column width (2⅝ inches) or when necessary, two column widths (5½ inches). A charge will be made by the publisher for that space used for tables and figures which exceeds one-half of the space used for the manuscript exclusive of tables and figures. A table or figure should be constructed so as to be intelligible without reference to the text. Lengthy tabulation of essentially similar data can often be avoided by giving the number of experimental results and their mean values, with standard deviations or ranges within which the values fall. Statements that significant differences exist between the mean values of two groups of data should be accompanied by indications of probability derived from the test of significance applied. Units of measure should be indicated clearly two spaces above the first value in a column.

Original drawings, with two reproductions, to be sent to reviewers, or in the case of photographs, the original and two glossy prints, should accompany the manuscript. They should be marked on the back in ink with the author's name, complete address, and with the figure numbers. Such drawings and photographs must not exceed 8½ × 11 inches in size and must be at least 5¼ inches wide in order to fit the 2⅝-inch single column width when reduced by one-half. When a complicated figure requires more space for clarity, a proportionately larger illustration will be acceptable. But two copies of *prints* should be submitted on sheets of the same size as the text. Or if prints are smaller, they should be affixed to sheets of manuscript size. Oversize or undersize figures are difficult to handle in editing. Legends (including any keys to symbols or charts) should appear on a separate sheet. Drawings should be on white or blue-white paper or bristol board — not cream-white. They should be prepared for reproduction as line or halftone engravings. Letters and numbers should be uniform and large enough so that no character will be less than 2 mm high after reduction. A line 0.4 mm wide reproduces satisfactorily when reduced by one-half.

Figures should be lettered (preferably by stencil) in *black* India ink. For any charts made on cross section paper, use India ink on paper printed in light blue only. Charts drawn in India ink should be so executed throughout, with no typewritten material included. Graphs and charts should be given consecutive figure numbers as they will appear in the text.

Page charge. For manuscripts received on and after December 1, 1962, a charge of $20.00 per printed page in the Journal of Nutrition will be made by the American Institute of Nutrition to authors or institutions whose research funds permit charges for publication. Authors will be notified of the cost after they receive the page proofs, and will have the opportunity at that time to signify whether the research reported was supported by the type of funds that permit such charge for publication. Ability to pay this charge will *in no way* prejudice the acceptance of a manuscript. Billing will be handled directly by AIN; the Editor's Office will not see these charges, nor be advised concerning the author's payment or nonpayment of the page charge. A separate standard charge is made by the publisher for reprints, excess tabular material and photoengraving, and for changes in proof that are considered excessive or unnecessary.

Action to institute a page charge for publication in the Journal of Nutrition was taken at the April 1962 meeting of the AIN. Proceedings of this meeting are printed in

[2] See footnote 1.

the Journal of Nutrition, 78: 120–132, 1962.

Reprints. Reprint order forms and rates will be sent with page proofs. Anyone other than an author desiring reprints must have orders approved by the author, and give written assurance that (1) distribution will be restricted to those interested in the science of nutrition, and (2) no advertising or imprinting will be attached to the reprints thus furnished.

American Documentation Institute. To conserve space in the Journal, lengthy descriptions of experimental procedures, detailed data such as that on individual animals or subjects, extra figures and other material considered important supplementary information, may be deposited with the American Documentation Institute, Library of Congress, through the Editor's Office with a footnote reference.

This Guide for Contributors has been reprinted and is available upon request to the Editor or The Wistar Institute Press, 3631 Spruce Street, Philadelphia, Pa. 19104. It is recommended that you obtain copies for the use of secretaries, associates and students.

Effect of Penicillin Added to an Unheated Soybean Diet on Cystine Excretion in Feces of the Rat [1]

RICHARD H. BARNES, EVA KWONG AND GRACE FIALA
Graduate School of Nutrition, Cornell University, Ithaca, New York

ABSTRACT Fecal pellets were collected by removing them directly from the large intestine of anesthetized rats. The feces were lyophilized and analyzed for nitrogen, cystine, and trypsin activity. On the basis that trypsin contains 8.7% cystine (a value obtained by analyzing a commercial crystalline trypsin sample), the contribution of cystine in trypsin to the total fecal cystine was calculated. The rats were fed a diet containing unheated soybeans either with or without penicillin and the effect of preventing coprophagy was also measured. Penicillin in the diet increased the excretion of cystine in feces and this increase could be entirely accounted for by an increased excretion of trypsin. Under such conditions the rat must eat its feces in order to return the increased fecal cystine to the body so as to help provide for the large requirement for cystine in the synthesis of pancreatic protein secretions.

The well-known increase in the dietary requirement for methionine that results from feeding unheated soybeans, as compared with properly heat-treated soybeans, has been found to be entirely accounted for by the animal's increased need for cystine (1). The remarkable effect of antibiotics in increasing the growth of rats fed unheated soybeans has never been adequately explained, although Carroll et al. (2) have probably provided the most useful experimental data in the development of an hypothesis for this unusual antibiotic effect. They reported that the cystine content of feces was markedly increased when chlortetracycline was added to a diet containing raw soybean meal, but that the antibiotic did not alter cystine excretion when a heat-treated soybean meal was fed. These authors suggested the possibility that the antibiotic preserved cystine from bacterial degradation and as a consequence more cystine was available for absorption from the gut. The increased fecal cystine, therefore, may not have been a reflection of poor absorption, but rather an indication of preservation from bacterial destruction. The implications relating cystine, antibiotics and unheated soybean have been investigated further in the present study.

METHODS

Whole soybeans (Clarke variety, seed grade), untreated in any manner, were ground to a fine flour and this flour was incorporated in a basal purified diet mixture as described in table 1. The level of soybean flour added to the diet was adjusted so that the final diet mixture contained 10% protein (N × 6.25). In certain studies penicillin was added as crystalline procaine penicillin at a level of 0.05% in the diet.

Cystine was determined by oxidation to cysteic acid with performic acid, followed by hydrolysis with 6 N HCl at 120° for 18 hours. The hydrolysate was separated by paper electrophoresis and the cysteic acid determined by densitometry after staining with ninhydrin (3). Trypsin was measured by a modification of Anson's method (4). A hemoglobin substrate at pH 7.6 was used to measure the tyrosine released per 10 minutes' incubation at 37°.

Coprophagy was prevented by the use of plastic tail cups as described by Barnes et al. (5). Feces that were collected for the special analyses were taken directly from the large intestine in ether-anesthetized rats. The rats were used for only a single collection and were then killed. The fecal pellets were immediately frozen and lyophilized. Analytical results are, therefore, expressed on the basis of concentra-

Received for publication August 25, 1964.

[1] This research was supported in part by funds provided through the State University of New York, a Public Health Service Research Grant no. A-3620 from the National Institutes of Health, and a research grant from the National Science Foundation.

tion in the dried sample. To determine non-protein nitrogen, feces were suspended in 10% trichloroacetic acid (TCA). After centrifuging, the precipitate was washed 3 times with TCA and the supernatant collections were combined. Nitrogen was determined by the all-glass micro-Kjeldahl apparatus after digestion with sulfuric acid.

TABLE 1

Diet composition

Major components	g
Soybean protein source	78.0
Glucose monohydrate [1]	78.0
Hydrogenated vegetable oil [2]	15.0
Salts [3]	4.0
Choline dihydrogen citrate	0.3
B-vitamins in sucrose	2.0
Fat-soluble vitamins in corn oil	1.0
Total	100.3
B-vitamins in 2.0 g sucrose	*mg*
Thiamine·HCl	0.40
Riboflavin	0.80
Pyridoxine·HCl	0.40
Ca pantothenate	4.00
Niacin	4.00
Inositol	20.00
Biotin	0.02
Folic acid	0.20
Vitamin B_{12}	0.003
Menadione	1.00
Fat-soluble vitamins in 1.0 g corn oil	*mg*
Vitamin A acetate	0.31
Vitamin D (calciferol)	0.0045
α-Tocopherol	5.00

[1] Cerelose (Corn Products Refining Company, Argo, Illinois) was added in an amount sufficient to make the total of protein source plus Cerelose equal to 78%.

[2] Primex, Procter and Gamble Company, Cincinnati.

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

EXPERIMENTAL AND RESULTS

Male weanling rats (Holtzman), 6 to a group, were fed semipurified diets (table 1) containing either unheated solvent-extracted soyflakes or a properly heat-treated commercial soybean meal.[2] Feces were collected from under wire screen-bottom, individual cages and analyzed for trypsin activity. The results shown in table 2 illustrate wide variability and lack of any consistent difference in the trypsin activity of feces from rats ingesting either heat-treated or unheated soybean-containing diets. Furthermore, the addition of 0.6% DL-methionine to the diets had no consistent effect upon trypsin activity of the feces. All of these results are in confirmation of the studies of Haines and Lyman (6).

It appeared logical that trypsin excretion in the feces might be reflected in cystine balance because it was known that the cystine content of crystalline trypsin was extremely high (8.7% as determined on a sample of crystalline trypsin in these laboratories). Furthermore, in the preliminary studies just described, the feces were permitted to accumulate under the cages for 24 hours and bacterial destruction of fecal trypsin might account for some of

[2] The white (unheated) flakes and commercial soybean meal were provided by the Central Soya Company, Chicago.

TABLE 2

Fecal trypsin

Diet	Week 1	Week 2	Week 3	Week 4
	mg/rat/day	*mg/rat/day*	*mg/rat/day*	*mg/rat/day*
Experiment 1				
50% Heated soybeans		2.2		
50% Unheated soybeans		16.2		
Experiment 2				
50% Heated soybeans	6.5	2.1	2.7	
50% Unheated soybeans	8.0	1.5	2.0	
70% Unheated soybeans	12.0	2.2	5.2	
Experiment 3				
25% Unheated soybeans	4.4	6.8	4.6	1.5
25% Unheated soybeans + 0.6% DL-methionine	4.4	8.0	7.9	5.8
50% Unheated soybeans	41.7	24.6	12.9	10.0
50% Unheated soybeans + 0.6% DL-methionine	16.7	4.2	4.4	2.2

TABLE 3

Partition of nitrogen and cystine in the feces of rats and the influence of coprophagy and penicillin

		Unheated soybeans	Unheated soybeans + fecal cups	Unheated soybeans + penicillin	Unheated soybeans + penicillin + fecal cups
		mg/100 mg dried feces		*mg/100 mg dried feces*	
a	Total nitrogen	5.67	6.21	6.17	6.30
b	Non-protein nitrogen	0.40	0.43	0.55	0.58
c	Protein nitrogen (*a* minus *b*)	5.27	5.78	5.62	5.72
d	Trypsin nitrogen (16%)	0.21	0.27	0.66	0.80
e	Non-trypsin, protein N (c minus *d*)	5.06	5.51	4.96	4.92
f	Total cystine	0.52	0.60	0.74	0.84
g	Non-protein cystine	0.04	0.03	0.08	0.08
h	Protein cystine (*f* minus *g*)	0.48	0.57	0.66	0.76
i	Trypsin cystine (8.7%)	0.12	0.15	0.36	0.43
j	Non-trypsin, protein cystine (*h* minus *i*)	0.36	0.42	0.30	0.33

the variability that had been observed. For these reasons, the trypsin excretion studies were repeated, but in addition, nitrogen and cystine analyses of feces were carried out. Since Carroll et al. (2) had observed that an antibiotic increased the cystine excretion in feces, penicillin was fed to one of the groups. Two groups of 12 male weanling rats each were fed the semipurified diet containing whole ground soybeans at a level that provided 10% protein in the diet. One group received a supplement of 50 mg of procaine penicillin per 100 g of diet. Each of these 2 groups was further divided into 2 subgroups. One subgroup from each diet treatment had fecal collection cups attached so as to prevent coprophagy. At the end of 4 weeks the rats were killed with ether, the abdomen was opened and the fecal pellets collected from the large intestine. These feces were immediately frozen with dry CO_2 and lyophilized. Total and trichloroacetic acid-soluble nitrogen and cystine were determined on the dried samples. Trypsin activity was measured and converted into nitrogen (assuming 16% N) and cystine (assuming 8.7% cystine). The various measurements for nitrogen and cystine, both calculated and directly determined, are shown in table 3.

The inclusion of penicillin in the diet appeared to increase the amount of non-protein nitrogen and cystine in the feces. However, the non-protein fractions were very small, and hence the net effect of penicillin on this fraction of the feces was of little significance. The most remarkable result of penicillin feeding was on trypsin excretion, which increased threefold. Since trypsin has an unusually high cystine content, this enzyme accounted for approximately one-half of the total cystine excreted in the feces in the groups fed penicillin.

DISCUSSION

The fact that pancreatic enzyme secretions in the rat are stimulated by unheated soybeans and by soybean trypsin inhibitor has been well established by the studies of Lyman and Lepkovsky (7). This stimulation is not necessarily reflected in an increase in fecal enzyme content (6), although in chicks this has been reported to be the case (8). Snook and Meyer (9), Pelot and Grossman (10) and Lepkovsky et al. (8) have illustrated the rapid destruction of pancreatic enzymes in the small intestine and, therefore, measurement of trypsin activity in feces might be expected to be a poor index of the level of original secretion from the pancreas. The present observation that penicillin increases the trypsin activity in feces argues in favor of a protection within the small intestine, although the penicillin effect could possibly be at some more distal section of the tract. Of particular interest is the interpretation of the current observations in the light of the hypothesis of Carroll et al. (2) that antibiotics preserve

cystine from bacterial destruction in the intestinal tract, and that this results in an increase in fecal cystine following the ingestion of chlortetracycline. The experiments reported here suggest the following explanation. Cystine-rich enzyme secretions of the pancreas, particularly trypsin and chymotrypsin, are produced in increased quantity when unheated soybeans are fed. Presumably this is due to the presence in the soybeans of trypsin inhibitor. Some of these enzymes are hydrolyzed by bacterial protease, thus releasing cystine which can be absorbed and reutilized for pancreatic protein synthesis. Some part remains intact throughout its transport through the intestinal tract and the cystine contained in this portion of the pancreatic secretions is not available for absorption and reutilization unless feces are ingested through the practice of coprophagy. Penicillin and other antibiotics help to keep the protein intact during its intestinal passage so that less of this pancreatic cystine is available for absorption. Again, coprophagy may make a large part of the fecal cystine available for protein synthesis.

The addition of an antibiotic to the diet preserves trypsin from bacterial destruction. To a smaller, but very significant extent, it preserves TCA-soluble cystine from destruction, as shown in table 3. Therefore, in part, the explanation of Carroll and his associates (2) for the antibiotic effect was correct, but this protection of free cystine is apparently far less important quantitatively than the preservation of the intact enzyme-bound cystine. In table 3 it will be noted that 100% of the total fecal cystine increase resulting from penicillin in the diet is accounted for by the cystine content of trypsin that is excreted in feces.

LITERATURE CITED

1. Kwong, E., and R. H. Barnes 1963 Effect of soybean trypsin inhibitor on methionine and cystine utilization. J. Nutrition, *81:* 392.
2. Carroll, R. W., G. W. Hensley, C. L. Sittler, E. L. Wilcox and W. R. Graham, Jr. 1953 Absorption of nitrogen and amino acids from soybean meal as affected by heat treatment or supplementation with aureomycin and methionine. Arch. Biochem. Biophys., *45:* 260.
3. Diehl, J. F. 1959 Quantitative determination of cysteic acid in protein hydrolyzates—rapid paper electrophoretic method. Analyt. Chem., *31:* 1204.
4. Anson, M. L. 1938 The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. J. Gen. Physiol., *22:* 79.
5. Barnes, R. H., G. Fiala and E. Kwong 1963 Decreased growth rate resulting from prevention of coprophagy. Federation Proc., 22 *(1):* 125.
6. Haines, P. C., and R. L. Lyman 1961 Relationship of pancreatic enzyme secretion to growth inhibition in rats fed soybean trypsin inhibitor. J. Nutrition, *74:* 445.
7. Lyman, R. L., and S. Lepkovsky 1957 The effect of raw soybean meal and trypsin inhibitor diets on pancreatic enzyme secretion in the rat. J. Nutrition, *62:* 269.
8. Lepkovsky, S., E. Bingham and R. Pencharz 1959 The fate of the proteolytic enzymes from the pancreatic juice of chicks fed raw and heated soybeans. Poultry Sci., *38:* 1289.
9. Snook, J. T., and J. H. Meyer 1964 Effect of diet and digestive processes on proteolytic enzymes. J. Nutrition, *83:* 94.
10. Pelot, D., and M. I. Grossman 1962 Distribution and fate of pancreatic enzymes in small intestine of the rat. Am. J. Physiol., *202:* 285.

Prevention of Coprophagy in the Rat and the Growth-stimulating Effects of Methionine, Cystine and Penicillin when Added to Diets Containing Unheated Soybeans [1]

RICHARD H. BARNES, GRACE FIALA AND EVA KWONG
Graduate School of Nutrition, Cornell University, Ithaca, New York

ABSTRACT The growth inhibition caused by feeding unheated soybeans to rats is partially overcome by supplementing the diet with methionine, cystine or penicillin. With conventional rats these supplements were effective when the diet contained either a large quantity (50%) of unheated, solvent-extracted soybean flakes or a small quantity (25%). When coprophagy was prevented completely by the use of fecal collection cups attached to the tail, the beneficial effect of penicillin was abolished at either level of unheated soybean in the diet. The beneficial effect of methionine was abolished by preventing coprophagy when the higher level of soybean flakes was fed, but not with the lower (25%) level. Prevention of coprophagy did not affect cystine-supplementing effects at either level of soybean in the diet. The results are interpreted to mean that a biologically active substance is excreted in the feces as a result of feeding an antibiotic and this substance exerts its beneficial effect only after ingestion of feces and thus cycling the substance to the upper part of the intestinal tract. It was concluded that this substance cannot be cystine, although fecal cystine undoubtedly contributes under these conditions. Cystine utilization is not impaired in the coprophagy-prevented rat, but some alteration in the metabolism of methionine is believed to be caused by the prevention of coprophagy.

In another study from this laboratory (1) it was shown that proteolytic enzymes secreted from the pancreas under the stimulation of feeding unheated soybeans remained as intact proteins during passage through the intestinal tract if an antibiotic was present so as to inhibit bacterial destruction. The passage of the cystine-rich enzyme, trypsin, into the feces and the possible importance of coprophagy in making this fecal cystine available to the rat, led to the present studies.

EXPERIMENTAL AND RESULTS

Male, weanling rats (Holtzman) were maintained individually in wire-bottom cages in a room that was temperature-controlled at 23°. A semipurified diet, described in detail in an accompanying paper (1), was fed for 4 weeks and weekly body weight measurements were made. Soybeans added to the diet were unheated, as solvent-extracted white flakes or whole, ground Clarke variety soybeans.[2]

In the first study, the results of which are shown in figure 1, rats were fed either 50 or 25% unheated soybean flakes and one-half of each of these groups was given penicillin at a level of 20 mg/100 g diet. The rats were further subdivided so that in one-half of each of the above groups coprophagy was prevented by the use of fecal collection cups (2). By this means of subdividing there were 8 groups, as illustrated in figure 1, each group consisting of 6 rats. As expected from previous studies, penicillin increased the growth rate of conventional rats receiving either 50 or 25% unheated soybean flakes in the diet. Unexpected, however, was the observation that prevention of coprophagy abolished the growth-stimulating effect of the antibiotic.

Next, the same type of subdivision into 8 groups of 6 rats each was carried out, but in this study 0.6% DL-methionine was added to the diet instead of penicillin.

Received for publication August 25, 1964.

[1] This research was supported in part by funds provided through the State University of New York, Public Health Service Research Grant no. A-3620 from the National Institutes of Health, and a research grant from the National Science Foundation.

[2] The white (unheated) flakes were provided by the Central Soya Company, Chicago. The whole ground soybeans were provided by Dr. Keith Steinkraus, New York State Agricultural Experiment Station, Geneva, New York.

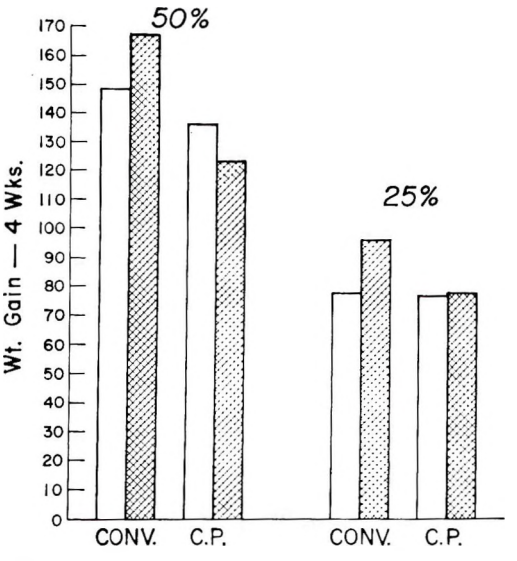


Fig. 1 Effect of coprophagy prevention (C.P.) on the growth response of rats fed unheated soybean flakes to the inclusion of penicillin in the diet. Open bars represent rats receiving unsupplemented, unheated soybean flakes at either 50 or 25% of the diet. Cross-hatched bars represent rats receiving penicillin-supplemented diets. Numbers on the ordinate are in grams.

The results are shown in figure 2. As expected from previous studies, methionine supplementation increased the growth rate of conventional rats receiving either 50 or 25% unheated soybean flakes. In the coprophagy-prevented rats receiving 25% unheated soybean flakes, methionine gave the expected stimulation of growth rate, but no such effect was observed with rats receiving a diet with 50% unheated soybean flakes. This observation was so unusual that it was repeated, but with step-by-step additions of 3 levels of methionine to the diet, each level being fed to a group of 6 rats over a 4-week period. The results in figure 3 show that with 25% unheated soybean flakes in the diet, the growth rate of both conventional and coprophagy-prevented rats increased and reached a maximal rate when 0.15% DL-methionine was added to the diet. With 50% unheated soybean flakes, growth rate increased and a maximal rate was reached when 0.3% DL-methionine was included in the diet with conventional rats, but when coprophagy was prevented, no significant increase in growth rate was observed at any level of supplemental methionine.

In an earlier study (3) it had been established that the supplemental effect of methionine in diets containing 50% soybean (25% protein) was due to the need for and consequent conversion to cystine. The normal requirement for methionine is satisfied at this dietary level of soybean protein and yet there is still a stimulation of growth rate when cystine is added to the diet. A possible explanation for the lack of methionine supplementation at the higher level of unheated soybean would be that prevention of coprophagy in some manner either blocks the conversion of methionine to cystine or prevents the utilization of cystine for growth promotion. The effectiveness of cystine supplementation was tested in the same model experiment that was used to establish figures 1 and 2. In this study, however, whole, ground soybeans were used and this accounts for the lower general growth rates (fig. 4) than were obtained in the previous studies. Diets were supplemented with 0.3% of L-cystine. It is apparent from figure 4 that a

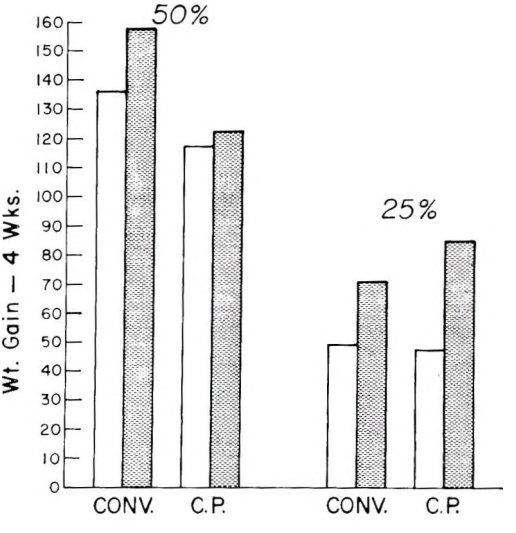


Fig. 2 The effect of coprophagy prevention (C.P.) on the growth response of rats fed unheated soybean flakes to the inclusion of methionine(0.6% DL-methionine) in the diet. The open bars represent rats receiving unsupplemented, unheated soybean flakes at either 50 or 25% of the diet. Shaded bars represent rats receiving methionine-supplemented diets. Numbers on the ordinate are in grams.

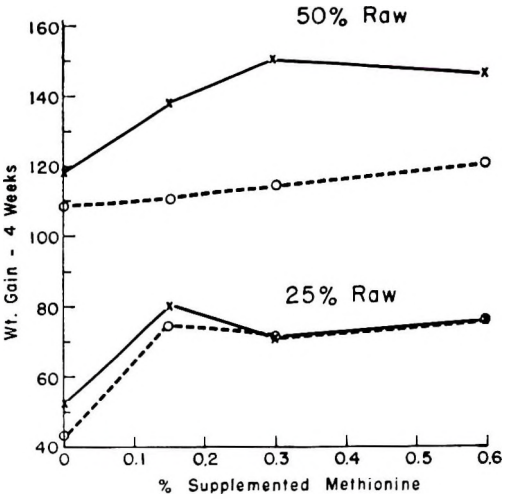


Fig. 3 Coprophagy-prevented (broken lines) and conventional rats (solid lines) fed unheated soybean flakes at either 50 or 25% of the diet. Growth response in grams at varying increments of supplemental DL-methionine in the diet.

normal stimulation of growth with cystine is obtained at both low and high levels of soybeans in the diet in both conventional and coprophagy-prevented rats. Cystine does not give the same pattern of results that was obtained with methionine supplementation. This means that the inhibition of stimulation of growth by methionine in the coprophagy-prevented rat ingesting a high soybean protein diet is not due to a block in cystine utilization. The possibility remains open that there is an interference in the conversion of methionine to cystine when coprophagy is prevented.

To extend the interpretation of the penicillin effect in stimulating growth to methionine and cystine requirements of the rat, multiple supplementations were conducted as described in table 1. The basal diet contained whole, ground soybeans, Clarke variety, in an amount sufficient to give a protein concentration (N × 6.25) of 10%. L-Cystine and DL-methionine were added at levels of 0.3% and procaine penicillin at a level of 50 mg/100 g diet. Certain parts of this experiment were conducted more than once. Each time a replicate study was carried out 6 rats per group were used; therefore the number of replications can be determined by dividing by six the total number of rats indicated in the table. Food intake was measured and protein efficiency ratios calculated, but since these results were parallel to the growth rates, only the latter are presented in the table. In one study coprophagy was prevented and, for comparative purposes, results with these animals are included in the table.

In attempting an interpretation of the penicillin effect on growth, the most striking observation presented here is that cystine plus penicillin gives a greater growth stimulation than cystine or penicillin alone, and methionine plus penicillin is better than methionine or penicillin alone. In the coprophagy prevention experiment, the penicillin stimulation of growth was abolished whether the penicillin was given alone or in combination with methionine. The methionine stimulation of growth was obtained despite the prevention of coprophagy, but since the diet contained a low level of unheated soybeans, this was to be expected.

DISCUSSION

Interference with penicillin-induced growth stimulation by prevention of coprophagy is presumed to mean that penicillin either initiates the excretion or increases

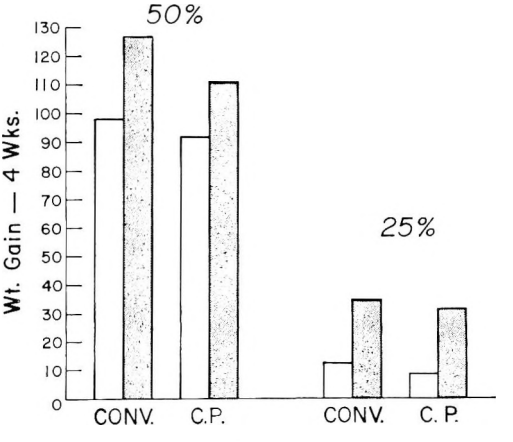


Fig. 4 The effect of coprophagy prevention (C.P.) on the growth response of rats fed unheated soybeans to the inclusion of cystine (0.3% L-cystine) in the diet. The open bars represent rats receiving unsupplemented, unheated soybean at either 50 or 25% of the diet. Shaded bars represent rats receiving cystine-supplemented diets. Numbers on the ordinate are in grams.

TABLE 1

Effects of cystine, methionine and penicillin, alone and in combination, on the growth of rats fed a diet containing 10% protein derived from ground whole soybeans

	No. rats	4-Week growth rate
		g/rat/4 weeks
Conventional		
Unheated soybean	24	30
Unheated soybean + cystine	12	43
Unheated soybean + methionine	24	56
Unheated soybean + penicillin	24	45
Unheated soybean + penicillin + cystine	12	53
Unheated soybean + penicillin + methionine	24	66
Coprophagy prevented		
Unheated soybean	6	22
Unheated soybean + methionine	6	47
Unheated soybean + penicillin	6	24
Unheated soybean + penicillin + methionine	6	46

the quantity of excretion of some substance which stimulates growth rate when the feces are ingested. It has been tempting to assume that the fecal growth stimulator was cystine. There is an increased requirement for cystine when unheated soybeans are fed and penicillin increases cystine excretion in the feces (1). The experiment described in table 1, makes it unlikely that this is the entire answer to the penicillin effect. Although both cystine and pencillin enhanced growth rate, a combination of the two gave a larger response than either supplement alone. This would not be expected if cystine in the feces was responsible for the penicillin effect. In an accompanying paper (1) it was observed that all of the increase in total fecal cystine that results from feeding an antibiotic can be accounted for on the basis of the cystine in the increased trypsin excretion in feces. The increased requirement for cystine that is associated with the ingestion of unheated soybeans is apparently closely tied to the increased protein formation and secretion, particularly trypsin, by the pancreas. It seems logical to conclude that when unheated soybeans are fed there is an increased requirement for cystine due to the increased synthesis and fecal excretion of pancreatic enzymes. When penicillin is added to the diet, an increased amount of intact pancreatic trypsin passes completely through the intestinal tract and is ultimately excreted in the feces, thus removing even more cystine from possibility of utilization by the animal (1). When coprophagy is prevented and penicillin is included in the diet, a biologically active compound, presumably containing cystine, is prevented from recycling through the intestinal tract. This substance might well be a protein such as trypsin that is secreted into the intestine. In any event, the excretion of amino acids, either free or protein-bound, cannot be the entire explanation for the poor nutritive value of unheated soybeans as postulated by Booth et al. (4) and Haines and Lyman (5). Amino acid supplementations alone do not return full nutritional quality to unheated soybeans (6). Antibiotics given in addition to amino acids increase nutritional quality beyond that obtainable with amino acids alone. The antibiotic effect is blocked when coprophagy is prevented. A biologically active substance such as a pancreatic enzyme may be the substance that is protected by the antibiotic and which is the beneficial component of feces when they are ingested.

The mechanism of the block in methionine stimulation of growth of rats receiving a relatively high protein diet derived from unheated soybeans, when coprophagy is prevented, remains unexplained. Since methionine stimulation of growth under these dietary conditions has been thought to be due to cystine formed from the methionine and not from the methionine per se, it is logical to believe that this conversion is in some way involved. Cystine utilization in the coprophagy-prevented rat is

not impaired. Therefore, if this is the metabolic area that is involved, it may be that coprophagy is necessary for the proper conversion of methionine to cystine under conditions of ingesting a large quantity of the inhibitory substance, possibly trypsin inhibitor, contained in unheated soybeans.

LITERATURE CITED

1. Barnes, R. H., E. Kwong and G. Fiala 1965 The effect of penicillin added to an unheated soybean diet on cystine excretion in feces of the rat. J. Nutrition, *85:* 123.
2. Barnes, R. H., G. Fiala and E. Kwong 1963 Decreased growth rate resulting from prevention of coprophagy. Federation Proc., 22 *(1):* 125.
3. Kwong, E., and R. H. Barnes 1963 Effect of soybean trypsin inhibitor on methionine and cystine utilization. J. Nutrition, *81:* 392.
4. Booth, A. N., D. J. Robbins, W. E. Ribelin and F. Deeds 1960 Effect of raw soybean meal and amino acids on pancreatic hypertrophy in rats. Proc. Scc. Exp. Biol. Med., *104:* 681.
5. Haines, P. C., and R. L. Lyman 1961 Relationship of pancreatic enzyme secretion to growth inhibition in rats fed soybean trypsin inhibitor. J. Nutrition, *74:* 445.
6. Barnes, R. H., G. Fiala and E. Kwong 1962 Methionine supplementation of processed soybeans in the rat. J. Nutrition, *77:* 278.

Manganese Metabolism in College Men Consuming Vegetarian Diets [1]

VIRGINIA M. LANG, BARBARA B. NORTH AND LURA M. MORSE
School of Home Economics, University of Minnesota, St. Paul, Minnesota

ABSTRACT Manganese balance was studied in eight college men consuming a vegetarian diet low in protein. The diet was supplemented with either a vegetable protein food or the vegetable protein food plus skim milk powder. The values for total mean daily manganese intake, excretion and retention were 7.07, 3.74 and 3.34 mg, respectively. The mean daily intake, excretion and retention for each subject by periods are reported. In addition, the mean daily intakes and retentions are expressed on the basis of body height (cm) and body weight (kg). All subjects were in positive balance throughout the study. Regression analyses of manganese retention on manganese intake indicated that correction of retention for either height or weight explains twice the variation in retention when compared with the uncorrected retention data. Due to the high correlation between height and weight, it was not possible in this study to determine which of the two has more influence on retention.

It has been demonstrated that manganese is an essential element for rats, chicks, mice, rabbits and guinea pigs. As a result of animal studies, attempts have been made to determine whether manganese is essential in human subjects and to establish a human requirement for this element. On the basis of balance studies, Everson and Daniels (1) in 1934 suggested 0.2 to 0.3 mg of manganese/kg of body weight for normal development in children 3 to 5 years old. Basu and Malackar (2) reported that 3 adult males could be kept in equilibrium on a manganese intake of 4.6 mg/day. North et al. (3), in a study of 8 college women, observed that positive balance was maintained with a mean daily manganese intake of 3.7 mg. No balance studies with adult men have been reported in the literature since the work of Basu and Malackar in 1940.

In addition Underwood (4) noted average intakes of 6 to 8 mg of manganese/day for adults consuming typical Australian diets, and Monier-Williams (5) calculated the intakes for adults with a typical English winter diet to be in this same range (6 to 8 mg). Kent and McCance (6) have observed average daily intakes of 2 adults to be 2.2 to 8.8 mg depending upon the source of calories.

Because of the lack of information on manganese balance in human subjects, the present study was undertaken to determine the manganese intake and retention of young adult men. A metabolism study designed to investigate nitrogen balance with a vegetarian diet low in protein presented the opportunity for the simultaneous study of manganese balance in young men.

PROCEDURE

For purposes of statistical analysis, the experiment was designed as an extra period Latin square. Eight young adult males, ranging in age from 20 to 29 years, were divided into 2 groups of 4 subjects each. The subjects were fed a basal vegetarian diet which was supplemented with either a vegetable protein food [2] or the vegetable protein plus skim milk powder. In some experimental periods, skim milk powder was combined with the vegetable protein so that 25% of the total protein intake was supplied from an animal source. A 10-day adjustment period (basal diet only) was followed by six 4-day experimental periods plus one post-experimental day.

Received for publication September 18, 1964.

[1] Paper 5475, Scientific Journal Series, Minnesota Agricultural Experiment Station.

[2] The authors wish to express their appreciation to General Mills, Incorporated, for their contribution of MPF (Toasted soy protein with added calcium carbonate, ascorbic acid, niacin, vitamin A, riboflavin, pyridoxine, thiamine, vitamin D, KI, vitamin B_{12}, analysis, in part, as follows: 50% protein, 6.5% ash, 0.5% calcium, 0.5% phosphorous and manganese 0.02 mg/g (our analysis).

Four menus were used in rotation during the study, with each menu occurring once during each of the six 4-day balance periods. A typical menu is shown in table 1. The basal diet was calculated to contain 34.1 g of protein with the supplements adjusted individually so that each subject received 0.75 g of protein/kg of body weight/day. After analysis for nitrogen, the mean daily protein intake was calculated to be 0.45 g/kg of body weight (range 0.37 to 0.54 g) for the basal diet and 0.75 g/kg (range 0.71 to 0.77 g) for the basal diet plus the protein supplements. All of the subjects were in negative nitrogen balance during the 10-day period when they were fed the basal diet alone. After the addition of protein supplements, the subjects remained in equilibrium or in positive nitrogen balance.

TABLE 1

Typical vegetarian menu

	g
Breakfast	
Banana	100
Cornmeal (dry)	25
Brazil nuts	10
Syrup	80
Margarine	14
Brewer's yeast	5
Orange juice	200
Lunch	
Fruit salad	
Dates	15
Orange	50
Cabbage	50
Banana	30
Lettuce	10
French dressing	23
Green beans	50
Mushrooms	25
Celery sticks	50
Vienna bread	20
Rum hard sauce	16
Baked apples	115
Margarine	14
Peanut butter	10
Fried potato	100
Dinner	
Rice (dry)	35
Tomato puree	60
Mushrooms	25
Fried potatoes	100
Lettuce	50
Avocado	50
French dressing	23
Margarine	14
Cashews	10
Fruit ice	142
Asparagus	100

Certain fluids such as coffee, tea, distilled water and a carbonated beverage (cola-type) were allowed ad libitum. In addition, hard candy and butterballs [3] were allowed ad libitum to meet the energy requirement of subjects whose energy needs were not met by the diet. Daily records were kept of the amounts of ad libitum fluids and foods consumed. The mean daily caloric intake was 3471 kcal (range 3140 to 3788 kcal). There was no observed net change in weight for any of the subjects.

Since it is difficult to insure adequate intakes of certain vitamins and minerals with many vegetarian diets, each subject received daily a vitamin B-complex capsule containing 3 mg thiamine hydrochloride, 2.5 mg riboflavin, 20 mg niacinamide, 0.5 mg pyridoxine and 1 mg calcium pantothenate. In addition, 10 μg of vitamin B_{12}, 1000 IU vitamin D and a calcium diphosphate capsule (which provided approximately 300 mg calcium and 250 mg phosphorous) were given each day to all subjects.

All food served to the subjects was prepared in the laboratory. Each portion was individually weighed on a torsion balance to the nearest one-tenth of a gram. A food aliquot was made each day which contained an amount equivalent to one-fourth of each serving of food. At the end of each 4-day period, composites were prepared from these food aliquots. The composites were weighed, homogenized in a Waring Blendor and stored in a freezer until analysis could be performed.

Each subject collected complete 24-hour urine and fecal specimens. The 24-hour urine collections were measured and one-fourth of the amount was saved. At the end of each 4-day period a 400-ml aliquot of the composite urine collection was acidified with 40 ml of concentrated HCl and saved for future analysis. Acid digests of each 4-day fecal and food composite were made according to the method of Leichsenring et al. (7) and saved until analysis could be performed.

[3] Butterballs consist of margarine plus powdered sugar and flavoring.

Approximate 25-g samples of the food digests and 10-g samples of the fecal digests were weighed and dry-ashed in quadruplicate and triplicate, respectively, in tared silica crucibles at 550° until a white ash was obtained. Triplicate 100-ml urine samples from the urine composites were dry-ashed in new porcelain evaporating dishes. All ash samples were acidified with concentrated HCl and made up to 100 ml.

Manganese determinations were carried out with a modification of the periodate method of the AOAC (8) and Milton (9). The method was used in earlier work by this laboratory and is described in a prior publication (3).

RESULTS AND DISCUSSION

Results of the food analyses showed that the basal diet provided a mean intake of 6.27 mg of manganese/day (range 4.20 to 7.61 mg/day). Despite considerable care in the purchase and preparation of the food, as well as preparation of the food composites, there was marked variation in the manganese content of the basal diet. This variation from period to period is not unexpected since Richards (10) and Hodges (11) demonstrated that plant foods vary considerably in manganese content, not only from plant to plant but also within tissues of the same plant. One explanation for the variation in the manganese content of the basal diet is that variation occurred in the sampling of the foods since the fresh fruits and vegetables which made up most of the diet were purchased weekly or more often as needed.

Analysis of the ad libitum foods indicated that coffee, tea, the carbonated beverage and butterballs contributed substantially to the manganese content of the diet. The results of the analyses of these ad libitum foods showed the following mean values for manganese: coffee per cup, 0.04 mg (0.8 g in 150 ml distilled water of powdered [instant] coffee); tea per cup, 0.09 mg (2 g tea in tea bag steeped 3 minutes in 150 ml distilled water); carbonated beverage per 355-ml bottle, 0.03 mg; butterballs per 14-g portion, 0.02 mg. Coffee and the carbonated beverage were consumed in large quantities by most of the subjects and therefore contributed not only to the variation in the managnese content of the diet but also provided significant amounts of this element in the intakes of those subjects who used them.

Although skim milk powder contained no manganese measurable by our methods, the vegetable protein supplement contained considerable manganese. Since the supplements were administered on the basis of body weight, the amount of manganese each subject received from the supplements varied appreciably (range per day from 0.09 to 1.64 mg). The total mean daily manganese intake by analysis was 7.07 mg/day with a range from 6.63 to 7.47 mg/day (table 2). These values fall within the range of average intakes reported by Underwood (4), Monier-Williams (5), and Kent and McCance (6). However, this intake level for all subjects is higher than the 3.70 mg/day which North et al. (3) reported as sufficient for maintenance of positive balance in women. This level of intake is also higher than the 4.6 mg of manganese/day suggested by Basu and Malackar (2) for maintenance of equilibrium in men. The higher intake reported here is due primarily to the use of a vegetarian diet, since animal foods are known to be lower and less variable in manganese content than are vegetable foods (10, 11).

During the 35 days of observation, the mean daily urinary excretion of manganese was 0.22 mg/day. Although these values are higher than those reported by Kehoe et al. (12) for adults, they agree well with the mean urinary manganese value of 0.20 mg/day reported by North et al. (3) for college women. The mean fecal excretion was 3.53 mg of manganese /day. These analyses show remarkably consistent values, and statistical analysis indicates there is no significant difference between the 2 groups of subjects or between periods in fecal excretion. Results of the analysis of urine and feces showed a mean total daily excretion of 3.74 mg of manganese.

