Invitation for Nominations for 1966 American Institute of Nutrition Awards

Nominations are requested for the 1966 annual awards administered by the American Institute of Nutrition to be presented at the next annual meeting. Nominations may be made by anyone, including members of the Nominating Committees and non-members of the Institute.

The following information must be submitted: (1) Name of the award for which the candidate is proposed. (2) A brief convincing statement setting forth the basis for the nomination. A bibliography and supporting letters are not to be submitted. (3) Five copies of the nominating letter must be sent to the chairman of the appropriate nominating committee *before October 1, 1965,* to be considered for the 1966 awards.

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

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

1966 Borden Award in Nutrition

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

Former recipients of this award are:

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

NOMINATING COMMITTEE:

L. D. WRIGHT, *Chairman*
E. L. HOVE
P. H. WESWIG

Send nominations to:

DR. L. D. WRIGHT
Graduate School of Nutrition
Savage Hall, Cornell University
Ithaca, New York

1966 Osborne and Mendel Award

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

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

Former recipients of this award are:

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

NOMINATING COMMITTEE:

NEVIN SCRIMSHAW, *Chairman*
M. O. SCHULTZE
ALEX BLACK

Send nominations to:

DR. NEVIN SCRIMSHAW
Department of Nutrition and Food Science
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

1966 Mead Johnson Award for Research in Nutrition

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

Former recipients of this award are:

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

NOMINATING COMMITTEE:

B. CONNOR JOHNSON, *Chairman*
P. L. DAY
A. R. KEMMERER

Send nominations to:

DR. B. CONNOR JOHNSON
Division of Animal Nutrition
University of Illinois
259 Animal Science Laboratory
Urbana, Illinois

1966 Conrad A. Elvehjem Award for Public Service in Nutrition

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

NOMINATING COMMITTEE:

B. S. SCHWEIGERT, *Chairman*
E. E. HOWE
F. W. QUACKENBUSH

Send nominations to:

DR. B. S. SCHWEIGERT, *Chairman*
Food Science Department
Michigan State University
East Lansing, Michigan 48823

Invitation for Nominations for 1966 American Institute of Nutrition Fellows

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

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

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

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

Fellows Committee:

CHARLOTTE M. YOUNG, *Chairman*
W. D. SALMON
S. L. HANSARD
PAUL E. JOHNSON
W. H. GRIFFITH

Send nominations to:

DR. CHARLOTTE M. YOUNG
Cornell University
Savage Hall
Ithaca, New York 14850

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

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

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

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

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

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

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

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

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

Committee on Honorary Memberships:

Paul György, *Chairman*
Paul Day
Grace Goldsmith

Send nominations to:

Dr. Paul György
University of Pennsylvania
Pennsylvania General Hospital
Philadelphia, Pennsylvania 19104

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

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

Effects of Copper, Molybdenum, and Zinc on Zinc-65 Tissue Distribution and Excretion in the Rat

KENNETH E. KINNAMON AND GEORGE E. BUNCE [1]
U. S. Army Medical Research and Nutrition Laboratory, Fitzsimons General Hospital, Denver, Colorado

ABSTRACT Tissue distribution and excretion of injected Zn^{65} have been studied in both male and female rats maintained with increased dietary levels of different combinations of 3 mineral nutrients: copper (100 mg/kg), molybdenum (800 mg/kg), and zinc (5000 mg/kg). Results obtained indicate that diets high in zinc caused a significant increase in bone retention and urinary excretion and a significant decrease in soft tissue retention of intraperitoneally injected Zn^{65}. Increased dietary levels of copper or molybdenum or both, did not elicit a similar effect. Significant growth retardation was observed only in animals maintained with a ration high in both molybdenum and zinc.

The metabolism of zinc has assumed greater importance with the advent of the nuclear age. Since atomic weapons testing began, radiozinc has been reported in many foodstuffs (1–5) as well as in the Marshall Island natives (6). Apparently, the isotope persists through food chains.