Since the vegetable protein supplement was primarily a soybean product and because it had been shown that the phytic acid present in soybeans will depress the absorption and retention of the divalent

TABLE 2

Mean daily manganese intake, excretion and re tention of 8 young men for eight 4-day periods

Subject	Height	Wt	Diet	Periods	Intake	Excretion		Retention
						Fecal	Urinary	
	cm	*kg*			*mg*	*mg*	*mg*	*mg*
WA	185	91.6	A [1]	pre-period	6.71	1.95	0.00	4.76
			A	pre-period	6.54	2.33	0.24	3.96
			B [2]	1	6.71	3.50	0.21	3.01
			B	2	8.44	4.47	0.27	3.70
			C [3]	3	8.60	3.75	0.43	4.41
			C	4	7.25	4.59	0.20	2.46
			C	5	6.07	3.53	0.00	2.55
			C	6	9.42	5.84	0.17	3.41
				mean	7.47	3.75	0.22	3.53
WC	173	67.9	A	pre-period	6.79	3.74	0.06	2.98
			A	pre-period	6.58	3.52	0.07	2.98
			B	1	6.13	2.99	0.12	3.02
			B	2	7.81	2.94	0.08	4.78
			C	3	7.90	4.18	0.08	3.64
			C	4	6.43	9.67	0.06	−3.30
			C	5	5.21	3.85	0.28	1.08
			C	6	8.63	5.22	0.08	3.34
				mean	6.93	4.51	0.11	2.32
DH	182	79.7	A	pre-period	6.78	2.39	0.15	4.24
			A	pre-period	6.51	2.04	0.13	4.34
			B	1	6.38	2.34	0.18	3.86
			B	2	8.15	3.12	0.18	4.85
			C	3	8.19	4.23	0.19	3.77
			C	4	6.84	3.66	0.18	3.00
			C	5	5.65	2.62	0.17	2.86
			C	6	8.99	3.91	0.26	4.82
				mean	7.19	3.04	0.18	3.97
KK	175	80.1	A	pre-period	6.83	3.06	0.00	3.77
			A	pre-period	6.46	2.46	0.16	3.85
			C	1	7.09	4.18	0.49	2.42
			C	2	8.82	3.84	0.16	4.82
			B	3 [4]	—	—	—	—
				mean	7.30	3.38	0.20	3.71
JM	175	76.2	A	pre-period	6.79	2.53	0.17	4.08
			A	pre-period	6.53	2.26	0.13	4.14
			C	1	6.98	2.94	0.20	3.84
			C	2	8.72	2.61	0.27	5.85
			B	3	7.44	2.67	0.24	4.52
			B	4	6.16	3.35	0.33	2.49
			B	5	4.91	2.51	0.18	2.22
			B	6	8.33	2.15	0.09	6.09
				mean	6.98	2.63	0.20	4.15
DR	176	63.1	A	pre-period	6.77	3.33	0.18	3.27
			A	pre-period	6.53	4.45	0.49	1.59
			C	1	6.52	4.09	0.16	2.27
			C	2	8.20	3.86	0.16	4.19
			B	3	7.05	5.52	0.20	1.33
			B	4	5.67	3.35	0.14	2.18
			B	5	4.42	2.89	0.41	1.13
			B	6	7.84	5.11	0.00	2.73
				mean	6.63	4.08	0.25	2.33

TABLE 2 (*Continued*)

Mean daily manganese intake, excretion and retention of 8 young men for eight 4-day periods

Subject	Height	Wt	Diet	Periods	Intake	Excretion		Retention
						Fecal	Urinary	
	cm	*kg*			*mg*	*mg*	*mg*	*mg*
DS	180	84.2	A	pre-period	6.72	2.64	0.23	3.86
			A	pre-period	6.50	2.16	0.16	4.18
			C	1	7.18	3.78	0.16	3.25
			C	2	8.88	3.68	0.09	5.10
			B	3	7.55	4.10	0.69	2.76
			B	4	6.16	5.55	0.19	0.41
			B	5	4.99	3.05	0.19	1.75
			B	6	8.38	3.03	0.16	5.20
				mean	7.05	3.50	0.23	3.31
AY	180	78.8	A	pre-period	6.63	2.38	0.13	4.12
			A	pre-period	6.39	1.75	0.26	4.38
			B	1	6.25	2.49	0.17	3.59
			B	2	7.96	3.76	1.07	3.13
			C	3	8.07	6.12	0.27	1.68
			C	4	6.69	3.20	0.15	3.34
			C	5	5.42	4.20	0.22	1.00
			C	6	8.84	2.83	0.00	6.01
				mean	7.03	3.34	0.33	3.41
	Mean of all				7.07	3.53	0.21	3.34

[1] Diet A, basal diet.
[2] Diet B, basal diet plus vegetable protein and skim milk powder supplements.
[3] Diet C, basal diet plus vegetable protein supplement alone.
[4] Subject left study.

calcium (13) and magnesium ions,[4] it was thought that perhaps a similar situation might exist with manganese. The variation in total mean daily absorption of manganese for the 2 treatments was: vegetable protein supplement alone, 3.55 mg (range 1.31 to 5.08 mg); vegetable protein plus skim milk powder supplement, 3.57 mg (range 2.03 to 4.53 mg). The difference between the treatments was not statistically significant. The total mean daily absorption of manganese was 3.56 mg (range 2.42 to 4.36). These data did not exhibit the magnitude of difference in manganese absorption between treatments B and C that was observed in the studies of magnesium absorption carried out with these same subjects.[5]

The mean daily retention was 3.34 mg of manganese (table 2). In addition, all of the subjects but one were in positive balance throughout the study. One subject (WC) showed a large negative balance during one of the periods. This was ascribed to the fact that he had an episode of diarrhea and a consequent loss of manganese. Statistical analysis of the retention data indicated no significant difference between treatments. However, as would be expected in a human balance study, the variation among individuals was significant ($P = 0.05$) and the variation in retention between periods was highly significant ($P = 0.01$). In addition a highly significant coefficient of correlation ($r = 0.55$) indicates that in this study, retention was proportional to the intake. This observation agrees with that of Everson and Daniels (1) who stated that the retention of manganese is proportional to intake in children. Consequently, it would be expected that the variation in retention between periods would be significant since there was an appreciable variation in intake. When mean daily retention for each subject was expressed as percentage of intake, the retention varied from 33 to 59% with a mean retention of 47%. North et al. (3) have reported a mean retention

[4] Carleton, I. R. 1964 A magnesium balance study of college men consuming a controlled low protein diet. M.S. Thesis, University of Minnesota.
[5] See footnote 4.

of 41% when the mean intake was 3.70 mg/day. It is possible that such retentions represent losses through the skin, hair and nails which were not determined. In addition, Mitchell et al. (14) have reported the presence of small amounts of manganese in sweat (3.2 to 7.4 μg/100 ml).

Because these subjects varied considerably in body size, intake and retention were expressed on the basis of height and weight (table 3). The calculations showed a mean daily intake of 0.09 mg of manganese/kg (range 0.08 to 0.10 mg/kg), and a mean daily retention of 0.04 mg/kg (range 0.03 to 0.05 mg/kg). When expressed on the basis of body height the mean daily intake was 0.04 mg of manganese/cm and mean daily retention was 0.02 mg of manganese/cm (range 0.01 to 0.02 mg/cm). The consistency of results when expressed on the basis of body size (table 3) may suggest a regulatory mechanism within the body which maintains the level of manganese on a basis which is related to some parameter of body size, such as bone size or bone weight. However, the protein supplements were based on the body weight of the subjects and the manganese intake would, therefore, have been increased with increasing body weight. Since retention has been noted to be proportional to intake, this may explain in part the consistent relationship of manganese retention to body size.

Since earlier work by North et al. (3) from this laboratory also indicated that a relationship may exist between retention and body size, a series of regression coefficients of manganese retention on manganese intake were calculated to determine the influence of height and weight on retention. The results of these analyses indicated that when retention was expressed as retention per kilogram body weight or as retention per centimeter of body height, twice the variation in retention could be accounted for by the relationship with the 3 independent variables (intake per day, height, weight) than when retention was expressed as total manganese per day. This was shown by an increase in the R^2 value from 0.18 to 0.40 when retention is expressed as per kilogram body weight as opposed to total retention and to 0.42 when retention is expressed as per centimeter body height as opposed to total retention (R^2 = the square of the multiple correlation coefficient and the proportion of the total variation in the dependent variable accounted for by its relationship with the independent variables). However, due to the high correlation between height and weight ($r = 0.79$) it is not possible to say which of the two had more influence on retention.

When the intake is expressed on a per kilogram basis and the retention is also expressed on a per kilogram basis, R^2 is equal to 0.41 as opposed to the previous 0.40. Similarly, when retention is expressed on a per centimeter of body height basis and intake as total intake per day, R^2 is equal to 0.42 and when both intake and retention are expressed on a per centimeter basis, R^2 is found to be equal to 0.43. Consequently, these analyses indicate that once the retention has been corrected for either height or weight, correction of the intake for height or

TABLE 3

Comparison of mean daily intake and retention of manganese in milligrams on the basis of height and weight

Subject	Intake/kg	Retention/kg	Intake/cm	Retention/cm
	mg	*mg*	*mg*	*mg*
WA	0.08	0.04	0.04	0.02
WC	0.10	0.03	0.04	0.01
DH	0.09	0.05	0.04	0.02
JM	0.09	0.05	0.04	0.02
KK	0.09	0.05	0.04	0.02
DR	0.10	0.04	0.04	0.01
DS	0.08	0.04	0.04	0.02
AY	0.09	0.04	0.04	0.02
Mean	0.09	0.04	0.04	0.02

weight does not explain a significantly larger portion of the variation in retention. The high retention of manganese (3.34 mg/day) suggests a need for further investigation of another possible pathway for excretion of manganese or a possible level of intake at which there are no further increases in retention.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Ardell Linnerud for assistance with the statistical analysis of the data, Mrs. Irene Carleton and Mary Parsons for technical assistance, and Mrs. Jean S. Gowan for calculation of the diets.

LITERATURE CITED

1. Everson, G. J., and A. L. Daniels 1934 Study of manganese retentions in children. J. Nutrition, *8:* 497.
2. Basu, K. P., and M. C. Malackar 1940 Iron and manganese requirements of the human adult. J. Ind. Chem. Soc., *17:* 317.
3. North, B. B., J. M. Leichsenring and L. M. Norris 1960 Manganese metabolism in college women. J. Nutrition, 72: 217.
4. Underwood, E. J. 1953 Biochemistry and Physiology of Nutrition, vol. 2, eds., G. H. Bourne and G. W. Kidder. Academic Press, Inc., New York, pp. 459–467.
5. Monier-Williams, G. W. 1949 Trace Elements in Foods. J. Wiley and Sons, Inc., New York, pp. 298–316.
6. Kent, N. L., and R. A. McCance 1941 The absorption and excretion of minor elements by man. 2. Cobalt, nickel, tin and manganese. Biochem. J., *35:* 877.
7. Leichsenring, J. M., A. Biester, H. Roberts, P. P. Swanson, M. R. Gram, R. M. Leverton, W. Brewer and L. M. Burrill 1958 Methods used for human metabolic studies in the North Central Region. Minnesota Agricultural Experiment Station Tech. Bull. 225, St. Paul.
8. Association of Official Agricultural Chemists 1955 Official Methods of Analysis, ed. 5. Washington, D. C.
9. Milton, R. F. 1955 Methods of Quantitative Microanalysis, eds., R. F. Milton and W. A. Waters. Edward Arnold Ltd., London, pp. 229–420.
10. Richards, M. B. 1930 Manganese in relation to nutrition. Biochem. J., *24:* 1572.
11. Hodges, M. A., and W. H. Peterson 1931 Manganese, copper, and iron content of serving portions of common foods. J. Am. Dietet. A., 7: 6.
12. Kehoe, R. A., J. Cholak and R. O. Story 1940 A spectrochemical study of the normal ranges of concentration of certain trace metals in biological materials. J. Nutrition, *19:* 579.
13. Palmer, N., and J. C. Mottram 1939 The influence of phytin and of fats on the production of rickets by a cereal diet. Biochem. J., *33:* 512.
14. Mitchell, H. H., T. S. Hamilton and W. T. Haines 1949 The dermal excretion under controlled environmental conditions, of nitrogen and minerals in human subjects with particular reference to calcium and iron. J. Biol. Chem., *178:* 345.

Effect on Nitrogen Retention of Men of Altering the Intake of Essential Amino Acids with Total Nitrogen Held Constant [1]

CONSTANCE V. KIES [2] AND HELLEN M. LINKSWILER
Department of Foods and Nutrition, School of Home Economics, University of Wisconsin, Madison, Wisconsin

ABSTRACT Nitrogen retention of 6 men fed semi-purified diets containing 5.80 g total nitrogen was affected significantly by the amount of essential amino acids ingested when the caloric intake was 45 kcal/kg of body weight. The inclusion of arginine, histidine, cystine and tyrosine appeared to have a beneficial effect on nitrogen retention when the essential amino acids contributed a major portion of the dietary nitrogen. Marked relationships exist among caloric intake, essential amino acid intake and nitrogen retention.

The effects of altering the ratio of essential amino acids to non-essential amino acids in the diets of animals have been reviewed recently by Stucki and Harper (1). However, relatively little work has been reported concerning the ratio of essential to non-essential amino acids in human nutrition. Snyderman et al. (2) reported that non-essential nitrogen is the most limiting factor in milk protein for growth. Kirk et al. (3) and Swendseid et al. (4, 5) fed amino acids in several patterns and reported that nitrogen retention in adults was improved when the level of nitrogen from the essential amino acids was increased at the expense of non-essential sources of nitrogen.

The purpose of the present investigation was to determine the effect on nitrogen retention in adult men of varying the intake of the essential or the essential plus semi-essential amino acids when total dietary nitrogen was constant. A preliminary study was also made to determine the effect of caloric intake on the nitrogen retention of men when the essential amino acids furnished a high, an intermediate, or a low percentage of the total dietary nitrogen.

PROCEDURE

In 2 separate studies human subjects were fed diets which contained 5.80 g nitrogen daily. Ordinary foods provided 0.44 or 0.57 g nitrogen, and crystalline amino acids and diammonium citrate supplied the remainder. Each study contained 6 experimental periods of 5 days each, and the amount of the essential amino acids fed differed for each of the experimental periods. At the end of study 2 there was an addendum of 12 days during which the effect of an altered caloric intake on nitrogen balance was determined at 3 levels of essential amino acid intake. During study 1 the 8 essential amino acids and the 4 semi-essential amino acids (histidine, arginine, tyrosine, and cystine) patterned as in egg protein were fed at 6 different levels, and during study 2 the 8 essential amino acids patterned as in egg protein were fed at 6 different levels.

Prior to the experimental periods the subjects were fed a semi-purified diet similar to that fed during the experimental periods until a stabilized nitrogen output was reached.

The sources and amounts of dietary nitrogen of each experimental period for both studies are shown in table 1. In study 1 the amounts of the 8 essential amino acids and the 4 semi-essential amino acids fed were equivalent to those in 15, 20, 30, 40, 50, and 60 g of egg protein; the nitrogen from these amino acids provided 25, 33, 50, 67, 83, and 100%, respectively, of the purified nitrogen (i.e., the nitrogen

Received for publication August 17, 1964.

[1] Published with the permission of the Director of the Wisconsin Agriculture Research Station.

[2] Present address: Department of Food and Nutrition, School of Home Economics, College of Agriculture and Home Economics, University of Nebraska, Lincoln.

TABLE 1

Sources and amounts of dietary nitrogen for each of the experimental diets with a total nitrogen intake of 5.80 g

Diet no.	EAA [1] N	SEAA [1] N	DAC [1] and glycine N	Ordinary food N [2]	Total N intake
	g/day	*g/day*	*g/day*	*g/day*	*g/day*
			Study 1		
1	0.81	0.50	3.92	0.57	5.80
2	1.07	0.67	3.49	0.57	5.80
3	1.61	1.00	2.62	0.57	5.80
4	2.14	1.34	1.75	0.57	5.80
5	2.68	1.67	0.88	0.57	5.80
6	3.22	2.01	0.00	0.57	5.80
			Study 2		
1	1.11 [3]	none	4.25	0.44	5.80
2	1.62 [3]	none	3.74	0.44	5.80
3	2.14	none	3 22	0.44	5.80
4	3.22	none	2.14	0.44	5.80
5	4.28	none	1.08	0.44	5.80
6	5.36	none	0.00	0.44	5.80

[1] EAA = essential amino acids; SEAA = semi-essential amino acids; DAC = diammonium citrate.
[2] Composed of the following: (g/day) applesauce, 100; peaches, 100; pears, 100; green beans, 100; tomatoes, 100. Other foods given were: wafers made of cornstarch, fat, and sugar; carbonated beverage; jelly; candy; sugar; butter oil; tea or coffee, or both. A vitamin supplement was given daily and contained the following: (in mg) niacinamide, 9; thiamine·HCl, 3; riboflavin, 3; D-pantothenyl alcohol, 4.5; pyridoxine·HCl, 2.5; folic acid, 0.6; biotin, 0.15; vitamin B_{12}, 0.001; (study 1 only) DL-methionine, 150; dl-α-tocopheryl acetate, 4.5; choline dehydrogen citrate, 180; ascorbic acid, 50; and 4500 USP units vitamin A. A mineral supplement given daily contained the following: (in g) Ca, 1.000; P, 1.001; Mg, 0.199; Fe, 0.015; Cu, 0.002; I, 0.00015; Mn, 0.002; Zn, 0.0009.
[3] Additional methionine given at the 2 lower intakes of essential amino acid to meet the "safe" level of intake of Rose (6).

from the amino acids and diammonium citrate). In study 2 the amounts of the 8 essential amino acids fed were equivalent to those in 20, 30, 40, 60, 80, and 100 g of egg protein (plus additional methionine at the 2 lower intakes to meet the "safe" levels of Rose (6)) and provided 20, 30, 40, 60, 80 and 100%, respectively, of the purified nitrogen. To maintain a constant nitrogen intake, glycine and diammonium citrate in isonitrogenous amounts were given in all periods except those in which the essential amino acids plus the semi-essential amino acids, or the essential amino acids contributed 100% of the purified nitrogen. Throughout study 1 the caloric intake for each individual subject was held constant at the amount required for weight maintenance as determined prior to the study and varied from 42 to 52 kcal/kg among the different subjects. During study 2 all subjects were given daily 45 kcal/kg of body weight. The caloric intake was systematically varied during the addendum to study 2; all 6 subjects were fed the essential amino acids in amounts which contributed 20% of the nitrogen from purified sources with a caloric intake of 55 kcal/kg of body weight; 3 of the subjects were fed the essential amino acids in amounts which contributed 60% of the purified nitrogen with caloric intakes at both 35 and 55 kcal, while the other 3 subjects were fed the essential amino acids in amounts which supplied 100% of the purified nitrogen with caloric intakes at both 35 and 55 kcal. Additional calories were provided during the periods of increased caloric intake by butterballs (a mixture of butter oil and confectioner's sugar) or cornstarch wafers, or both. During the periods of reduced caloric intake sugar, hard candy, and butter oil were removed from the diets in appropriate amounts so as to maintain a constant fat-to-carbohydrate ratio during all periods.

Random arrangement of experimental diets. To eliminate the possible influence of order of presentation of the diets and the factor of time, the sequence of the diets fed during the experimental periods was arranged at random for each individual subject.

TABLE 2

Age, height, weight, and caloric intakes of the subjects

Subject	Age	Weight		Height	Daily caloric intake
		Initial	Final		
	year	*kg*	*kg*	*cm*	*kcal/kg body wt*
		Study 1			
A	21	58	58	168	52
B	20	75	76	185	42
C	34	72	71	178	42
D	20	74	74	183	44
E	21	72	70	190	42
		Study 2 [1]			
A	24	68	70	180	45
B	24	75	74	180	45
C	35	70	70	178	45
D	25	82	81	183	45
E	22	60	59	168	45
F	25	69	69	175	45

[1] These men were subjects during the addendum to study 2 also.

Subjects. The subjects were 11 young men, students at the University of Wisconsin; 5 participated in study 1 and 6 participated in study 2. Pertinent information regarding the subjects is presented in table 2. All were in good health as shown by physical examinations conducted by a physician.

Collection and handling of metabolic products. Complete 24-hour urine collections were made throughout the studies. Analyses for nitrogen (7) and creatinine (8) were made daily on fresh urine samples. Food composites and feces were collected, treated and analyzed for nitrogen as described by Jones et al. (9).

RESULTS

Average values for nitorgen balance per period for each subject as well as the mean for all subjects in both studies are shown in figures 1 and 2. Increasing the amounts of essential amino acids or essential amino acids plus the semi-essential amino acids resulted in an increase in nitrogen retention. These increases in nitrogen retention are statistically significant ($P < 0.005$ for study 1; $P < 0.01$ for study 2).

During study 1 each subject showed improved nitrogen retention with each step-by-step increase in essential amino acids plus semi-essential amino acids with the exception of subject B (fig. 1). All subjects showed marked increases in nitrogen retention between the lowest and highest intakes of essential amino acids plus semi-essential amino acids; increments in nitrogen retention were 1.45, 0.94, 1.29, 1.72, and 2.74 g/day for subjects A, B, C, D, and E, respectively. All subjects achieved positive nitrogen balance sometime during the experiment. Subjects A and B were in positive balance while consuming the 3 highest intakes of essential amino acids plus semi-essential amino acids, and all subjects were in positive balance at the 2 highest intakes.

In study 2 all subjects showed progressive increases in nitrogen retention as the nitrogen furnished by the essential amino acids was increased from 20 to 80% with increments in nitrogen retention being 1.09, 1.25, 0.80, 1.31, 0.56, and 2.29 g/day for subjects A, B, C, D, E, and F, respectively (fig. 2). However, the effect on nitrogen retention was less clearly defined when the percentage of nitrogen furnished by the essential amino acids was increased from 80 to 100%. Subjects A and B retained less nitrogen; subjects C, E, and F retained more; and subject D retained similar amounts. Subjects A, B, and D achieved positive nitrogen balance at a lower essential amino acid intake than did the other 3 subjects.

The caloric intake required to maintain nitrogen balance with an intake of 5.80 g nitrogen daily appears to be affected by the amount of essential amino acids fed (fig. 3). Increasing the caloric intake

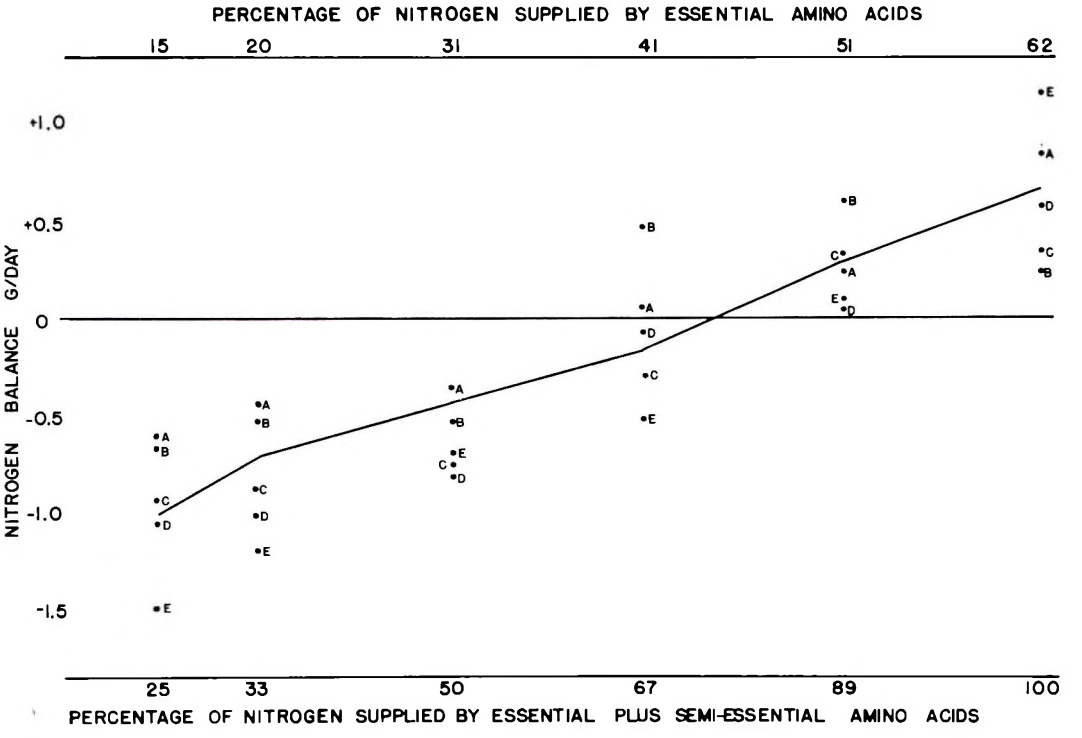


Fig. 1 Effect of varying the intake of essential plus semi-essential amino acids on nitrogen balance of men fed diets containing 5.80 g nitrogen daily. The percentage nitrogen indicated on the graph applies only to that furnished by crystalline amino acids and diammonium citrate and does not include the 0.57 g furnished by ordinary foods. The points with letters refer to the daily nitrogen balance of individual subjects; the line, to the mean daily nitrogen balance for all subjects.

from 45 to 55 kcal/kg of body weight resulted in average increments in nitrogen retention of 0.67 g daily when the essential amino acids contributed 20% of the purified nitrogen ($P < 0.025$); 0.40 g when the essential amino acids contributed 60%; and 0.06 g when the essential amino acids contributed 100% of the purified nitrogen. The latter 2 increments in nitrogen retention are not statistically significant.

DISCUSSION

The results of the studies described indicate that the nitrogen retention of men fed semi-purified diets supplying 5.80 g total nitrogen and approximately 45 kcal/kg of body weight daily is significantly affected by the amount of essential amino acids ingested. During both studies each step-by-step increase in essential amino acids resulted in improved nitrogen retention. When the semi-essential amino acids were included in the diet, increasing the intake of essential amino acid nitrogen from 0.81 to 3.22 g daily caused an average increment in nitrogen retention of 1.63 g daily; when the semi-essential amino acids were not included in the diet, increasing the essential amino acid nitrogen from 1.11 to 4.28 g resulted in an average increment in nitrogen retention of 1.22 g.

A comparison of the data from studies 1 and 2 indicates that the semi-essential amino acids may have an effect on nitrogen retention when the intake of the essential amino acids is high. Average nitrogen balances of subjects fed the essential amino acids plus the semi-essential amino acids in study 1 or the 8 essential amino acids in study 2 were similar at low intakes. For example, at the 20- and 30-g egg protein equivalents the average nitrogen balances during study 1 were −0.79

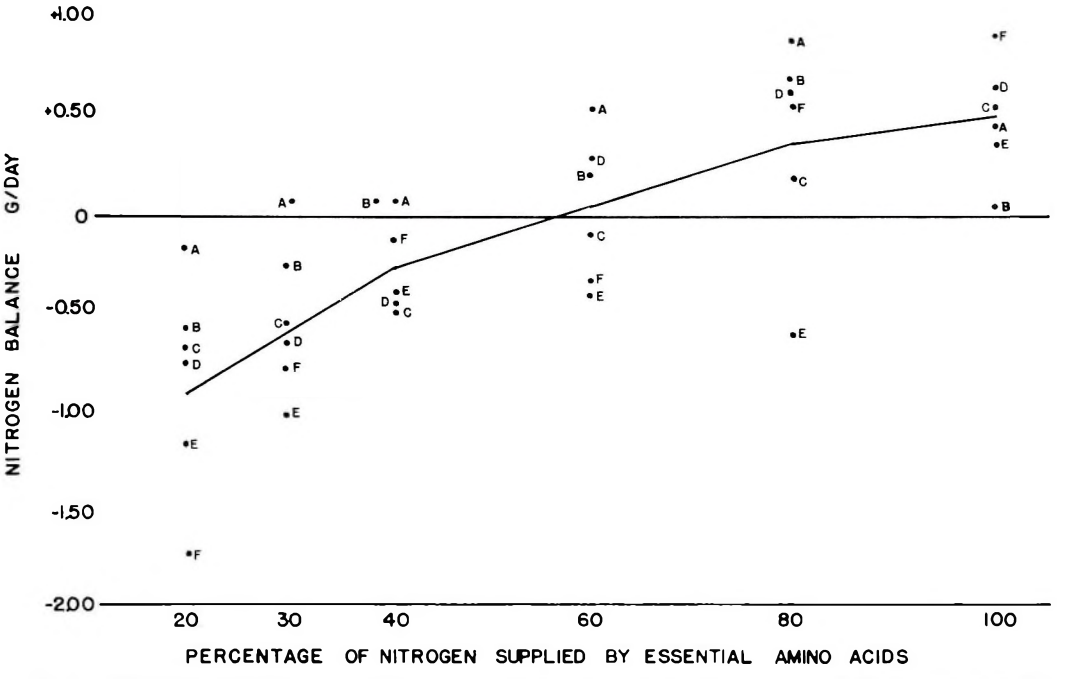


Fig. 2 Effect of varying the intake of essential amino acids on nitrogen balance of men fed diets containing 5.80 g nitrogen daily. The percentage nitrogen indicated on the graph applies only to that furnished by crystalline amino acids and diammonium citrate and does not include the 0.44 g furnished by ordinary foods. The points with letters refer to the daily nitrogen balance of the individual subjects; the line, to the mean daily nitrogen balance of all subjects.

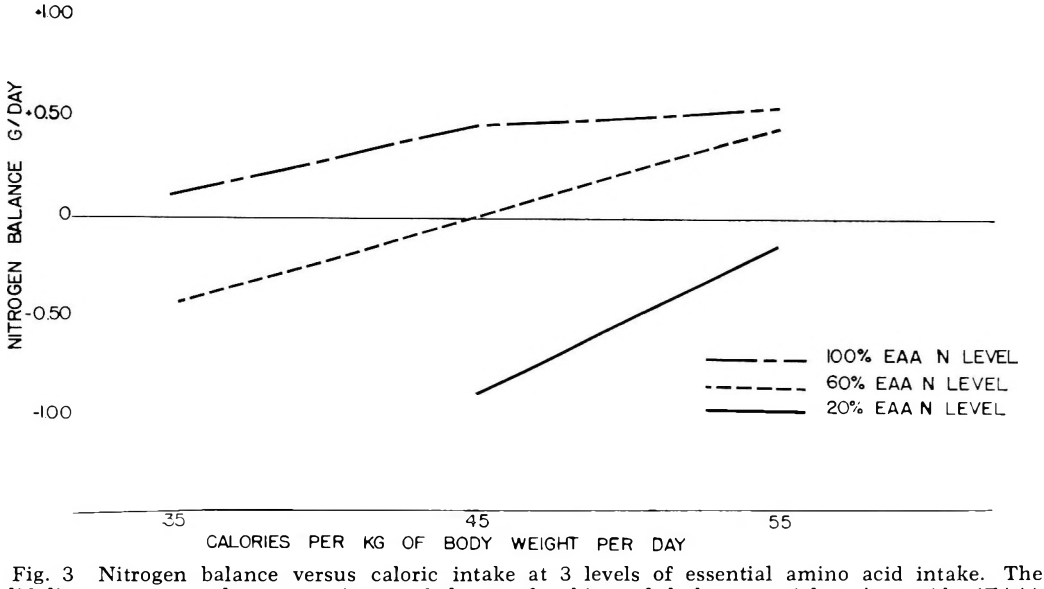


Fig. 3 Nitrogen balance versus caloric intake at 3 levels of essential amino acid intake. The solid line represents the mean nitrogen balance of subjects fed the essential amino acids (EAA) in amounts that furnished 20% of the nitrogen from purified sources; the evenly dashed line that of subjects fed the essential amino acids in amounts that furnished 60% of the purified nitrogen and the unevenly dashed line that of the subjects fed essential amino acids in amounts that furnished 100% of the purified nitrogen.

and −0.41 g, respectively, and in study 2 were −0.84 and −0.52 g. However, during high intakes, greater nitrogen retention was achieved by subjects fed the essential amino acids plus the semi-essential amino acids than by those fed the 8 essential amino acids. At the 60-g egg protein equivalent intake during study 1, subjects retained an average of 0.67 g nitrogen and in study 2 the average retained was 0.04 g. Although a higher intake of essential amino acids was used in study 2 than in study 1, the amount of nitrogen retained by the subjects in study 2 was never as great as that retained by the subjects in study 1.

Marked relationships appear to exist among caloric intake, essential amino acid intake and nitrogen retention. Increasing the caloric intake from 45 to 55 kcal/kg of body weight increased nitrogen retention by 0.67 g daily when the essential amino acids contributed 1.11 g nitrogen daily but had no effect when the essential amino acids furnished 5.36 g. Furthermore, subjects given 5.36 g essential amino acid nitrogen retained nitrogen (0.28 g daily) while ingesting 35 kcal/kg body weight, but subjects given 1.11 g essential amino acid nitrogen lost nitrogen (0.17 g daily) while ingesting 55 kcal/kg body weight.

The fact that the amount of essential amino acids ingested affects so markedly the caloric intake required to bring about a state of positive nitrogen balance suggests that a critical appraisal be made of the criteria used in assessing amino acid requirements. With a total daily nitrogen intake of 5.80 g and with a caloric intake of 35 kcal/kg of body weight, men retained nitrogen when given essential amino acids equivalent to those present in 100 g egg protein (5.36 g essential amino acid nitrogen). When the amounts of essential amino acids given were equivalent to those in 60 g egg protein (3.22 g essential amino acid nitrogen), 45 kcal/kg of body weight were required; and when the equivalent in 20 g egg protein (1.11 g essential amino acid nitrogen) was fed, more than 55 kcal/kg of body weight were required.

LITERATURE CITED

1. Stucki, W. P., and A. E. Harper 1962 Effects of altering the ratio of indispensable to dispensable amino acids in diets for rats. J. Nutrition, *78:* 278.
2. Snyderman, S. E., L. E. Holt, Jr., J. Dancis, E. Roitman, A. Boyer and M. E. Balis 1962 "Unessential" nitrogen: a limiting factor for human growth. J. Nutrition, *78:* 57.
3. Kirk, M. C., N. Metheny and M. S. Reynolds 1962 Nitrogen balances of young women fed amino acids in the FAO reference pattern, the milk pattern and the peanut pattern. J. Nutrition, *77:* 448.
4. Swendseid, M. E., J. H. Watts, C. L. Harris and S. G. Tuttle 1961 An evaluation of the FAO amino acid reference pattern in human nutrition. I. Studies with young men. J. Nutrition, *75:* 295.
5. Swendseid, M. E., C. L. Harris and S. G. Tuttle 1962 An evaluation of the FAO amino acid pattern in human nutrition. II. Studies with young women. J. Nutrition, *77:* 391.
6. Rose, W. C. 1957 The amino acid requirements of adult man. Nutrition Abstr. Rev., *27:* 631.
7. Scales, F. M., and A. P. Harrison 1920 Boric acid modification of the Kjeldahl method for crop and soil analysis. J. Ind. Eng. Chem., *12:* 350.
8. Peters, J. H. 1942 The determination of creatinine and creatine in blood and urine with the photoelectric colorimeter. J. Biol. Chem., *146:* 179.
9. Jones, E. M., C. A. Baumann and M. S. Reynolds 1956 Nitrogen balances of women maintained on various levels of lysine. J. Nutrition, *60:* 549.

Influence of Citrus Pectin Feeding on Lipid Metabolism and Body Composition of Swine

HOMER D. FAUSCH [1] AND THOMAS A. ANDERSON [2]
Animal Science Department, California State Polytechnic College, Pomona, California and Biochemistry Department, School of Medicine, University of South Dakota, Vermillion, South Dakota

ABSTRACT Pigs receiving 5% citrus pectin in the ration for 4 weeks had significantly higher serum cholesterol, triglyceride and phospholipid levels, more backfat and higher liver cholesterol levels than pigs receiving the control ration. The addition of 5% fat to the ration resulted in significantly higher serum and hepatic cholesterol and increased backfat. The inclusion of 5% pectin plus 5% added fat in the ration caused a highly significant increase in serum cholesterol, phospholipid and triglycerides, hepatic cholesterol and backfat thickness compared with that of the animals fed either fat or pectin alone. There appeared to be a definite synergistic effect to the addition of both fat and pectin to the ration. The increases in serum and hepatic lipid levels noted as the result of feeding pectin to swine are exactly opposite to the hypocholesterolemic activity of this compound which has been reported in man and the rat.

Citrus pectin has been shown by Keys et al. (1) to possess a slight but statistically significant hypocholesterolemic effect when administered to patients at a level of 15 g/day. Similarly, Wells and Ershoff (2) have reported that citrus pectin fed to male rats at a level of 5% of the diet was effective in counteracting the increased plasma and liver cholesterol levels induced by cholesterol feeding. Further studies by these authors (3) indicated that pectin feeding had essentially no effect on plasma and liver cholesterol levels in the cholesterol-fed rabbit, guinea pig and hamster.

Because of this apparent species difference in the effect of pectin on cholesterol metabolism, it seemed worthwhile to study the influence of this compound on the lipid metabolism of swine since this animal in many ways resembles man in nutrient requirements and digestive physiology. An additional objective of this study was to determine what effect pectin might have on counteracting the anticipated increase in serum and liver cholesterol reported by several workers (4–6) to result from the addition of fat to swine rations.

MATERIALS AND METHODS

Twenty Minnesota no. 3 weanling barrow swine were allotted at random to 4 pens, each with identical feeding and watering facilities. The 4 groups of 5 animals each were fed the rations shown in table 1. Pectin was added to rations C and D during the last 4 weeks of the 12-week feeding period. The computation of net energy values is difficult because it is not known to what extent pectin contributes to the caloric content of the ration. It has been suggested that colon bacteria can hydrolyze the partially methoxylated polygalacturonic acid chain of pectin, forming galacturonic acid and short-chain fatty acids (7). Since it has not been shown that appreciable absorption of these hydrolytic products occurs, no caloric value has been assigned to the 5% pectin added to rations C and D, although it is probable that pectin does make some small contribution to the net energy of the rations.

Body weights and blood samples via heart puncture were obtained initially and at 4, 8, 10 and 12 weeks. At the end of the experiment all animals were processed through the College meats laboratory at which time blood, liver, fat and muscle samples were obtained for lipid analysis, and the following carcass measurements obtained: back fat thickness, *longissimus*

Received for publication August 24, 1964.

[1] Animal Science Department, California State Polytechnic College, Pomona, California.

[2] Biochemistry Department, School of Medicine, University of South Dakota, Vermillion, South Dakota.

TABLE 1

Composition of experimental rations

	Control (A)	Control + 5% fat (B)	Control + 5% pectin (C)	Control + 5% fat + 5% pectin (D)
	% of ration	*% of ration*	*% of ration*	*% of ration*
Milo	69.5	62.8	62.8	57.4
Soybean meal	7.0	7.8	7.8	8.0
Cottonseed meal	7.0	7.9	7.9	8.1
Fish meal	2.5	2.5	2.5	2.5
Molasses	5.0	5.0	5.0	5.0
Dehydrated alfalfa meal	5.0	5.0	5.0	5.0
Trace mineralized salt [1]	0.5	0.5	0.5	0.5
Bentonite	2.0	2.0	2.0	2.0
Limestone flour	0.5	0.5	0.5	0.5
Vitamin mix [2]	1.0	1.0	1.0	1.0
Citrus pectin	—	—	5.0 [3]	5.0 [3]
Hydrogenated vegetable oil	—	5.0	—	5.0
	100.0	100.0	100.0	100.0
Proximate analysis of diet				
Crude protein, %	16.1	16.7	16.9	16.6
Ether extract, %	2.8	6.4	2.4	6.7
Estimated net energy/kg feed, kcal	1591	1729	1505	1643

[1] The percentage composition of the trace mineralized salt was: NaCl, 96.7–98.9; and not less than the following amounts of the remaining ingredients were used: manganese, 0.2; iron, 0.16; copper, 0.033; cobalt, 0.01; iodine, 0.007; and zinc, 0.005.

[2] The vitamin mix contained per 454 g: (in milligrams) vitamin B_{12}, 0.8; niacin, 750; riboflavin, 50; pantothenic acid, 250; choline, 5000; chlortetracycline, 1000; butylated hydroxy toluene, 5675; and (in grams) $ZnSO_4$, 136.2; and vitamin A, 150,000 IU, and vitamin D_3, 150,000 IU.

[3] Citrus pectin was added to the ration the last four weeks of the 12-week feeding period. This material was kindly provided by Mr. Grant Palmer, Research Division, Sunkist Growers, Corona, California.

dorsi area, ham weight and percentage of dressing. Serum cholesterol was determined according to the method of Pearson and associates (8), tissue cholesterol by a modification of the technique of Mann (9), triglycerides by the method of Van Handel and Zilversmit (10) and phospholipids according to the procedure of Zilversmit and Davis (11).

RESULTS AND DISCUSSION

The average initial serum cholesterol values between swine in the 4 pens were quite similar; however, by the eighth week the effect of feeding 5% added fat in rations B and D was becoming noticeable in a slight but nonsignificant increase in the serum cholesterol of the pigs receiving these rations (table 2). This observation is in agreement with the work of Barnes et al. (4, 5) and Reiser et al. (6) who have demonstrated an increase in the serum and tissue cholesterol levels of swine following the addition of fat to the ration. At the beginning of the eighth week citrus pectin was added to rations C and D (table 1). Two weeks later the animals receiving 5% pectin in the ration had significantly higher serum cholesterol levels than those pigs fed ration A ($P < 0.01$), with the pigs receiving fat plus pectin showing a significantly higher serum cholesterol level than those pigs fed pectin alone ($P < 0.01$). At the end of the 12-week experimental period, animals receiving the control ration had significantly lower serum cholesterol levels than those in any other treatment group ($P < 0.01$). Feeding pectin had approximately the same effect on serum cholesterol levels as feeding 5% added fat. Feeding 5% added fat plus 5% pectin resulted in a marked increase in serum cholesterol compared with the effect of feeding 5% added fat alone.

Serum triglyceride and phospholipid determinations on blood taken at the time of slaughter indicated an almost identical pattern to that of serum cholesterol levels (table 2). The pigs receiving pectin in

TABLE 2

Influence of feeding citrus pectin and fat on serum and tissue lipid levels of swine

Variable	Ration				SE of mean
	A	B	C	D	
Serum cholesterol, mg/100 ml					
Initial	94	90	91	98	± 6.3
8 weeks	99	108	100	107	± 9.7
10 weeks	103	112	122	141 **	± 5.1
Final	91	109	107	144 **	± 6.6
Serum triglycerides, mg/100 ml	30.5	32.5	41.0	47.3 **	± 3.43
Serum phospholipids, mg/100 ml	76.0	94.0	99.6	113.6 *	± 11.1
Adipose tissue cholesterol, mg/g	0.269	0.260	0.241	0.269	± 0.0150
Muscle cholesterol, mg/g	0.570	0.544	0.577	0.600	± 0.0166
Liver cholesterol, mg/g	3.01	3.29	3.25	3.39 *	± 0.075
Liver triglycerides, mg/g	5.08	4.69	4.50	4.51	± 0.565
Liver phospholipids, mg/g	16.10	20.71	19.52	20.69	± 1.880

* $P < 0.05$.
** $P < 0.01$.

the ration, either alone or with 5% added fat, had significantly elevated serum triglyceride and phospholipid levels compared with those of the control animals or with those of pigs fed 5% added fat in the ration ($P < 0.01$).

The response of tissue lipid levels to the various treatments was less pronounced. Adipose tissue and muscle cholesterol levels were unaffected by the inclusion of fat or pectin in the ration. Phospholipid and triglyceride determinations were not made on these tissues.

The hepatic cholesterol level of the pigs receiving both pectin and fat (ration D) was significantly greater than that of the control animals ($P < 0.05$) but not significantly different from pigs fed 5% added fat or 5% pectin in the ration. No treatment differences were noted in the liver triglyceride or phospholipid levels.

Body composition was altered by the inclusion of fat and pectin into the ration, although body weight gain, feed consumption and feed efficiency for the 4 treatment groups were essentially identical throughout the 12-week feeding period (table 3). Measurements of backfat thickness closely paralleled the serum lipid levels, with the pigs fed ration D having significantly thicker backfat than those in any other treatment group ($P < 0.01$) and the control animals (ration A) having significantly less backfat than those in the other groups ($P < 0.01$). As noted with serum cholesterol the backfat of the pigs fed either 5% added fat or 5% pectin did not differ significantly from each other. Tanner (12) has reported a similar relation between serum cholesterol and physique in young men, finding a statistically significant relationship between choles-

TABLE 3

Influence on feeding citrus pectin and fat on body composition of swine

Variable	Ration				SE of mean
	A	B	C	D	
Body weight, kg					
Initial	23.6	23.6	23.6	23.6	± 0.50
Final	100.2	103.0	99.3	99.3	± 3.36
Average daily gain, kg	0.93	0.94	0.90	0.90	± 0.014
Feed conversion, kg feed/kg gain	3.54	3.46	3.76	3.55	— [1]
Backfat thickness, mm	31.24	34.80	37.08	39.37 **	± 0.940
Longissimus dorsi area, cm^2	23.35	22.45	22.84	20.13	± 1.360
Ham weight, kg	8.2	8.0	8.1	8.0	± 0.24
Dressing, %	72.9	74.3	73.8	74.4	± 0.57

** $P < 0.01$.
[1] Not applicable, pigs fed in groups of 5.

terol level in the blood and subscapular subcutaneous tissue thickness.

The *longissimus dorsi* area followed closely the pattern of backfat thickness, with the leanest animals (ration A) having the largest *longissimus dorsi* area and the fattest pigs (ration D) having the smallest. Although there was a 3.22-cm^2 difference in *longissimus dorsi* area between the control group and the pigs receiving ration D, the difference was not statistically significant.

Pigs receiving fat in the ration (rations B and D) had slightly lighter hams and a slightly higher percentage of dressing.

Taking into consideration the normal biological variation usually noted in a group of 20 animals such as those used in this study, it is unusual that so many parameters of lipid metabolism were so significantly altered by the treatments imposed. This is particularly unexpected when the marked effects of pectin feeding on lipid levels reported here are in a direction exactly opposite to those reported previously in man (1) and rats (2). It appears that although feeding pectin to swine increases the levels of serum and tissue lipids, the feeding of fat plus pectin results in a synergistic response much higher than noted with the feeding of either fat or pectin alone. The possibility that the differing calorie-protein ratios of the rations may have had some influence on the results of the experiment does not appear to be significant, because the 2 rations containing pectin (rations C and D), which were fed for the last 4 weeks of the experiment, actually had a lower energy-to-protein ratio than comparable rations with no added pectin. The pigs receiving these lower energy rations might have been expected to be leaner and have lower serum cholesterol levels than animals fed ration B but the opposite effect was noted. The validity of this explanation, however, rests on the assumption that pectin does not make an appreciable contribution to the energy content of rations C and D.

The results of this experiment indicate that there is a definite species difference in the effect of pectin on lipid metabolism. Wells and Ershoff (3) suggest that the "anticholesterol" effect of pectin may be confined to nonherbivorous species since the hamster, guinea pig and rabbit do not respond to pectin feeding by a lowering of serum or liver cholesterol levels. These workers reported that the cholesterol-fed rabbit actually exhibited slight but nonsignificant elevations in plasma and liver total cholesterol when fed 5% citrus pectin (3). Since the pig is an omnivore, it was thought that the response of lipid levels to the feeding of pectin, in this animal, would be similar to that observed in man; however, the rather unexpected results of this study have shown this theory to be without foundation.

For pectin to exert such a divergent action on lipid metabolism, depending upon the species involved, suggests the need for future investigations on the mechanism of action by which these species differences might be mediated.

LITERATURE CITED

1. Keys, A., F. Grande and J. T. Anderson 1961 Fiber and pectin in the diet and serum cholesterol concentration in man. Proc. Soc. Exp. Biol. Med., *106:* 555.
2. Wells, A. F., and B. H. Ershoff 1961 Beneficial effects of pectin in prevention of hypercholesterolemia and increase in liver cholesterol in cholesterol-fed rats. J. Nutrition, *74:* 87.
3. Wells, A. F., and B. H. Ershoff 1962 Comparative effects of pectin N.F. administration on the cholesterol-fed rabbit, guinea pig, hamster and rat. Proc. Soc. Exp. Biol. Med., *111:* 147.
4. Barnes, R. H., E. Kwong, G. Fiala, M. Rechcigl, R. N. Lutz and J. K. Loosli 1961 Dietary fat and protein and serum cholesterol, I. Adult swine. J. Nutrition, 69: 261.
5. 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.
6. Reiser, R., M. F. Sorrels and M. C. Williams 1959 Influence of high levels of dietary fats and cholesterol on atherosclerosis and lipid distribution in swine. Circ. Res., *7:* 833.
7. Reviews and News of Citrus Pharmaceuticals in Health and Disease 1963 Sunkist Growers, Ontario, California. Nutritional significance of citrus pectin, 2(1): 2.
8. Pearson, S., S. Stern and T. H. McGavack 1953 A rapid, accurate method for the determination of total cholesterol in serum. Analyt. Chem., *25:* 813.

9. Mann, G. V. 1961 A method for measurement of cholesterol in blood serum. Clin. Chem., 7: 275.
10. Van Handel, E., and D. Zilversmit 1957 Micromethod for the direct determination of serum triglycerides. J. Lab. Clin. Med., 50: 152.
11. Zilversmit, D.. and A. Davis 1950 Microdetermination of plasma phospholipids by trichloroacetic acid precipitation. J. Lab. Clin. Med., 35: 155.
12. Tanner, J. M. 1951 The relation between serum cholesterol and physique in healthy young men. J. Physiol., *115:* 371.

The Absence of Thyroid Hormones in a Growth Factor of Duodenal Powder [1]

C. J. ACKERMAN
Department of Biochemistry and Nutrition, Virginia Polytechnic Institute, Blacksburg, Virginia

ABSTRACT Three fractions prepared from the acid-insoluble residue after acid hydrolysis of duodenal powder were effective in reinstating growth of growth-arrested, sulfaguanidine-fed rats. One fraction was studied more extensively than the others to determine whether the growth response to this fraction was due to thyroxine or triiodothyronine. The stability of this fraction to acid hydrolysis, its insolubility in acid, its behavior on paper chromatograms and its failure to react with ninhydrin contrasted with the behavior of thyroxine or triiodothyronine. It was therefore concluded that duodenal powder contains an unidentified substance effective in reinstating growth of growth-arrested, goitrogen-fed rats.

Previous reports (1, 2) have demonstrated that dietary duodenal powder is effective in reinstating growth of growth-arrested, goitrogen-fed rats. A water-soluble fraction prepared from enzymatically hydrolyzed duodenal powder reinstated growth of growth-arrested thyroidectomized, hypophysectomized, sulfaguanidine-fed or thiouracil-fed rats (3). Although the data suggested that the observed growth responses were not due to thyroid hormones, unequivocal evidence for the presence or absence of thyroid hormones in this fraction was needed.

The present paper reports evidence that the growth response of growth-arrested sulfaguanidine-fed rats produced by a fraction obtained from duodenal powder is not due to thyroxine or triiodothyronine. In addition, certain observations suggest that more than one growth factor is present in duodenal powder.

EXPERIMENTAL

Materials and methods. "Active" duodenal powder, "inactive" duodenal powder, and thyroid powder (3 × USP) were obtained from commercial sources.[2] The "active" material was the same material as that used in earlier experiments (2) and when fed at a level of 4 g/100 g of diet, the 2-week growth response of growth-arrested, sulfaguanidine-fed rats was 39 to 49 g. Another preparation of duodenal powder which had no ability to reinstate growth was defined as "inactive." When tested under the same conditions as the active material, the 2-week growth response to the inactive powder was − 1 to + 1 g/2 weeks. This material was used as a diluent of thyroid powder and of triiodothyronine to provide the same experimental conditions when the stability of thyroid hormones was being compared with that of the growth factor of the "active" duodenal powder. L-Thyroxine (T_4) and 3, 5, 3′-triiodo-L-thyronine (T_3) were recrystallized from hot HCl (4, 5); mp 231 to 233° and 202 to 204°, respectively. These were dried over H_2SO_4. All solvents were reagent or C.P. grade, but solvents used for paper chromatography were distilled once. Acidified methanol: 95 ml of methanol plus 5 ml of 2 N HCl; acidified acetone: 95 ml of acetone plus 5 ml of 2 N HCl.

Paper chromatography was conducted in the dark in an all-glass chromatography jar at 5° (descending) unless otherwise specified. Whatman 3 MM paper was used throughout these experiments. Three solvents were used: solvent 1, 0.1 N HCl; solvent 2, methanol:0.2 M ammonium acetate adjusted to pH 4.5 with acetic acid (1:1); solvent 3, acetone:0.2 M ammonium acetate adjusted to pH 4.4 with

Received for publication August 6, 1964.

[1] Supported in part by a grant, G13165, from the National Science Foundation, Washington, D.C.

[2] "Active" duodenal powder purchased from Cudahy Laboratories, Omaha, Nebraska. "Inactive" duodenal powder was a gift from Wilson Laboratories, Chicago. Thyroid powder was purchased from Nutritional Biochemicals Corporation, Cleveland.

acetic acid (4:6). The fractions obtained from duodenal powder were streaked evenly in a band from edge to edge because the nature of these fractions was such as to retard the solvent flow. When the samples were applied as spots or in bands shorter than the width of the paper, the solvents tended to encircle, or by-pass the samples.

Iodinated compounds on the chromatograms were detected by the ceric sulfate-arsenious acid technique of Kono et al (6).

Growth - arrested, sulfaguanidine - fed male rats of the Sprague-Dawley strain (2) were used for the growth assays. Such animals may vary in body weight from 100 to 160 g, but their body weight gain is negligible after the fifth week of goitrogen feeding (2). For example, 50 rats selected at random from 600 rats so treated, gained an average of + 0.34 g ± 2.98 (SD) in 2 weeks. The maximal gain of any rat in this group was + 5 g/2 weeks (one rat). Therefore it was feasible to use one rat per assay to assess qualitatively the growth activity of the various fractions. Unless otherwise specified, the sample to be assayed (usually a methanolic solution) was mixed into 15 to 20 g of the diet containing sulfaguanidine (2), air-dried for 3 to 5 hours, and then fed ad libitum to one rat until it was consumed. Feeding of the usual sulfaguanidine-containing diet was then resumed, and the body weight gain at the end of 14 days was used to assess the potency of the growth factor in the sample tested.

Stability to HCl and NaOH hydrolysis. For these experiments, a definite quantity of the material to be assayed was fed to one rat in 100 g of diet. This was consumed in 11 to 12 days. The usual sulfaguanidine-containing diet was then fed for the remainder of the 14-day period. In this way, the effect of the growth factor in duodenal powder could be directly compared with that of a definite quantity of thyroid powder or T_3. Also, the recovery of the growth factor or thyroid hormones after acid hydrolysis could be evaluated by comparing the growth responses to the hydrolyzed samples with the growth responses to the samples before hydrolysis.

Active duodenal powder (12 g) mixed into 288 g of the sulfaguanidine diet was assayed with 3 rats. Twenty-four grams of active duodenal powder were refluxed for 22 hours with 100 ml of 5 N HCl. The cooled hydrolysate was filtered and the insoluble residue was washed twice with 10 ml of 1 N HCl. One-half of this residue mixed into 300 g of diet was assayed with 3 rats. The other half of the insoluble residue was refluxed with 8 ml of 2 N NaOH for 16 hours. The entire hydrolysate was neutralized with HCl, mixed into 300 g of diet and assayed with 3 rats.

Three levels of thyroid powder, 135, 270 and 405 mg, were each diluted to 36 g with "inactive" duodenal powder. These were then treated in exactly the same way as was the active duodenal powder, namely, 12 g of the mixture were assayed and 24 g were hydrolyzed with HCl as described above. Hydrolysis with NaOH was omitted. Thus, each rat consumed 100 g of diet containing 15, 30 or 45 mg of thyroid powder or the acid-insoluble fraction obtained from these quantities of thyroid powder. In addition to the above, 315 mg of thyroid powder in 24 g of "inactive" duodenal powder were hydrolyzed with HCl, washed and filtered as described above. One-half of the insoluble residue in 300 g of diet was assayed with 3 rats. Also, 30 μg of T_3 were mixed with 36 g of inactive duodenal powder. Twelve grams in 288 g of diet were assayed with 3 rats (3.3 μg of T_3/rat). Twenty-four grams were hydrolyzed with HCl, filtered, and one-half of the insoluble residue was assayed as described above.

Preparation of the growth factors. Four hundred grams of active duodenal powder were refluxed for 22 hours in 3000 ml of 5 N HCl. The cooled hydrolysate was filtered through paper and the acid-insoluble residue was air-dried for 16 hours and then extracted with acetone in Soxhlet extractors for 6 hours. An equal volume of benzene was added to the acetone extract. This mixture separated into 2 phases with a black precipitate at the interface. After separating the precipitate and the lower phase from the benzene (upper) phase, the benzene phase was washed 3 times with 30 ml of 10% aqueous acetone and then discarded.

All the washings and the insoluble material were combined with the lower

phase. This was evaporated to a black viscous oil at reduced pressure and then dissolved by adding 2 N NaOH until the pH was approximately 12. It was diluted to 2 liters with water and then filtered through paper. The insoluble material was discarded. The filtrate was acidified to pH 1 with HCl, cooled to room temperature and then filtered through paper. The acid-insoluble residue was again dissolved in NaOH and the solution was acidified to pH 5 with acetic acid. After filtering, the precipitate was air-dried for 16 hours and then extracted in a Soxhlet extractor with 95% ethanol for 6 hours. The amber alcohol extract was evaporated to dryness. The residue was dissolved in 100 ml of 0.1 N NaOH, which was then acidified to pH 10 with acetic acid, cooled overnight and filtered. The filtrate was acidified to pH 8 with acetic acid, cooled overnight and filtered. The pH 8 precipitate was dissolved in 100 ml of acidified methanol (fraction 1), and 2 ml were assayed with one rat.

After fraction 1 had been allowed to stand for one week at room temperature in a well lighted room, evaporation of the methanol left a residue which was no longer completely soluble in NaOH. Therefore, the alkaline solution was acidified to pH 4 with acetic acid and extracted with an equal volume of benzene, and 3 times with one-half volumes of benzene. One forty-ninth of this solution was assayed with one rat. Also, another aliquot in 10 ml of methanol:acetate buffer, pH 5 (1:1) was heated for 10 minutes in a boiling water bath with 5 mg of ninhydrin. This was assayed with one rat.

After the pH 8 precipitate (fraction 1) was removed by filtration, the filtrate was adjusted to pH 6 with acetic acid, cooled overnight at 5° and then filtered. The red precipitate was extracted for 6 hours with methanol in a Soxhlet extractor. This was fraction 2 in a volume of 130 ml. When air-dried, 1 ml of this solution contained 14 mg of an oily black residue. The final steps of the isolation procedure, beginning with the pH 5 precipitate, are illustrated in figure 1. Also shown on figure 1 is the subsequent division of fraction 2 into 2 active fractions, which was observed after the tests described below were conducted on fraction 2.

Three milliliters of the solution of fraction 2 were effective in reinstating growth of one growth-arrested rat. Therefore, multiples of 3-ml aliquots of fraction 2 were treated as follows and then assayed with the appropriate number of rats so that the effect of the treatment could be evaluated by comparing the growth responses before and after treatment:

(a) Two 3-ml aliquots were evaporated to dryness in test tubes to which were added 2 ml of 2 N NaOH and 1 mg of thiouracil as an antioxidant (7). The tubes were evacuated with a water aspirator and filled with nitrogen 3 times. The tubes were sealed and heated in a boiling water bath for 16 hours. The hydrolysates were neutralized with HCl and then assayed with 2 rats. This entire procedure was

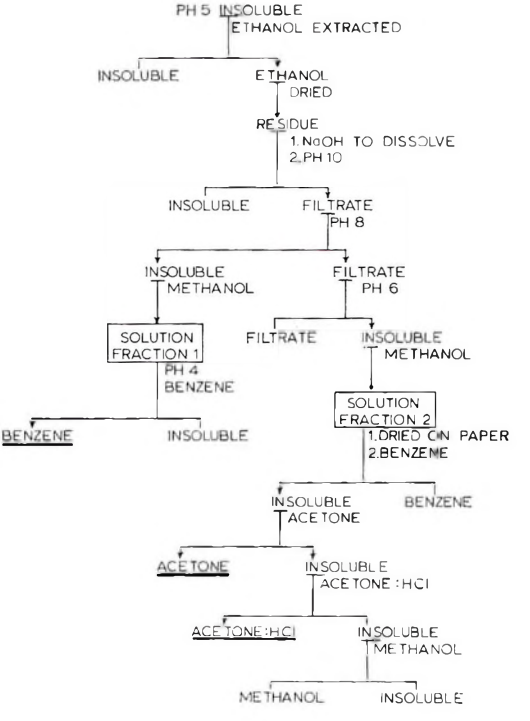


Fig. 1 A diagram of the preparation of Fractions 1 and 2 from the pH 5 precipitate which was obtained from the acid-insoluble residue of acid-hydrolyzed duodenal powder. Further treatment of Fractions 1 and 2 resulted in 3 fractions (heavy underline) which were effective in reinstating growth of growth-arrested, sulfaguanidine-fed rats.

repeated except that the evacuation and filling of the tubes with nitrogen were omitted.

(b) Six milliliters were diluted to 12 ml with methanol to which were added 50 mg of palladium black. This was shaken for 7 hours at room temperature with hydrogen at a pressure of 2.8 kg/cm^2. The methanol was decanted and the palladium was washed 3 times with 5 ml of acidified methanol. The methanol extracts were combined and assayed with 2 rats.

(c) The growth response to 3 ml of fraction 2 was comparable to that produced by 5 μg of T_4[3] or 3.3 μg of T_3. These quantities are readily soluble in dilute HCl (8) and washing fraction 2 with HCl may be expected to decrease its activity if these hormones are present. Therefore, 9 ml of fraction 2 were evaporated to dryness, and the residue was dissolved in 9 ml of 0.1 N KOH. To this was added 1 ml of 12 N HCl. The precipitate was redissolved in KOH and precipitated with acid 2 times, and the last HCl solution, containing the precipitate, was extracted once with an equal volume of benzene. The yellow benzene and the acid extracts were discarded. The precipitate, dissolved in methanol, was assayed with 3 rats.

(d) If present in fraction 2, thyroid hormones could be bound in a form resistant to acid hydrolysis. To test this possibility and demonstrate the solubility of thyroxine in HCl, 6 ml of fraction 2, and 6 ml of fraction 2 to which had been added 10 μg of T_4, were evaporated to dryness in separate test tubes. These were hydrolyzed in 4 ml of 2 N NaOH as described in experiment (a) above. The alkaline hydrolysates were acidified to pH 1 with HCl and centrifuged. The precipitates were dissolved in 9 ml of 0.1 N NaOH and reprecipitated with HCl. This was repeated once. The combined HCl extracts and the insoluble precipitates were assayed with 2 rats for each fraction.

(e) Two 3-ml aliquots were each applied onto 2 large sheets of filter paper (56 × 47 cm) in bands 56-cm long (0.05 ml of sample/cm). On 2 separate strips 2-cm wide, 2 μg of T_4 and 2 μg of T_3 in 0.11 ml of fraction 2 were applied in bands 2-cm wide. The chromatograms were developed with solvent 1 at room temperature for 16 hours. The T_4 and T_3 were located on the narrow strips with ceric sulfate-arsenious acid.

The narrow orange bands at the origin of the large chromatograms were cut off, eluted with methanol in Soxhlet extractors for 3 hours and the methanol extracts were assayed with 2 rats. The remainder of the chromatograms (R_f 0.03 to 1.0) were also eluted with methanol and the extracts were assayed with 2 rats. This procedure was repeated except that 10 μg of T_4 were co-chromatographed with 6 ml of fraction 2 to demonstrate that the growth factor could be separated from thyroid hormones in this manner.

(f) Experiment (e) was repeated except that the chromatograms were developed with solvent 2 and then divided into 4 sections each of which were eluted with acidified methanol. The extracts were assayed with 2 rats each.

(g) Three milliliters on filter paper in a band 32-cm wide were developed with solvent 3. Three sections of the chromatogram were extracted and each extract was assayed with one rat.

(h) Three milliliters in a band 32-cm wide on filter paper were developed with solvent 3. The section R_f 0.85 to 1.0 was sprayed on both sides with 0.3% ninhydrin in acetone. When the paper had dried, it was again sprayed on both sides with ninhydrin and then allowed to stand 20 hours at room temperature. The section R_f 0.85 to 1.0 was cut off, eluted with acidified methanol, and the extract was assayed with one rat

(i) The residue from 3 ml was dissolved in methanol:0.1 N acetate buffer, pH 5 (1:1). To this were added 5 mg of ninhydrin which was heated for 10 minutes in a boiling water bath. The red solution was assayed with one rat.

(j) Fifteen micrograms of T_4 on filter paper (10 × 5 cm) were treated with ninhydrin as described above (exp. (h)). The paper was extracted with acidified methanol which was then assayed with one rat. Also, 400 μg of T_4 treated with ninhydrin as described in experiment (i) above, were assayed with 2 rats.

(k) After drying 15 ml of fraction 2 on filter paper (10 × 15 cm), the paper was

[3] Unpublished experiments.

extracted for 3 hours with benzene in a Soxhlet extractor. The benzene was removed and when the paper was air-dried, it was extracted with acetone for 3 hours. This was followed by a 3-hour extraction with acidified acetone and finally by a 3-hour extraction with methanol. Two-fifths of each extract were assayed with 2 rats each. The preparation of these fractions is illustrated in figure 1.

RESULTS

Because of the limited number of rats used in the experiment summarized in table 1, it was not intended to quantitatively assess the growth response to duodenal powder, thyroid powder or T_3. However, the results show that 4 g of duodenal powder produced a growth response approximately equivalent to that produced by 15 mg of thyroid powder (3 × USP) or 3.3 μg of T_3. If duodenal powder contained thyroid hormones in amounts 3 times that amount present in 15 mg of thyroid powder, they would not be recovered in the acid-insoluble residue after acid hydrolysis. The acid-insoluble residue from 45 mg of thyroid powder produced little, if any, growth response, whereas the acid-insoluble residue from 4 g of duodenal powder produced a growth response almost equivalent to that of the untreated duodenal powder. The acid-insoluble residue from the higher level of thyroid powder (52 mg) did retain some ability to reinstate growth but triiodothyronine at the level tested, was not recovered after acid hydrolysis.

The growth factor in the acid-insoluble residue obtained from active duodenal powder was partially destroyed by NaOH hydrolysis. The growth response of 3 rats to the alkaline hydrolysate (see Experimental section) was 15, 19 and 22 g/2 weeks. Since the acid-insoluble residues obtained from thyroid powder were inactive, NaOH hydrolysis of these fractions was omitted.

After acid hydrolysis of active duodenal powder, 2 fractions effective in reinstating growth were recovered from the acid-insoluble residue. Table 2 summarizes the growth response to these fractions before and after various treatments. Fraction 1 (precipitated at pH 8) became soluble in benzene (fig. 1) and this fraction lost its ability to reinstate growth after it had been treated with ninhydrin. Further work on this fraction will not be reported here. Emphasis was placed on fraction 2 (the pH 6 precipitate) to determine whether the growth response produced by this fraction was caused by thyroid hormones.

The instability of the growth factor to alkaline hydrolysis suggested a method for distinguishing the growth factor from the thyroid hormones which are relatively stable to alkaline hydrolysis under the proper conditions. However, fraction 2 was stable to alkaline hydrolysis when air was excluded although it lost all activity when air was present (table 2, (a)). This, and its instability to catalytic reduction (table 2, (b)) did not distinguish the growth factor from the thyroid hormones.

TABLE 1

Growth response of growth-arrested [1] rats to acid-hydrolyzed duodenal powder, thyroid powder and triiodothyronine

Sample fed/rat	Results [2]	
	Untreated	HCl-hydrolyzed acid-insoluble [3]
	g/2 weeks	*g/2 weeks*
4 g duodenum	39, 40, 45	32, 36, 38
15 mg Thyroid powder [4]	40, 44, 49	0, 2, 3
30 mg Thyroid powder [4]	48, 49, 51	2, 2, 5
45 mg Thyroid powder [4]	44, 53, 59	6, 8, 10
52 mg Thyroid powder [4]	—	15, 23, 25
3.3 μg Triiodothyronine [4]	36, 44, 47	0, 3, 4

[1] No rat gained more than 4 g in the 2 weeks prior to these experiments.
[2] Each value represents the body weight gain of one rat that had been fed the indicated sample in 100 g of diet.
[3] The acid-insoluble residue after duodenal powder, or thyroid powder mixed with "inactive" duodenal powder, was boiled for 22 hours with 5 N HCl.
[4] Thyroid powder and triiodothyronine diluted with an inactive preparation of duodenal powder, were mixed into the sulfaguanidine-containing diet. This was consumed in 11 to 12 days.

However, little growth activity was lost after extraction of fraction 2 with HCl and benzene (table 2, (c)) and it was concluded that neither T_4 nor T_3 was present in a free form. That neither T_4 nor T_3 were present in a bound form was demonstrated when alkaline hydrolysis, and washing with acid failed to decrease the growth activity of the acid-insoluble residue (table 2, (d)). Thyroxine added to this fraction before alkaline hydrolysis was recovered in the acid-soluble extract, demonstrating that it would have been removed from fraction 2 if it had been present.

That the growth factor differs from T_4 and T_3 was also demonstrated by paper chromatography. After 16 hours' development with solvent 1, the solvent front had moved off the end of the paper, but T_4 and T_3 had moved 20 and 33 cm, respectively, from the origin (fig. 2). However, the R_f of the growth factor was zero, and 10 μg of T_4 co-chromatographed with fraction 2 were recovered in the area R_f 0.03 to 1.0 (table 2, (e)).

With solvent 2, the R_f of the growth factor was again zero and T_4 added to fraction 2 was recovered in R_f section 0.3 to 0.65 (fig. 2 and table 2, (f)).

When fraction 2 was developed on paper chromatograms with solvent 3, an orange band moved with the solvent front, and this contained all of the growth activity of the sample (table 2, (g)). The R_f's of T_4 and T_3 with this solvent were 0.80 and 0.85, respectively, but when another chromatogram developed with this solvent was sprayed with ninhydrin, the growth response (table 2, (h)) indicated that the growth factor was neither T_4 nor T_3, and the growth factor probably contains no free amino group. The reaction with ninhydrin was tested again by treating fraction 2 in solution with ninhydrin (table 2 (i)), and again the growth response indicated that the growth factor was neither T_4 nor T_3. Large excesses of T_4 on paper or in solution were readily destroyed by ninhydrin (table 2, (j)).

As shown in figure 1 further purification of fraction 2 by extraction with various solvents produced 2 additional active fractions (table 2 (k)). The benzene extract was yellow but inactive. The acetone extract was a bright yellow solution and elicited a growth response somewhat weaker than that of the original sample. It was sufficiently pure to be applied in a band 3.5-cm wide on a paper chromatogram, and when developed with solvent 2, no iodine could be detected (fig. 2). The acetone:HCl extract produced a growth response comparable to that of the original sample, but it could not be chromatographed in a sufficiently small area to determine whether iodine was present in this fraction. A weak response was also obtained with the methanol extract. It was observed that the sum of the growth effects produced by the separate fractions was greater than that of the original sample, which suggested that 2 active substances were present in fraction 2.

DISCUSSION

That more than one fraction, capable of reinstating growth of growth-arrested rats, was present in duodenal powder was re-

Fig. 2 Paper chromatograms of Fraction 2, thyroxine and of triiodothyronine. Solvent front: the top edge of the paper except as indicated. 0: origin. The growth factor remained at the origin of all chromatograms shown. 1. Thyroxine (2 μg) co-chromatographed with 0.11 ml of Fraction 2 using solvent 1 (0.1 N HCl), 16 hours. 2. Triiodothyronine (2 μg) co-chromatographed with 0.11 ml of Fraction 2 using solvent 1, 16 hours. The solvent front of 1 and 2 is beyond the top edge of these strips. Residual chloride from the solvent also reacted with ceric sulfate-arsenious acid (6) and produced a streaked, mottled appearance on 1 and 2. 3. Thyroxine (2 μg) co-chromatographed with 0.15 ml of Fraction 2 using solvent 2 (methanol:0.2 M ammonium acetate, pH 4.5; 1:1). The slower moving substance is thyroxine. The origin and the 3 sections indicated by the horizontal lines to the right of this chromatogram, were those which were cut out, eluted and assayed as described in the text (also table 2, (f)). 4. This is the image of chromatogram 3. By the technique of Kono et al. (6), the chromatogram, pressed onto another strip of paper saturated with ceric sulfate-arsenious acid, leaves an image of all iodinated compounds on this paper. The band at the origin which is in reality orange, leaves no image, thus demonstrating the absence of detectable amounts of iodine at the origin. This was also true of chromatograms 1 and 2. 5. The acetone extract of Fraction 2 (table 2, (k)) developed with solvent 2. 6. The image of a chromatogram on which 1 μg of triiodothyronine was co-chromatographed with the acetone extract of Fraction 2 using solvent 2. The band at the origin (as seen on 5) was again not detectable indicating that the band at the origin does not contain iodine.

Figure 2

TABLE 2

Growth response of growth-arrested [1] rats to the growth factors of duodenal powder and to thyroxine after various treatments

Treatment	Results [2]		
	Growth factor with T_4 [3]	Growth factor without T_4	Without growth factor
	g gained/2 weeks	*g gained/2 weeks*	*g gained/2 weeks*
Assay of fraction 1 (2 ml assayed/rat)			
None		32	
Benzene extract of fraction 1		27	
Benzene extract plus 5 mg of ninhydrin		5	
Assay of fraction 2 (3 ml assayed/rat)			
None		40, 43	
(a) NaOH hydrolysis under nitrogen		38, 39	
NaOH hydrolysis under air		0, 4	
(b) Reduced with hydrogen-palladium		minus 2, 4	
(c) Extracted with HCl and benzene		32, 35, 40	
(d) NaOH hydrolysis under nitrogen [4]			
HCl-soluble	22, 26	minus 2, minus 3	
HCl-insoluble	32, 36	34, 41	
(e) Paper chromatography, solvent 1 [5]			
R_f 0.0	39,43	35, 38	
R_f 0.03–1.0	29, 33	10, 12	
(f) Paper chromatography, solvent 2 [6]			
R_f 0.0	34, 38	40, 42	
R_f 0.05–0.3	9, 14	3, 8	
R_f 0.3–0.65	33, 37	8, 8	
R_f 0.65–1.0	0, 1	3, 4	
(g) Paper chromatography, solvent 3 [7]			
R_f 0.0		3	
R_f 0.0–0.85		0	
R_f 0.85–1.0		45	
(h) Paper chromatography, solvent 3			
R_f 0.85–1.0 sprayed with ninhydrin		30	
(i) Heated in solution with ninhydrin		33	
Assay of thyroxine			
(j) 15 μg of T_4 on filter paper sprayed with ninhydrin			2, 4
400 μg of T_4 heated in solution with ninhydrin			7, 9
Assay of extracts of fraction 2 [8]			
(k) Benzene extract		minus 2, 1	
Acetone extract		23, 27	
Acetone-HCl extract		38, 40	
Methanol extract		10, 15	

[1] No rat had gained more than 5 g during the 2 weeks prior to its use in an assay.

[2] Each value represents the 2-week body weight gain of one rat which had been fed the sample treated as indicated. For each rat, the sample was mixed into 15–20 g of the sulfaguanidine-containing diet and this was fed until it was consumed. The sulfaquanidine-containing diet was then fed for the remainder of the 2-week period. The quantity of sample used for each treatment was equal to that of the untreated sample so that the response to the treated sample may be compared to that before treatment.

[3] T_4: L-thyroxine.

[4] Six milliliters of fraction 2 were hydrolyzed with and without 10 μg of T_4. Upon acidification, each of the soluble and insoluble fractions was mixed into 40 g of diet which was divided into 2 parts and assayed with 2 rats.

[5] Six milliliters of fraction 2, with and without 10 μg of T_4, were developed on paper with 0.1 N HCl at 26°. The indicated R_f sections were cut out, extracted and assayed with 2 rats.

[6] Six milliliters of fraction 2, with and without 10 μg of T_4, were developed on paper with methanol:0.2 M ammonium acetate at pH 4.5 (1:1) at 5°. The indicated R_f sections were cut out, extracted and assayed with 2 rats.

[7] Three milliliters of fraction 2 were developed on paper with acetone:0.2 M ammonium acetate at pH 4.4 (4:6) at 5°. The indicated R_f sections were cut out, extracted and assayed.

[8] Fifteen milliliters of fraction 2 were dried on filter paper and then extracted as indicated. Two-fifths of each extract were assayed with 2 rats.

ported briefly elsewhere.[4] The data presented in this report indicate that 3 active fractions were separated from the acid-insoluble residue of duodenal powder after acid hydrolysis. These were a fraction insoluble at pH 8 and 2 fractions separated from a fraction that was insoluble at pH 6. The possibility exists that these growth factors are chemically modified forms of the same active agent although numerous attempts to isolate a growth factor from duodenal powder has consistently produced from 3 to 5 active fractions depending on the procedure or technique used.

It was concluded that fraction 2 contained neither thyroxine nor triiodothyronine. The stability of the growth factors in this fraction to acid hydrolysis is strong evidence to support this conclusion. Haga (9) reported that 86% of L-thyroxine was decomposed by boiling with HCl for 12 hours, and this decomposition was accelerated if carbonyl compounds such as glucose or xylose were present. The more drastic conditions (5 N HCl for 22 hours) used here and filtration of the acid hydrolysate would be expected to remove most, if not all, thyroid hormones from the acid-insoluble residue. More conclusively, the R_f of the growth factors in fraction 2 on paper chromatograms differed from that of the thyroid hormones. In addition, ninhydrin did not destroy the growth factors as it did thyroxine.

The metabolic significance and the relationship of the growth factors in duodenal tissue to thyroid hormone function remains to be determined.

LITERATURE CITED

1. Ackerman, C. J., and V. Tsou 1961 Effect of various tissues on the growth of sulfaguanidine-fed rats. J. Nutrition, *74:* 84.
2. Ackerman, C. J. 1963 Effect of potassium iodide and duodenal powder on the growth and organ weights of goitrogen-fed rats. J. Nutrition, *79:* 140.
3. Ackerman, C. J., and V. Mah 1963 Response of thyroid hormone-deficient rats to a growth factor in duodenal tissue. J. Nutrition, *80:* 263.
4. Gross, J., and R. Pitt-Rivers 1953 3,5,3'-Triiodothyronine. I. Isolation from thyroid gland and synthesis. Biochem. J., *53:* 645.
5. Harington, C. R. 1926 Chemistry of thyroxin. I. Isolation of thyroxin from the thyroid gland. Biochem. J., *20:* 293.
6. Kono, T., L. Van Middlesworth and E. B. Astwood 1960 Chemical identification of iodine containing compounds in human serum. Endocrinology, *66:* 845.
7. Taurog, A., G. D. Potter and I. L. Chaikoff 1955 Conversion of inorganic I^{131} to organic I^{131} by cell free preparations of thyroid tissue. J. Biol. Chem., *213:* 119.
8. Kendall, E. C. 1929 Thyroxine. The Chemical Catalog Company, Inc., New York, p. 41.
9. Haga, T. 1958 Improved method of protein acid hydrolysis. XX. Effect of mineral acid and carbonyl compounds on the decomposition of L-thyroxine. Nagasaki Igakkai Zasshi, *33:* 1426. (Cited in Chem. Abstr., *53:* 5366c, 1959).

[4] Ackerman, C. J. 1960 Effect of certain tissue extracts on the growth of goitrogen-fed rats and hypophysectomized rats. Fifth International Congress on Nutrition, Washington, D.C. Abstracts, p. 27.

Protein Utilization in Growing Rats [1]

I. RELATIVE GROWTH INDEX AS A BIOASSAY PROCEDURE

D. M. HEGSTED AND YET-OY CHANG
Department of Nutrition, Harvard School of Public Health, Boston, Massachusetts and the Division of Home Economics, University of Wyoming, Laramie, Wyoming

ABSTRACT The application of standard bioassay procedures for the evaluation of the nutritive value of proteins with growing rats has been examined. The slope-ratio technique using gain as the response and nitrogen intake as the measure of dose appears to be most satisfactory. The "relative growth index" is proposed as a satisfactory measure of nutritive value, this being the slope of the regression between dose and response expressed as a percentage of the slope obtained with a protein of maximal nutritive value. It seems apparent that biological value of rather poor quality proteins recorded in the literature overestimates the nutritive value of proteins for growing rats. The biological value of wheat gluten, for example, is recorded as 40, whereas it appears to have a relative growth index of only 20. Since estimates of protein requirements involve the use of biological values, current estimates may be too low. The need for accurate and appropriate estimates of the nutritive value of proteins is stressed. The inadequacies of protein efficiency ratio and its variants as a bioassay are emphasized.

There are basically two biologic procedures in common use for the evaluation of the nutritive value of proteins. Biological value as determined by the method of Thomas (1) as modified by Mitchell (2) and Mitchell and Carmen (3) is defined as the "percentage of absorbed nitrogen retained" and is generally considered the method of choice. Justification of the value or correctness of chemical score (4) or protein score (5) was inferred from the correlation between biological value and the computed values. Biological value can be determined only when the animal is depleted of nitrogen and the intake of protein is low so that the animal is in negative nitrogen balance or near balance. The "nitrogen balance index" of Allison and co-workers (6) is a modification of this method.

The other method is the protein efficiency ratio originally proposed by Osborne and co-workers (7). In this method the gain per gram of protein eaten is computed in young rats fed diets in which protein is limiting, usually 9 to 10% of the diet. This method has been criticized (8) since the protein efficiency ratio is highly correlated with weight gain. The protein efficiency ratio is thus not characteristic of the protein but of the rate of gain of the animals consuming the diet. The protein efficiency ratio is also not a true measure of efficiency since not all the protein is utilized for growth. The recent development of net protein utilization (9) attempts to account, at least in part, for the maintenance requirement by the inclusion of a negative control fed no protein.

The purpose of a biologic assay is to determine the *relative potency* of some test material compared with a standard. It is important to stress this fact since, otherwise, it is known only that the proteins tested have a different nutritive value. It is generally believed that biological value does this. If so, when the requirement for one protein is known, the requirement for other proteins can be computed (10). This is the procedure adapted by the Expert Committee on Protein Requirements of FAO (5) and the Committee on Amino Acids of the NRC (11). It forms the basis of the recommended dietary allowances for protein of the NRC (12). Similar use cannot be

Received for publication August 31, 1964.
[1] This work was supported by Public Health Service Research Grant no. 5K6-AM 18,455-02 from the National Institutes of Health, the Nutrition Foundation, New York, and the Fund for Research and Teaching, Department of Nutrition, Harvard School of Public Health.

made of protein efficiency ratio values and the equivalent protein content of foods cannot be estimated.

The principles involved in the development and execution of satisfactory bioassays have been thoroughly discussed (13). Some function must be developed which yields a linear regression between dose and response. This may require transformation of either the measure of dose or of response or both. In the most common assays the log dose-response curve is linear and the curves for the standard and unknown must be parallel. The slope-ratio technique has been developed for assays in which parallel regression lines are not obtained. For the satisfactory application of this technique, the regression lines must be linear and have a common origin.

In all adequate bioassays, a standard must be run with the unknown and comparisons between the standard and unknown should be made *at equally effective doses*. This is emphasized because it is rarely done in protein assays. The necessity for it will be apparent from the discussion.