The functions of mineral nutrients and their interrelationships are so complex that it is difficult to evaluate the requirements of man or animal for any given one without considering the effects of others present. Such functional interrelationships exist between copper, molybdenum, and zinc. For example, molybdenum and zinc synergestically retard growth (7–9). Copper supplementation has been shown to prevent anemia, increase growth, and raise the liver catalase and liver and heart cytochrome oxidase to normal or greater than normal levels in rats suffering from zinc toxicity (10–12). Molbydenum toxicity in rats (13) and cattle (14) has been treated effectively with dietary copper. Dick (15) has even demonstrated the characteristic lesions of copper deficiency, despite adequate dietary copper, in the wool of sheep given high doses of molybdenum. In the present investigation the effects of increased dietary levels of different combinations of copper, molybdenum, and zinc were studied with respect to the affected growth and Zn^{65} distribution and excretion in rats.

MATERIALS AND METHODS

Rats of the Sprague-Dawley strain,[2] weighing initially 104 to 143 g, were selected at random and placed in 8 treatment groups of 6 animals each (3 males and 3 females). They were allowed free access to water and their respective ration for 7 weeks. Then, using a weighed syringe technique they were given a 5-μc intraperitoneal injection of aqueous $Zn^{65}Cl_2$ solution [3] containing 350 μg of zinc/ml and of specific activity 1380 μc/mg. The rats were then placed in metabolism cages and urine and feces were collected daily until the animals were killed [4] 4 days later. Weight gain and feed consumption for each rat were recorded daily throughout the experimental period.

The animals were housed individually for the first 7 weeks in stainless steel suspension-type cages having dimensions 25 cm long, 20 cm wide, and 18 cm high. During the last 4 days of the study the metabolism cages used were essentially the same construction as described above except the dimensions were 15 cm long, 11 cm wide, and 11 cm high. Food containers for the initial 7 weeks were glass

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[1] Present address: Dept. of Biochemistry and Nutrition, Virginia Polytechnic Institute, Blacksburg, Va.
[2] Obtained from Sprague-Dawley, Inc., Madison 5, Wisconsin.
[3] Obtained from Abbott Laboratories, Oak Ridge, Tennessee.
[4] The Principles of Laboratory Animal Care as promulgated by the National Society for Medical Research were observed.

cylinders, 9 cm in diameter and 10 cm deep, equipped with white lacquered screw-cap metal lids having 3.2-cm hole stamped through the middle to minimize spillage of food. Distilled-demineralized water for drinking was dispensed ad libitum from glass bottles equipped with stainless steel outlet tubes.

A finely ground commercial laboratory ration [5] containing 18 mg/kg copper, 70 mg/kg zinc, and less than 1 mg/kg molybdenum was used as the basal diet. One or more of the following ingredients was added to the basal diet: 100 mg/kg copper as $CuSO_4 \cdot 5H_2O$; 800 mg/kg molybdenum as $Na_2MoO_4 \cdot 2H_2O$; and 5000 mg/kg zinc as $ZnCO_3$. These levels of supplementation were utilized to obtain the following 8 treatments: 1) basal ration (control); 2) Cu; 3) Mo; 4) Zn; 5) Cu + Mo; 6) Cu + Zn; 7) Mo + Zn; and 8) Cu + Mo + Zn.

At the end of the experimental period all animals were killed with ether and the desired tissues were removed and weighed. These tissues, along with daily urine and fecal collections, were assayed for radiozinc utilizing a well-type sodium iodide scintillation counter. Diets were analyzed for total zinc by the method of Vallee and Gibson (16) using the ashing procedure described by Reitz et al. (17), and for copper and molybdenum as described in AOAC (18).

RESULTS

Growth and feed consumption. Significant weight reduction ($P < 0.01$) was observed in both male and female rats fed the diet with excess Cu + Mo + Zn and in the male rats fed the high Mo + Zn diet. Growth was also retarded in the females from the latter ration though to a lesser extent ($P < 0.05$). Feed consumption was reduced ($P < 0.05$) only in the male rats fed the high Mo + Zn combination (table 1).