As many as 1,500 investigations have been reported dealing with the nutritive value of proteins (14). Most of these studies have dealt with protein efficiency ratio. It is not unfair to say that the protein efficiency ratio has none of the characteristics of a good bioassay. The relationship between dose and response is complex and ill-defined, and those of different proteins are certainly not parallel. The assay is often carried out without a standard. Comparisons are made at equal doses of the test protein rather than at equal responses. Perhaps the best that can be said of the protein efficiency ratio is that it distinguishes a difference between proteins, is simple to carry out, and has been in use for a long time.

The assessment of the nutritive value of proteins by growing rats has progressed little beyond the contribution of Osborne and co-workers (7). Similar techniques applied to vitamins were discarded long ago and biostatisticians have evaluated critically nearly every conceivable vitamin assay (13, 15). On the other hand, both nutritionists and statisticians have for all practical purposes ignored the problems involved in the evaluation of protein quality. For example, the recent publication, "Evaluation of Protein Quality" (16) fails to mention even the principles involved in satisfactory bioassays or their application to the assessment of protein quality. Other recent publications (17, 18) have also dealt almost entirely with the reproducibility of the protein efficiency ratio rather than its inherent limitations.

The data presented in this paper were obtained for another purpose. The experimental conditions were not ideal since the several assays were carried out at different times. Nevertheless these observations demonstrate the inadequacies of current methods and suggest more appropriate procedures. Also these results point up the possible unreliability of biological value as a measure of the relative nutritive value of proteins, as well as of the inadequacy of the procedures used to derive protein requirements.

EXPERIMENTAL

Four experiments were carried out over a period of several months. Each experiment used 7 groups of weanling male rats of the Sprague-Dawley strain that were fed at graded levels of protein. There were 6 animals per group in 3 of the experiments, 5 animals per group in the other. The protein replaced cornstarch in the diets so the diets were isocaloric. The diets contained 8% fat,[2] 4% salt mixture,[3] 2% cod liver oil, 1% of a vitamin mix[4] and the remainder was cornstarch or the protein being tested. The animals received these diets over a 3-week period during which time the food consumption was measured and the animals were weighed weekly. The proteins studied were lactalbumin, casein, soy protein, and wheat gluten.[5]

During the third week of the study the feces were collected and analyzed for nitrogen by the micro-Kjeldahl method. Protein sources were analyzed in a like man-

[2] Hydrogenated cottonseed oil (Crisco, Procter and Gamble, Cincinnati).
[3] USP mixture XIV.
[4] Supplied: (mg/kg of ration) thiamine, 5; pyridoxine, 5; menadione, 5; cobalamin concentrate (3 mg/g), 5; riboflavin, 10; *p*-aminobenzoic acid, 10; nicotinic acid, 20; Ca pantothenate, 20; *d*-α-tocopheryl succinate, 75; folic acid, 5.5; biotin, 0.3; inositol, 400; and choline chloride, 1,000.
[5] Nutritional Biochemicals Corporation, Cleveland.

ner, with the protein calculated as N × 6.25. At the end of the experimental period the animals were killed with ether, and each whole carcass was placed in a sealed jar containing 300 ml of 6 N HCl and autoclaved 3 hours at 120°. The solution was extracted once with ether to remove fat and diluted to one liter. Aliquots were taken for nitrogen analysis. The total carcass nitrogen in 4 weanling rats, killed at the start of each experiment, was obtained. Data were also obtained upon a group of animals fed a nitrogen-free diet for 3 weeks.

RESULTS

The levels of protein studied, the nitrogen intake, and the resulting weight gains are shown in table 1. The calculated protein efficiency ratio values are also shown. Figures 1 and 2 are presented to demonstrate further the inconsistency inherent in the use of the protein efficiency ratio. By this method the relative activity of different proteins is determined by the level of protein included in the diet. The relative potency of the 4 proteins at 10% in the diet, considering albumin as 100, are 68 for casein, 32 for soy protein, and approximately zero for gluten (fig. 1). At 20% protein in the diet, the relative values are: albumin, 100; casein, 93; soy protein, 48; and gluten, 16. Whether there is any particular merit in obtaining values at the 10% dietary level is problematical. Similarly the suggestion that maximal values be compared is of unknown usefulness and impossible in practice.

Log dose-response assay. The log dose-response curves with gain as the response [6] are shown in figures 3 and 4. The sigmoid nature of these curves is apparent. Inspection indicates however that the central portions of these curves may be nearly linear and parallel. The elimination of points which are above or below the linear portion of the curve is necessary in evaluating bioassays since it is recognized that the curves are never linear indefinitely. Some prior knowledge of the approximate activity is in fact required in order to establish a satisfactory assay, and levels outside the range of the assay must be eliminated.

The covariance analysis of Finney (13) was applied. Five dosage levels for each of the proteins were included in the analysis. The more points that can be used, the greater is the stability of the regression lines, and the more discriminating the assay will be. With repetition of the assay, more appropriate levels could have been selected than those which were chosen in this instance.

When log % protein was used as the measure of dose, the departure of the regression lines from linearity is highly significant ($P < 0.01$). Inspection of figure 3 demonstrates that this is largely due to the curve obtained with casein. Also the regression lines are not parallel ($P < 0.01$). The data for casein and gluten are different in this respect.

When the data were treated as log N intake, the regression lines did not depart significantly from linearity, ($P > 0.10$), but again are not parallel ($P < 0.01$). Inspection of figure 4 shows that this latter observation is undoubtedly due primarily to the data obtained with gluten.

The data fail to meet the requirements of an ideal assay, although the log N intake is a more satisfactory metameter of dose than log % protein, as might be expected. The departure from parallelism means that the relative potency depends somewhat upon the levels at which protein was fed. Whether the data obtained with gluten are typical of very poor quality proteins or only representative of some unusual occurrence in this particuular study, can only be determined by additional evidence. The calculated relative potencies (table 2) are not entirely correct in view of the failure of the data to fulfill the requirements of an entirely satisfactory assay. They are presented, nevertheless, since they are at least reasonable estimates.

Slope-ratio assay. In the slope-ratio assay the potency is proportional to the slopes of the regression lines relating dose and response. In figures 5 and 6 the gain

[6] The original purpose of these studies was to investigate the efficiency of nitrogen utilization at different levels of intake, particularly as maximal growth is approached. Knowledge of this is important in attempting to arrive at proper estimates of protein requirements (see Hegsted (26)) and will be discussed in another publication. Total body nitrogen or nitrogen retention might have been used as the metameter of response rather than body weight. The merit of this is not obvious from a preliminary examination of the data and it is doubtful that it is worthwhile in routine assays.

TABLE 1

Summary of experimental data

Protein fed	Protein level in diet [1]	Nitrogen intake	Wt gain	PER [2]
	%	*g/day*	*g*	
Lactalbumin	2.21	0.0190	−6.5	—
	3.69	0.0469	18.3	3.0
	5.16	0.0864	44.7	3.94
	7.38	0.1630	89.0	4.12
	9.59	0.1940	92.7	3.62
	11.80	0.2297	109.5	3.62
	14.75	0.3086	111.7	2.75
Casein	3.47	0.0271	−3.3	—
	6.08	0.0566	13.3	1.79
	8.69	0.1132	41.8	2.80
	12.16	0.2247	87.5	2.97
	15.64	0.2963	116.7	3.0
	19.11	0.3532	125.5	2.71
	26.06	0.4817	138.5	2.19
Soy protein	8.51	0.1561	27.0	1.32
	11.92	0.2463	48.8	1.52
	15.32	0.3748	75.8	1.53
	18.73	0.4661	86.2	1.40
	25.54	0.6143	106.8	1.32
	34.05	0.8255	114.2	1.06
	42.56	0.9950	116.4	0.68
Wheat gluten	11.38	0.1123	3.2	0.22
	14.63	0.1693	8.3	0.42
	17.88	0.2206	16.7	0.62
	21.13	0.2925	28.3	0.73
	24.38	0.3550	30.7	0.66
	32.50	0.6061	54.7	0.69
	40.63	0.7169	64.3	0.69
None	—	—	−16.0	—

[1] Nitrogen × 6.25.
[2] Protein efficiency ratio.

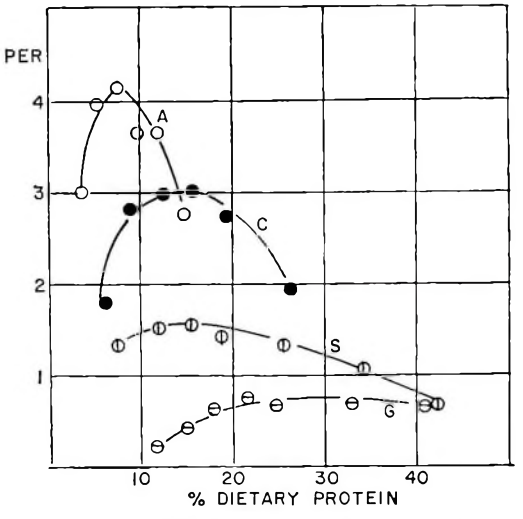


Fig. 1 Protein efficiency ratio versus % dietary protein. Lactalbumin, casein, soy protein, and wheat gluten are identified as A, C, S, and G.

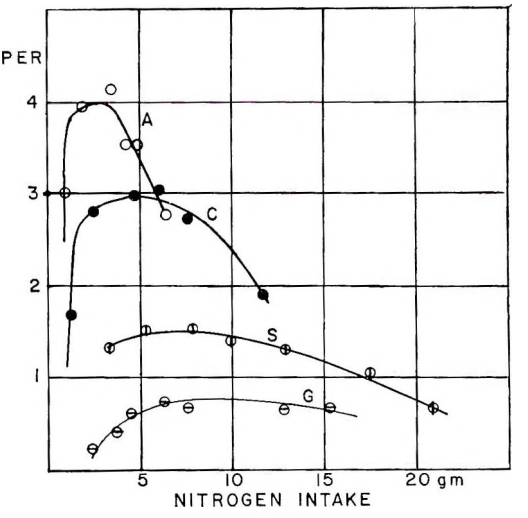


Fig. 2 Protein efficiency ratio versus nitrogen intake. Proteins identified as in figure 1.

in weight of each group has been plotted with the 2 metameters of dosage, percentage of dietary protein, and nitrogen intake. The linear regression lines were calculated using the first 5 or 6 groups which appear to be linear or nearly so. The data from the group fed the protein-free diet were included in calculating each line. The characteristics of the regression equations are shown in table 3.

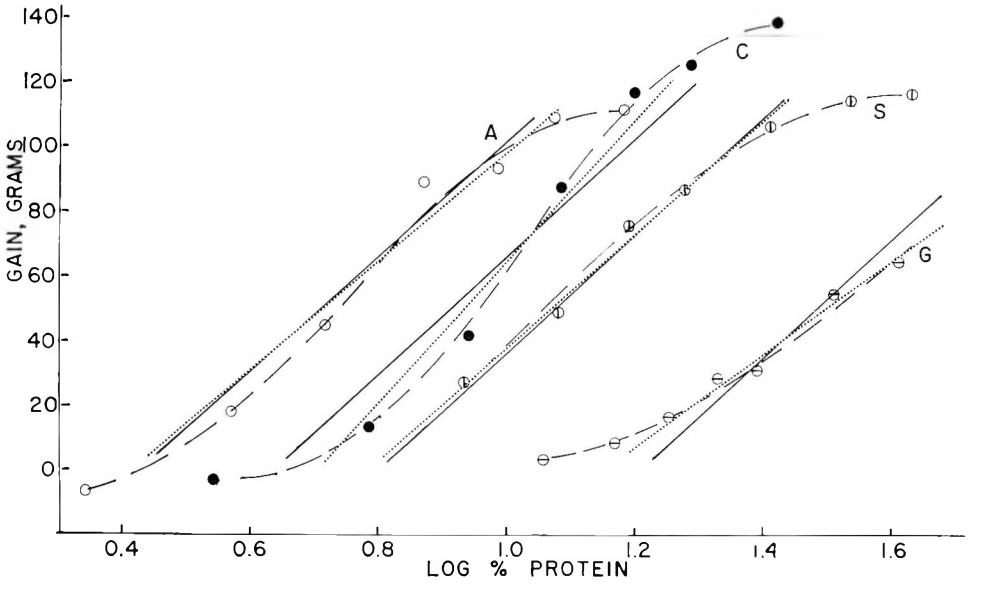


Fig. 3 The relationship between gain in weight and log % protein. The sigmoid curves have been drawn by inspection. The dotted lines are the individual regression lines calculated for each protein. The solid lines have the mean slope calculated from the combined data.

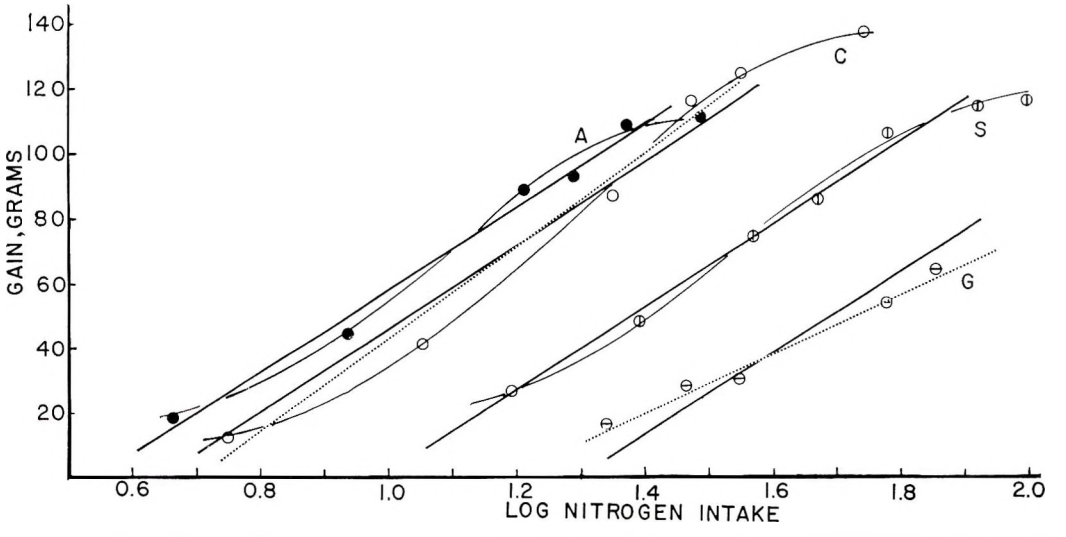


Fig. 4 The relationship between gain in weight and log N intake. The sigmoid curves were drawn by inspection. The dotted lines are the individual regression lines calculated through the lower 5 points for each protein. The solid lines are regression lines with the same mean slope calculated from the combined data. The 2 regression lines for lactalbumin and soy protein are essentially superimposed which accounts for the omission of the dotted line.

When gain in weight is plotted against the percentage of dietary protein (fig. 5) the data appear to follow a sigmoid distribution (broken lines in fig. 5). Although the correlation coefficients are very high (table 3), the calculated intercept tends to be lower than that actually found in the negative control group which lost 16 g of body weight. It appears likely, therefore, that there is an appreciable departure from linearity in the data near the intercept. The standard deviations of the slopes range from 3.6 to 5.5% of the respective slopes. Assuming that albumin has a potency of 100,[7] the relative potency of casein is 69.2%, of soy protein, 43.3%, and of gluten, 16.5%.

When the same data are plotted against nitrogen intake (fig. 6) the fit is better, as expected. The correlation coefficients reach very high levels, the intercepts approach

TABLE 2

Parallel line assays

Protein	Metameter of dosage	
	Log N intake relative potency	Log % dietary protein relative potency
	%	%
Albumin	100	100
Casein	81.3	63.1
Soy protein	36.4	43.6
Gluten	17.7	16.9

[7] The total carcass analyses show that the nitrogen from albumin was very efficiently utilized at the lower levels of intake. Data are not available to show that it is maximally utilized but it must approach maximal values.

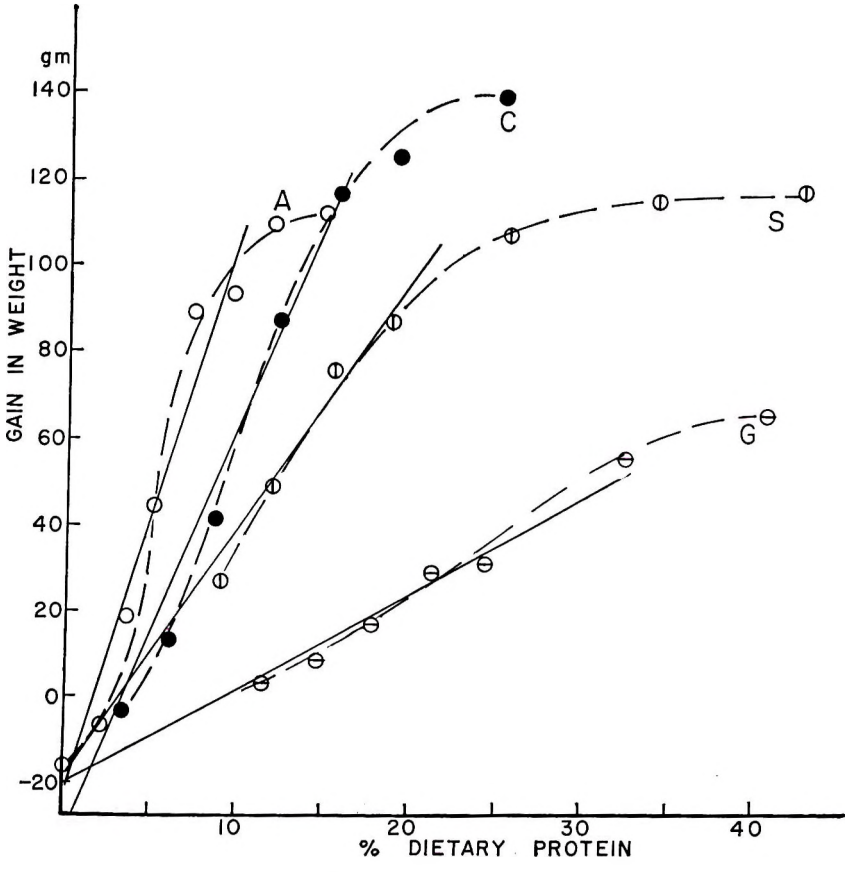


Fig. 5 Relationship between weight gain and % dietary protein. Broken curves drawn by inspection. Solid lines are regression lines calculated through the points which appear to be in the linear range (see table 3).

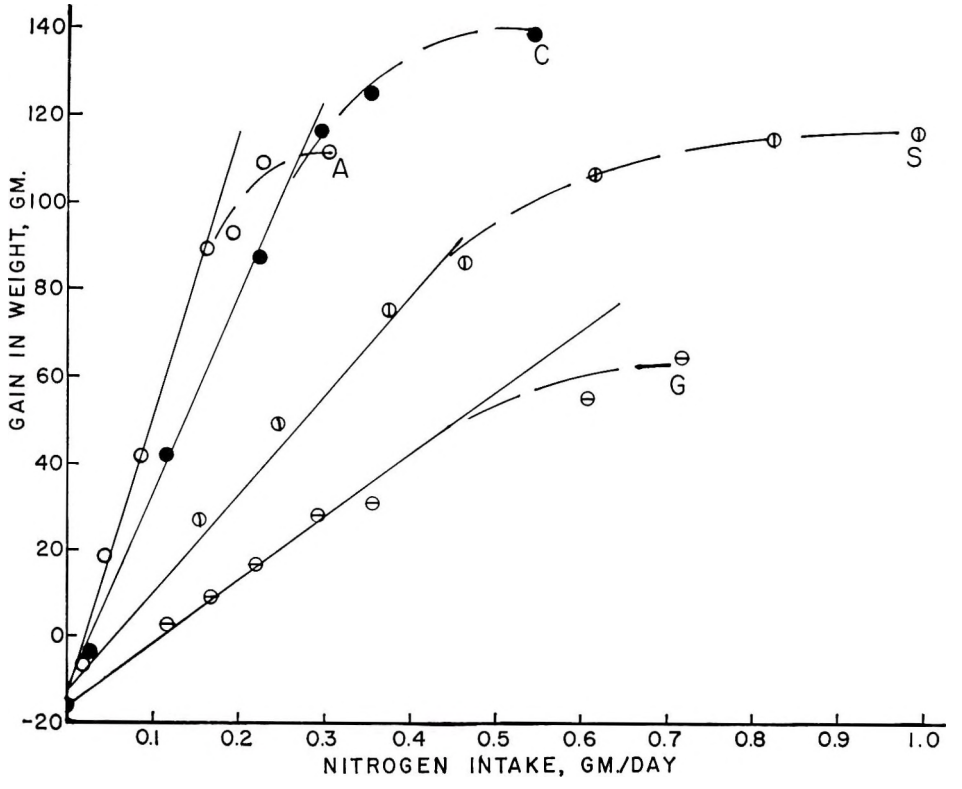


Fig. 6 Relationship between weight gain and nitrogen intake. Broken lines drawn by inspection. Solid lines are the regression lines calculated through the points which appear to be in the linear range (see table 3).

TABLE 3

Slope-ratio assay

	Albumin	Casein	Soy protein	Gluten
Dose, as % dietary protein				
No. of animals	36	36	26	42
Correlation coefficient	0.955	0.978	0.988	0.965
Intercept	−24.1	−29.7	−17.5	−20.0
Slope	13.09	9.08	5.68	2.17
SD of slope as %	5.3	3.6	3.2	4.3
Relative potency	100	69.2	43.3	16.5
Dose, as N intake				
No. of animals	30	30	26	30
Correlation coefficient	0.989	0.981	0.975	0.983
Intercept	−15.5	−13.9	−11.2	−14.9
Slope	656	460	221	143
SD of slope as %	2.4	2.7	4.7	3.5
Relative potency	100	70.0	33.7	21.8

the value found experimentally, and the errors in the slopes tend to be smaller (table 3). Again assuming albumin to have a relative potency of 100, the values for casein, soy protein, and gluten are 70, 34, and 22%, respectively.[8]

DISCUSSION

The data and analysis presented indicate that accurate and inherently valid growth assays of the nutritive value of proteins are feasible. The use of the slope-ratio assays appears to be most useful since the regression lines are substantially linear over a considerable range and little difficulty is anticipated in selecting appropriate doses for assay. Should the procedure prove acceptable in more extensive tests, an assay utilizing two or three groups and a negative control would appear to be most appropriate.

Parallel line assays could probably also be used if the doses of the test proteins were carefully selected. However, it would require considerable testing to select the appropriate doses of each protein and many animals would be wasted. With some products it might be difficult or impossible to feed sufficient protein to reach the linear portion of the log-dose response curve. It appears unlikely that efforts expended in developing such assays would be worthwhile.

The slope-ratio assays based upon nitrogen intake appear to fulfill the requirements of a good bioassay. Those based upon the percentage of dietary protein appear somewhat less satisfactory. However, the estimated potency was similar with the 2 procedures except for soy protein which was found to have 33 or 43% of the potency of lactalbumin. The assay of soy protein might have been improved by the inclusion of groups supplied with less protein which would yield values nearer the origin of the regression line. Since the measurement of food intake is the most time consuming, and therefore expensive, part of the assay, there would be considerable practical gain if the measurement of food intake could be eliminated without affecting the accuracy and reproducibility of the assay. There is substantial evidence that "animals do not eat for protein" (19, 20) and therefore the measurement of food eaten might not be critical for a good assay procedure. This should be investigated further.

As has been indicated, the experimental conditions were not ideal since these studies were made at different times. The animals came from the same source but the mean starting weights were 56 g for the animals used in the albumin and casein studies, 71 g for the soy protein studies, and 62 g for the gluten studies. Food consumption is a function of body weight (19, 20) and the larger size of the animals fed the soy protein may, in part, account for the discrepancy between the 2 assay procedures. The animals fed the casein appeared to have considerably more growth potential than those in the other experiments. In adequately designed assays the animals must be distributed at random among groups so that animal variation is minimized.

The data suggest that the most appropriate values, assuming lactalbumin has a relative value of 100, are casein, 70%; soy protein, 34%; and wheat gluten, 22%. The reported biological values and protein scores according to the FAO publication (5) are as follows: albumin, not given; casein, 69 and 80; soy protein, 75 and 73; and wheat gluten, 40 and 40. Allison (21) has presented nitrogen growth indexes for egg albumin of 33; casein, 25; and wheat gluten, 7. The significance of these values can be appreciated only when they are expressed in relative terms. Thus, considering egg albumin as 100, casein would have a relative growth index of 76 and gluten of 21. These values are in essential agreement with the values presented in this paper and samples may vary in their nutritional value.

It has long been recognized that the biological value appears to overestimate the nutritive value of poor quality proteins. Gelatin, for example, which will support no growth appears to have a biological

[8] Finney has described more complex methods of evaluating the data (13, p. 189). However, the solution of 5 simultaneous equations is extremely tedious. Furthermore, the distribution of the variation between regression, blanks, intersection, and curvature, as outlined by Finney, cannot be made when nitrogen intake is the metameter of dose since there is significant regression within groups. The proper estimate of the errors in the estimates requires the solution of an inverse matrix which is again tedious unless a computer program can be utilized for data of this kind.

value of approximately 25 (22). Bender (23) has reported that with most amino acids, nitrogen retention is not proportional to the amounts of amino acids supplied at low levels. He observed this to be true particularly of lysine which may be one of the more important limiting amino acids under practical conditions. Diets lacking lysine were reported to have net protein utilization values of 30 to 40%. Such data would apparently demonstrate some conservation mechanism which is called into play when deficiencies are imposed. On the other hand the data of Calhoun et al. (24) with growing rats are directly contradictory to Bender's conclusion that growth and nitrogen retention are not proportional to lysine intake, and suggest that lysine might be determined by a slope-ratio assay. This is confirmed by the report of Carpenter et al. (25) with chicks. In any event there is no direct evidence which demonstrates that the ability of proteins to maintain animals over long periods is directly related to biological value, and it is doubtful that gelatin or lysine-free proteins will in fact maintain weight or nitrogen equilibrium.

Regardless of the true explanation, there is no doubt that diets completely devoid of essential amino acids will not support growth. Since the relative nutritive values of proteins have been classified by biological values, and chemical scores are based largely upon correlations with biological value measurements, the status of this whole area is open to question. Protein efficiency ratio values are useless for this purpose since it is admitted that they do not provide a measure of the relative nutritive value of proteins.

The significance of accurate estimates of the nutritive value of proteins for growth needs emphasis. Hegsted (10) indicated that if corn grits and white bread have biological values of 54 and 47, as reported, an adequate caloric intake from these sources would be expected to provide approximately the estimated minimal protein needs of infants and children. However, if these values are serious overestimates of the true nutritive value, as appears likely, these products would not be able to supply the protein needs. The National Research Council (12) and FAO (5) have also made corrections based upon biological values. Thus the estimated requirements, after correction for biological value may be too low.

LITERATURE CITED

1. Thomas, K. 1909 Ueber die biologische Wertigkeit der stickstoff-substanzen in verschiedenen Nahrungsmitteln. Arch. Physiol., 219.
2. Mitchell, H. H. 1924 A method of determining the biological value of protein. J. Biol. Chem., *58:* 873.
3. Mitchell, H. H., and G. G. Carmen 1926 The biological value of the nitrogen of mixtures of patent white flour and animal foods. J. Biol. Chem., *68:* 183.
4. Block, R. J., and H. H. Mitchell 1946–47 The correlation of the amino acid composition of proteins with their nutritive value. Nutrition Abstr. Rev., *16:* 249.
5. Food and Agriculture Organization 1957 Protein Requirements. FAO Nutritional Studies no. 16 (1955), Report of the FAO Committee, Rome, Italy.
6. Allison, J. G. 1955 Biological evaluation of proteins. Physiol. Rev., *35:* 664.
7. Osborne, T. B., L. B. Mendel and E. L. Ferry 1919 A method of expressing numerically the growth-promoting value of proteins. J. Biol. Chem., *37:* 223.
8. Hegsted, D. M., and J. Worcester 1947 A study of the relation between protein efficiency and gain in weight on diets of constant protein content. J. Nutrition, *33:* 685.
9. Miller, D. S. 1963 A procedure for determination of NPU using rats body N technique. In Evaluation of Protein Quality, pub. 1100. National Academy of Sciences — National Research Council, Washington, D. C.
10. Hegsted, D. M. 1957 Theoretical estimates of the protein requirements of children. J. Am. Dietet. A., *33:* 225.
11. National Research Council, Food and Nutrition Board 1957 Evaluation of Protein Nutrition, pub. 711. National Academy of Sciences — National Research Council, Washington, D. C.
12. National Research Council, Food and Nutrition Board 1964 Recommended Dietary Allowances, ed. 6, pub. 1146. National Academy of Sciences — National Research Council, Washington, D. C.
13. Finney, D. J. 1952 Statistical Methods in Biological Assay. Hafner Publishing Company, New York.
14. Kuppnswamy, S., M. Srinivasan and V. Subrahmanyan 1958 Proteins in Foods. Special Report Series no. 33. Indian Council of Medical Research, New Delhi, India.
15. Bliss, C. I. 1951 Statistical methods in vitamin research. In Vitamin Methods, vol. 2, ed. P. György. Academic Press, Inc., New York.
16. National Research Council, Food and Nutrition Board 1963 Evaluation of Protein Quality, pub. 1100. National Academy of

Sciences — National Research Council, Washton, D. C.
17. Derse, P. H. 1962 Evaluation of protein quality (biological method). J. A. Off. Agr. Chem., *45:* 418.
18. Campbell, J. A. 1963 Methodology of Protein Evaluation, pub. 21. American University of Beirut, Beirut, Lebanon.
19. Hegsted, D. M., and V. K. Haffenreffer 1949 Calorie intakes in relation to the quantity and quality of protein in the diet. Am. J. Physiol., *157:* 141.
20. Carpenter, K. J. 1953 The concept of an "appetite quotient" for the interpretation of ad libitum feeding experiments. J. Nutrition, *51:* 435.
21. Allison, J. B. 1959 The efficiency of utilization of dietary proteins. In Protein and Amino Acid Nutrition, ed., A. A. Albanese. Academic Press, Inc., New York, p. 97.
22. Mitchell, H. H., J. R. Beadles and J. H. Kruger 1927 The relation of the connective tissue content of meat to its protein value in nutrition. J. Biol. Chem., 73: 767.
23. Bender, A. E. 1961 Determination of the nutritive value of proteins by chemical analyses. In Progress in Meeting Protein Needs of Infants and Preschool Children, pub. 843. National Academy of Sciences — National Research Council, Washington, D. C., p. 407.
24. Calhoun, W. K., F. N. Hepburn and W. B. Bradley 1960 The availability of lysine in wheat flour, bread and gluten. J. Nutrition, 70: 337.
25. Carpenter, K. J., B. E. March, C. K. Milner and R. C. Campbell 1963 A growth assay with chicks for the lysine content of protein concentrates. Brit. J. Nutrition, *17:* 309.
26. Hegsted, D. M. 1959 Protein requirement of man. Federation Proc., *18:* 1130.

Effect of Protein Malnutrition on the DNA Content of Rat Liver [1,2,3]

ROBERTO UMAÑA
Institute of Nutrition of Central America and Panama (INCAP), Guatemala, Central America

ABSTRACT The constancy of the DNA concentration in normal tissues has suggested the possibility of using this cellular component as a fixed point of reference to express the results of tissue analysis. In the field of nutrition, the constancy of this concentration in the liver has been questioned in those cases in which the rats have been fed protein-deficient diets. Some investigators who have studied the average DNA content of the isolated liver cell nucleus under conditions of protein deficiency, have not been able to show any significant increase. On the other hand, histochemical studies as well as DNA analysis of whole liver homogenates of the same type of animals have demonstrated a significant increase in the average DNA content of the nucleus and in the DNA concentration of the liver, respectively. In the present study, weanling and young adult rats were fed protein-deficient diets and the average DNA content of the isolated liver cell nuclei, as well as the DNA concentration of the whole liver homogenate, were studied. The results obtained strongly support previous observations that protein-deficient diets produce in the rat a significant increase in the average DNA content of the liver cell nucleus and in the DNA content of the liver.

It has been shown that the deoxyribonucleic acid (DNA) content of diploid nuclei in interphase is a constant quantity for all the cells of a given animal or plant species (1–4). Based on this observation, it has been proposed (3, 5–8) that the DNA concentration of a given organ be used as a point of reference to demonstrate any changes in the concentration of other cellular constituents induced by the experimental manipulation of the animal.

In the last decade, there has been much controversy in the field of nutrition with respect to the constancy of the average DNA content of the liver cell nucleus under conditions of protein deficiency. Thomson et al. (3), McIndoe and Davidson (9), Fukuda and Sibatani (10) and Campbell and Kosterlitz (11–13) have all reported that no significant increase in the average DNA content of the liver cell nuclei can be demonstrated when these organelles are isolated from the livers of rats fed protein-deficient diets. On the other hand, Ely and Rose (14) and Lecomte and DeSmul (15), using histochemical methods, have shown that a significant increase in the DNA content of the liver cell nucleus occurs when the rats are fed nitrogen-free or protein-deficient diets. Moreover, studies carried out on total liver homogenates (16–18) showed also that there is a slight increase in the DNA concentration of the liver of protein-malnourished rats.

The present study was carried out in an attempt to clarify this controversy, and because of the importance that the results may have on the elucidation of the effect of protein malnutrition on cellular composition and metabolism.

MATERIAL AND METHODS

One group of young adult rats (190 to 250 g) and one group of weanling female rats (35 to 45 g) of the Sprague Dawley strain were used in this study. Each group was divided into subgroups having a similar average weight. The animals were placed in individual cages with raised wire-screen bottoms. Four of the subgroups of adult rats were then fed each of the following diets, ad libitum, for 3 weeks: a) 20% casein (vitamin-free);[4] b) 5% zein;[5] c) 5% total corn protein (as ground yel-

Received for publication July 20, 1964.
[1] This investigation was supported by Public Health Service Research Grants no. AM-00981 and AM-4731 from the National Institutes of Health.
[2] The author gratefully acknowledges the technical assistance of Daniel Fernández.
[3] INCAP Publication I-336.
[4] "Vitamin Free" Casein, Nutritional Biochemicals Corporation, Cleveland.
[5] Obtained from Nutritional Biochemicals Corporation.

low corn); and d) N-free diet, prepared by replacing all the protein by cellulose.[6] The other constituents of the diets were the following: 10 ml cottonseed oil, 1 ml cod liver oil, 4 g minerals (19), and enough cornstarch to complete 100 g. Four milliliters of a vitamin solution were added to every 100 g of diet. The composition of this solution has been described previously (20). The remaining subgroup of the adult rats was starved for 8 days. Three subgroups of the weanling rats were fed diets *a*, *b*, and c, respectively. During the experiment, all the animals were allowed free access to water.

After the experimental period (3 weeks) the animals were decapitated, bled and the liver removed and weighed.

An approximately 10% homogenate was made in water with a Dounce homogenizer for the analysis of DNA and the determination of dry weights. Liver nuclei were isolated by a slight modification of the method of Dounce (21) in which the homogenates were made with the Dounce homogenizer instead of the Waring Blendor.

The total number of nuclei in each preparation was determined by direct counting of a very dilute suspension of nuclei in 0.05 M citric acid in a Levy-type homocytometer chamber. Three freshly loaded chambers were counted for each nuclei preparation with a reproducibility of around 5%.

DNA was extracted from the liver homogenates and from the isolated nuclei by the method of Schneider (22) and colorimetrically determined by the diphenylamine reaction of Dische (23), using highly polymerized calf thymus DNA[7] as a standard. The analytical results were expressed as milligrams of DNA per gram of dry liver. The average DNA content of the nuclei was expressed as micromicrograms per nucleus.

The statistical significance of the difference between mean values found in the concentration of DNA in the liver homogenates or in the average DNA content of the nuclei was determined by the *t* test.

RESULTS

Body weight changes. The changes in body weight produced by the different diets in the group of adult rats during the experimental period were the following: the weight of rats in the control group increased 17% of original weight; those animals fed the zein diet lost 20%; the animals fed the corn diet gained 6%; the animals in the N-free diet lost 20%; and the animals in starvation lost 30% of their initial weight. In the weanling group, the changes in body weight were the following: rats in the control group gained 300% of their original weight; the animals in the zein diet lost 34%, and the animals in the corn diet did not show any change in their original weight.

DNA content of isolated nuclei. The data on the average DNA content of the isolated nuclei are presented in table 1. These data show that in the adult rat, all the dietary conditions used produced a significant increase in the average DNA content of the nuclei when compared with the 20% casein group. In the group of

[6] Alphacel, Nutritional Biochemicals Corporation.
[7] Obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.

TABLE 1

Changes in the average DNA content of nuclei isolated from the livers of rats fed various rations or starved

Diet	Weanling rats		Adult rats	
	No.[1]		No.[1]	
		μμg/nucleus		*μμg/nucleus*
20% Casein	8	12.1 ± 0.7 [2]	8	10.8 ± 2.5
5% Zein	7	11.5 ± 0.7	14	15.5 [3] ± 1.6
5% Corn	8	11.9 ± 1.8	8	14.1 [3] ± 0.9
N-free		—	8	15.8 [3] ± 1.7
Starvation (8 days)		—	7	14.7 [3] ± 2.1

[1] The number of rats in each group is cumulative from various independent replicate trials designed to be of 2 rats each.
[2] Mean ± SD.
[3] $P < 0.01$ when compared with the 20% casein group.

the weanling rats, no significant increase in the average DNA content of the nuclei could be demonstrated.

DNA concentration in total liver homogenates. Table 2 shows the results of the analysis of the total liver homogenates. In the group of adult rats, the corn diet, the N-free diet and the period of starvation produced a significant increase in the DNA content of the homogenates. The increase produced by the zein diet did not reach statistical significance owing to the high variance resulting.

In the group of weanling rats, both diets studied produced a significant increase in the DNA content of the liver homogenates.

The comparison between the DNA concentration in the livers of both groups studied shows that the adult rat has a higher concentration of DNA than the weanling rat. This is in accord with previous observations (8, 10).

Changes in total liver content of DNA in relation to liver weight changes. One of the outstanding effects of a protein-deficient diet is a retarded rate of growth; therefore, animals of a given age fed an appropriate diet will be heavier than animals of the same age fed a protein-deficient diet. If it is postulated that the difference between the 2 types of animals is only a matter of size and that the relative composition of the organs has remained unchanged, the ratio, total organ weight (deficient)/total organ weight (normal), should be the same as the ratio, total organ constituent (deficient)/total organ constituent (normal). If the latter ratio is found to be greater or smaller, it would be necessary to conclude that the content of the constituent in question in the organ from the deficient animal is larger or smaller than that in the organ from the normal animal. This type of calculation reveals changes in the composition of the organ with respect to the total mass regardless of any changes that might have occurred in other constituents.

The data from our experiments were calculated in this form and are presented in table 3. It is evident from these calculations that both groups of animals re-

TABLE 2

Changes in the DNA concentration in homogenates made from the livers of rats fed various rations or starved

Diet	Weanling rats		Adult rats	
	No.[1]	*mg DNA/g dry tissue*	No.[1]	*mg DNA/g dry tissue*
20% Casein	13	8.24 ±1.0 [2]	15	14.60 ±2.0
5% Zein	6	11.92 [3] ±1.9	13	17.75 ±2.7
5% Corn	11	9.99 [4] ±2.8	12	18.95 [3] ±2.0
N-free		—	12	17.00 [4] ±1.0
Starvation (8 days)		—	6	23.15 [4] ±2.5

[1] The number of rats in each group is cumulative from various independent replicate trials designed to be of 2 rats each.
[2] Mean ± SD.
[3] $P < 0.01$.
[4] $P < 0.05$, when compared with the 20% casein group.

TABLE 3

DNA changes in the livers of rats fed various diets or starved

Ratios [1]	5% Zein		5% Corn		N-free	Starvation
	Adults	Weanling	Adults	Weanling	Adults	Adults
$\frac{\text{Liver wt (exp.)}}{\text{Liver wt (control)}}$	0.66 ±0.03 [2]	0.32 ±0.03	0.71 ±0.19	0.62 ±0.10	0.59 ±0.06	0.59 ±0.06
$\frac{\text{Total DNA (exp.)}}{\text{Total DNA (control)}}$	0.71 [3] ±0.02	0.37 [3] ±0.10	0.81 [4] ±0.10	0.76 [4] ±0.10	0.77 [4] ±0.21	0.86 [4] ±0.12

[1] The total number of animals studied in each group was 6.
[2] Mean ± SD.
[3] $P = 0.01$.
[4] $P \leqq 0.05$, when compared with the liver ratio.

sponded to the dietary treatment with a significant increase in the DNA content of the liver.

DISCUSSION

The values found for the average DNA content of nuclei isolated from normal adult rats (10.8 ± 2.5 μμg/nucleus) agree with values reported previously in the literature (3, 5, 9, 11, 14, 18). The average DNA content of nuclei isolated from the liver of the weanling control group was 12.1 ± 0.73 μμg/nucleus, in contrast with the values of 6.7 to 7.2 μμg/nucleus reported by Thomson et al. (3). This discrepancy might be explained by the fact that during the 3-week experimental period the weanling control group increased from 30 g to about 100 g of body weight, and it has been shown by Fukuda and Sibatani (10) that the average DNA content of the rat liver nucleus increases from 5.9 to 11.2 μμg/nucleus when the body weight increases from 25 to 100 g.

Table 4 summarizes some of the data available in the literature on the effect of protein deficiency on the DNA content of the liver cell nucleus. It is evident from this table that except for the results of Ely and Ross (14), the other investigators who studied isolated nuclei were unable to demonstrate any change in the DNA content of the protein-deficient organelles. In a previous publication (24), it has been shown that the use of the Waring Blendor for homogenization abolishes the difference between the DNA content of normal and of protein-deficient nuclei. Therefore, it can be concluded that the negative results obtained by the investigators cited in table 4 should be ascribed to the type of blender used for homogenization.

The histochemical studies of Ely and Ross (14) and Lecomte and De Smul (15), as well as the DNA determinations of Cooper (16), Muntwyler (17) and Villela (18), showed an increase in the DNA content of the liver cell nuclei and the liver homogenates of protein-deficient rats. These results are in good agreement and are in confirmation of our results.

In the weanling group, it was not possible to show any increase in the average DNA content of the isolated nuclei even

TABLE 4

Summary of the data in the literature on the effect of protein deficiency on the average DNA content of the liver cell nuclei

Reference	Wt of rat	Diets				Method
		Normal	N-free	Protein-deficient	Starvation	
	g	*μμg DNA/nucleus*		*μμg DNA/nucleus*		
(9)	—	8.70			9.27	citric acid, Waring Blendor
(10)	200	11.20			11.20	citric acid, Waring Blendor
(14)	130–160	10.20	12.0	16.70 [1]		citric acid, Waring Blendor
(2)	—	8.0			8.0	citric acid, Waring Blendor
(3)	195–250	9.28	9.90		10.26	citric acid, Waring Blendor
(11)	325	10.8	11.0			citric acid, Waring Blendor
(15)	60–65	582.4 [2]		638.6 [2,3]		histochemical
(14)	130–160	0.723 [4]	1.090 [4]	0.961 [1,4]		histochemical

[1] 12% casein.
[2] Arbitrary units.
[3] 3.2% casein.
[4] Extinction coefficients.

though the DNA concentration of the liver homogenates and the calculated ratios of total DNA content and of liver weight between groups clearly indicated an increase in the DNA content of the livers. The reason for this discrepancy has not been found.

The mechanism of the increase in the DNA content of the livers of rats fed protein-deficient diets cannot be ascertained from the data presented. Nevertheless, if it is accepted that this increase could be ascribed to the presence in the livers of malnourished rats of a greater proportion of tetra- and octaploid-type of nuclei as suggested by Cunningham et al. (25) and Lecomte and De Smul (15), it would be necessary to conclude that under the conditions of protein deficiency used, the biosynthesis of DNA was not affected, whereas general protein synthesis was impaired in such a way that the mitotic process was blocked.

From the practical point of view, the increase in the DNA content of the liver of malnourished rats impedes its use as a reference point to express the liver composition in situations of protein deficiency.

LITERATURE CITED

1. Swift, H. 1950 The constancy of desoxyribose nucleic acid in plant nuclei. Proc. Natl. Acad. Sci., *36:* 643.
2. Mirsky, A. E., and H. Ris 1951 The desoxyribonucleic acid of animal cells and its evolutionary significance. J. Gen. Physiol., *34:* 451.
3. Thomson, R. Y., F. C. Heagy, W. C. Hutchison and J. N. Davidson 1953 The deoxyribonucleic acid content of rat cell nucleus and its use in expressing the results of tissue analysis, with particular reference to composition of liver tissue. Biochem. J., *53:* 460.
4. Vendrely, R. 1955 The deoxyribonucleic acid content of the nucleus. In: The Nucleic Acids, vol. 2, eds. E. Chargaff and J. N. Davidson. Academic Press, Inc., New York, p. 155.
5. Dounce, A. L., G. H. Tishkoff, S. R. Barnett and R. M. Freer 1950 Free amino acids and nucleic acid content of cell nuclei isolated by a modification of Behren's technique. J. Gen. Physiol., *33:* 629.
6. Waterlow, J. C., J. Cravioto and J. M. L. Stephen 1960 Protein malnutrition in man. Adv. Protein Chem., *15:* 131.
7. Waterlow, J. C., and T. Weisz 1956 The fat, protein and nucleic acid content of the liver in malnourished human infants. J. Clin. Invest., *35:* 346.
8. Campbell, R. M., and H. W. Kosterlitz 1950 The effects of growth and sex on the composition of the liver cells of the rat. J. Endocrinol., *6:* 308.
9. McIndoe, W. M., and J. N. Davidson 1952 The phosphorus compounds of the cell nucleus. Brit. J. Cancer, *6:* 200.
10. Fukuda, M., and A. Sibatani 1953 Relation between the body weight and the average DNA content of liver nuclei in postnatal growth of the rat. Exp. Cell Res., *4:* 236.
11. Campbell, R. M., and H. W. Kosterlitz 1952 The absence of dietary effects on the DNA content of liver nuclei of the adult rat. Science, *115:* 84.
12. Campbell, R. M., and H. W. Kosterlitz 1947 Ribonucleic acid as a constituent of labile liver cytoplasm. J. Physiol., *106:* 12P.
13. Campbell, R. M., and H. W. Kosterlitz 1948 The effect of dietary protein on the turnover of phospholipids, ribonucleic acid, and desoxyribonucleic acid in the liver. J. Biol. Chem., *175:* 989.
14. Ely, J. O., and M. H. Ross 1951 Desoxyribonucleic acid content of rat liver nuclei influenced by diet. Science, *114:* 70.
15. Lecomte, C., and A. De Smul 1952 Effet du régime hypoprotéique sur la teneur en acide désoxyribonucléique des noyaux hépatiques chez le rat jeune. C. R. Acad. Sci. (Paris), *234:* 1400.
16. Cooper, J. A. D. 1953 Effect of low protein diet and fasting on nucleic acid metabolism in the rat. J. Biol. Chem., *200:* 155.
17. Muntwyler, E., S. Seifter and D. M. Harkness 1950 Some effects of restriction of dietary protein on the intracellular components of liver. J. Biol. Chem., *184:* 181.
18. Villela, G. G. 1952 Effect of diet on the desoxyribonucleic acid of rat liver. Rev. Brasil. Biol., *12:* 321.
19. Hegsted, D. M., R. C. Mills, C. A. Elvehjem and E. B. Hart 1941 Choline in the nutrition of chicks. J. Biol. Chem., *138:* 459
20. Manna, L., and S. M. Hauge 1953 A possible relationship of vitamin B_{13} to orotic acid. J. Biol. Chem., *202:* 91.
21. Dounce, A. L. 1943 Further studies on isolated cell nuclei of normal rat liver. J. Biol. Chem., *151:* 221.
22. Schneider, W. C. 1945 Phosphorus compounds in animal tissues. 1. Extraction and estimation of desoxypentose nuclei acid and of pentose nucleic acid. J. Biol. Chem., *161:* 293.
23. Dische, Z. 1930 Mickrochemie, 8: 4. (cf. Leslie, I. 1955 In: The Nucleic Acids, vol. 2, eds., E. Chargaff and J. N. Davidson. Academic Press, Inc., New York, p. 3).
24. Umaña, R. 1965 The effect of the method of nuclei isolation on the average DNA content of the nucleus. Canad. J. Biochem., (in press).
25. Cunningham, L., A. C. Griffin and J. M. Luck 1950 Polyploidy and cancer. The desoxypentosenucleic acid content of nuclei of normal, precancerous, and neoplastic rat tissues. J. Gen. Physiol., *34:* 59.

Effect of Excess of Vitamin A on Sulfur Metabolism in the Rat [1]

K. RODAHL, B. ISSEKUTZ, JR., AND D. MERRITT SHUMEN
Division of Research, Lankenau Hospital, Philadelphia, Pennsylvania

ABSTRACT The effect of acute excess of vitamin A on sulfur metabolism was studied in nondeficient rats, using $Na_2S^{35}O_4$, methionine-S^{35} and cystine-S^{35}, and the liquid scintillation technique to measure specific activity (SA) of the $BaS^{35}O_4$ precipitates in the urine. Both in hypervitaminotic and control rats given methionine-S^{35} or cystine-S^{35}, the same amount (75 to 80%) of the excreted radioactivity appeared in the inorganic SO_4 fraction of the urine. When single or repeated vitamin A injections were given to rats treated previously with methionine-S^{35}, a larger amount of inorganic $S^{35}O_4$ was excreted than in the controls. After injection of $Na_2S^{35}O_4$, vitamin A-treated rats showed a more rapid decrease in the SA of urinary inorganic SO_4 than control rats, suggesting an increased breakdown of body protein. It therefore appears that vitamin A did not inhibit the oxidation of SH or S-S groups, but that large overdosage had a proteolytic effect in intact rats.

In man, single large doses of vitamin A cause acute toxic symptoms in the form of violent headache and nausea, followed by peeling of the skin (1). Chronic overdosage of vitamin A causes a variety of symptoms. In young growing animals, the most striking changes are retarded growth, hemorrhage and bone fragility (2).

Several hypotheses have been advanced to explain the mechanism of the effect of excess vitamin A. Russian investigators (3–5) have suggested that vitamin A is an antioxidant that inhibits the oxidation of sulfhydryl groups to sulfates. Fell (6) and Thomas and co-workers (7) have suggested that an excess of vitamin A activates a proteolytic enzyme which has an effect similar to papain. Finally, Wolf and Varandani (8) have advanced the hypothesis that vitamin A regulates the formation of mucopolysaccharides, in that excess vitamin A causes an increase in the mucus type of mucopolysaccharides.

The purpose of the present study was to test the first two of these hypotheses in intact nondeficient rats and specifically to clarify the in vivo effect of acute excess of vitamin A on the sulfur metabolism by using $Na_2S^{35}O_4$, methionine-S^{35} and cystine-S^{35} and the liquid scintillation technique to measure urinary specific activity of the $BaS^{35}O_4$ precipitate.

METHODS

The experiments were carried out on 100- to 120-g male Carworth rats [2] (CFN strain). The animals were divided into groups of 8; each group was kept in a metabolic cage divided into 8 compartments by Plexiglas walls. On a total of 35 groups, 3 types of experiments were carried out. In 2 types of experiments (types 1 and 2), S^{35}-labeled L-methionine or cystine was used. In a third type (type 3), $Na_2S^{35}O_4$ was used. Each experiment consisted of 1 or 2 treated groups and of one simultaneous control group. In the type 1 experiments, the treated animals (9 groups) received daily by stomach tubing 50,000 units of vitamin A acetate, dissolved in 0.5 ml ethyl laurate, for 3 days. The controls (6 groups) were given the same amount of solvent. On the third day all rats were given intraperitoneally the radioactive amino acid. The S^{35}-labeled methionine (0.1 to 0.3 μc/rat) was administered to 7 vitamin A-treated groups and to 5 control groups, and S^{35}-labeled cystine (0.27 μc/rat) was used in 2 vitamin A-treated groups and one control group.

Received for publication July 23, 1964.

[1] This investigation was supported by Public Health Service Research Grant no. AM-06212-03 NTN from the National Institute of Arthritis and Metabolic Diseases.

[2] Carworth Farms, New City, Rockland County, New York.

In the type 2 experiments, the animals were pretreated with intraperitoneal injections of S^{35}-labeled methionine (0.21 to 0.35 μc/day/rat) for 2 consecutive days. On the third day, vitamin A dissolved in 0.25 ml ethyl laurate was administered intraperitoneally for 1 to 3 days in doses of 25,000 to 100,000 units/day/rat, as shown in figure 1.

The third type of experiment was similar to type 1, except that following the 3-day pretreatment with vitamin A, $Na_2S^{35}O_4$ (0.05 to 0.15 μc/rat) was given intraperitoneally instead of the S^{35}-labeled amino acids.

Unless othterwise stated, the animals were maintained with commercial laboratory chow [3] ad libitum. Starting after the injection of the radioactive material, the urine was collected for a total of 48 to 72 hours. Each collection period lasted for 23 hours during which the rats fasted; this was followed by a one-hour feeding period. The purpose of this schedule was to prevent food particles from contaminating the urine. Drinking water containing 1% glucose was available to the rats at all times.

Radiochemical determinations. At the end of each collection period the cages were rinsed with a few milliliters of distilled water and the excretion of radiosulfur was measured in 3 fractions, each determination being carried out in duplicate. In the first fraction, inorganic SO_4 was determined by direct precipitation with $BaCl_2$. In the second fraction, total SO_4 was determined by precipitation with $BaCl_2$ after acid hydrolysis (boiling in 0.15 N HCl for 25 minutes). In the third fraction, total sulfur was determined by precipitation with $BaCl_2$ after oxidation according to the procedure described by Jeffay et al. (9). The $MgSO_4$ formed during the oxidation was dissolved in 2 ml of 10% HCl. In each of these fractions the amount of urine used was about 10 to 15 ml, or sufficient to provide 20 to 40 mg $BaSO_4$. In each case, the samples, at the end of the procedure described above, were placed in pre-weighed vials of a Tricarb liquid scintillation counter. The vials were placed in a warm water bath, and 2 ml of a 5% $BaCl_2$ solution were added slowly. The precipitate was then centrifuged, washed twice with distilled water and once with 96% alcohol. The final supernatant fraction was tested with sulfuric acid for traces of $BaCl_2$. The precipitate was then dried and kept for one hour in a furnace at 400°. After cooling, the $BaSO_4$ precipitate was weighed, and 10 to 12 stainless steel balls (diameter, 1.6 mm) were placed into the vials and 2 ml scintillator toluene containing 0.5% 2,5-diphenyloxazole (PPO) and 0.03% 1,4-bis-2-(4-methyl-

[3] Purina Rat Chow, Ralston Purina Company, St. Louis.

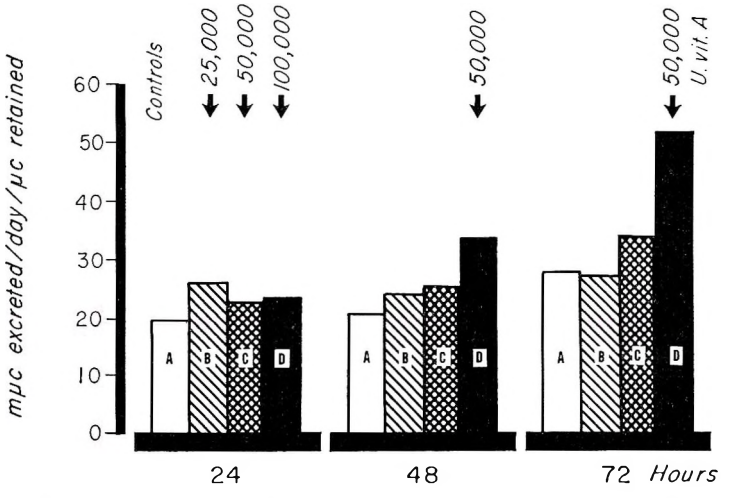


Fig. 1 Effect of excess vitamin A on the urinary excretion of inorganic $S^{35}O_4$ of rats pretreated with methionine-S^{35}. For detailed explanation, see text.

5-phenyloxazolyl)-benzene (dimethyl POPOP) added. The caps were screwed on tightly and the vials were then shaken vigorously for 10 to 20 minutes until the precipitate was broken up and finely dispersed. Following this, about 15 ml Cab-O-Sil thixotropic gel (5 g Cab-O-Sil in 100 g scintillator toluene) were added, and the vial was shaken for 10 minutes. After removing the air bubbles (by slowly rotating the vials) the radioactivity was counted either in a Tracerlab liquid scintillation counter at −12° (efficiency of about 70%) or in a Tricarb liquid scintillation counter at +2° (efficiency 63%).

This method has the advantage that no separate aliquots are used for sulfur determination and counting, and thus no transfer of the precipitate is needed. For this reason the method proved to be particularly useful for the determination of specific activity.

The radioactivity in the material injected into the rats was determined as $BaSO_4$ in an aliquot after adding the appropriate carrier to it. In experiments with methionine-S^{35}, nonradioactive methionine was the carrier and the $BaSO_4$ precipitate was obtained after complete oxidation. When $Na_2S^{35}O_4$ was used, this was precipitated along with added Na_2SO_4 and counted in the same way as the urine samples.

The reliability of the described procedure was tested in the following ways:

(A) Increasing amounts of $Na_2S^{35}O_4$ of a fixed specific activity were placed into vials to provide approximately 20, 40 or 60 mg $BaS^{35}O_4$, respectively. The same specific activity was obtained regardless of the amount of precipitate, showing that within the above range "self-absorption" was not an interfering factor (table 1, section A). A similar observation was reported by Nathan et al. (10) using a Thixin thixotropic gel for counting $BaC^{14}O_3$ precipitate. In amounts up to 200 mg $BaCO_3$ there was no measurable "self-absorption."

(B) Increasing amounts of nonradioactive Na_2SO_4 were precipitated to provide 25, 50, 75 or 100 mg $BaSO_4$, respectively. The precipitate was brought into suspension and 0.1 ml of C^{14}-toluene standard was added to each vial. The increasing

TABLE 1

Assessment of reliability of radioisotope determinations: [1] *effect of amount of precipitate on specific activity ("self-absorption") (A); quenching effect of increasing amount of precipitate on the C^{14} toluene (B); and "trapping" of methionine by precipitated inorganic SO_4 (C)*

	Source of radioactivity	Carrier	$BaSO_4$ precipitate	Radioactivity	Specific activity
			mg/vial	*dpm* [2] */vial* ($\times 10^4$)	*dpm/mg $BaSO_4$* ($\times 10^3$)
A	$Na_2S^{35}O_4$	Na_2SO_4	20.8	4.29	2.063
		Na_2SO_4	41.6	8.36	2.008
		Na_2SO_4	61.2	12.29	2.007
B	Toluene-C^{14}	none	—	4.29	—
		Na_2SO_4	24.3	4.19	—
		Na_2SO_4	50.0	4.29	—
		Na_2SO_4	75.6	4.38	—
		Na_2SO_4	100.0	4.27	—
C	Methionine-S^{35}	Methionine	66.4	28.48	4.289
		Na_2SO_4	73.6	0.32	0.043
		Methionine	42.7	33.22	7.780
		Na_2SO_4	72.4	0.34	0.048
		Methionine	72.9	41.98	5.758
		Na_2SO_4	73.1	0.09	0.011
		Methionine	57.5	44.27	7.700
		Na_2SO_4	75.0	0.35	0.046

[1] For details see text.
[2] dpm = disintegrations per minute.

amount of precipitate did not have any quenching effect on the C^{14}-toluene (table 1, section B).

(C) Two aliquots were taken from a methionine-S^{35} solution; sodium sulfate was added as a nonradioactive carrier to one of the aliquots, and methionine to the other. The former was precipitated with $BaCl_2$ directly, the latter after complete oxidation. When Na_2SO_4 was used as a carrier, the measured specific activity (dpm/mg $BaSO_4$) was usually less than 1% of that found after oxidation of the methionine-methionine-S^{35} mixture (table 1, section C). This shows that the inorganic SO_4, when precipitated, does not carry with it (absorbed or trapped) any appreciable amount of methionine.

RESULTS

1. *Effect of vitamin A on the oxidation of methionine-S^{35} and cystine-S^{35} (experiments, type 1).* When labeled methionine was administered intraperitoneally, only a relatively small portion (4 to 29%) of the injected material was excreted in the urine during the first 48 hours (table 2). There was no difference in this respect whether the rats received 50,000 IU vitamin A daily for 3 days (7 groups) or only the solvent (5 groups). As shown in table 2, there was no consistent difference between the vitamin A-treated and the control groups in the urinary excretion of S^{35}. However, in 6 out of 7 experiments, 76 to 91% of the excreted S^{35} appeared in the $S^{35}O_4$ fraction, the major portion of it as inorganic $S^{35}O_4$. Again, there was no difference between treated and control groups.

Essentially the same results were obtained when cystine-S^{35} was used instead of labeled methionine. Of the injected radioactivity, 23 to 33% was excreted and most of it in SO_4 form during the first 48 hours. No difference was found between control and vitamin A-treated rats. It may therefore be justified to conclude from these experiments that vitamin A did not inhibit the oxidation of the SH group of methionine or the S-S group of cystine.

2. *Proteolytic effect of vitamin A (experiments, type 2).* Theoretically the most direct way to demonstrate a proteolytic

TABLE 2

Urinary excretion of S^{35} in 48 hours in hypervitaminotic and control rats, expressed as percentage of the administered radioactive dose of S^{35}

Group no.	Radioactive dose administered		Urinary excretion of S^{35} in 48 hours							
			Hypervitaminotic rats [1]				Control rats			
	Source of radioactivity	Dose	Total radiosulfur (S^{35}) [2]	Radiosulfate ($S^{35}O_4$)			Total radiosulfur (S^{35}) [2]	Radiosulfate ($S^{35}O_4$)		
				Inorganic $S^{35}O_4$ [3]	Total $S^{35}O_4$ [4]			Inorganic $S^{35}O_4$ [3]	Total $S^{35}O_4$ [4]	
		μc/rat		*% of total excreted S^{35}*						*% of total excreted S^{35}*
1	L-Methionine-S^{35}	0.09	26.1	17.0	23.7	90.9	13.2	9.8	12.5	94.7
2		0.10	29.2	22.8	26.2	89.7	19.6	14.8	15.7	80.1
3		0.10	17.3	11.6	14.0	90.2				
4		0.19	12.4	9.0	10.0	80.6	21.4	15.6	18.5	86.4
5		0.21	12.5	7.7	9.5	76.0	9.2	4.5	5.9	64.1
6		0.21	8.4	4.3	5.1	60.7				
7		0.30	4.0	3.1	3.6	90.0	18.2	12.2	14.2	78.0
8	Cystine-S^{35}	0.27	33.3	24.8	30.1	90.4	26.3	18.1	21.4	81.4
9		0.27	22.5	15.5	18.3	81.3				

[1] Given 50,000 IU vitamin A/rat daily for 3 days.
[2] Total S^{35}: after oxidation with perchloric acid and precipitation with $BaCl_2$.
[3] Inorganic $S^{35}O_4$: direct precipitation with $BaCl_2$.
[4] Total $S^{35}O_4$: inorganic plus organic sulfate; precipitation with $BaCl_2$ after acid hydrolysis.

effect would be to measure the nitrogen balance. This approach, however, leads to great technical difficulties, because excess vitamin A when administered for a prolonged period of time decreases the food intake of the rats, which in itself leads to a negative balance. Short-term experiments, such as those described here, on the other hand, cannot give reliable balance values since neither the measurement of food intake nor the determination of urinary nitrogen output (loss of nitrogen in the form of ammonia during the collection period) can be carried out with the required accuracy. However, the fact that 71 to 96% of the injected methionine-S^{35} was retained in the body 48 hours after injection provided a possibility to study the proteolytic effect of a single or repeated doses of vitamin A. In this study (fig. 1), 96 rats received methionine-S^{35} for 2 consecutive days; the urine was collected, and the total excreted radioactivity was measured. The difference between this value and the injected dose was taken as the amount of radioactivity present in the rats at the end of the 48 hours when the vitamin A was given intraperitoneally. Eight animals received 25,000 IU vitamin A (group B), 24 animals received 50,000 IU in a single injection (group C), and 32 rats were given 100,000 IU once and 50,000 IU daily for the 2 following days (group D). Finally, 32 animals (group A) served as controls, receiving the solvent only. The excreted inorganic $S^{35}O_4$ (mμc/24 hours) was related to the amount of radioactivity (μc) present in the animals at the beginning of each collection period. At the end of the 3 days, vitamin A showed a marked proteolytic effect only in those rats which received altogether 200,000 IU in form of repeated injections (fig. 1, group D). During the third collection period, they excreted in the inorganic sulfate fraction 52 mμc for each microcurie present in the body at the beginning of this period, compared with 28 mμc excreted by the control rats, representing an increase of 86% in the hypervitaminotic rats. Fifty thousand international units of vitamin A in a single dose (group C) increased the excretion of $S^{35}O_4$ only about 23% above that of the controls (group A), whereas 25,000 units had no effect (group B).

3. *Experiments with* $Na_2S^{35}O_4$ *(experiments, type 3).* The effect of an excess of vitamin A on the breakdown of body protein could be shown also by using the isotope dilution technique. Five groups of rats were given 50,000 IU vitamin A/rat/day for 3 days, followed by an intraperitoneal injection of $Na_2S^{35}O_4$. The urine was collected for two 23-hour periods, and the specific activity of the inorganic $S^{35}O_4$ was determined. The advantage of this approach is that it does not require an accurate measurement of the daily urine output; hence any loss of urine, whether it be soaked into the pelt of the animal or trapped elsewhere in the cage, does not cause any error. Furthermore, this method as outlined above, measures directly the specific activity of the $BaSO_4$ precipitate present in the counting vial.

When the specific activity obtained in the second collection period is expressed as percentage of the specific activity obtained in the first 23 hours ($= 100 \times 10^3$ dpm/mg $BaSO_4$), this value depends only on the dilution of radioactivity by the unlabeled SO_4. It is assumed that the major part of the unlabeled SO_4 is derived from the oxidation of sulfur-containing amino acids, partly from the food and partly from the body proteins. A protein-free diet would be expected to increase this value because in this instance the only source of unlabeled SO_4 is the body protein. On the other hand, administration of unlabeled methionine should cause a more rapid decrease of the specific activity, since the oxidation of unlabeled methionine yields non-isotopic sulfate. The results obtained with 5 control groups were consistent with these assumptions (table 3). The specific activity of urinary inorganic sulfate decreased less with the protein-free diet than with ordinary laboratory chow, and decreased more rapidly in the group treated with nonradioactive methionine. As shown in table 3, the specific activity decreased more rapidly in the vitamin A-treated groups than in the corresponding controls, whether they were fed the regular or the protein-free diet. This indicates that vitamin A increased

TABLE 3

Effect of orally administered vitamin A on the specific activity ($SA = 10^3$ dpm/mg $BaSO_4$) of urinary inorganic $S^{35}O_4$ during the second 24-hour period after intraperitoneal injection of $Na_2S^{35}O_4$, expressed as percentage of the specific activity in the first 24-hour urine collection

Hypervitaminotic rats [1]		Control rats		Remarks
No. of groups of rats	SA	No. of groups of rats	SA	
5	9.52	3	14.17	$P < 0.02$
1	19.52	1	24.10	Fed protein-free diet
1	7.80	1	6.80	Nonradioactive methionine 3×33 mg/rat ip

[1] Given 50,000 IU vitamin A/rat daily for 3 days.

the breakdown of body proteins and that the liberated sulfur diluted the excreted isotope. When the rats were pretreated with nonradioactive methionine for 3 days, the specific activity of the $S^{35}O_4$ was very low in both groups, with no difference between them. This suggests again that excess of vitamin A does not inhibit the oxidation of the sulfhydryl groups of methionine to the inorganic SO_4.

DISCUSSION

This study showed that even large doses of vitamin A did not inhibit the oxidation of methionine or cystine, for when S^{35}-labeled amino acids were used, they were readily oxidized to inorganic $S^{35}O_4$. When $Na_2S^{35}O_4$ was administered along with nonradioactive methionine, the latter considerably decreased the specific activity of the excreted inorganic $S^{35}O_4$. Therefore, vitamin A cannot be considered as an antioxidant of the SH or S-S groups of amino acids.

The proteolytic effect of excess vitamin A was shown in 2 ways. In rats pretreated with methionine-S^{35}, massive doses of vitamin A increased the excretion of inorganic $S^{35}O_4$ considerably, and when $Na_2S^{35}O_4$ was used, the specific activity of it decreased in the urine of the treated animals more rapidly than in the urine of controls. This latter phenomenon is presumably due to an increased breakdown of body proteins in general, followed by the oxidation of the sulfur-containing amino acids or by a loss of already preformed sulfate of the mucopolysaccharides, or both. A loss of chondroitin sulfate after large doses of vitamin A has been demonstrated (7) in the articular and epiphyseal cartilages of the rabbit, an effect closely resembling that of papain proteinase. Fell and Dingle (11) have shown that the cartilaginous limb-bone rudiments from chick embryo cultivated in the presence of vitamin A (3.3 μg/ml) released an acid protease into the culture medium. Recently, Chung and Houck (12) reported that an excess of vitamin A decreased the hexosamine content of the connective tissue of the rat, and they concluded that this represented the loss of acid mucopolysaccharides.

However, in our experiments, as well as in the studies of others, referred to above, this proteolytic effect of vitamin A could be demonstrated only with massive overdoses, in the range of one million units/kg or more, and it is not at all certain that this effect is responsible for the symptoms of hypervitaminosis A in man.

LITERATURE CITED

1. Rodahl, K. 1949 The toxic effect of polar bear liver. Norsk Polarinstitutt Skrifter Nr. 92. Jacob Bybwad, Publishers, Oslo.
2. Rodahl, K. 1950 Hypervitaminosis A in the rat. J. Nutrition, *41:* 399.
3. Balakhovskii, S. D., and N. N. Drozdova 1956 Mechanism of action of carotenoids (vitamin A, retinene, and copper). Dokl. Akad. Nauk., *109:* 355.
4. Balakhovskii, S. D., and N. N. Drozdova 1957 Mechanism of action of vitamin A (retinol); copper-polyene antagonism and keratization of the epithelial tissue. Biokhimiya, 22: 330.
5. Balakhovskii, S. D., and I. V. Kuznetsova 1958 Carotenoidal polyenes and oxidative processes in the organism. The deficiency of retinol and oxidation of sulfur in the organism. Dokl. Akad. Nauk., *118:* 331.
6. Fell, H. B., and L. Thomas 1960 Comparison of the effects of papain and vitamin A on cartilage. II. The effects on organ cultures of embryonic skeletal tissue. J. Exp. Med., *111:* 719.

7. Thomas, L., R. T. McCluskey, J. L. Potter and G. Weissmann 1960 Comparison of the effects of papain in vitamin A on cartilage. I. The effects in rabbits. J. Exp. Med., *111:* 705.
8. Wolf, G., and P. T. Varandani 1960 Studies on the function of vitamin A in mucopolysaccharide biosynthesis. Biochem. Biophys. Acta, *43:* 501.
9. Jeffay, H., F. O. Olubajo and W. R. Jewell 1960 Determination of radioactive sulfur in biological materials. Analyt. Chem., *32:* 306.
10. Nathan, D. G., J. D. Davidson, J. G. Waggoner and N. I. Berlin 1958 The counting of barium carbonate in a liquid scintillation spectrometer. J. Lab. Clin. Med., *52:* 915.
11. Fell, H. B., and J. T. Dingle 1963 Studies on the mode of action of excess of vitamin A. 6. Lysosomal protease and the degradation of cartilage matrix. Biochem. J., *87:* 403.
12. Chung, A. C., and J. C. Houck 1964 Connective tissue. IX. Effects of hypervitaminosis A upon connective tissue chemistry. Proc. Soc. Exp. Biol. Med., *115:* 631.

Variations in the Urinary Creatinine Excretion of Rats Fed Diets with Different Protein and Amino Acid Content [1]

HANS FISHER

Nutrition Laboratories, Department of Animal Sciences, Rutgers, The State University, New Brunswick, New Jersey

ABSTRACT A series of experiments was carried out with adult male rats to study the effect of varying the dietary protein and amino acid content in relation to the daily creatinine excretion. It was observed that creatinine excretion was not constant and varied with protein intake and amino acid content. On the basis of these experiments, no pattern which would permit prediction of creatinine excretion as it relates to the level of dietary N or of amino acids could be distinguished. With a free amino acid-containing diet which provided the least amount of dietary N offered in these experiments, daily creatinine excretion was highest, the excretion being approximately 3 times as high as with a diet which provided 15% protein. Considerable variation in creatinine excretion of individual rats fed any given diet was observed, particularly following a change from one diet to another. The problems inherent in the use of creatinine excretion values for the calculation of body composition and other metabolic values are discussed.

During the course of a series of nitrogen (N) balance studies in men, in which urinary N constituents were routinely determined, we noted, upon increasing the dietary N content for the same subjects from 9 to 19 g/day, a 31% increase (1.82 to 2.38 g/day) in urinary creatinine, which was highly significant ($P < 0.001$).

Since Folin's (1) classic studies on protein metabolism, the concept that the creatinine excretion in adult animals and man was essentially constant and not subject to change as a result of dietary protein manipulation has been widely accepted. Only a few divergent reports suggesting the inconstancy of creatinine excretion have appeared. Commenting on Folin's original observations, Paton (2) reported a marked change in the creatinine excretion of dogs fed protein at varying levels. During the 1930's and early 1940's Beard published a series of papers as well as a review monograph (3) in which he criticized Folin's concept of a constant creatinine excretion and gave considerable evidence in support of a lack of constant creatinine excretion. Beard's studies have often been quoted, but dismissed as insignificant. Recently, Chow et al. (4) observed that their experience during the past 10 years did not support the concept of a constant creatinine excretion in man; indeed, creatinine excretion was just as variable as any other urinary component.

The results of a number of studies (5–7) in which the constancy of creatinine excretion had been claimed, do not support such a conclusion. The recent report by Nakagawa et al. (8) on the amino acid requirements of children shows not only a clear variation, but also a distinct pattern in urinary creatinine excretion upon changing the subjects from their "normal diet" to the experimental diet.

In view of the importance which has been attached to relationships between creatinine excretion and body composition (9), basal metabolism (10), and to the evaluation of dietary protein quality (11), we considered it important to study directly the effect of dietary protein and amino acid variation on the creatinine excretion in the rat. Our results point to considerable variation as a result of dietary protein or amino acid alteration, or both.

Received for publication September 14, 1964.

[1] Paper of the Journal Series, New Jersey Agricultural Station, Rutgers, The State University, New Brunswick, New Jersey. Supported in part by Grants-in-aid from the National Science Foundation and the U.S. Public Health Service.

EXPERIMENTAL

Albino male rats of the Wistar strain, weighing between 210 and 250 g, were maintained in individual metabolism cages. For 5 days of every week, urine, and in some instances, feces, was collected daily for N and creatinine analysis. Food intake was measured daily, and body weight measurements were usually taken at the start and thereafter at weekly intervals. Nitrogen was determined on food and on 5-day dried fecal composites by Kjeldahl digestion followed by colorimetric determination of the ammonium ion on the AutoAnalyzer according to a modification of Ferrari's (12) method. Urinary N was determined daily by the direct AutoAnalyzer method (12). Daily creatinine was determined in duplicate by the method of Owen et al. (13), which has been used successfully by a number of recent investigators (14, 15). In our experiments with rat urine this method permitted a recovery of a 94.1 ± 3.6%.

The composition of the diets used is shown in table 1. The 15% protein, corn-soya diet was found to contain 43 μg creatinine/g, which, based on the food consumption values in the experiments to be cited, added an insignificant amount to the rat's intake. The amino acid mixture used to study amino acid deficiencies relative to creatinine excretion was based upon Nasset's maintenance requirements for adult rats (16).

TABLE 1

Composition of diets

	%
Stock diet	
Ground oats	10.0
Corn meal	36.5
Wheat middlings	10.0
Ground wheat	10.0
Soybean meal (50% protein)	15.0
Meat meal (50% protein)	5.0
Alfalfa meal	3.0
Limestone	6.3
Trace mineral mix [1]	0.1
Dicalcium phosphate	1.2
Corn distillers solubles	2.5
Salt	0.4
Vitamins [2]	+
	%
Protein-free diet	
Cornstarch	38.5
Corn dextrin	20.0
Corn oil	12.0
Fiber	5.0
Minerals [3]	4.0
Vitamins [3]	0.2
Choline chloride (70%)	0.1
Sucrose	to 100.0
	% of diet
Amino acid mix	
L-Arginine·HCl	0.40
L-Histidine·HCl·H_2O	0.13
DL-Isoleucine (allo-free)	0.44
L-Leucine	0.31
DL-Phenylalanine	0.40
DL-Tryptophan	0.11
DL-Valine	0.47
L-Lysine·HCl	0.30
DL-Methionine	0.25
DL-Threonine	0.32
L-Glutamic acid	1.20

[1] Contained: (in per cent) manganese, 6.0; iodine, 0.12; iron, 2.0; copper, 0.2; zinc, 0.01; cobalt, 0.02; and calcium, 26.5.
[2] Supplied per kg diet: vitamin A, 600 IU; vitamin D_3, 150 IU; riboflavin, 0.15 mg; vitamin B_{12}, 1.5 μg.
[3] For composition, see Summers and Fisher (17).

RESULTS

Experiment 1. Ten rats were fed either the corn-soya, 15% protein diet (stock diet) or this diet diluted in different proportions with protein-free diet (table 1) for 15 weeks. During the first 9 weeks food intake was held essentially constant at 15 g/day with the N intake varying between 400 and 200 mg/day (table 2).

During the first 6 weeks there was a decrease in urine volume (table 2) but no consistent concomitant change in creatinine excretion. Only during the first week, while the rats received the stock diet diluted 50% with the protein-free diet, was there a marked (and significant) change in the creatinine excretion, despite marked changes in urinary N excretion. When, however, the stock diet was diluted further, urinary creatinine values more than doubled. Upon returning the animals to the undiluted stock diet, (10th week) with food intake decreased to 10 g/day, creatinine excretion decreased slightly during the 10th and 11th week but more sharply and significantly so during the 12th week. A further decrease occurred when the stock diet was again diluted, but, despite a significant decrease in body weight, creatinine excretion did not return to the low levels experienced during the first 6 weeks of the experiment.

Experiment 2. As marked changes in creatinine excretion were observed when the rats were gradually given less dietary

TABLE 2

Effect of varying protein intake on creatinine excretion of rats (exp. 1)

Experimental period	Diet	Body wt	Daily intake		Daily urine			N balance
			Food	N	Volume	N	Creatinine	
week		*g*	*g*	*mg*	*ml*	*mg*	*mg*	*mg*
1	Stock, 15% protein	238 ± 5 [1]	14.1 ± 0.3	387	9.0 ± 0.8	221.4 ± 7.0	3.5 ± 0.05	65.0
2		264 ± 2	14.9 ± 0.02	410	11.8 ± 1.3	216.7 ± 8.1	3.7 ± 0.14	92.7
3		—	14.9 ± 0.02	410	9.8 ± 1.0	221.7 ± 3.3	3.2 ± 0.18	87.7
4	Stock, diluted 50% [2]	255 ± 2	15.0 ± 0.1	213	8.4 ± 0.8	138.2 ± 7.5	4.3 ± 0.18	27.2
5		—	14.9 ± 0.1	211	8.1 ± 0.8	145.2 ± 4.5	3.3 ± 0.19	18.2
6		271 ± 2	14.9 ± 0.02	211	5.4 ± 0.6	81.3 ± 3.2	3.7 ± 0.20	82.1
7	Stock, diluted 75% [2]	274 ± 2	14.9 ± 0.01	90	8.4 ± 1.1	108.3 ± 2.9	5.8 ± 0.26	—
8		270 ± 2	14.9 ± 0.01	90	5.9 ± 0.7	56.5 ± 2.5	6.2 ± 0.08	—
9		271 ± 1	14.9 ± 0.02	90	7.0 ± 0.5	59.9 ± 3.4	7.4 ± 0.41	—
10	Stock, 15% protein	250 ± 1	9.9 ± 0.01	272	7.0 ± 0.8	136.3 ± 15.2	6.9 ± 0.18	35.1
11		249 ± 2	9.9 ± 0.01	272	9.5 ± 0.7	158.8 ± 12.1	6.9 ± 0.27	40.4
12		221 ± 2	9.9 ± 0.01	272	6.5 ± 0.5	185.5 ± 18.2	5.7 ± 0.26	−45.8
13	Stock, diluted 50% [2]	220 ± 2	9.9 ± 0.01	139	8.4 ± 0.8	77.3 ± 7.1	5.2 ± 0.21	8.7
14		216 ± 2	9.9 ± 0.01	139	8.1 ± 0.9	67.3 ± 8.1	4.7 ± 0.23	28.4
15		200 ± 2	9.9 ± 0.01	139	10.7 ± 1.2	96.8 ± 10.2	5.5 ± 0.23	−2.5

[1] Mean ± SE for 10 rats; body weights were recorded weekly, food intake daily, and other measurements taken 5 days of each week.
[2] Dilution of the stock diet was carried out with the protein-free diet (table 1).

TABLE 3

Effect of varying protein intake on creatinine excretion of rats (exp. 2)

Experimental period	Diet	Body wt	Daily intake		Daily urine			N balance
			Food	N	Volume	N	Creatinine	
week		*g*	*g*	*mg*	*ml*	*mg*	*mg*	*mg*
1	Stock, 15% protein	241 ± 4 [1]	14.6 ± 0.3	401	8.2 ± 1.1	183.0 ± 32.4	3.44 ± 0.13	114.4
2	Protein-free	215 ± 1	14.1 ± 0.4	tr	10.3 ± 1.0	93.6 ± 5.4	3.9 ± 0.15	—
3		—	11.7 ± 0.6	tr	8.4 ± 1.9	92.6 ± 4.9	2.3 ± 0.15	—
4		192 ± 3	10.1 ± 0.4	tr	6.8 ± 1.3	67.4 ± 2.7	2.2 ± 0.10	—
5		183 ± 3	10.4 ± 0.4	tr	9.0 ± 2.9	62.4 ± 1.0	4.3 ± 0.36	—
6		162 ± 4	9.4 ± 0.4	tr	8.1 ± 0.3	35.8 ± 1.2	4.2 ± 0.14	—
7		145 ± 3	9.2 ± 1.0	tr	7.8 ± 1.5	32.0 ± 1.6	3.8 ± 0.24	—
8	Stock, 15% protein	142 ± 4	9.6 ± 0.7	264	7.4 ± 0.7	140.2 ± 24.8	3.6 ± 0.41	23.2
9		159 ± 5	9.8 ± 0.3	270	6.2 ± 0.9	152.6 ± 15.9	3.0 ± 0.37	37.8
10		148 ± 4	9.8 ± 0.02	270	6.3 ± 0.6	176.4 ± 13.7	2.8 ± 0.31	−23.9
11	Stock, diluted 50% [2]	153 ± 4	9.7 ± 0.4	138	8.3 ± 1.1	49.2 ± 7.2	2.5 ± 0.33	36.8
12		159 ± 4	9.8 ± 0.02	139	9.5 ± 1.0	49.7 ± 7.5	2.7 ± 0.28	47.8
13		150 ± 5	9.8 ± 0.02	139	11.9 ± 1.7	68.3 ± 6.9	3.5 ± 0.33	25.6

[1] Mean ± SE for 10 rats; body weights were recorded weekly, food intake daily, and other measurements taken 5 days each week.
[2] Dilution of the stock diet was carried out with the protein-free diet (table 1).

protein over a prolonged feeding period, the second experiment was designed to study sudden reversals in shifting between the protein-free and the stock diet.

Another group of 10 rats was given, in succession: the stock diet for one week; the protein-free diet for 6 weeks; the stock diet again for 3 weeks; and finally the stock diet diluted 50% with the protein-free diet for 3 weeks.