Tissue distribution of Zn^{65}. A summary of results presenting the percentage of injected Zn^{65} retained 4 days after administration per gram of wet weight is shown in table 2. Within any treatment group, greatest activity was observed in liver and spleen, with pancreas, kidney, lung, and bone slightly less active. Least activity was observed in skeletal muscle and brain. The activities of heart and testes were intermediate. These observations for relative values are similar to those reported by other investigators (19–21) although the mean values reported here for bone are slightly higher.

An elevation in dietary zinc resulted in a decreased retention of the injected radiozinc in soft tissue in almost every instance regardless of the level of copper or molybdenum. Retention in bone, however, was significantly increased, in both male ($P < 0.05$) and female ($P < 0.01$) rats when the high zinc diet alone was fed; this effect was intensified ($P < 0.01$) in the males and reduced in the females by supplements of molybdenum or copper, or both.

[5] Purina Laboratory Chow, Ralston Purina Company, Davenport, Iowa.

TABLE 1

Average body weight, weight gain and feed consumption of the various treatment groups

	Treatment	1 Final body weight		2 Weight gain		3 Feed consumption	
		Males	Females	Males	Females	Males	Females
		g	*g*	*g*	*g*	*g*	*g*
1	Basal ration (control)	345	222	217	111	1143	842
2	Mo	314	238	192	120	928	822
3	Cu	332	233	211	125	1011	808
4	Zn	321	222	198	109	1147	928
5	Cu+Mo	318	220	193	112	1086	828
6	Mo+Zn	178	204	58 [1]	92 [2]	883 [2]	843
7	Cu+Zn	319	222	200	112	1050	901
8	Cu+Mo+Zn	272	179	152 [1]	69 [1]	961	782

[1] Indicates a significant difference at the 1% level when compared with control animals (t test).
[2] Indicates a significant difference at the 5% level when compared with control animals (t test).

TABLE 2

Percentage per gram of retained Zn^{65} present 96 hours after administration

Tissue	Treatment							
	Basal ration (controls)	Mo	Cu	Zn	Cu + Mo	Mo + Zn	Cu + Zn	Cu + Mo + Zn
Bone (right femur)								
Male	0.456	0.574	0.572	0.643 [1]	0.522	0.934 [2]	0.645 [2]	0.670 [2]
Female	0.617	0.651	0.663	0.833 [2]	0.584	0.689	1.181	0.582
Brain								
Male	0.187	0.146	0.133	0.075 [2]	0.139	0.098 [1]	0.076 [2]	0.075 [2]
Female	0.187	0.193	0.196	0.090 [2]	0.180	0.109 [2]	0.114 [2]	0.086
Heart								
Male	0.368	0.446	0.372	0.147 [2]	0.376	0.195 [1]	0.143 [2]	0.148 [2]
Female	0.541	0.551	0.548	0.215 [2]	0.518	0.192 [2]	0.125 [2]	0.194 [2]
Kidney								
Male	0.463	0.466	0.528	0.290 [2]	0.499	0.323 [1]	0.316 [1]	0.232 [2]
Female	0.624	0.644	0.648	0.349 [2]	0.685	0.267 [2]	0.444 [1]	0.309 [2]
Liver								
Male	0.643	0.699	0.735	0.435	0.729	0.511 [1]	0.513	0.403
Female	0.795	0.870	0.841	0.633 [1]	0.864	0.482 [2]	0.768	0.645
Lung								
Male	0.433	0.484	0.424	0.172 [2]	0.392	0.295	0.178 [2]	0.147 [2]
Female	0.672	0.591	0.605	0.243 [2]	0.634	0.155 [2]	0.164 [2]	0.190 [2]
Muscle								
Male	0.109	0.125	0.115	0.057 [2]	0.119	0.103	0.057 [2]	0.056 [2]
Female	0.164	0.189	0.228	0.070 [2]	0.140	0.055 [2]	0.054 [2]	0.070 [2]
Pancreas								
Male	0.469	0.558	0.451	0.590	0.388	0.578	0.573	0.593
Female	0.574	0.495	0.591	0.486	0.595	0.725	0.648	0.639
Spleen								
Male	0.493	0.501	0.516	0.193 [2]	0.495	0.323 [1]	0.173 [2]	0.183 [2]
Female	0.699	0.677	0.685	0.278 [2]	0.707	0.209 [2]	0.169 [2]	0.230 [2]
Testes	0.419	0.390	0.380	0.223 [2]	0.487	0.374	0.193 [2]	0.224 [2]

[1] Indicates a significant difference at the 5% level when compared with control animals (*t* test).
[2] Indicates a significant difference at the 1% level when compared with control animals (*t* test).

Excretion of Zn^{65}. No significant differences in fecal elimination of Zn^{65} were demonstrated among the treatment groups. Despite the variability within groups, there was no doubt that fecal excretion is the chief means of radiozinc elimination, presumably via pancreatic secretion.

Although the amount of activity excreted in the urine is small when compared with fecal excretion, the urinary radiozinc excretion was shown to be increased in all animals maintained with an elevated zinc ration (table 3). When compared with controls, the increased elimination was highly significant ($P < 0.01$) in all high zinc groups except in females of the Cu + Mo + Zn treatment.

TABLE 3

Percentage of injected Zn^{65} excreted in the urine 96 hours after administration

		Males	Females
		%	%
1	Basal ration (control)	0.873	0.609
2	Mo	1.109	0.988
3	Cu	0.976	0.833
4	Zn	3.648 [1]	3.078 [1]
5	Cu+Mo	0.715	0.508
6	Mo+Zn	2.940 [1]	2.814 [1]
7	Cu+Zn	3.185 [1]	4.641 [1]
8	Cu+Mo+Zn	2.701 [1]	4.419 [2]

[1] Indicates a significant difference at the 1% level when compared with control animals (*t* test).

[2] Indicates a significant difference at the 5% level when compared with control animals (*t* test).

However, in the latter the increased excretion was significant ($P < 0.05$). All diets were equally effective in accelerating elimination (t test).

DISCUSSION

Growth inhibition reported to occur with dietary molybdenum alone (22–26) or zinc alone (27–29) was not observed in this study (table 1). However, in evaluating the lack of effects with either of these mineral additives alone, it is necessary to consider: 1) the large size (104 to 143 g) of the animals at the beginning of the experiment, and 2) the relatively high level of copper (18 mg/kg) in the basal ration used in this study. With this intake of copper, a greater tolerance for either molybdenum or zinc should be expected. The fact that only the male rats of the Mo + Zn group showed reduced feed consumption (table 1) indicates that the observed growth depression may be due in part, but not entirely to reduced food intake. In the male rats consumption of the non-toxic (as determined by weight gain) ration containing only molybdenum was not significantly different from that of controls, yet consumption of the toxic molybdenum-zinc diets (i.e., Mo + Zn and Cu + Mo + Zn) was reduced. Monty and Click (25) have demonstrated that molybdate-containing diets are not rejected per se — only the toxic ones.

This study has shown that dietary zinc, but not molybdenum or copper, significantly influences tissue distribution and excretion of injected tracer zinc in adult non-pregnant rats. This "zinc-influence" upon tracer zinc is not altered by either copper or molybdenum being present also. The dichotomy between the effect of zinc and that of copper or molybdenum could be considered as an argument for the discriminatory capabilities on the part of the body's transport and storage mechanism. These data suggest that commonly recognized zinc interactions between either Cu or Mo should not be attributed to a lack of discrimination within the body. The observed overall lower Zn^{65} tissue retention and increased excretion in zinc-fed rats could be explained by a difference in the rate of zinc turnover or by an active participation of a homeostatic control mechanism for zinc reported by Cotzias et al. (30, 31), or both.

Inspection of table 1, column 1, shows that the group differences in body weight at the time of death present an additional potentially significant variable. In view of this, all radiozinc retention data were subjected to 2 additional statistical analyses using 2 types of weighted values. The first additional analysis was made by comparing values that were obtained by weighting the percentage of retained Zn^{65}/g with day-of-death mean body weight ratios of animals in the group being compared and, the second, on tissues only, by simply comparing the percentage of retained radiozinc per organ. Analysis of either type of weighted value resulted in conclusions which did not differ from those obtained by analyzing the "non-weighted" information presented in table 2.