Table 3 shows that creatinine excretion decreased significantly during the 3rd and 4th week to a value less than one-third the highest value observed in experiment 1. This decrease was followed by a virtual doubling of the creatinine excretion during weeks 5 and 6, followed by a gradual return to the starting levels between weeks 8 through 13. In view of the very marked changes in body weight as a result of feeding the protein-free diet, the changes in creatinine excretion do not justify consideration of a direct relationship between creatinine excretion and body size.

At the termination of experiments 1 and 2, the 10 rats from each group were killed with chloroform, and body water and N were determined. The results of these analyses are shown in table 4. There was little difference in the percentage body water and N content between the rats from each of these 2 experiments.

A comparison of the total carcass N in the average rat from each of the 2 groups might suggest, at least for the final week of the 2 experiments, that a relationship between creatinine excretion and body

TABLE 4

Body composition and creatinine excretion of rats

Measurments	Group [1]	
	A	B
Body wt, g	200 ± 2 [2]	150 ± 5
Carcass composition		
Water, %	67.4 ± 0.1	66.7 ± 0.3
Nitrogen:		
% dry wt	10.8 ± 0.1	10.2 ± 0.3
Total, g	7.1 ± 0.1	5.1 ± 0.3
Creatinine excretion,[3] mg/day	5.5 ± 0.2	3.5 ± 0.3

[1] The detailed diet and excretion pattern for group A rats is given in table 2; that for group B rats is listed in table 3.
[2] Mean ± SE for 10 rats/group.
[3] During last week prior to killing.

TABLE 5

Effect of different amino acid deficiencies and a free amino acid-containing diet on creatinine excretion of rats (exp. 3)

Measurement	Diet [1]			
	Complete	Lysine-deficient	Methionine-deficient	Threonine-deficient
Body wt, g				
Start	218 ±3 [2]	235 ±0	241 ±2	232 ±2
After 3 weeks	212 ±7	211 ±6	195 ±5	185 ±6
Food intake, g/day				
1st week	16.2±1.4	15.1±0.6	13.5±0.9	12.0±0.6
2nd and 3rd week	12.1±1.1	11.5±0.5	11.5±0.5	10.2±0.7
Nitrogen intake, mg/day				
1st week	136	119	113	100
2nd and 3rd week	102	91	97	85
Urine:				
Volume, ml/day				
1st week	9.7±1.7	7.6±0.9	8.4±0.8	9.4±1.6
2nd and 3rd week	7.1±1.5	3.3±0.2	5.5±1.3	6.3±1.5
Nitrogen, mg/day				
1st week	34.7±3.7	39.5±4.4	50.3±9.9	43.3±4.0
Creatinine, mg/day				
1st week	11.2±0.7	11.4±0.3	10.7±0.3	10.4±0.4
2nd and 3rd week	10.5±0.4	6.8±1.1	7.8±0.2	7.2±1.0

[1] The lysine and methionine-deficient diets were totally devoid of these amino acids; the threonine-deficient diet provided 0.03% DL-threonine as a percentage of diet.
[2] Mean values ± SE for 5 rats over the period of time indicated.

weight does exist. It is clear, however, that these values are fortuitous since a comparison of the body weight and creatinine excretion values within as well as between the 2 experiments does not support this suggestion.

Experiment 3. This experiment was carried out to determine whether, and to what extent, specific amino acid deficiencies might influence the urinary creatinine excretion pattern. The results (table 5) show that creatinine excretion of the rats on all treatments was approximately 3 times as high with these amino acid diets as those values obtained with the stock diets or the protein-free diet in experiments 1 and 2 (even though the rats were of similar origin and body weight). During the second and third weeks that the rats received their respective diets, the animals' creatinine excretion decreased significantly particularly with the amino acid-deficient diets, the excretion with the 3 deficient diets being significantly less ($P < 0.01$) than that with the complete diet. There appeared to be little difference among the specific amino acid deficiencies studied.

The coefficients of variation in daily creatinine excretion of individual rats were calculated for various diets throughout these experiments. These coefficients varied from a low of 6% for a rat fed the undiluted stock diet continuously for 3 weeks to a coefficient as high as 73% for a rat during a second period when fed the 50% diluted stock diet. For most rats the coefficient of variation for daily excretion values was between 10 and 20% and was always higher following a major change in the dietary protein pattern.

DISCUSSION

These studies illustrate that dietary protein and amino acid content influence the creatinine excretion of adult rats and do so in a manner which is not easily predictable. Thus, creatinine excretion did not increase with an increase in protein intake, since in experiment 3 the free amino-acid diet provided much less N than any of the other experimental diets. It may be, as Beard has suggested (3), that free amino-acids stimulate creatine synthesis, and subsequently creatinine excretion, more readily than proteins.

In view of the changes in creatinine excretion observed, considerable caution is needed in the interpretation and utilization of creatinine excretion values for evaluations of body composition and other related calculations such as basal metabolism.

The lack of constant creatinine excretion herein observed casts particular doubt on some of the underlying assumptions in the classical determination of the biological value of proteins. Thus, in the present study, the creatinine excretion decreased significantly during the protein-free feeding period only to double upon continuation of this feeding regimen. It is, however, possible that during the relatively short time during which biological value determinations are carried out, such changes will not occur. Nevertheless, the possibility does exist and sufficient anomalies have been recorded in the literature for unaccountably high or low biological values to suggest that the endogenous N excretion, as determined from a protein-free feeding regimen, might not always be a true reflection of the endogenous excretion that occurs when dietary protein is available to the animal.

ACKNOWLEDGMENTS

The author wishes to acknowledge the technical assistance of Mrs. Nilda Prieto in these investigations, and is also grateful to Dr. M. K. Brush for many helpful discussions relative to the preparation of this manuscript.

LITERATURE CITED

1. Folin, O. 1905 A theory of protein metabolism. Am. J. Physiol., *13:* 117.
2. Paton, D. N. 1905 On Folin's theory of proteid metabolism. J. Physiol., *33:* 1.
3. Beard, H. H. 1943 Creatine-Creatinine Metabolism. Chemical Publishing Company, Brooklyn, New York.
4. Chow, B. F., H. Holljes, S. D. J. Yeh, A. Horonick, J. M. Hsu, L. Calkins and H. Eberspaecher 1963 Studies on urinary excretion of nitrogen and electrolytes. Am. J. Clin. Nutrition, *13:* 40.
5. Tolbert, B., and J. H. Watts 1963 Phenylalanine requirement of women consuming a minimal tyrosine diet and the sparing effect of tyrosine on the phenylalanine requirement. J. Nutrition, *80:* 111.
6. Lee, M., and S. P. Lucia 1963 Some relationships between caloric restriction and body weight in the rat. IV. Fecal nitrogen, fat, energy and ash and urinary creatinine. J. Nutrition, *81:* 117.
7. Kiriyama, S., and K. Ashida 1964 Effect of the quality of dietary protein on nitrogen compounds in the urine of rats. J. Nutrition, *82:* 127.
8. Nakagawa, I., T. Takahashi, T. Suzuki and K. Kobayashi 1964 Amino acid requirements of children: nitrogen balance at the minimal level of essential amino acids. J. Nutrition, *83:* 115.
9. Picon-Reategui, E. 1962 Creatinine excretion and body composition. Am. J. Clin. Nutrition, *10:* 128.
10. Moreland, F. B., and A. E. Gurgiolo 1955 The use of the urinary pigment: Creatinine ratio as a measure of basal metabolism rate and thyroid activity. J. Lab. Clin. Med., *45:* 352.
11. Murlin, J. R., A. D. Hayes and K. Johnson 1953 Correlation between the biological value of protein and the percentage of creatinine N in the urine. J. Nutrition, *51:* 149.
12. Ferrari, A. 1960 Nitrogen determination by a continuous digestion and analysis system. Ann. N. Y. Acad. Sci., *84:* 792.
13. Owen, J. A., B. Iggo, F. J. Scandrett and C. P. Stewart 1954 The determination of creatinine in plasma or serum, and in urine; a critical examination. Biochem. J., *58:* 426.
14. Van Niekerk, B. D. H., A. Bensadoun, O. L. Paladines and J. T. Reid 1963 A study of some of the conditions affecting the rate of excretion and stability of creatinine in sheep urine. J. Nutrition, *79:* 373.
15. Kumar, I., D. G. Land and A. W. Boyne 1959 The determination of body composition of living animals. Brit. J. Nutrition, *13:* 320.
16. Nasset, E. S. 1957 Comparison of amino acid mixtures and egg protein as sources of dietary nitrogen. J. Nutrition, *61:* 555.
17. Summers, J. D., and H. Fisher 1961 Net protein values for the growing chicken as determined by carcass analysis: Exploration of the method. J. Nutrition, *75:* 435.

Influence of Dietary Fat on Triglyceride Structure in the Rat [1]

O. S. PRIVETT, M. L. BLANK AND B. VERDINO
The Hormel Institute, University of Minnesota, Austin, Minnesota

ABSTRACT The structure of the triglycerides of adipose, liver and kidney tissues of rats fed a fat-free diet and diets supplemented with corn oil, lard and menhaden oil was investigated. The triglyceride composition of the tissues had no direct relationship to that of the dietary fat. Quantitative differences in the triglyceride composition of the tissues were produced by the different dietary fats as related to their fatty acid compositions. Differences between the triglyceride composition of the tissues in each group were indicated by the pattern of the distribution of the fatty acids. Such differences were especially apparent in the animals fed menhaden oil, as evidenced by high concentrations of triglycerides containing polyunsaturated fatty acids in the liver and kidney tissues when compared with the epididymal fat pads. Structural analysis of the triglycerides of all tissues indicated that a competitive interrelationship existed for the β-position, increasing, in order, with palmitic, oleic and linoleic acids. The higher polyunsaturated fatty acids of menhaden oil did not take precedence over other fatty acids for the β-position. The effect of switching the diets containing the different fatty supplements indicated that the normal turnover of lipid in the rat was a gradual process requiring at least 6 months.

The influence of dietary fat on the fatty acid composition of animal depot fat has been known for many years. Information on the structure of triglycerides has been limited mostly to oils and fats from composite natural products as opposed to individual tissues because of the lack of methodology required for such analyses. The possibility that the distribution of fatty acids among triglycerides of natural fats follows a simple mathematical pattern, which could form the basis of a simple analytical method, has been investigated extensively (1–3). Recently, Vander Wal (3) proposed a method based on a new set of calculations for the determination of triglyceride structure. Perkins (4) applied this method to the analysis of rat carcass fat and demonstrated that the major triglyceride types of this fat approximated a random distribution. However, it was also shown via lipase hydrolysis, in accordance with the studies by Mattson et al. (5) and Tove et al. (6), that the fatty acids were not distributed randomly among the triglycerides and that unsaturated fatty acids were esterified preferentially in the β-position in animals fed diets containing corn oil, hydrogenated vegetable-animal fat shortenings or triricinolein.

In the present study, triglyceride structural analyses were carried out by a direct method of analysis (7) on several tissues of rats fed a fat-free diet and diets supplemented with corn oil, lard and menhaden oil. The effect of switching the diets of each group to that of the other groups, on triglyceride structure, fatty acid and lipid composition, was also determined.

EXPERIMENTAL

Materials and methods. The fats used in these studies were obtained from commercial sources. Their triglyceride structure and fatty acid composition are reported in the results.

Fatty acid composition was determined on methyl esters by gas-liquid chromatography (GLC) with an F & M Scientific Corporation instrument equipped with a hydrogen flame detector (Model 609) and a 240 × 0.6 cm column packed with 8% ethylene glycol succinate polyester phase (EGSS-X, Applied Science Laboratory) on Chromosorb P, mesh size 100–120, at 175° and a carrier gas flow of 60 ml/minute.

Triglycerides and other lipids were interesterfied with methanol by heating 1 to 10 mg of sample, sealed in a glass ampule with about 3 ml of 5% dry HCl in metha-

Received for publication August 31, 1964.
[1] Supported in part by NSPS Grant no. A 4942.

nol, in a boiling water bath for one hour. The methyl esters were recovered by extraction with petroleum ether in the usual manner.

Quantitative analysis of the lipid classes was carried out by thin-layer chromatography (TLC) as described previously (8), using as reference compounds highly purified cholesterol, cholesteryl oleate, triolein, oleic acid and hydrogenated egg lecithin, obtained from The Hormel Institute.

The method for the determination of triglyceride composition has also been described (7). It was carried out by quantitative thin-layer chromatography, using plates coated with silver nitrate impregnated silicic acid and, using methyl pentadecanoate as an internal standard for quantification by GLC. The precision of this method was demonstrated by the analyses of standard mixtures resulting in a relative error of less than 3%.

Nutritional experiments. Four groups of male weanling rats of the Sprague-Dawley strain were fed ad libitum a basic fat-free diet consisting of 16% vitamin-test casein, 74% sucrose, 4% α-cellulose, 4% salt mixture [2] and 1% casein containing vitamins in the required amounts. Vitamin A and E were mixed into the diet in a diethyl ether solution and the ether was evaporated under reduced pressure. The diets of three of the groups of animals were supplemented with 10% lard, corn oil and menhaden oil, respectively. The fourth group was maintained with the fat-free diet. Five animals of each group were killed after the second, fourth and sixth month. At the 6-month period the remainder of the animals in each group were divided into 2 groups. The 2 groups receiving the lard diet were switched to the corn oil and menhaden oil diets, respectively; the 2 groups receiving the corn oil diet were switched to the lard and menhaden oil diets, respectively; and the 2 groups receiving the menhaden oil were switched to the lard and corn oil diets, respectively. All groups were continued on these switched dietary regimens for an additional 6 months and then killed.

The animals were killed by exsanguination by withdrawal of the blood from the aorta while they were under a light ether anesthetic. The tissues were excised immediately, frozen in dry ice and stored at −20° until they could be extracted. The epididymal fat pads, livers and kidneys were pooled within each group and extracted in an Omin-mixer 3 times with about 100 ml of chloroform:methanol (2:1 v/v). This extract was filtered and the solvent removed under reduced pressure. The residue was extracted with chloroform dried over sodium sulfate, filtered and the solvent evaporated again under reduced pressure.

Each sample of blood was centrifuged immediately, after the addition of a small amount of sodium heparin to prevent coagulation, to separate the plasma from the cells. The plasma was then extracted 3 times with 100 ml of ethanol:ether (2:1 v/v) and the lipid recovered in the usual manner.

The lipid extracts were analyzed quantitatively by thin-layer chromatography, for cholesteryl esters, triglycerides, free fatty acids, cholesterol and polar lipids. The polar lipids consisted mostly of phospholipids and, henceforth, this fraction is designated as phospholipids. The sterol and steryl esters may also contain a mixture of compounds, but they consisted mostly of cholesterol and cholesteryl esters and are so designated. The triglyceride and phospholipid fractions were isolated by preparative TLC or silicic acid column chromatography. The fatty acid composition of each phospholipid fraction and the structural analysis, as well as fatty acid composition of each triglyceride fraction was determined.

RESULTS

The data for the 2- and 4-month-old groups of animals indicated that the lipids of the tissues were still undergoing changes in composition related to the diet at the end of the 4-month feeding period. The differences between the 4- and 6-month-old animals were small, however, and thus the latter are reported to show the nature of the influence of the dietary fat.

Fatty acid analysis. The distribution of fatty acids between the phospholipids

[2] Wesson, L. G. 1932 A modification of the Osborne-Mendel salt mixture containing only inorganic constituents. Science, 75: 339.

and triglycerides assumed fairly well-defined patterns which were influenced to only a minor degree by the dietary fatty acid composition as shown in table 1. The results may be summarized briefly as follows:

Myristic acid, a minor but widely distributed component, was consistently higher in the triglycerides than in the phospholipids.

Palmitic acid was fairly evenly distributed between the triglycerides and phospholipids in the tissues of all groups.

Palmitoleic acid was concentrated in the triglycerides, although a small amount was present in the phospholipids, especially in the fat-free group.

Stearic acid was observed predominantly in the phospholipids of all tissues.

Oleic acid was observed in higher concentrations in the triglycerides than in the phospholipids.

Linoleic acid varied considerably in the various groups but was distributed fairly evenly in both triglycerides and phospholipids in most tissues.

TABLE 1

Fatty acid composition of the dietary fats, the triglycerides and the phospholipids of the tissue of each group at the end of 6 months

	Diet	Liver		Kidney		Epididymal	Plasma	
		Triglycerides	Phospholipids	Triglycerides	Phospholipids	Triglycerides	Triglycerides	Phospholipids
	% by wt	*% by wt*	*% by wt*	*% by wt*	*% by wt*	*% by wt*	*% by wt*	*% by wt*
Lard group								
14:0	1.4	2.1	tr	2.1	tr	1.8		
16:0	23.4	29.1	19.8	30.1	32.2	26.6		
16:1	3.2	7.6	1.6	7.1	tr	9.1		
18:0	11.7	2.9	30.3	6.4	23.0	4.1		
18:1	48.7	53.0	11.2	48.2	15.5	52.6		
18:2	11.6	5.3	9.4	6.1	7.0	5.8		
18:3	—	—	—	—	tr	—		
20:3	—	—	2.1	—	—	—		
20:4	—	—	25.6	—	22.3	—		
Corn oil group								
14:0	tr	1.4	0.4	1.6	tr	1.0		
16:0	11.6	22.5	25.1	23.7	19.9	19.0		
16:1	tr	8.1	1.0	4.7	tr	6.4		
18:0	2.3	2.5	31.4	4.2	25.5	2.3		
18:1	28.7	48.5	9.6	31.3	11.8	34.9		
18:2	57.4	11.8	14.0	34.5	15.3	35.6		
18:3	tr	1.4	1.9	—	—	0.8		
20:3	—	0.4	1.2	—	2.4	—		
20:4	—	3.4	15.4	—	25.1	—		
Menhaden group								
14:0	9.2	2.6		4.5		6.7		
16:0	24.9	26.4		32.3		35.2		
16:1	13.5	13.2		12.1		21.1		
18:0	4.9	2.9		5.9		3.8		
18:1	20.6	25.3		34.5		31.9		
18:2	1.2	1.4		2.1		1.3		
18:3	2.2	1.3		—		—		
Others [1]	23.5	26.9		8.6		—		
Fat-free group								
14:0		1.0	0.4	2.0	—	1.6	1.6	0.5
16:0		29.8	24.2	33.3	10.4	24.7	28.3	23.9
16:1		10.4	8.3	11.0	6.3	17.0	15.8	7.6
18:0		4.2	17.4	5.2	21.6	1.6	3.1	16.7
18:1		53.5	25.0	48.5	28.8	55.1	49.9	28.3
18:2		1.1	1.0	—	tr	—	1.3	1.5
20:3		—	21.2	—	20.5	—	—	19.8
20:4		—	2.5	—	12.4	—	—	1.7

[1] Long-chain polyunsaturated fatty acids and minor constituents.

The higher polyunsaturated fatty acids also varied considerably in the various groups but were generally concentrated in the phospholipids.

Lipid class analysis. The composition of the lipid classes conformed to characteristic patterns in each tissue and the variations in the different groups were unexpectedly small as shown in table 2.

The cholesteryl esters were generally lowest in the kidney, a minor component in the liver and a major component of the plasma lipids.

Triglycerides were the major component of the epididymal fat pads. The livers of all groups contained almost twice as much triglyceride as the kidney and plasma lipids.

TABLE 2

Lipid class composition of tissues

	Lard group			Corn oil group		Menhaden group		Fat-free group		
	Liver	Kidney	Plasma	Liver	Kidney	Liver	Kidney	Liver	Kidney	Plasma
	% by wt			% by wt		% by wt		% by wt		
Cholesteryl esters	2.9	tr	21.4	1.8	tr	5.0	tr	5.1	2.8	29.2
Triglycerides	33.4	18.2	16.4	26.4	16.7	27.4	23.0	33.4	19.9	14.2
Free fatty acids	tr	3.7	5.7	tr	7.9	4.9	4.2	tr	3.3	1.4
Cholesterol	4.2	19.8	17.4	6.5	15.0	5.1	14.8	5.7	10.7	8.6
Phospholipids	59.5	58.3	39.1	65.3	60.4	57.6	58.0	55.8	63.3	46.6

TABLE 3

Triglyceride composition of diets and tissues

Triglycerides [1]	Lard group				Corn oil group			
$a\beta a'$	Diet	Epididymal	Liver	Kidney	Diet	Epididymal	Liver	Kidney
	% by wt	% by wt	% by wt	% by wt	% by wt	% by wt	% by wt	% by wt
SSS	2.4	2.3	1.8	2.2	tr	0.6	tr	2.0
SMS	tr	15.9	10.3	24.2	1.6	1.3	5.2	10.1
SSM	24.0	4.5	6.9			2.5	2.0	
SMM	3.5	31.9	51.3	33.5	4.6	7.5	34.7	12.4
MSM	29.6	2.4	1.4			1.7	1.7	
SDS	0.1	2.2	0.5	3.5	2.7	5.1	1.3	10.0
SSD	4.5		0.7			1.8	0.8	
MMM	8.5	25.0	11.0	18.4	3.9	8.1	12.9	5.6
SMD	0.1	1.2	3.9	9.4	5.6	6.3	6.0	23.5
SDM	0.3	6.0	6.3		7.7	16.2	10.6	
DSM	14.8	0.8	0.4		0.6	2.0	0.2	
MDM	2.3	3.1	1.2	5.2	5.2	11.9	2.8	12.9
MMD	4.3	2.7	1.4		8.0	8.8	2.9	
SDD	5.6	2.0	2.9	3.6	13.9	9.7	18.9	10.9
DSD					0.5	0.3		
MDD					19.2	12.6		10.4
DMD					5.0	2.5		
DDD					21.5	1.1		tr
Others	—	—	—		—	—	—	2.2

	Menhaden group				Fat-free group			
	Diet	Epididymal	Liver	Kidney	Epididymal	Liver	Kidney	Plasma
	% by wt	% by wt	% by wt	% by wt	% by wt	% by wt	% by wt	% by wt
SSS	3.1	9.3	1.7	6.2	2.5	2.7	3.7	1.7
SMS	2.0	11.7	8.9	28.7	12.9	15.0	29.0	11.6
SSM	15.9	23.0			4.2	4.2		6.3
SMM	3.5	11.5	15.7	33.1	40.1	57.1	47.3	50.6
MSM	11.5	24.9			3.5	2.7		3.3
SDS	0.7	—	2.1	1.3	—	—	—	2.1
SSD	—	—			—	—		
MMM	2.6	16.1	6.9	8.9	36.8	18.3	20.0	23.1
Others	60.7	3.5	64.7	21.8	—	—	—	1.3

[1] S, M and D indicate saturated, monoenoic and dienoic acids, respectively.

The free fatty acids were a variable minor component and considered to be largely an artifact of the treatment in the process of recovery of the lipid despite the precautions taken to avoid hydrolysis.

Cholesterol was generally highest in the kidney lipids; a trace component of epididymal lipid and a minor component of the liver. However, it may be varied considerably by dietary factors, as has been well demonstrated in many nutritional studies (9).

The percentage of phospholipid varied little in the kidney and liver lipids and was a major component of all of the tissues except the epididymal fat pad. The lower values for phospholipid in the plasma lipid may be due to the different method of extraction used to recover the lipid from the tissue.

Triglyceride analysis. The results of these analyses (table 3) showed that although the same types of triglycerides were present in the fats of all groups, the amounts of each type of triglyceride varied considerably in relation to the fatty acid composition of the diet. The diets with the more highly unsaturated fat produced greater amounts of the unsaturated types of triglycerides. However, there was little resemblance between the structures of the triglycerides of the dietary fat and that of the tissues. This divergence in composition is well illustrated by the results in the lard group in table 4, as well as in the general summary of the results presented in table 3.

TABLE 4

Comparison of selected triglyceride isomers of lard and tissues of the animals fed lard

Triglycerides [1] $\alpha\beta\alpha'$	Lard	Epididymal	Liver
	% by wt	% by wt	% by wt
SMS	tr	15.9	10.3
SSM	24.0	4.5	6.9
SMM	3.5	31.9	51.3
MSM	29.6	2.4	1.4
SMD	0.1	1.2	3.9
SDM	0.3	6.0	6.3
DSM	14.8	0.8	0.4

[1] S, M. and D indicate saturated, monoenoic and dienoic acids, respectively.

Differences also existed in the structure of the triglycerides in the various tissues as illustrated by the results presented in table 5 as well as in table 3. For example, the results in table 5 showed that whereas the distribution of the fatty acids in the triglycerides of the epididymal and kidney fat corresponded closely to the pattern predicted by the Vander Wal method of calculation (3), it deviated from this pattern in the triglycerides of the plasma and especially in those of the liver. The results in tables 3 and 5 also showed that the deviation of the experimental results from the calculated values were too great to permit the determination of triglyceride structure by methods based on mathematical patterns of distribution.

In keeping with the observation that the distribution of the fatty acids did not conform to a strict mathematical pattern was the formation of certain triglyceride

TABLE 5

Structure of tissue triglycerides from the fat-free group of animals

Triglycerides [1] $\alpha\beta\alpha'$	Epididymal		Liver		Kidney		Plasma	
	Found	Calculated [2]	Found	Calculated [2]	Found	Calculated [2]	Found	Calculated [2]
	% by wt	% by wt	% by wt	% by wt	% by wt	% by wt	% by wt	% by wt
SSS	2.5	1.4	2.7	2.1	3.7	5.4	1.7	2.4
SMS	12.9	12.0	15.0	20.9	29.0	30.4	11.6	18.9
SSM	4.2	4.9	4.2	4.5			6.3	5.5
SMM	40.1	41.5	57.1	45.5	47.3	44.6	50.6	44.1
MSM	3.5	4.2	2.7	2.4			3.3	3.2
SDS	—	—	—	—	—	—	2.1	—
SSD	—	—	—	—	—	—		—
MMM	36.8	36.0	18.3	24.6	20.0	19.6	23.1	25.9
Others	—	—	—	—	—	—	1.3	—

[1] S, M and D indicate saturated, monoenoic and dienoic acids, respectively.
[2] Calculated according to Vander Wal (3).

TABLE 6

Percentage of total fatty acids esterified at the β-position in the total liver and epididymal triglycerides determined via lipase hydrolysis (10) [1]

Fatty acid	Lard group	Corn oil group	Menhaden group		Fat-free group	
	Epididymal	Epididymal	Epididymal	Liver	Epididymal	Liver
	% by wt	*% by wt*	*% by wt*	*% by wt*	*% by wt*	*% by wt*
14:0	18.5	14.3	22.4	29.5	20.8	23.3
16:0	12.3	15.3	18.2	12.1	12.8	7.6
16:1	34.1	22.9	40.4	51.8	37.5	36.2
18:0	tr	7.0	7.9	9.2	tr	15.2
18:1	42.9	28.4	48.2	58.7	42.6	49.4
18:2	70.1	52.3	87.2	88.1	—	57.6
20:5	—	—	—	11.6	—	—

[1] A value of 33% corresponds to a random distribution in the β-position; a value below 33% indicates esterification mostly in the α- and α′-positions and a value above 33% indicates a preference for the β-position.

isomers in preference to others. For example, in the lard and corn oil groups, linoleic acid took precedence over all other fatty acids for the β-position. In those glycerides containing only oleic, palmitoleic, palmitic and stearic acids as the major components, oleic acid had a preference for the β-position. These observations indicated that there was a preferential placement of fatty acids in the molecule and that it increased for the β-position, in order, with palmitic, oleic and linoleic acids. This relationship was not based simply on the degree of unsaturation because the higher polyunsaturated fatty acids of the menhaden oil group appeared to be preferentially distributed in the α- and α′-positions. The distribution of the fatty acids located in the β-position is illustrated by selected analysis via the lipase hydrolysis technique (10) shown in table 6.

The analyses reported in the above tables for kidney triglycerides were limited by the amount of sample. Only limited triglyceride analyses could also be performed on the lipid of the animals receiving the menhaden oil because of the complexity of the fatty acid composition of this oil. A fairly complete analysis of the triglycerides of plasma was possible for the fat-free group because blood from animals fed this diet became available from other studies.

Switched diet experiments. The results on the analyses of the animals receiving the switched diets show the same relationships as described above, as indicated by selected fatty acid composition, lipid class and triglyceride composition analyses in tables 7, 8, and 9. These results also show that, although the values approach the originals, they had not been attained after the switched diets had been fed for 6 months. For example, the linoleic acid-containing triglycerides for animals fed the corn oil diet was 78.3%, but animals fed the lard diet and then switched to corn oil had only 64.7% of the triglycerides containing linoleic acid. Thus, the turnover of the lipid apparently had not been completed after 6 months of feeding the switched diets.

TABLE 7

Effect of switching diets on the composition of epididymal triglyceride

Triglyceride [1,2]	Lard to corn oil	Corn oil to lard
	% by wt	*% by wt*
S_3	0.9(0.6) [3]	0.4 (2.3) [4]
S_2M_1	7.7(3.8)	14.7 (20.4)
S_1M_2	16.5(9.2)	29.3 (34.3)
S_2D_1	3.2(6.9)	2.6 (2.2)
M_3	10.2(8.1)	17.1 (25.0)
SMD (and isomers)	20.7(24.5)	16.8 (8.0)
M_2D_1	16.8(20.7)	12.3 (5.8)
S_1D_2	7.1(10.0)	2.1
M_1D_2	14.3(15.1)	4.1 (2.0)
D_3	2.0(1.1)	tr
Others	0.6 —	0.6

[1] The number subscripts denote the number of fatty acids in each triglyceride class without respect to position.

[2] S, M, and D indicate saturated, monoenoic, and dienoic acids, respectively.

[3] Percentage in parentheses indicates the triglyceride structure of corn oil groups from table 3.

[4] Percentage in parentheses indicates the triglyceride structure of lard groups from table 3.

TABLE 8

Effect of switching diets on liver fatty acid composition

Fatty acid	Lard to corn oil		Corn oil to lard	
	Triglycerides	Phospholipids	Triglycerides	Phospholipids
	% by wt	*% by wt*	*% by wt*	*% by wt*
14:0	1.6	1.2	2.1	0.8
16:0	25.1	20.8	26.6	24.5
16:1	8.9	3.8	9.7	3.9
18:0	1.0	15.9	1.9	18.7
18:1	33.9	13.3	51.2	18.7
18:2	29.5	19.4	8.5	11.7
20:3	—	2.6	—	1.5
20:4	—	23.0	—	20.2

TABLE 9

Effect of switching diets on lipid class composition

	Lard to corn oil		Corn oil to lard	
	Liver	Kidney	Liver	Kidney
	% by wt	*% by wt*	*% by wt*	*% by wt*
Cholesteryl esters	3.7	tr	4.1	tr
Triglycerides	27.1	15.8	21.5	20.6
Free fatty acids	7.6	4.1	3.1	4.7
Cholesterol	15.8	14.0	8.3	14.0
Phospholipids	45.8	66.1	63.0	60.7

DISCUSSION

In accordance with the general observations of other investigators (11) the present study illustrates the profound influence of the fatty supplement of the diet on the fatty acid composition of the rat. The relatively small influence of the dietary fat on the general lipid class composition, particularly of the liver and kidney, is noteworthy in view of the large differences that may be effected in plasma cholesterol by changes in dietary fat (9).

Although much is known about the processes of digestion and absorption of fat which affords an insight into the general nature of the synthesis of triglycerides in the intestinal mucosa, the factors involved in the synthesis of triglycerides of specific structures is not known. The distribution of fatty acids in the synthesis of triglycerides in the intestinal mucosa, which involves the acylation of absorbed monoglycerides or the phosphatidic acid pathway, is believed to follow closely a random pattern (13). However, our studies on the composition of intestinal lymph (14) indicated that there is a preferential incorporation of fatty acids into specific positions of the triglycerides. The lymph triglycerides differed from those of other tissues in that they contained relatively high concentrations of polyunsaturated fatty acids, even in animals fed fat-free or low unsaturated fat diets. They resembled the triglycerides of the dietary fat in the respect that the β-skeleton of a large portion of the dietary triglycerides was retained. In lard-fed animals, lard having about 65% triglycerides of the type containing palmitic acid in the β-position, the lymph triglycerides were characterized by having a relatively large number of triglycerides of the β-palmito type. However, the present study shows that this type of triglyceride is very low in other tissues, indicating, together with other considerations, that the metabolism of the triglycerides occurs in fairly specific patterns in each tissue. Linoleic acid occurred only in trace amounts in β-palmito triglycerides in the lymph; that is, as LPO, LPL or LPS, even in lard-fed animals, illustrating its preference for the β-position in the triglycerides of this tissue as well as in those analyzed here.[3]

[3] The 3-letter combinations denote fatty acids esterified at the α-, β- and α′-positions of glycerol moiety in that respective order. L, P, and O indicate linoleic, palmitic, and oleic acids, respectively.

The preference of linoleic acid for the β-position of triglycerides has also been observed in studies on mouse fat (6), rat carcass fat (4) and thoracic lymph (12). All unsaturated fatty acids do not exert a preference for the β-position as illustrated by the triglyceride composition of the tissues of the animals fed menhaden oil in the present study. The higher polyunsaturated fatty acids were preferentially esterified in the α- and α′-positions in this group. The analysis of the individual triglyceride types and simple mixtures, into which the total triglyceride of each fat was fractionated, indicated that the preference for the β-position depended on the particular triglyceride. Linoleic acid appeared to be esterified preferentially in the β-position in all triglycerides of which it was a constituent, including the higher polyunsaturated fatty acids of menhaden oil which were concentrated in the α- and α′-positions. In triglycerides containing saturated fatty acids instead of linoleic acid, oleic acid was esterified predominantly in the β-position. In general, lipase analysis indicated that stearic and palmitic acid were esterified in the α- and α′-positions, but again the distribution is probably related to the constituent fatty acids, and the precise relationship will have to be determined by more specifically designed experiments. In general, the order of preference for the β-position appeared to be palmitic, oleic and linoleic acids. However, these observations, taken together with that of differences in triglyceride structure from tissue to tissue, indicated that the fatty acids were turning over in a regulated manner and that the processes follow specific patterns in each tissue. The general reaction may be regarded as a competitive relationship between fatty acids for lipid hosts as well as positional arrangement within molecules leading to triglycerides and phospholipids of specific structures, dependent, however, on the type and amount of fat in the diet.

Delineation of the specific patterns of triglyceride compostion will have to await further development in methodology which will permit a determination of the distribution of the fatty acids specifically in the α- and α′-positions, as well as the β-positions, thereby providing a complete definition of each molecular species. At present, much attention is being given to the application of multiple chromatographic procedures for the isolation of individual triglycerides as a basis for a complete structural analysis of these compounds (15).[4] Methods based on mathematically derived relationships are not generally applicable for such analysis as illustrated by the comparison of analyses by these techniques with the experimental values on liver and plasma triglycerides in the present study. Moreover, it is evident that lipase methods must be interpreted with caution when not performed on individual triglycerides or simple mixtures. For example, whereas a lipase analysis on the total fat may show that both oleic acid and linoleic acid are preferentially esterified in the β-position, an analysis of individual components will demonstrate that the oleic acid is only preferentially in the β-position in triglycerides in which it is associated with saturated fatty acids.

Since there are individual tissue differences in triglyceride structure in animals and probably plants, the analysis of processed fats, such as lard, for example, which consists of a mixture of tissue fats, provides only limited information on the metabolism of triglycerides. Analysis of tissue triglycerides, likewise, may not be nearly as informative as that of subcellular particles into which tissues may be fractionated.

The experiments, in which the fatty supplements were switched, demonstrated further the relationships delineated above. The analysis of the tissues from animals killed at intermediate periods (not reported) showed that the turnover of the lipid was fairly slow with some tissues requiring at least 6 months, which is a large portion of the life span of the rat. Nevertheless, it is evident that the metabolism of all lipid components are in equilibrium with each other in a highly regulated manner in relation to the fatty acid composition of the dietary fat.

[4] Litchfield, C., and R. Reiser 1964 Analysis of triglycerides by multiple chromatographic techniques. Presented at the World Fat Congress, Hamburg.

ACKNOWLEDGMENT

The authors wish to express their appreciation to Francis Lightly, David Schlichting and Patricia Igou for their interest and technical assistance during the course of this work.

LITERATURE CITED

1. Vander Wal, R. J. 1963 The determination of glyceride structure. J. Am. Oil Chemists' Soc., *40:* 242.
2. Youngs, C. G. 1959 Glyceride structure of fats. J. Am. Oil Chemists' Soc., *36:* 664.
3. Vander Wal, R. J. 1960 Calculation of the distribution of the saturated and unsaturated acyl groups in fats from pancreatic lipase hydrolysis data. J. Am. Oil Chemists' Soc., *37:* 18.
4. Perkins, E. G. 1964 The effect of dietary fat on the glyceride structure of rat carcass fat. J. Am. Oil Chemists' Soc., *41:* 285.
5. Mattson, F. H., and E. S. Lutton 1958 Specific distribution of fatty acids in glycerides of animal and vegetable fats. J. Biol. Chem., *233:* 868.
6. Tove, S. B., and F. H. Smith 1960 Changes in the fatty acid composition of the depot fat of mice induced by feeding oleate and linoleate. J. Nutrition, *71:* 264.
7. Blank, M. L., B. Verdino and O. S. Privett 1964 Determination of triglyceride structure by silver nitrate-TLC. J. Am. Oil Chemists' Soc., in press.
8. Blank, M. L., J. Schmit and O. S. Privett 1964 Quantitative analysis of lipids by thin-layer chromatography. J. Am. Oil Chemists' Soc., *41:* 371.
9. Alfin-Slater, R. B., and R. Schull 1963 Fats, fatty acids and sterols. In New Methods of Nutritional Biochemistry, ed., A. A. Albanese. Academic Press, Inc., New York, p. 411.
10. Mattson, F. H., and R. A. Volpenhein 1961 The use of pancreatic lipase for determining the distribution of fatty acids in partial and complete glycerides. J. Lipid Res., *2:* 58.
11. Carroll, K. K. 1964 Diet and fatty acid composition of lipids. Quantitative Methodology in Lipid Research Symposium, Pennsylvania State University, August 3–7. J. Am. Oil Chemists' Soc., in press.
12. Whyte, H. M., D. S. Goodman and A. Karmen 1962 Specificity in fatty acid esterification during fat absorption. Proc. 7th International Conference on Biochemical Problems in Lipids, Birmingham, p. 229.
13. Mattson, F. H., and R. A. Volpenhein 1962 Rearrangement of glyceride fatty acids during digestion and absorption. J. Biol. Chem., *237:* 53.
14. Verdino, B., M. L. Blank and O. S. Privett 1964 Studies on the lipid composition of the intestinal lymph of rats. J. Lipid Res., in press.
15. Kaufmann, H. P., and H. Wessels 1964 Die Struktur der Triglyceride: Theorien und Bestimmungsmethoden. Fette, Seifen, Anstrichmittel, *66:* 13.

Action of Linolenic and Docosahexaenoic Acids upon the Eicosatrienoic Acid Level in Rat Lipids

RODOLFO R. BRENNER [1] AND PEDRO JOSÉ
Cátedra de Bioquímica, Instituto de Fisiología, Facultad de Ciencias Médicas, La Plata, Argentina

ABSTRACT Weanling rats were fed a fat-free diet, and a fat-free diet supplemented with 4-deoxypyridoxine·HCl. The fatty acid composition of the lipids of heart, liver, epididymal fat, liver phospholipids and triglycerides were analyzed after feeding linoleate, linolenate and docosahexaenoate. The structure of octadecadienoate, eicosatrienoate and eicosatetraenoate was recognized by reductive ozonolysis. The levels of eicosa-5,8,11-trienoate were markedly lowered either by linoleate, linolenate or docosahexaenoate, thus showing a reduced conversion of oleate into eicosa-5,8,11-trienoate. Docosahexaenoate was readily incorporated into the phospholipids and appeared to be a direct regulator of eicosa-5,8,11-trienoic level, whereas linolenate was very slightly incorporated and appeared to produce, at least partially, the same effect through its conversion into docosahexaenoate. This phenomenon is thought to be related not only to a competition in the desaturating and elongating step of oleic, linoleic and linolenic acids, but also to phospholipid synthesis.

Reports from this laboratory have shown that although dietary linoleate, as well as arachidonate, decreases the concentration of eicosa-5,8,11-trienoic acid in heart and liver lipids of the rat, arachidonate may be a specific regulator of that effect, whereas linoleate appears to act mainly through its conversion into arachidonic acid (1). These results led to our investigation on the interrelationship between linolenate and docosahexaenoate on eicosa-5,8,11-trienoic level. The effect of linolenate and docosahexaenoate on the disappearance of eicosa-5,8,-11-trienoic acid was studied in rats reared with a fat-free diet or this same diet supplemented with 4-deoxypyridoxine·HCl (DP).

MATERIALS AND METHODS

Methyl linolenate [2] (18:3 ω3) [3] was more than 95% pure. No linoleate (18:2 ω6) was detected by gas-liquid chromatography (GLC). Methyl docosahexaenoate (59% pure) (22:6 ω3) was obtained from an industrial fish oil [4] (mackerel) by saponification, fractional crystallization (2) and vacuum distillation. It contained 0.1% eicosatetraenoic acid (20:4), 6.3% eicosapentaenoic acid (20:5 ω3) and was free from linolenate, as estimated by GLC. Methyl linoleate 95% pure was obtained from sunflower seed oil by the method of urea adducts (3) and vacuum distillation.

Weanling 21-day-old male albino rats from the strain of the Institute were maintained under the conditions detailed in table 1. All the rats except the control group received the fat-free diet until they reached a plateau in growth. One group of animals was then fed the fat-free diet without pyridoxine but supplemented with 450 mg 4-deoxypyridoxine·HCl/kg of diet, whereas the other group continued to receive the fat-free diet. In both groups of rats, the effect of daily administration of 100 mg of methyl linolenate, methyl docosahexaenoate and methyl linoleate was studied. Methyl esters were administered orally by microsyringe in 2 doses. To protect the esters from oxidation, 0.1% α-tocopherol (4) was incorporated.

The effect of pyridoxine on the animals fed the fat-free diet plus the DP supplement was also studied. For 7 additional days, 5 animals of this group were maintained with the same diet supplemented daily with 0.35 mg of pyridoxine/rat. Pyridoxine was administered in solution in 2 doses.

Received for publication June 15, 1964.

[1] Member of the Carrera del Investigador Científico, Consejo Nacional de Investigaciones Científicas y Técnicas, República Argentina.

[2] Fluka, A. G., Buchs, Switzerland.

[3] The number before the colon denotes the number of carbon atoms, and the number after the colon, number of double bonds. The ω-number denotes the position of the double bond nearest to the methyl end and points out family relationship; i.e., all fatty acids ω3 belong to the linolenic acid family.

[4] Gift of S. A. Copemar, Mar del Plata, Argentina.

TABLE 1

Periods the various diets were fed to experimental groups of rats

		Fat-free diet (with pyridoxine)						Fat-free diet (without pyridoxine) + DP [1]							
	Control [2]	Fat-free	+ 18:2 [3]	+ 18:3		+ 22:6		Fat-free + DP	+ Pyridoxine	+ 18:2	+ 18:3			+ 22:6	
Group no.	C(6) [4]	1(3)	2(2)	3(3)	3′(2)	4(3)	4′(3)	5(5)	6(5)	7(5)	8(5)	8′(3)	8″(1)	9(5)	9′(4)
	days	*days*	*days*	*days*	*days*	*days*	*days*	*days*	*days*	*days*	*days*	*days*	*days*	*days*	*days*
Fat-free diet [5]		90	83	83	83	83	83	73	73	73	73	73	73	73	73
Fat-free diet without pyridoxine								7	7	7	7	7	7	7	7
Fat-free diet without pyridoxine + DP								10	10	3	3	3	3	3	3
Fat-free diet + 18:2	90		7												
Fat-free diet + 18:3				7	10										
Fat-free diet + 22:6						7	10								
Fat-free diet without pyridoxine + DP + 18:2										7					
Fat-free diet without pyridoxine + DP + 18:3											7	10	14		
Fat-free diet without pyridoxine + DP + 22:6														7	10
Fat-free diet + 35 mg pyridoxine + DP									7						

[1] 4-Deoxypyridoxine·HCl.

[2] Animals received the fat-free diet plus 1% methyl linoleate.

[3] Fatty acid; the number before the colon denotes number of carbon atoms, and the number after the colon, number of double bonds.

[4] Numbers in parentheses indicate the number of rats per group.

[5] Fat-free diet: (in grams) fat-free casein, 19; sucrose, 77; McCollum-Davis salt mixture no. 4 (1), 4; and vitamins. Each kilogram of diet contained the following vitamins: (in milligrams) thiamine, 10; riboflavin, 10; pyridoxine, 10; Ca pantothenate, 50; niacin, 25; inositol, 1000; biotin, 2.0; *p*-aminobenzoic acid, 10; folic acid, 2.0; choline chloride, 1000; vitamin B_{12}, 0.1; *α*-tocopherol, 30; menadione, 50; vitamin A, 3000 IU; and vitamin D, 800 IU.

In comparison with our previous work (1) low mortality was observed in all the pyridoxine-deficient rats. From the pyridoxine-deficient groups 3 animals died, whereas only one died from the other group. However all fat-deficient animals fed the DP supplement showed a marked decrease in their weights when compared with the fat-deficient groups, showing an additional effect of deoxypyridoxine in this respect. For instance, the growth coefficient expressed as $(Wf - Wi/Wi) \times 100$ (where Wi means initial weight of the rats 3 days before the fat supplementation or at the beginning of DP administration; Wf means final weight after 10 days, when the rats were killed) reached −0.69 for the fat-deficient animals (group 1) and −8.16 for fat-deficient rats receiving the DP supplement (group 5). The administration of pyridoxine (group 6) increased the growth coefficient to −3.77. The supplementation of polyunsaturated acids provoked a favorable growth response in all the animals, but this effect was unable to compensate completely for the sharp decrease in weight caused by the DP. These results are shown clearly by comparison of the high growth coefficients 2.99 and 3.53 of fat-deficient rats receiving linolenate (group 3) and docosahexaenoate (group 4), respectively, to the low values −3.61 and −3.65 of similar fat-pyridoxine-deficient rats fed the supplement with the same acids.

After the periods shown in table 1, the animals were killed by ether anesthesia. The hearts, livers and epididymal fat were quickly removed, weighed and pooled according to groups. The tissues were immediately homogenized and the lipids extracted with chloroform:methanol (2:1) by the procedure of Folch et al. (6). From the washed extracts, the total lipid content was estimated in aliquots by evaporation under reduced pressure. Phosphorous (7) and cholesterol (8) were determined in liver extracts. The liver lipids were dissolved in chloroform and fractionated by adsorption in activated silicic acid.[5] Triglycerides and cholesterol esters were washed away with chloroform. Crude phospholipids were eluted with methanol. Pure triglycerides from groups 8 and 9 were separated by thin-layer chromatography on silicia gel G with petroleum ether. ethyl ether, and acetic acid (90:10:1) (9).

All the total lipids and lipid fractions separated were transesterified by refluxing 3 hours under nitrogen with 3N HCl in methanol.[6] The methyl esters were purified by sublimation (10) and analyzed by G.L.C. using a Pye apparatus with argon ionization detector. Glass columns 122-cm long with a 4-mm i.d. packed with 10% polyethylene-glycol-adipate in Chromosorb P (100–120 mesh) and 10% Apiezon N in Celite 80–120 mesh at 190 and 200° were used. The percentage compositions were calculated from the surface of the peaks by triangulation. The individual esters, palmitate, palmitoleate, stearate, oleate, linoleate, linolenate, arachidonate, eicosatrienoate and docosahexaenoate, were identified by comparison of their adjusted retention times relative to stearate with those of known standards. Docosatrienoate, docosatetraenoate, docosapentaenoate (22:5 ω6) and docosapentaenoate (22:5 ω3) were tentatively identified by the carbon number (11) and by the procedure of Ackman (12) (13). The structure of 20:3 recovered from the liver phospholipids of fat-deficient rats (group 1) and fat-deficient animals receiving DP plus linoleate (group 7) was investigated by ozonolysis. Unsaturated fatty acids were separated from the phospholipids and concentrated by micro-crystallization (14) in acetone at −25 and −65°. Pure (> 99%) 20:3 methyl ester was recovered by preparative GLC in the Pye apparatus with a 1-cm i.d. column packed with 10% polyethylene - glycol - adipate. Eicosatrienoate was ozonized at −65° in 0.5 ml methylene chloride, reduced by triphenylphosphine (15) and the products of cleavage identified by GLC in 10% polyethylene-glycol-adipate. Aldehydes of 9, 7 and 6 carbon and aldehyde methyl esters of 5, 7, and 8 carbons were recognized. These components correspond with 5,8,11-, and 7,10,13- and 8,11,14-eicosatrienoic acids belonging to the oleic, palmitoleic and linoleic acids, respectively. However the proportion of the different isomers was different in both groups of animals. In group 1, the approximate composition was 93, 5 and 2%

[5] Baker Analyzed Reagent, J. T. Baker Chemical Company, Phillipsburg, New Jersey.
[6] Van Gent, C. M., personal communication.

of 5,8,11-, 7,10,13- and 8,11,14-acids, respectively. In group 7, receiving linoleate, the relative proportions were 81, 5 and 14%. This means that the relative proportion of the 8,11,14-isomer depends upon the amount of dietary linoleate, but it does not mean that the total amount of this acid is higher in the animals fed linoleate. The structure of 20:4 separated from the phospholipids of the animals of group 7 (fat-deficient + DP + linoleate) was investigated in a similar way. The main component was arachidonic acid, containing minimal amounts of eicosa-7,10,13,16-tetraenoic acid (palmitoleic family).

In the fat-deficient rats the linoleate peak of the gas-liquid chromatogram was immediately followed by small amounts of another ester of C_{18} (recognized by hydrogenation). This peak was concentrated and separated together with the linoleate by preparative GLC and studied by reductive ozonolysis. Aldehydes and aldehyde esters corresponding to 9,12-, 8,11-, 6,9- and 5,8-octadecadienoates were observed, proving the existence of small amounts of isomers belonging to the linoleic, palmitoleic, oleic and vaccenic series, respectively.

RESULTS

The primary effects of the dietary changes were noted in the fatty acids of liver (table 2) and heart (table 3). Liver phospholipids (table 4) were very sensitive to the presence and type of polyenoic acids in the diet. Triglycerides from the liver and epididymal fat, on the contrary, were not affected as much by these acids. Only linoleate was readily incorporated into the triglycerides in rather large amounts (19% in epididymal fat from group 2). Linolenate was incorporated in minimal proportions (0.7% and 1%, respectively, in epididymal fat and liver triglycerides of group 8). This low incorporation of 18:3 ω3 into the depot fat of the rat agrees with results of Mohrhauer and Holman (16). Furthermore, these investigators proved that the amount incorporated is proportionally related to the amount in the diet. Similar results were obtained in fish by Reiser et al. (17). Dietary docosahexenoate was also incorporated in small amounts in triglycerides (1% in epididymal fat of group 9). Polyunsaturated fatty acids (20:3, 20:4, 20:5, and 22:6) synthesized by the rats from the corresponding precursors, were observed in minor amounts in the depot fat.

The conversion of dietary 18:2 ω6 into 20:4 ω6 and 18:3 ω3 into 20:5 ω3, 22:5 ω3 and 22:6 ω3, was clearly shown by comparing the compositions of total lipids of heart and liver (tables 2 and 3, groups 2 and 3 vs. 1) and liver phospholipids (table 4, groups 2 and 3 vs. 1). Notwithstanding, an important difference was observed between the linoleate and linolenate series.

TABLE 2

Fatty acid composition of liver lipids

		Fat-free diet				Fat-free diet without pyridoxine + DP				
	Control	Fat-free	+ 18:2	+ 18:3	+ 22:6	Fat-free + DP	+ Pyridoxine	+ 18:2	+ 18:3	+ 22:6
Group no.	C	1	2	3	4	5	6	7	8	9
Fatty acids [1]	%	%	%	%	%	%	%	%	%	%
16:1 [2]	4.1	6.5	4.1	4.1	4.3	7.5	6.0	7.4	5.4	4.2
18:1	18.4	32.5	23.8	23.5	24.7	44.0	43.7	36.4	37.0	27.7
18:2	6.9	1.2	5.5	0.3	0.9	0.2	0.5	3.6	0.3	0.9
18:3	—	0.2	—	0.9	—	—	—	—	0.6	0.4
20:3	0.4	14.0	2.6	3.8	2.9	9.8	11.6	2.7	3.1	2.9
20:4	19.5	4.8	17.7	2.8	3.2	3.1	3.4	9.0	1.7	2.6
20:5	—	—	—	8.0	5.3	—	—	0.4	5.5	4.0
22:5 ω 3	0.3	tr	0.4	1.9	tr	tr	tr	tr	0.5	0.4
22:6	0.6	0.5	2.8	7.0	8.3	tr	tr	0.5	3.8	8.0

[1] Principal unsaturated acids only.
[2] The number before the colon denotes number of carbon atoms, and the number after the colon, number of double bonds; the ω denotes position of double bond nearest to the methyl end.

TABLE 3

Fatty acid composition of heart lipids

		Fat-free diet				Fat-free diet without pyridoxine + DP				
	Control	Fat-free	+ 18:2	+ 18:3	+ 22:6	Fat-free + DP	+ Pyridoxine	+ 18:2	+ 18:3	+ 22:6
Group no.	C	1	2	3	4	5	6	7	8	9
	%	%	%	%	%	%	%	%	%	%
Fatty acids [1]										
16:1 [2]	2.5	4.8	3.0	3.1	4.2	5.1	3.9	3.0	3.7	3.5
18:1	17.7	27.4	21.2	24.7	24.3	36.8	30.7	22.5	28.5	26.7
18:2	14.5	2.6	14.5	2.8	1.9	1.0	1.1	17.6	1.8	2.4
18:3	0.6	tr	0.3	2.8	—	0.3	0.2	0.1	1.6	0.4
20:3	0.7	17.4	8.2	11.5	11.0	14.6	18.3	10.4	13.2	10.5
20:4	19.5	5.4	10.9	8.5	8.1	4.8	6.9	9.6	6.5	7.1
20:5	—	0.1	tr	3.2	1.4	tr	—	—	2.0	1.1
22:5 ω 3	tr	tr	0.4	1.2	tr	tr	tr	tr	1.0	0.3
22:6	1.2	0.6	2.0	2.7	7.4	0.6	1.4	0.8	2.4	7.1

[1] Principal unsaturated acids only.
[2] The number before the colon denotes number of carbon atoms, and the number after the colon, number of double bonds; the ω denotes position of double bond nearest to the methyl end.

TABLE 4

Fatty acid composition of liver phospholipids

		Fat-free diet				Fat-free diet without pyridoxine + DP				
	Control	Fat-free	+ 18:2	+ 18:3	+ 22:6	Fat-free + DP	+ Pyridoxine	+ 18:2	+ 18:3	+ 22:6
Group no.	C	1	2	3	4	5	6	7	8	9
	%	%	%	%	%	%	%	%	%	%
Fatty acids [1]										
16:1 [2]	2.8	4.5	3.6	3.1	3.2	7.2	5.5	4.4	4.9	4.9
18:1	10.0	21.0	13.9	17.7	11.5	30.5	25.1	19.0	19.4	19.1
18:2	7.7	1.0	5.4	0.8	0.7	0.5	0.6	3.5	0.4	0.4
18:3	tr	—	tr	0.9	—	—	—	tr	0.6	tr
20:3	0.5	16.3	3.1	4.3	4.3	15.0	17.8	4.7	5.3	3.2
20:4	27.8	8.9	22.1	3.5	4.8	5.8	7.7	20.1	2.8	3.7
20:5	—	0.1	tr	8.9	7.5	—	tr	—	8.7	5.8
22:5 ω 3	0.1	tr	tr	2.2	tr	tr	tr	0.1	1.5	0.3
22:6	1.2	1.3	2.7	9.5	17.8	0.7	0.9	1.1	9.0	12.9
Phospholipids, % (P % × 25)	3.22	2.60	2.95	3.18	2.70	2.63	2.83	2.39	2.93	3.23

[1] Principal unsaturated acids only.
[2] The number before the colon denotes number of carbon atoms, and the number after the colon, number of double bonds; the ω denotes position of double bond nearest to the methyl end.

Linoleate was readily incorporated in total lipids and phospholipids in large amounts and at the same time, it was substantially converted into arachidonate. Linolenate was incorporated only to a small degree, but a large proportion was converted into the corresponding pentaenoic and hexaenoic acids. In 7 days of feeding 18:3 ω3, this conversion did not reach the steady state, since in 3 additional days liver 20:5 ω3 and 22:6 ω3 increased from 8.0 to 10.1% and 7.0 to 8.1%, respectively.

Eicosa-5,8,11-trienoic acid increased to high levels in the liver and heart of fat-deficient rats. These high values were effectively reduced by 18:2 ω6 and 18:3 ω3 (tables 2, 3 and 4), although the latter was incorporated only slightly into the lipids. Morhrhauer and Holman (16, 18) observed similar results, reporting that the level of 20:3 was lowered by 18:3 in the same manner as when 18:2 or 20:4 was fed. A more effective and even more efficient decrease of eicosa-5,8,11-trienoic

TABLE 5

Amount of liver fatty acids

		Fat-free diet				Fat-free diet without pyridoxine + DP				
	Control	Fat-free	+ 18:2	+ 18:3	+ 22:6	Fat-free + DP	+ Pyridoxine	+ 18:2	+ 18:3	+ 22:6
Group no.	C	1	2	3	4	5	6	7	8	9
Fatty acids [1]	*mg* [2]	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
16:0 [3]	89.9	26.4	51.9	72.1	75.6	56.3	43.4	50.5	62.9	72.7
16:1	14.4	7.8	11.3	10.8	10.6	21.1	13.2	15.6	13.1	11.0
18:0	71.4	18.7	58.0	46.7	46.4	38.6	32.1	27.8	37.1	52.3
18:1	64.5	39.0	65.7	64.8	61.7	123.8	96.3	77.6	89.9	72.6
18:2	24.2	1.4	15.2	0.8	2.2	0.6	1.1	7.6	0.7	2.4
18:3	—	0.2	—	2.4	—	—	—	—	1.5	1.0
20:3	1.4	16.7	7.2	10.0	7.2	27.6	25.5	5.7	7.5	7.6
20:4	68.2	5.7	49.0	7.4	7.9	8.7	7.5	19.0	4.1	6.8
20:5 ω 3	—	—	—	21.1	13.1	—	—	0.8	13.3	10.5
22:5	1.1	—	1.1	5.0	—	—	—	—	1.2	1.0
22:6	2.1	0.6	7.7	18.5	20.5	—	—	1.1	9.2	20.9
Lipid, %	4.67	4.12	5.63	5.16	5.00	5.87	4.38	4.32	5.22	4.56
Cholesterol, %	0.145	0.232	0.192	0.191	0.178	0.481	0.254	0.205	0.271	0.207

[1] Principal fatty acids.
[2] Per liver.
[3] The number before the colon denotes number of carbon atoms, and the number after the colon, number of double bonds.

acid was observed when 22:6 was included in the diet (tables 2, 3 and 4, group 4). Rahm and Holman (19) recently reported the same results. They showed that not only 22:6 ω3 and 20:4 ω6 but also 22:5 ω3 are more effective than 18:2 ω6 and 18:3 ω3 in reducing 20:3 ω9 content in the rat. The percentage of oleate was decreased either by 18:2 ω6, 18:3 ω3 or 22:6 ω3, whereas 20:4 ω6 content was reduced in the liver by feeding 18:3 ω3 or 22:6 ω3. All rats fed linoleate showed a slight increase in 22:5 ω6 compared with those animals receiving a fat-free diet. This increase was also observed by Rahm and Holman (19). The variations noted were from 0.4 to 1.8% and 0.7 to 1.0% in liver and heart total lipids, respectively. In liver phospholipids the increase calculated was from 0.7 to 2.3%.

The 4-deoxypyridoxine·HCl (DP) in the diet was included to complement this first part of the experiment. The DP produced an antipyridoxine effect (tables 2 and 3, group 6 vs. 5) in the rats, and lower percentages of 20:4 ω6, 20:5 ω3 and 22:6 ω3 were apparently synthesized from linoleate and linolenate, respectively (tables 2 and 3, groups 7 and 8 vs. 2 and 3). However, the amounts of 20:4 ω6, 20:5 ω3 and 22:6 ω3 produced in the presence of DP were high enough to reduce the percentage of 20:3 ω9 to very low values, thus confirming the previous results. The percentage of eicosatrienoic acid was also reduced in this experiment by DP supplementation alone (tables 2 and 3, group 5 vs. 1 and 6). This result does not appear to agree with our previous work (1), but similar results might be obtained when the total amount of 20:3 is calculated per liver, multiplying the percentages of that fatty acid by the amount in milligrams of total non-cholesterol lipid per liver. The quantity of eicosatrienoic acid calculated in this way for the liver of fat-deficient rats fed the DP supplement was similar to that estimated in fat-deficient animals receiving pyridoxine (table 5, group 5 vs. 6 and 1). Although the amount of 20:3 ω9 was not decreased by the addition of DP, oleic acid increased markedly, thus suggesting that not only the transformation of 18:2 ω6 into 20:4 ω6 and of 18:3 ω3 into 20:5 ω3 and 22:6 ω3 are lowered by DP, but also the transformation of 18:1 ω9 into 20:3 ω9. In general it might be postulated that the polyunsaturated acid formation is partially decreased by the lack of pyridoxine.

DISCUSSION

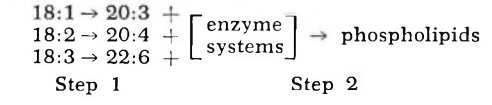
The comparison between all these results and those obtained previously (1) on linoleate and arachidonate, leads us to consider that the eicosa-5,8,11-trienoic level is not reduced by the direct displacement by linoleate or linolenate but rather by their conversion into more unsaturated acids, namely, arachidonic, eicosapentaenoic and docosahexaenoic acids.

In the case of linoleate, Dhopeshwarkar and Mead (20) have proposed that this effect might be due to a competition between oleic and linoleic acids for the enzymes involved in their transformation into polyunsaturated fatty acids. Mohrhauer and Holman (21) have also agreed with this explanation in the case of feeding linoleate and linolenate. Furthermore, they have shown that not only the conversion of oleate into eicosatrienoate, but also the conversion of linoleate into arachidonate, is likewise patrially inhibited by dietary linolenate. They attribute this effect to a substrate competition, and that linoleate and linolenate competitively occupy the enzymes responsible for dehydrogenation and chain lengthening. However, either dietary 20:4 ω6 (1) or 22:6 ω3 readily and effectively decrease the 20:3 ω9 level. Therefore, these acids are directly responsible for the inhibition in the formation of 20:3 ω9. Since the inclusion of linoleate and linolenate in the diet produces 20:4 ω6 and 22:6 ω3 synthesis, respectively, the inhibition caused in the formation of 20:3 ω9 from oleic, may be due not only to a competitive action between 18:2 ω6 or 18:3 ω3 desaturation and elongation, on the one hand, and 18:1 on the other, but also to a direct effect of synthetized 20:4 ω6 and 22:6 ω3. Considering that 20:4 ω6, 22:5 ω3 and 22:6 ω3 are more effective than 18:2 ω6 and 18:3 ω3 in reducing 20:3 ω9 content, we may extrapolate for those acids the observation of Mohrhauer and Holman (21) that the order of affinity for the enzyme sites apparently depends on the degree of unsaturation of the acid (the order of affinity was

linolenate > linoleate > oleate). Arachidonate and docosahexaenoate, in such a case, would probably inhibit its own synthesis from linoleate and linolenate. To avoid this effect, 20:4 ω6 and 22:6 ω3, to the extent that they are synthesized, must be removed from the system by some mechanism. We consider that this mechanism involves the incorporation of fatty acids into phospholipids.

In previous experiments (22, 23) we have shown that 20:3 ω9, 20:4 ω6 and 22:6 ω3 are preferentially directed, in vivo, to the same position of phosphatidyl choline and phosphatidyl ethanolamine. Lands and Merkl (24, 25) have also shown that, in vitro, rat liver microsomes preferentially incorporate linoleate or oleate in the β-carbon and stearate into the α′-carbon of acylglycerophosphoryl choline and acylglycerophosphoryl ethanolamine. Furthermore, these investigators have provided evidence that of the unsaturated acids, oleic and linoleic, linoleic was a better substrate than oleic acid for the β-acyl transferase. Lorch et al. (26) have recently reported that microsomal systems, in vitro, actually synthesize fatty acids which appear directly as phospholipids, the microsomal enzymes being also responsible for the dehydrogenation of linoleic (27, 28), oleic[7,8] and other fatty acids (29) into polyunsaturates. Thus, polyunsaturated fatty acids are preferentially incorporated into the β-position of glycerophosphatides, and the synthesis of phospholipids can be produced in the same subcellular particle where the desaturation takes place.

Comparing these results we believe that marked competition between 20:3 ω9, 20:4 ω6 and 22:6 ω3 may take place in their incorporation into the β-carbon of phospholipids and this competition may be interrelated with polyunsaturated fatty acid synthesis. The following simplified diagram shows the possible mechanism.



In the intermediate reactions of step 1 there appears to be competition in the desaturation and elongation of the 3 acids or at least a different rate of conversion, but step 2 is, in our opinion, another important place where the level of polyunsaturated fatty acids may be controlled. Competition of the polyunsaturated fatty acids for the step-2 enzyme systems (probably lysophospholipid β-fatty acyl transferase) may remove preferentially certain fatty acids, favoring the corresponding desaturating reaction (step 1) to go further and both steps 1 and 2, working together displace one polyunsaturated fatty acid for other. We prefer the lysophospholipid β-acyl transferases as the enzymes responsible for this effect considering the work of Lands and Merkl (24, 25) as well as the observation of Stein et al. (30). These investigators showed that in aortic homogenates linoleic acid was confined to the β-position of lecithin when lysolecithin was used, whereas it was incorporated in both α′- and β-position with α-glycerophosphate.

ACKNOWLEDGMENT

The authors are grateful to Marta Maysonave for her technical assistance.

LITERATURE CITED

1. Peluffo, R. O., R. R. Brenner and O. Mercuri 1963 Action of linoleic and arachidonic acids upon the eicosatrienoic acid level in rat heart and liver. J. Nutrition, *81*: 110.
2. Brown, J. B. 1955 Fractional solvent crystallization. J. Am. Oil Chemists' Soc., *32*: 646.
3. Swern, D., and W. E. Parker 1953 Application of urea complexes in the purification of fatty acids esters and alcohols. III. Concentrates of natural linoleic and linolenic acids. J. Am. Oil Chemists' Soc., *30*: 5.
4. Moore, R. N., and W. G. Bickford 1952 A comparative evaluation of several antioxidants in edible fats. J. Am. Oil Chemists' Soc., *29*: 1.
5. Holman, R. T. 1956 Function and metabolism of essential fatty acids. In Biochemical Problems of Lipids, eds., G. Popjak and E. Le Breton. Butterworths Scientific Publications, London, p. 463.
6. Folch, J., M. Lees and G. H. Sloane-Stanley 1957 A simple method for the isolation and purification of total lipids of animal tissues. J. Biol. Chem., *226*: 497.
7. Allen, R. J. L. 1940 The estimation of phosphorous. Biochem. J., *34*: 858.
8. Abell, L. L., B. B. Levy, B. B. Brodie and F. E. Kendall 1952 A simplified method for the estimation of total cholesterol in

[7] De Tomás, M. E., and R. R. Brenner, unpublished data.

[8] Nugteren, D. H. 1963 The enzymic conversion of γ-linolenic acid into homo-γ-linolenic acid. Meeting of the Biochemical Society, Oxford, England.

serum and demonstration of its specificity. J. Biol. Chem., *195*: 357.
9. Malins, D. C., and H. K. Mangold 1960 Analysis of complex lipid mixtures by thin-layer chromatography and complementary methods. J. Am. Oil Chemists' Soc., *37*: 576.
10. Stoffel, W., F. Chu and E. H. Ahrens 1959 Analysis of long chain fatty acids by gas liquid chromatography. Micromethod for preparation of methyl esters. Analyt. Chem., *31*: 307.
11. Woodford, F. P., and C. M. van Gent 1960 Gas liquid chromatography of fatty acid methyl esters: The "carbon number" as a parameter for comparison of columns. J. Lipid Res., *1*: 188.
12. Ackman, R. G. 1963 Structural correlation of unsaturated fatty acid esters through graphical comparison of gas-liquid chromatographic retention times on a polyester substrate. J. Am. Oil Chemists' Soc., *40*: 558.
13. Ackman, R. G. 1963 An analysis of separation factors applicable in the gas-liquid chromatography of unsaturated fatty acid methyl esters on a polyester substrate. J. Am. Oil Chemists' Soc., *40*: 564.
14. Schlenk, H. 1961 Crystallization of fatty acids. J. Am. Oil Chemists' Soc., *38*: 728.
15. Stein, R. A., and N. Nicolaides 1962 Structure determination of methyl ester of unsaturated fatty acids by gas-liquid chromatography of the aldehydes formed by triphenyl phosphine reduction of the ozonides. J. Lipid Res., *3*: 476.
16. Mohrhauer, H., and R. T. Holman 1963 The effect of dietary essential fatty acids upon composition of polyunsaturated fatty acids in depot fat and erythrocytes of the rat. J. Lipid Res., *4*: 346.
17. Reiser, R., B. Stevenson, M. Kayama and R. B. R. Choudhury 1963 The influence of dietary fatty acids and environmental temperature on the fatty acid composition of teleost fish. J. Am. Oil Chemists' Soc., *40*: 507.
18. Mohrhauer, H., and R. T. Holman 1963 The effect of dose level of essential fatty acids upon fatty acid composition of the rat liver. J. Lipid Res., *4*: 151.
19. Rahm, J. J., and R. T. Holman 1964 The relationship of single dietary polyunsaturated fatty acids to fatty acid composition of lipids from subcellular particles of liver. J. Lipid Res., *5*: 169.
20. 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.
21. Mohrhauer, H., and R. T. Holman 1963 Effect of linolenic acid upon the metabolism of linoleic acid. J. Nutrition, *81*: 67.
22. De Tomás, M. E., R. R. Brenner and R. O. Peluffo 1963 Position of eicosatrienoic acid in the phosphatidyl choline and phosphatidyl ethanol amine from essential fatty acid deficient rats. Biochim. Biophys. Acta, *70*: 472.
23. Brenner, R. R. 1964 Posición en la que se incorporan los ácidos linoleico, linolénico y polietilénicos derivados en la fosfatidil etanol amina y fosfatidil colina. Notas de la Comisión de Investigaciones de la Provincia de Buenos Aires. La Plata, Argentina, in press.
24. Lands, W. E. M., and J. Merkl 1963 Metabolism of glycerolipids. III. Reactivity of various acyl esters of coenzyme A with α'-acylglycerophosphorylcholine and positional specificities in lecithin synthesis. J. Biol. Chem., *238*: 898.
25. Merkl, I., and W. E. M. Lands 1963 Metabolism of glycerolipids. IV. Synthesis of phosphatidylethanolamine. J. Biol. Chem., *238*: 905.
26. Lorch, E., S. Abraham and I. L. Chaikoff 1963 Fatty acid synthesis by complex systems. The possibility of regulation by microsomes. Biochim. Biophys. Acta, *70*: 627.
27. Stoffel, W. 1961 Biosynthesis of polyenoic fatty acids. Biochem. Biophys. Res. Com., *6*: 270.
28. Nugteren, D. H. 1962 Conversion *in vitro* of linoleic acid into γ-linolenic acid by rat-liver enzymes. Biochim. Biophys. Acta, *60*: 656.
29. Holloway, P. W., R. O. Peluffo and S. J. Wakil 1963 On the biosynthesis of dienoic fatty acid by animal tissues. Biochem. Biophys. Res. Comm., *12*: 300.
30. Stein, Y., O. Stein and B. Shapiro 1963 Enzymic pathways of glyceride and phospholipid synthesis in aortic homogenates. Biochim. Biophys. Acta, *70*: 33.

Environmental Temperature and Growth Inhibition of Weanling Rats Fed Raw Soybean Rations [1]

RAYMOND BORCHERS
Department of Biochemistry and Nutrition, University of Nebraska, Lincoln, Nebraska

ABSTRACT Weanling rats were fed heated and raw soybean rations at environmental temperatures of 10° to 30°. At 30°, the growth rates with the raw soybean were markedly less than with the heated; at 10° the growth rates with raw meal were only slightly less than with the heated meal. Furthermore, animals fed the raw meal at 10° grew more rapidly than at 30°, contrary to the usual temperature effect on growth rate, whereas those fed the heated meal grew more slowly at 10° than at 30°. The supplementary effect of amino acids added to raw soybean rations was observed at 30° in confirmation of previous results.

Separation of the growth inhibitor from raw soybean meal is an objective of this laboratory. During the course of a series of rat growth assays of raw soybean fractions for growth inhibiting effects, variable growth responses were observed when aliquots of the same fraction were fed in successive experiments over a period of several months. An examination was made of the date of the feeding experiments with respect to the extent of growth inhibition by the soybean fraction. This suggested that greater growth inhibition occurred at those times when the environmental temperature in the animal room might have been higher, insignificant growth depression occurred during times of lower environmental temperature in the animal room. To test for a possible relationship between growth inhibition by raw soybean and environmental temperature, the growth rates of weanling rats fed heated and raw soybean meal were determined at several environmental temperatures.

PROCEDURE AND RESULTS

Weanling rats of the Holtzman strain were fed 25% heated and raw soybean meal rations following the procedure described in an earlier publication (1). The animals were housed at constant environmental temperature. Studies were made at 5° increments from 10° to 30°.

The rats fed heated soybean meal and housed at 30° made the most rapid growth with a decline in growth rate as the temperature was lowered. The opposite effect was noted with raw soybean meal feedings. The lowest growth rate was obtained at 30° with an increase in rate as the temperature was lowered to 10°. The growth rates at 10° and at 30° are presented in table 1 for 3 different soybean meal samples. Since the growth rates at intermediate temperatures approximated a straight line relationship, these are omitted for brevity. A comparison of the growth rates on heated and raw soybean rations indicates clearly that at low environmental temperatures, little growth inhibition is shown by raw soybeans. On the other hand, at higher environmental temperatures the growth inhibition by raw soybeans is distinctly accentuated over that noted previously (1, 2).

Rats housed at the low temperature and fed raw soybean meal consumed essentially the same amount of food as those fed the heated meal. Because the food consumption by rats fed the raw meal at 30° was markedly less than those fed the heated meal, a paired-feeding experiment was conducted at this temperature. According to the results presented in table 1, approximately one-half the growth differential between heated and raw soybean meal at 30° appears to be due to the reduced food intake of animals fed the raw soybean meal. A further calculation of the food intake with respect to body weight was made from the record of daily food intake and daily animal weight. This value was

Received for publication September 24, 1964.

[1] Published with the approval of the Director as paper no. 1582, Journal Series, Nebraska Agricultural Experiment Station. Supported in part by Public Health Service Research Grant no. A-2018 from the National Institute of Arthritis and Metabolic Diseases.

TABLE 1

Environmental temperature and the growth rate of weanling rats fed soybean meal rations [1]

	Soybean sample	Environmental temperature					
		10°			30°		
		Wt gain	Raw / Heated	Food intake	Wt gain	Raw / Heated	Food intake
		g/day	*%*	*g/day*	*g/day*	*%*	*g/day*
1	Heated	3.63	90	10.2	4.65	55	8.6
	Raw	3.29		10.2	2.54		6.5
2	Heated	3.42	97	10.7	4.27	54	8.1
	Raw	3.33		10.8	2.29		6.0
3	Heated	3.55	90	9.8	4.17	58	8.4
	Raw	3.18		10.1	2.41	76	6.4
	Heated, pair-fed with raw				3.15		6.5
3	Heated + 0.6% DL-threonine + 0.2% DL-valine				4.02	91	8.2
	Raw + 0.6% DL-threonine + 0.2% DL-valine				3.66		7.9

[1] All rations contained 25% of soybean meal and 0.6% of DL-methionine. The heated meal was prepared from the raw meal by autoclaving at 120° for 30 minutes. Each ration was fed to 4 female and 4 male weanling rats for 20 days. Animals were paired as to litter, sex, and initial weight.

12.0 g food consumed /100 g body weight /day for the rats fed the heated soybean meal ration, 11.1 g for those fed the raw meal, and 10.4 g for those fed the heated meal but pair-fed with the animals receiving the raw meal. These data indicate that depression of food intake by raw soybeans is not the major factor in the growth depression. Also, pair-feeding under these conditions imposes an unfair disadvantage to animals fed the heated soybean meal ration.

Data reported previously (2) indicated that amino acid supplements tended to equalize the growth rates with heated and raw soybean rations. Since these feeding experiments were conducted at a temperature of approximately 22° (range, 18° to 26°), these studies were repeated at 30°. The results presented in table 1 establish that an amino acid supplement is similarly effective at 30°.

DISCUSSION

The importance of conducting nutrition experiments at a known, constant, and optimal temperature is well recognized. The chance directing of our attention to the effect of environmental temperature has fortunately given different and apparently anomalous results with animals fed raw soybean meal. Animals fed raw soybean grew more rapidly at lower environmental temperatures, an effect which is contradictory to that observed with the usual ration (3–5). Furthermore, the growth differential between raw and heated soybean meal was much diminished in animals housed at 10°. A somewhat similar effect has been reported by Klain et al. (5) with animals fed amino acid imbalanced diets. These authors suggest that the severe metabolic derangements occurring in amino acid imbalance feeding in warm environments do not occur in colder environments. Such an hypothesis is an attractive one to consider in animals fed raw soybeans. The animals at low environmental temperature make metabolic adaptations, other than mere metabolic rate changes, which either bypass or otherwise render inocuous the heat-labile growth inhibitor of raw soybeans.

LITERATURE CITED

1. Borchers, R., D. Mohammad-Abadi and J. M. Weaver 1957 Antibiotic growth stimulation of rats fed raw soybean oil meal. Agr. Food Chem., *5:* 371.
2. Borchers, R. 1961 Counteraction of the growth depression of raw soybean oil meal by amino acid supplements in weanling rats. J. Nutrition, *75:* 330.
3. Swift, R. W. 1944 The effect of feed on the critical temperature for the albino rat. J. Nutrition, *28:* 359.
4. Heitman, H., and E. H. Hughes 1949 The effects of air temperature and relative humidity on the physiological well being of swine. J. Animal Sci., *8:* 171.
5. Klain, G. J., D. A. Vaughan and L. N. Vaughan 1962 Interrelationships of cold exposure and amino acid imbalances. J. Nutrition, *78:* 359.

Excretion of Total Nitrogen, Lysine and Methionine by Rats as Affected by the Type of Carbohydrate in the Diet [1,2]

YET-OY CHANG
Department of Biochemistry, Wyoming Experiment Station, University of Wyoming, Laramie, Wyoming

ABSTRACT The effect of various dietary carbohydrates on the urinary and fecal excretion of nitrogen, methionine and lysine in rats was determined. Rats fed raw-potato starch and dextrin diets excreted a larger amount of fecal nitrogen and amino acids than rats fed sucrose, glucose or cornstarch. Rats fed raw-potato starch excreted less urinary nitrogen and more urinary methionine and lysine than rats fed carbohydrate from the other sources. During the growth period, rats fed raw-potato starch and dextrin exhibited lowest methionine availability compared with the rats fed sucrose, cornstarch, or glucose. During the protein repletion period, there were no significant differences in methionine availability among the various groups. The low protein utilization by rats fed raw-potato starch was accompanied by lowered excretion of urinary nitrogen and by increased excretion of urinary methionine and lysine.

Investigators have demonstrated that certain starches, mainly those derived from tubers, were more poorly utilized than the starches derived from cereals. Harper and Katayama (1) postulated that the slower growth rate of rats fed a raw-potato starch diet was caused by the decrease in the utilization of dietary protein. Booher et al. (2) and Jelinek et al. (3) hypothesized that a heat-labile digestive-resistant substance present in raw-potato starch was responsible for the swift passage of food through the digestive canal, resulting in low protein utilization.

Folin (4) investigated the metabolism of protein by analyzing the urinary nitrogen compounds. In more recent studies, Kiriyama and Ashida (5) observed that the nutritive value of protein is reflected in the relative amounts of nitrogen compounds in the urine of rats. Other investigators (6–8) have reported that the urinary excretion of amino acids depends upon the quality and quantity of dietary protein. Feeding protein of low biological value was associated with the excretion of larger amounts of amino acids in the urine.

Previous workers (9–11) have postulated that the effect of different carbohydrates upon the utilization of dietary protein is related to the rate of the passage of the carbohydrate through the gastrointestinal tract. The slower movement of the more complex carbohydrates might, therefore, improve the digestion and absorption of protein. This relationship of dietary carbohydrate to protein digestion and to the process of protein absorption and catabolism in the animal body is not thoroughly understood.

In the present study the effect of carbohydrates on the excretion of urinary nitrogen, lysine, and methionine was investigated. In addition, the availability of methionine for rats in wheat gluten as affected by the nature of carbohydrates is also reported.

EXPERIMENTAL

Five experimental diets were prepared using the following carbohydrates: raw-potato starch, cornstarch, sucrose, dextrin, and glucose. The composition of diets is shown in table 1. Weanling, male rats of the Sprague-Dawley strain were divided into 5 groups of 12 according to body weight and litter origin. The experimental diets were fed to the rats when they were 4 weeks old and weighed 70 to 80 g. Food and water were provided ad libitum. Body weight and food consumption for each ani-

Received for publication August 17, 1964.
[1] Published with approval of the Director, Wyoming Experiment Station, as Journal Paper no. 240.
[2] Supported in part by funds from the Regional Project W-57 of the Western Agricultural Experiment Stations.

TABLE 1

Composition of diets

	%
Wheat gluten	24
Carbohydrate [1]	61
Hydrogenated fat	8
Salt mixture USP XIV	4
Cod liver oil, USP	2
Vitamin mix [2]	1

[1] Carbohydrate sources: potato starch (raw), cornstarch, sucrose, dextrin, glucose.
[2] Vitamin mix supplied: (in mg/kg of diet) thiamine, 5; pyridoxine, 5; menadione, 5; cobalamin concentrate (3 mg/g) 5; riboflavin, 10; nicotinic acid, 20; Ca pantothenate, 20; *d*-tocopheryl succinate, 75; folic acid, 5.5; biotin, 0.3; inositol, 400; and choline chloride, 1,000.

mal were recorded weekly during the 15-week study. The feces and urine from the rats were collected during 3 successive periods: 1) the growth period (5 days beginning the fourth week); 2) the protein-depletion period (5 days beginning the twelfth week); and 3) the protein-repletion period (5 days beginning the fifteenth week). During the protein-depletion period, the rats were fed nitrogen-free diets in order to measure endogenous urinary nitrogen and metabolic fecal nitrogen respectively. After the protein-depletion period, each group was fed its previous test ration, and the feces and urine of each rat were again collected for the determination of fecal and urinary nitrogen, and of amino acids in the protein-repletion period. In changing from one diet to another, 3 days were allowed to elapse before excreta were again collected.

During the collections, the animals were housed in metabolic cages; the feces and urine were collected daily, refrigerated and composited for the 5-day collection period. Toluene was used as a preservative for the urine. At the end of the collection, the cages, screens, and funnels were washed with distilled water, and the washings were added to the collected urine. The urine from each rat was evaporated to 50 ml, and a 20-ml aliquot was added to 20 ml of 8 N HCl. The sealed sample was autoclaved for 6 hours at 120°. The HCl was evaporated to dryness under reduced pressure. The residue was dissolved in distilled water, decolorized with 0.5 g of activated charcoal, filtered with suction and diluted to volume. The fecal samples were preserved with 30 ml of 2 N HCl. The composited fecal samples were homogenized and diluted to 100 ml with distilled water. Ten milliliters of the homogenate were added to 10 ml of 12 N HCl and autoclaved under the same conditions as the urine. Total nitrogen in the feces and urine was determined by the micro-Kjeldahl method (12); lysine and methionine were determined microbiologically (13). Differences in means were tested using Duncan's multiple range test (14).

RESULTS AND DISCUSSION

The total weight gain and the food consumption for rats as affected by the type of dietary carbohydrate have been published in a previous paper (15). In the present paper, the data given concern only the excretion of total nitrogen, lysine and methionine. The influence of carbohydrate source on fecal and urinary nitrogen excretion is shown in table 2; the excreted nitrogen is expressed as a percentage of the ingested nitrogen. During the growth period, the protein-depletion period, and the protein-repletion period, the fecal nitrogen excreted by rats fed raw-potato starch and dextrin was significantly higher ($P < 0.05$) than the fecal nitrogen excreted by rats fed the other carbohydrate sources. The experiment determined that the fecal nitrogen excreted by rats fed raw-potato starch was not related to the amount of nitrogen ingested. Rather the nature of the carbohydrate determined the amount of fecal nitrogen excretion. A study of the data shows that rats fed raw-potato starch always excrete the highest percentage of fecal nitrogen, whether or not protein is fed with the raw potato starch. Furthermore, during the protein-repletion period, the total nitrogen intake by rats fed dextrin was the highest, but the dextrin-fed rats excreted less fecal nitrogen than the rats fed raw-potato starch.

On the other hand, rats fed raw-potato starch excreted less urinary nitrogen than rats fed carbohydrate from the other sources during the growth, protein-depletion, and protein-repletion periods (table 2). The low excretion of urinary nitrogen by rats fed raw-potato starch may have been a reflection of reduced protein turnover. Perhaps these turnover rates are

TABLE 2

Total fecal and urinary nitrogen excreted by rats fed various carbohydrate diets [1,2]

Carbohydrate source	Total food intake	Nitrogen intake	Urinary nitrogen	Urinary N/N intake	Fecal nitrogen	Fecal N/N intake
	g	*g*	*g*	%	*g*	%
			Growth period			
Raw potato starch	50.7	1.677	0.775	46.21 ± 3.0^{a} [3,4]	0.412	24.55 ± 2.5^{a}
Cornstarch	41.1	1.389	0.876	63.30 ± 2.1^{c}	0.066	4.75 ± 0.6^{c}
Sucrose	40.5	1.372	0.850	61.95 ± 1.4^{c}	0.067	4.88 ± 0.5^{c}
Dextrin	47.5	1.660	0.919	57.43 ± 1.1^{b}	0.114	7.12 ± 0.5^{b}
Glucose	43.4	1.440	0.933	64.79 ± 2.2^{c}	0.067	4.64 ± 0.4^{c}
			Protein depletion period			
Raw potato starch	52.8	—	0.138^{a}	—	0.158^{a}	—
Cornstarch	30.5	—	0.173^{b}	—	0.048^{c}	—
Sucrose	36.5	—	0.186^{b}	—	0.027^{c}	—
Dextrin	43.0	—	0.208^{b}	—	0.083^{b}	—
Glucose	38.2	—	0.199^{b}	—	0.054^{c}	—
			Protein repletion period			
Raw potato starch	65.8	2.094	1.208	57.69 ± 2.3^{a}	0.339	16.18 ± 1.7^{a}
Cornstarch	57.6	1.880	1.418	75.43 ± 1.8^{c}	0.131	6.91 ± 0.1^{b}
Sucrose	57.0	1.910	1.440	75.39 ± 1.4^{c}	0.112	5.86 ± 0.8^{b}
Dextrin	68.8	2.320	1.581	68.14 ± 1.2^{b}	0.178	7.67 ± 0.5^{b}
Glucose	65.6	2.187	1.636	74.80 ± 2.4^{c}	0.128	5.85 ± 0.9^{b}

[1] Twelve rats/group.
[2] Five-day periods.
[3] Mean ± SE.
[4] Means having the same superscript are not significantly different ($P < 0.05$).

related to protein utilization since the excretion of urinary nitrogen is high when protein utilization is high and vice versa; also the low excretion of urinary nitrogen by rats fed raw-potato starch may be related to the deamination of amino acids into urea, which is low when the protein digestion and utilization are low. Schimke (16) demonstrated that the urinary urea increased as the dietary level of protein increased. However, in this study, dietary protein level was constant.

The lysine and methionine concentrations in the urine of rats fed the various carbohydrate diets are presented in table 3. The excreted lysine and methionine are also expressed as percentages of the ingested amino acids. In contrast with the urinary nitrogen excretion, significantly larger amounts of lysine and methionine ($P < 0.05$) were excreted in the urine of rats fed raw-potato starch during the growth, the protein-depletion, and the protein-repletion periods. Apparently the high urinary lysine and methionine excreted by rats fed raw-potato starch was not related to the amount of protein intake because during the protein-depletion period, protein was not included in the diets, and also during the protein-repletion period, the highest food intake was observed in rats fed dextrin as the carbohydrate source. The greater urinary excretion of lysine and methionine by rats in the raw-potato starch group may have been caused by a failure of the kidneys to reabsorb the filtered amino acids. The increased quantity of amino acids in the urine may also indicate a limited capacity of the rats to catabolize amino acids. It appears that the type of dietary carbohydrate affects not only the digestion and absorption of protein through the gastrointestinal tract, but also the protein assimilation and catabolism as well. This study points out that the urinary composition is indicative of the utilization of dietary protein.

The apparent methionine availability for rats was calculated as the ratio of the total fecal methionine absorbed and the total methionine ingested. The results are shown in table 4. During the growth period, the apparent availability of methionine was significantly lower ($P < 0.05$) when rats were fed raw-potato starch than when the rats were fed carbohydrates from

TABLE 3

Urinary lysine and methionine excretion in rats fed various carbohydrate diets [1]

Carbohydrate source	Methionine intake	Urinary methionine	Urinary methionine/ methionine intake	Lysine intake	Urinary lysine	Urinary lysine/ lysine intake
	mg	*mg*	*%*	*mg*	*mg*	*%*
			Growth period			
Raw potato starch	169.0	2.0	1.18 ± 0.16^{a} [2,3]	164.3	7.4	5.07 ± 0.80^{a}
Cornstarch	136.0	0.8	0.63 ± 0.06^{b}	133.1	2.9	2.17 ± 0.16^{b}
Sucrose	137.0	0.5	0.36 ± 0.09^{b}	134.2	2.9	1.86 ± 0.10^{b}
Dextrin	157.0	0.9	0.59 ± 0.07^{b}	154.0	3.4	2.15 ± 0.18^{b}
Glucose	143.6	0.8	0.56 ± 0.01^{b}	140.5	2.6	1.86 ± 0.18^{b}
			Protein depletion period			
Raw potato starch	—	0.6^{a}	—	—	4.3^{a}	—
Cornstarch	—	0.4^{b}	—	—	2.6^{b}	—
Sucrose	—	0.4^{b}	—	—	2.7^{b}	—
Dextrin	—	0.7^{a}	—	—	3.5^{b}	—
Glucose	—	0.4^{b}	—	—	2.9^{b}	—
			Protein repletion period			
Raw potato starch	218.0	5.1	2.33 ± 0.25^{a}	213.4	10.3	4.90 ± 0.50^{a}
Cornstarch	190.0	2.3	0.81 ± 0.05^{cd}	186.7	4.6	2.39 ± 0.10^{b}
Sucrose	188.8	1.9	1.00 ± 0.16^{bc}	184.7	4.8	2.51 ± 0.22^{b}
Dextrin	227.7	3.1	1.27 ± 0.17^{b}	222.8	6.6	2.97 ± 0.18^{b}
Glucose	217.4	1.4	0.64 ± 0.05^{d}	212.7	3.7	2.13 ± 0.09^{b}

[1] Five-day period.
[2] Means ± SE.
[3] Means having the same superscript are not significantly different ($P < 0.05$).

TABLE 4

Methionine availability for rats fed various carbohydrate diets [1] *(growth period)*

Carbohydrate source	Methionine intake	Methionine in feces	Methionine [2] absorbed	Apparent methionine availability [3]
	mg	*mg*	*mg*	*%*
Raw potato starch	169.0	16.6	152.4	90.1 ± 1.4^{a} [4,5]
Cornstarch	136.0	2.7	133.3	98.0 ± 0.8^{b}
Sucrose	137.1	1.6	135.5	98.8 ± 0.1^{b}
Dextrin	157.0	6.8	150.2	95.6 ± 0.8^{c}
Glucose	143.6	1.9	141.7	99.0 ± 0.4^{b}

[1] Five-day collection period.
[2] Methionine absorbed = methionine intake minus methionine in feces.
[3] Apparent methionine availability = methionine absorbed/methionine intake.
[4] Mean ± SE.
[5] Means having the same superscript are not significantly different ($P < 0.05$).

all other sources. The apparent methionine availability for rats of the dextrin group was lower than that for rats in the raw-potato starch group but higher than that of rats in the other 3 groups.

The apparent amino acid availability is only an approximate measure for the utilization of amino acids in protein since the fecal amino acids derived from food and those derived from the intestinal tract and its secretions were not determined separately. Following the method of Mitchell (17), the true methionine availability for rats as affected by the nature of the carbohydrates was also investigated. The data are presented in table 5. The true methionine availability for rats fed raw-potato starch was slightly lower, although not statistically significant, than that of rats fed the other types of carbohydrate.

Compared with the true lysine availability results (15), considerably less methionine than lysine was excreted in the feces when the rats were fed carbohydrate from the 5 sources. In a previous experiment (15), the true lysine availability for

TABLE 5

Methionine availability for rats fed various carbohydrate diets [1] (protein repletion period)

Carbohydrate source	Methionine intake	Total fecal methionine	Metabolic fecal methionine	Fecal food methionine [2]	Total methionine absorbed [3]	True methionine availability [4]
	mg	*mg*	*mg*	*mg*	*mg*	%
Raw potato starch	218.1	15.7	10.9	4.8	213.3	97.79 ± 0.9[a] [5,6]
Cornstarch	190.8	5.6	1.3	3.3	187.5	98.20 ± 0.4[a]
Sucrose	188.8	2.7	1.5	1.2	187.6	99.36 ± 0.3[a]
Dextrin	227.7	6.0	5.3	0.7	227.0	99.69 ± 0.8[a]
Glucose	217.4	1.8	1.1	0.7	216.4	99.55 ± 0.1[a]

[1] Five-day collection.
[2] Fecal food methionine = total fecal methionine minus metabolic fecal methionine.
[3] Total methionine absorbed = total methionine intake minus fecal food methionine.
[4] True methionine availability = total methionine absorbed/total methionine intake.
[5] Mean ± SE.
[6] Means having the same superscript are not significantly different ($P < 0.05$).

rats fed raw-potato starch was 70.5%, whereas in the present experiment the true methionine availability for rats fed the same carbohydrate was 97.8%. This evidence indicates that a significantly larger amount of methionine in wheat gluten was metabolized by the rats. The higher availability of methionine in wheat gluten might explain the increase in the growth of rats fed wheat gluten with lysine, whereas the addition of lysine and methionine to the wheat gluten diet was no more effective in promoting growth than the addition of lysine alone.[3] This result was unexpected because methionine is the second limiting amino acid in wheat gluten.

Investigators (18–20) have studied the availability of individual amino acids for man and animals. They have found that the analytically determined amino acids in a protein may not be available for assimilation. The present study establishes that in wheat gluten lysine is less available for assimilation than methionine. Nevertheless, more information about the quantitative aspects of amino-acid availability in food proteins would be useful in the calculation of supplementary amino acid requirements for unbalanced proteins.

The stimulating effect of dextrin on the growth of rats and chicks has been observed by many investigators. Monson et al. (10, 11) reported that chicks fed a dextrin diet grew faster than those supplied with a sucrose diet. These workers suggested that the superiority of dextrin to sucrose may have been caused by the better utilization of dietary protein or of amino acids as a result of the prolonged residual time of the dextrin in the gastrointestinal tract. This study and our previous studies do not support this hypothesis, since, with respect to the digestibility of protein and the availability of lysine in rats, dextrin was found inferior to cornstarch, sucrose and glucose. According to the data obtained in our laboratory, the food intake by rats fed dextrin was usually higher than that of rats fed sucrose or glucose. It, therefore, appears likely that the results obtained by previous investigators showing a superiority of dextrin to sucrose could have been caused by a greater intake of food by rats in the dextrin group (table 2). This greater food intake may depend directly upon the physical properties of dextrin rather than upon an indirect nutritional effect of this carbohydrate. Such a conclusion corresponds with Spivey's observations (21).

ACKNOWLEDGMENT

The author wishes to express her appreciation to Mrs. Emma Rice for the care of the animals and to Fumi Yokota, Thomas Miller and Joan Henricksen McConnell for technical assistance.

LITERATURE CITED

1. Harper, A. E., and M. C. Katayama 1953 The influence of various carbohydrates on the utilization of low protein rations by the white rat. J. Nutrition, 49: 261.
2. Booher, L. E., I. Behan, E. McMeans and H. M. Boyd 1951 Biological utilization of unmodified and modified food starches. J. Nutrition, 45: 75.

[3] Unpublished data.

3. Jelinek, B., M. C. Katayama and A. E. Harper 1952 The inadequacy of unmodified potato starch as dietary carbohydrate for the albino rat. Canad. J. Med. Sci., 30: 447.
4. Folin, O. 1905 Laws governing the chemical composition of urine. Am. J. Physiol., *13:* 66.
5. Kiriyama, S., and K. Ashida 1964 Effect of the quality of dietary protein on nitrogen compounds in the urine of rats. J. Nutrition, 82: 127.
6. Sauberlich, H. E., and C. A. Baumann 1946 Effect of dietary protein upon amino acid excretion by rats and mice. J. Biol. Chem., *166:* 417.
7. Steele, B. F., M. Reynolds and C. A. Baumann 1950 Effect of diet on amino acids in blood and urine of mice of various ages. Arch. Biochem. Biophys., 25: 124.
8. Heddin, P. A., and M. O. Schultz 1961 Excretion and metabolism of amino acids in rats. J. Nutrition, 74: 131.
9. Monson, W. J., L. S. Dietrich and C. A. Elvehjem 1950 Studies on the effect of different carbohydrates on chick growth. Proc. Soc. Exp. Bio. Med., 75: 256.
10. Monson, W. J., E. A. Harper, D. A. Benton and C. A. 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.
11. Stokstad, E. L. R., T. H. Jukes and W. L. Williams 1953 The growth promoting effect of aureomycin on various types of diet. Poultry Sci., 32: 1054.
12. Association of Official Agricultural Chemists 1960 Official Methods of Analysis, ed. 9, Washington, D. C.
13. Dunn, M. S., M. N. Camien, E. A. Murphy and P. J. Reiner 1949 Percentages of twelve amino acids in blood, carcass, heart, kidney, liver, muscle, and skin of eight animals. University of California, Publ. Physiol., *8:* 327.
14. Steel, R. G. D., and J. H. Torrie 1960 Principles and Procedures of Statistics. McGraw-Hill Book Company, Inc., New York.
15. Chang, Yet-Oy 1962 Effect of carbohydrates on utilization of protein and lysine by rats. J. Nutrition, 78: 21.
16. Schimke, R. T. 1962 Adaptive characteristics of urea cycle enzymes in the rat. J. Biol. Chem., 237: 459.
17. Mitchell, H. H., and M. H. Bert 1954 The determination of metabolic fecal nitrogen. J. Nutrition, 52: 483.
18. Gupta, J. D., A. M. Dakroury, A. E. Harper and C. A. Elvehjem 1958 Biological availability of lysine. J. Nutrition, 64: 259.
19. Deshpande, P. D., A. E. Harper, M. Collins and C. A. Elvehjem 1957 Biological availability of isoleucine. Arch. Biochem. Biophys., 67: 341.
20. Schweigert, B. S., and B. T. Guthneck 1954 Utilization of amino acids from foods by the rat. III. Methionine. J. Nutrition, 54: 333.
21. Spivey, E. E., M. C. Katayama, M. Yoshida and A. E. Harper 1958 Significance of the "protein-sparing effect of dextrin." Am. J. Physiol., *193:* 479.

Influence of Dietary Protein on Complement, Properdin, and Hemolysin in Adult Protein-depleted Rats [1]

MARY ALICE KENNEY, LOTTE ARNRICH, EVELYN MAR AND CHARLOTTE E. RODERUCK

Food and Nutrition Department, Iowa State University of Science and Technology, Ames, Iowa

ABSTRACT Three immunoproteins were measured in normal and in protein-depleted adult male rats and in animals which had been depleted of protein for 4 weeks and then repleted for 2 or 3 weeks with a diet containing 9 or 18% casein, 9 or 18% egg protein, 9% soy α-protein, 9% soy α-protein plus 0.25% methionine, 9% wheat gluten, 9% wheat gluten plus 0.35% lysine, or with stock ration. One milliliter 2% sheep erythrocytes was injected 2 weeks before the end of the experiment, and antibody titer was measured at intervals thereafter. Complement and properdin were titrated in serum obtained at autopsy. Titers of antibody, but not of complement or properdin, decreased significantly in the first 6 weeks of protein depletion. Weight gain and repletion of nitrogen in liver and in serum were rapid in animals repleted with stock ration, 18% egg protein or 18% casein, and slowest in those fed 9% soy α-protein or 9% wheat gluten. In contrast, properdin and antibody titers were inversely related to rate of gain and also to the total amount of sulfur amino acids in the diet.

Behavior of serum globulins in protein malnutrition is paradoxical. The impairment of antibody formation by protein deprivation (1, 2) may not be accompanied by lowered concentration of the antibody-containing γ-globulin fraction of serum (3). Moreover, protein depletion does not influence resistance to various organisms in the same way (4, 5).

Formation of antibody to *Salmonella paratyphi* B in growing rats was optimal if the diet contained 23% protein (mostly casein), in comparison with diets furnishing 7 or 43% (6). Similarly, repletion of hypoproteinemic rats with 18% egg protein allowed greater production of hemolysin than feeding 9%.[2] Hemolysin titers were higher in depleted animals fed beef for 7 days before immunization than in those repleted only 2 days with the same diet, but titers were still below those of normal controls (1). In adult human subjects, 20 g egg protein/day were superior to an equivalent amount of milk protein for production of typhoid and tetanus antibodies (7). However, when milk plus egg supplied 58 or 143 g protein/day, titers were generally lower than when the same amount of protein was provided by milk alone.

Among the factors other than antibody which contribute to resistance are various naturally occurring serum proteins such as properdin (8, 9) and the components of complement (10). Complement activity was significantly less in young adult rats fed 7% protein than in those fed 23% (6). Although no effect was noted in saline-injected animals, properdin titers of streptomycin-treated adult rats were raised by the addition of a commercial baby milk food plus vitamins A and D_2 to a stock diet consisting of wheat and Bajra flours, milk, and "vegetables" (11). Since titers of properdin have been increased by giving vitamin A to rats slightly deficient in that nutrient (12), and since supplementary milk and vitamins were not tested singly in the streptomycin-treated animals, it is not possible to determine whether the added protein may have influenced pro-

Received for publication August 31, 1964.

[1] Journal Paper no. J-4925, Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa, Project no. 1427. This investigation was supported in part by Public Health Service Research Grant no. AM-05885 from the National Institute of Arthritis and Metabolic Diseases.

[2] Glabais, J. J. 1946 The biological value of egg proteins. IV. Nutritional effectiveness of the proteins of dehydrated whole eggs as measured by hemolysin formation in hypoproteinemic rats. Unpublished M.S. Thesis. Library, Iowa State University of Science and Technology, Ames, Iowa.

perdin titers. Thus the effects of dietary protein on complement and properdin have not been studied extensively, although measurements of immunoproteins have been suggested as a sensitive measure of changes in nutritional state (12, 13).

The present study relates formation of specific immunoproteins to the course of protein metabolism after partial repletion of adult protein-depleted rats, by comparing titers of anti-sheep hemolysin, properdin, and complement with weight gain and nitrogen content of serum and of liver.

PROCEDURE

Groups of 6 to 14 male Wistar rats averaging 450 g were depleted of nitrogen for 4 weeks with a low nitrogen diet [3] and then repleted for 2 or 3 weeks. Repleted animals were compared with controls fed stock ration [4] continuously and with animals depleted for 3, 6, or 10 weeks. Repletion diets included stock and semi-purified diets supplying 9 or 18% protein from casein (C, CC) or hexane-extracted egg (E, EE), or the equivalent of 9% protein from soy α-protein (A) or soy α-protein plus methionine (AM) or wheat gluten (G) or wheat gluten plus lysine (GL) (table 1). Because the diets containing egg protein were studied separately, a group fed the diet containing soy α-protein plus methionine was repeated with the egg experiment to facilitate comparisons between experiments.

Rats were housed individually in hanging wire cages and given the appropriate diet and distilled water ad libitum. Supplementary vitamins [5] were fed separately each day to animals receiving semi-purified diets. Two weeks before the end of the experiment (after zero or 7 days of repletion) 1 ml 2% washed sheep erythrocytes was injected into a lateral caudal

[3] Composed of the following: (in %) hydrogenated vegetable oil (Crisco, Procter and Gamble, Cincinnati) 10; Hawk-Oser salts (General Biochemicals Inc., Chagrin Falls, Ohio), 4; NaCl, 1; non-nutritive fiber, 2; and corn dextrin (Fisher Scientific Co., Chicago), 83. The salt mixture contained: (in %) calcium carbonate, 6.86; calcium citrate, 30.83; calcium phosphate, monobasic, 11.28; ferric citrate, 1.53; magnesium carbonate, 3.52; magnesium sulfate, anhydrous, 3.83; manganous sulfate, 0.020; potassium aluminum sulfate, 0.009; potassium chloride, 12.47; potassium iodide, 0.004; potassium phosphate, dibasic, 21.88; sodium chloride, 7.71; sodium fluoride, 0.051.

[4] Composed of the following: (in %) cornmeal, 48; linseed meal, 14; dried whole milk, 14; wheat germ, 8.6; yeast (part irradiated), 8.6; casein, 4.3; alfalfa meal, 1.7; $CaCO_3$, NaCl, and trace elements, 0.8. Supplements to this diet mixture totaled 150 mg cod liver oil, 15 g lean ground beef, 20 g raw carrots, and 10 g fresh cabbage/rat/week.

[5] Including 50 mg cod liver oil, 0.75 mg α-tocopherol, and 500 mg of a mixture of crystalline vitamins in dextrin, furnishing the following: (in mg/day/rat) thiamine·HCl, 0.04; riboflavin, 0.06; pyridoxine·HCl, 0.04; Ca pantothenate, 0.10; nicotinic acid, 0.50; folic acid, 0.008; biotin, 0.001; vitamin B_{12}, 0.00075; ascorbic acid, 1.0; choline chloride, 5.0; inositol, 10.0; *p*-aminobenzoic acid, 10.0.

TABLE 1

Sources and amounts of protein in experimental diets

Diet	Protein		Amino acid
	Source		
		% [1]	
	Experiment A		
C	vitamin-free casein [2]	9	—
CC	vitamin-free casein	18	—
A	soy α-protein [3]	9	—
AM	soy α-protein	9	0.25% DL-methionine [2]
G	wheat gluten [2]	9	—
GL	wheat gluten	9	0.35% L-lysine [3]
	Experiment B		
E	hexane-extracted whole egg [2]	9	—
EE	hexane-extracted whole egg	18	—
AM	soy α-protein	9	0.25% DL-methionine

[1] To provide the equivalent of 9 or 18% protein (including amino acid), based on N analysis of the protein source, which was substituted for an equal weight of dextrin in the basal low nitrogen diet described in footnote 3 in the text.

[2] General Biochemicals Inc., Chagrin Falls, Ohio.

[3] Nutritional Biochemicals Corporation, Cleveland.

vein, and hemolysin was titrated in serum obtained from the tail 4, 5, 6, 7, 10 and 14 days after injection. Antibody was measured by incubating double dilutions of serum for 30 minutes at 37° with 2 units of guinea pig complement and 0.5 ml 2% sheep cells, in a final volume of 2.5 ml. All dilutions were made with 0.85% NaCl containing 100 mg $MgCl_2 \cdot 6H_2O$/liter. The endpoint of the antibody titration was determined by visual comparison with saponin-lysed cells representing 50% hemolysis.

Animals were fasted 6 hours and anesthetized with sodium pentobarbital before killing. Blood drawn from the abdominal aorta was allowed to clot at room temperature and was centrifuged at 5° for 30 minutes. Except for a portion reserved for complement titration on the same day, serum was frozen immediately. Complement activity was measured in serum from one-half the rats in experiment A and from all those in experiment B, by incubating at 37° for 30 minutes 0.5 ml of each of 6 dilutions of serum and 1 ml 1% sheep cells sensitized with rabbit anti-sheep hemolysin. Veronal buffer (14) was used for all washings and dilutions. Serum obtained at autopsy was analyzed for properdin by the zymosan assay [6] (14), titrating to a 50% endpoint.[7] Total nitrogen in dried aliquots of serum was measured by the Dumas procedure using an automatic nitrogen analyzer. Aliquots of homogenates of whole liver were analyzed for nitrogen by Kjeldahl procedure.

Correlation coefficients and t values for comparing means were calculated by methods of Snedecor (15). Titers of immunoproteins were transformed to logarithms before means and ranges of one standard deviation from the mean were calculated. These values were then converted back to reciprocal volume units and reported as means and ranges.

RESULTS AND DISCUSSION

Marked loss of body weight and hepatic nitrogen and decreased concentration of nitrogen in serum were observed after 6 weeks of protein depletion (table 2). These changes continued up to 10 weeks, but the rate of depletion reflected in these measurements diminished as depletion progressed. Decrease in antibody production

[6] Zymosan, human RP and R3 reagents, and purified human properdin kindly supplied by Mr. Earl Todd, Institute of Pathology, Western Reserve University, Cleveland.

[7] Because of difference in endpoint, and hence in definition of the unit of activity, titers of properdin reported here are about twice those originally reported for the rat (8).

TABLE 2

Body weights, serum and hepatic N, and titers of complement, properdin, and hemolysin in animals depleted of protein for zero, 3, 6, or 10 weeks

Weeks depleted	No. of rats	Body wt	Hepatic N	Serum N	Complement	Properdin	Hemolysin
		g	*mg*	g/100 ml	*units/0.1 ml* [1]	*units/ml*	*units/0.01 ml* [1]
				Experiment A			
0	13	522 ± 35 [2]	508 ± 79	0.94 ± 0.06	24 [3] (17–35)	40 (19–86)	80 (40–160)
		** [4]	**	**			**
6	14	332 ± 31	240 ± 44	0.78 ± 0.06	20 (15–28)	51 (27–98)	32 (16–63)
				Experiment B			
3	6	386 ± 28	233 ± 30	0.79 ± 0.05	19 (17–22)	57 (40–82)	13 (8–21)
		**		**	**		
10	10	310 ± 26	200 ± 34	0.73 ± 0.03	14 (12–17)	35 (21–60)	10 (6–14)

[1] Titer of antibody or complement represents the reciprocal of the greatest dilution of serum, 0.5 ml of which gave 50% lysis of 0.5 ml 2% sheep cells in the presence of an excess of guinea pig complement (in 2.5 ml total volume) or of rabbit hemolysin (in 1.5 ml).

[2] Arithmetic mean ± SD.

[3] Geometric mean; range (in parentheses) = antilog (mean log titer ± SD).

[4] Significant difference between groups in the same experiment ($P < 0.01$).

TABLE 3

Weight gains, serum and hepatic N, and titers of complement, properdin, and hemolysin in rats repleted with various proteins, arranged in each experiment in decreasing order of weight gain

	No. of rats		Wt gain [1]	Hepatic N	Serum N	Complement		Properdin		Hemolysin	
Days repleted	14	21		21	21	14	21	14	21	(5–6)[2]	(12–13)[2]
			g/week	*mg*	g/100 ml	*units/0.1 ml*		*units/ml*		*units/0.01 ml*	
					Experiment A						
Diet: [3]											
Stock	12	13	50 ± 14 [4]	450 ± 61	0.93 ± 0.05	32 [5] (24–42)	27 (22–34)	25 (14–43)	38 (20–73)	45 (25–80)	40 (18–91)
CC	12	12	38 ± 7	402 ± 54	0.95 ± 0.06	22 (16–30)	26 (21–31)	27 (16–47)	31 (17–55)	39 (21–72)	28 (17–47)
C	13	12	32 ± 8	379 ± 62	0.95 ± 0.06	27 (21–34)	22 (18–27)	40 (17–96)	45 (25–82)	35 (23–52)	41 (19–89)
GL	13	13	30 ± 9	381 ± 39	0.89 ± 0.08	24 (20–29)	20 (13–33)	43 (24–77)	43 (29–62)	40 (15–104)	42 (24–74)
AM	12	10	28 ± 8	370 ± 38	0.90 ± 0.06	23 (15–35)	27 (19–37)	34 (16–70)	36 (19–67)	50 (27–93)	38 (19–75)
G	13	12	16 ± 5	301 ± 47	0.86 ± 0.05	28 (22–34)	21 (16–28)	45 (19–104)	52 (29–93)	41 (26–66)	37 (18–76)
A	13	13	6 ± 6	300 ± 46	0.86 ± 0.05	20 (14–27)	20 (16–26)	55 (34–90)	60 (43–84)	55 (30–102)	56 (37–84)
					Experiment B						
EE	0	11	43 ± 8	399 ± 28	0.94 ± 0.07	—	19 (16–35)	—	45 (30–69)	—	44 (21–90)
E	12	12	41 ± 10	372 ± 44	0.93 ± 0.06	20 (16–25)	16 (14–17)	52 (34–80)	46 (29–74)	29 (15–57)	30 (14–66)
AM	0	6	32 ± 6	331 ± 20	0.84 ± 0.02	—	16 (14–18)	—	48 (28–82)	—	38 (20–72)

[1] Actual + average weight lost by comparable animals continued with depletion diet.
[2] Maximal titers, observed on the 5th or 6th day following injection of antigen.
[3] See table 1 for explanation of diets.
[4] Arithmetic mean ± SD.
[5] Geometric mean; range (in parentheses) = antilog (mean log titer ± 1 SD).

also was noted in depletion, although differences between experiments [8] make it difficult to describe this change with respect to time. Complement titers decreased slowly as depletion was continued. Properdin titers tended to be slightly higher after 3 or 6 weeks of depletion than in stock control animals, but neither this increase nor the decrease at 10 weeks was statistically significant.

In repletion, gains in weight, in hepatic nitrogen, and in concentration of nitrogen in serum were most rapid when animals were fed the stock, EE, or CC rations (table 3). Diets E and C also allowed repletion of serum nitrogen within 3 weeks, but data not shown here indicated that concentrations after 2 weeks of repletion were normal with diets furnishing 18% protein, but were still low in rats fed 9% protein. Diets G and A were inferior to all others with respect to gain in weight and repletion of nitrogen in liver and in serum.

Titers of properdin and of hemolysin generally were inversely related to rate of gain, except perhaps in animals fed the EE or stock rations, which appeared to produce near-maximal rates of gain and intermediate concentrations of hemolysin. On the other hand, complement titers did not appear to be related to weight gain.

Examination of the means for pairs of groups fed the same source of protein shows that increasing the amount of protein or addition of the limiting amino acid increased the rate of gain, the deposition of nitrogen in liver, and the concentration (or rate of repletion) of serum nitrogen. Effects of these dietary alterations on complement were small or inconsistent, except that 18% egg protein was superior to 9% ($P < 0.01$). Properdin titers were affected adversely by increased amounts of casein ($P < 0.10$ at 14 days, < 0.05 at 21 days) or by inclusion of methionine in the soy protein diet ($P < 0.025$ at 14 days, < 0.01 at 21 days), whereas increasing the amount of egg or inclusion of lysine in a gluten diet had no effect on properdin. Maximal titers of antibody differed little between diets with the same source of protein except that after 12 or 13 days of repletion (in groups injected with sheep

[8] Animals in the 2 experiments were from the same strain and the same colony originally, but had been raised in different laboratories and had received slightly different stock rations during growth. Rats in experiment A had greater resistance to respiratory infections and lower concentration of serum γ-globulin and of hepatic fat than rats in experiment B.

TABLE 4

Percentages of requirements for certain essential amino acids supplied by the mean daily intake of diets containing various proteins [1]

Diet [2]	Limiting amino acid	% of requirement			
		Limiting amino acid	Methionine-cysteine [3]	Lysine	Tryptophan
		Experiment A			
C	Methionine-cysteine	71	71	102	75
CC	—	> 100	137	195	144
A	Methionine-cysteine	25	25	62	38
AM	Tryptophan	50	81	80	50
G	Lysine	17	71	17	47
GL	Threonine	42	73	69	53
		Experiment B			
E	Tryptophan	75	117	89	75
EE	—	> 100	200	170	144
AM	Tryptophan	56	90	89	56

[1] Requirement estimated for 450-g rats from data of Steffee et al. (16) for requirements for repletion of adult rats; amino acid intake was calculated from analyses of soya assay protein reported by General Biochemicals Inc., Chagrin Falls, Ohio, and of other proteins as given by Block and Weiss (17).

[2] See table 1 for explanation of diets.

[3] Based on millimoles of S amino acids, assuming cysteine could furnish up to 80% of requirement.

cells after 7 days and repleted a total of 21 days), maximal titers of antibody were lowered, although not significantly, by "improvement" of diets containing soy protein or casein. Thus, mean hemolysin and properdin titers after about 2 weeks of repletion were generally higher in groups fed diets A or C than in those fed diets AM or CC, whereas increasing the amount of egg or the addition of lysine to gluten did not decrease properdin or antibody response. Both diets A and C were limiting in sulfur-containing amino acids (table 4), which were supplied in diet AM by amino acid supplementation and in diet CC by increasing the total amount of protein. Diet E was high in sulfur amino acids but probably was limited by a slight deficit of tryptophan. Diet G was low in lysine, and GL in threonine. Although concentrations of individual amino acids in the different diets were not varied independently, "correlation coefficients" were calculated from group means as an indication of closeness of relationships among weight gain, immunoproteins, and amino acid intake (table 5). Of the comparisons made, gain in weight and titers of immunoproteins appeared to be most closely related to sulfur amino acid content of the diet, even though these amino acids theoretically were limiting in only 2 of the 8 diets. In all cases, coefficients were positive for weight gain and complement titer and were negative for properdin and hemolysin titers, when related to intakes of various amino acids.

Figure 1 shows mean titers of immunoproteins and weight gain, expressed as percentages of the values for rats fed diet AM, plotted against the percentage of estimated methionine-cysteine requirement (16) met by the diet. Frequently divergent from the trend of other points are those for rats fed diet EE, which provided about 200% of the requirement for sulfur amino acids. Although consumption of stock ration was not measured in this experiment (and hence not included in fig. 1), it can be estimated that this diet also provided about twice the requirement for these amino acids. The data for groups repleted with stock ration and with diet EE are notably similar when expressed as the percentage of values for the reference group fed diet AM. Thus, the upper limit of the range wherein the intake of sulfur amino acids appeared to influence these factors lay between the estimated 137% of requirement (0.81 mmoles/day) provided by diet CC and the 200% (1.18 mmoles/day) furnished by diet EE; perhaps methionine in excess of that limit of about 1 mmole/day could not be utilized for growth and, hence, effectively promoted synthesis of properdin and hemolysin.

It is evident from these experiments that, in protein repletion, some globulins (specifically hemolysin and properdin) were not always maintained at the expense of so-called labile nitrogen stores of the body. Properdin and hemolysin titers were inversely related to liver nitrogen, serum nitrogen, and body weight during repletion. We are now attempting to determine whether methionine or perhaps some other amino acid does specifically influence immunoproteins, as suggested by these data, or whether the relationship observed here may be largely secondary to rate of gain.

TABLE 5

Correlation coefficients [1] relating titers of immunoproteins and weight gain to certain dietary essential amino acids

Amino acid	Wt gain	Complement	Properdin	Hemolysin
Limiting [2]	+0.87	+0.74	−0.82	−0.43
Methionine-cysteine	+0.89	+0.78	−0.88	−0.67
Tryptophan	+0.80	+0.66	−0.71	−0.46
Lysine	+0.70	+0.62	−0.68	−0.21

[1] Calculated from group means for weight gain and immunoprotein titers of rats repleted 3 weeks (expressed as % of values for rats fed diet AM in the same experiment) and log % amino acid requirement met by food consumed (from table 4). Because variances of individual means were not considered in computations, no attempt is made to assign P values.

[2] Based on the percentage of that essential amino acid in each diet which was present in least amount relative to repletion requirements (see table 4).

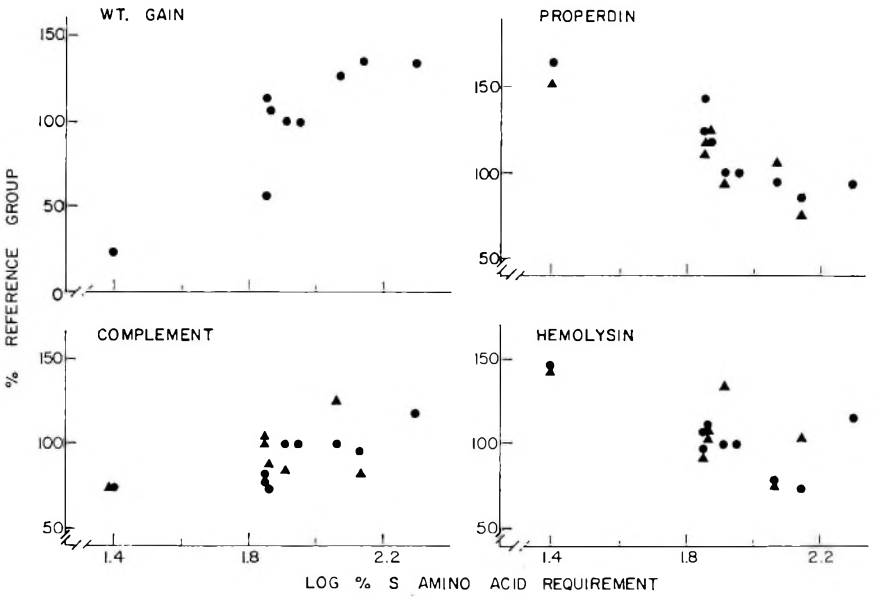


Fig. 1 Relationships of weight gain and titers of properdin, complement, and hemolysin to sulfur amino acid intake of rats repleted for 2 (triangle) or 3 (circle) weeks with various proteins.

LITERATURE CITED

1. Wissler, R. W., R. L. Woolridge, C. H. Steffee and P. R. Cannon 1946 The relationship of the protein-reserves to antibody-production. II. The influence of protein repletion upon the production of antibody in hypoproteinemic adult white rats. J. Immunol., *52:* 267.
2. Wissler, R. W. 1947 The effects of protein-depletion and subsequent immunization upon the response of animals to pneumococcal infection. II. Experiments with male albino rats. J. Infect. Dis., *80:* 264.
3. Weimer, H. E. 1961 The effects of protein depletion and repletion on the concentration and distribution of serum proteins and protein-bound carbohydrates of the adult rat. Ann. N. Y. Acad. Sci., *94:* 225.
4. Scrimshaw, N. S., C. E. Taylor and J. E. Jordan 1959 Interactions of nutrition and infection. Am. J. Med. Sci., *237:* 367.
5. Boyd, F. M., and H. M. Edwards 1963 The effect of dietary protein on the course of various infections in the chick. J. Infect. Dis., *112:* 53.
6. Trnka, Z. 1956 The influence of protein deficiency and excess in the diet on immunity response. Folia Biol., *2:* 306.
7. Hodges, R. E., W. B. Bean, M. A. Ohlson and R. E. Bleiler 1962 Factors affecting human antibody response. I. Effects of variations in dietary protein upon the antigenic response of men. Am. J. Clin. Nutrition, *10:* 500.
8. Pillemer, L., L. Blum, I. H. Lepow, O. A. Ross, E. W. Todd and A. C. Wardlaw 1954 The properdin system and immunity. I. Demonstration and isolation of a new serum protein, properdin, and its role in immune phenomena. Science, *120:* 279.
9. Lepow, I. H. 1959 Natural resistance to infection: The properdin system. Trans. Am. Acad. Ophth., *1958:* 628.
10. Wardlaw, A. C. 1962 The complement-dependent bacteriolytic activity of normal human serum. I. The effect of pH and ionic strength and the role of lysozyme. J. Exp. Med., *115:* 1231.
11. Sen, R., P. D. Mathur and A. C. Majumdar 1963 Some observations in relation to properdin system. IV. Relation of serum complement activity and properdin level of normal albino rats to streptomycin therapy and diet. Ann. Biochem. Exp. Med., *23:* 577.
12. Isliker, H., F. Weber and O. Wiss 1960 Die Beeinflussung des Properdins im Serum durch Vitamin A, beta-Carotin, beta-Apo-8'-carotinal (C_{30}) und Lycopin bei der Ratte. Hoppe-Seyler's Ztschr., *320:* 126.
13. Rice, C. E., P. Boulanger and E. Annau 1954 Serological effects of ethionine in the rabbit. Canad. J. Biochem. Physiol., *32:* 126.
14. Pillemer, L., L. Blum, I. H. Lepow, L. Wurz and E. W. Todd 1956 The properdin system and immunity. III. The zymosan assay of properdin. J. Exp. Med., *103:* 1.
15. Snedecor, G. W. 1956 Statistical Methods, ed. 5. Iowa State College Press, Ames.

16. Steffee, C. H., R. W. Wissler, E. M. Humphreys, E. P. Benditt, R. W. Woolridge and P. R. Cannon 1950 Studies in amino acid utilization. V. The determination of minimum daily essential amino acid requirements in protein-depleted adult male albino rats. J. Nutrition, 40: 483.
17. Block, R. J., and K. W. Weiss 1956 Amino Acid Handbook. Charles C Thomas, Springfield, Ill.