The increase rather than decrease in radiozinc retention in bones of animals fed elevated zinc diets was an unexpected result. The fact that bone is rather sensitive to changes in dietary zinc levels has been shown by Hove et al. (32). These investigators showed that the zinc content of soft tissues is normal, but that of bone is lowered in zinc-deficient animals. However, it could be presumed that bone would act in a manner similar to that of other tissues and present values indicating lower rather than higher Zn^{65} retention with increasing levels of added zinc. Martin and Patrick (33), for example, have noted this to be true in chicks, maintained with rations of low but varying zinc content. Extensive ion exchange appears unlikely in view of other results (19, 20, 34). Apparently zinc is very tightly bound in mineralized bone, perhaps in a metalloprotein complex (20). Rubini et al. (20) have reported that in mice Zn^{65} at a very low concentration in bone is observed initially, but owing to its ability to successfully compete with excretion, a large portion of administered radiozinc is ultimately transferred to bone. Increased bone concentration of Zn^{65}, in the present study, occurred in those groups of animals showing increased radiozinc urine excretion. An explanation for this mechanism of competition for excreted zinc and how

it is related to animals fed high zinc diets is lacking. Other studies bearing on this and some other related unanswered questions are under investigation.

ACKNOWLEDGMENTS

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Effects of Imbalances or Antagonisms among Nonessential Amino Acids on Growth and Nitrogen Utilization by Rats [1,2]

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ABSTRACT A series of studies with weanling rats was conducted to determine the effect on growth rate and nitrogen utilization of adding various combinations of nonessential amino acids to a methionine-supplemented, 14.3% casein basal diet. Amino acids were added at levels to make the diet resemble a 30.5% gelatin diet with respect to the amino acids added. The addition of 7.1% of glycine to the basal diet resulted in a reduction in growth rate of about 20%, whereas the addition of 4.0% of hydroxyproline or 3.0% of proline had smaller and inconsistent effects on growth rate. The concomitant addition of glycine and the 2 imino acids caused a much more severe depression in growth rate which was not altered significantly by supplemental arginine, alanine, or glutamic acid but was partially alleviated by the addition of aspartic acid or serine, or more effectively by a combination of these 5 amino acids. Efficiency of nitrogen utilization was greatly decreased by supplemental nonessential amino acids, but efficiency of feed utilization for the 20-day studies was not altered significantly by dietary treatment.

Several investigators have demonstrated that the ratio of essential to nonessential amino acids in a diet will affect the nutritive value of protein. Yet, few investigators have published work indicating that imbalances or antagonisms among nonessential amino acids may be created. Savage and Harper (1) suggested that the balance between hydroxyproline and other nonessential amino acids may affect growth rate in rats. These workers also observed that liver pyridine nucleotide concentrations were altered by certain combinations of nonessential amino acids. Hier et al. (2) reported that the growth rate of rats consuming all of the essential amino acids in excess of their requirements was depressed by supplemental gelatin. These authors attributed this growth-inhibiting property of gelatin to its high concentration of glycine and proline. A similar effect of gelatin was observed by Speirs et al.[3] working with preadolescent girls. They noted that the inclusion of gelatin in diets which supplied 2 or 3 g of protein/kg of body weight/day was associated with decreased nitrogen retention. The same girls also excreted lower levels of niacin metabolites when gelatin was included in the diets, even though the diets supplied adequate levels of tryptophan and niacin (3).

The purposes of the experiments reported here were to investigate the effects on growth rate and nitrogen retention of imbalances, antagonisms, or toxicities created by adding various combinations of nonessential amino acids to a basal diet which would support a high rate of growth and nitrogen retention in weanling rats.

EXPERIMENTAL PROCEDURE

Male weanling rats[4] were used in these studies. In each of 4 studies, 4 or 5 animals/treatment were placed in individual wire-bottom cages in a room maintained at approximately 25°. During the 10- or 20-day experiments, food and water were given ad libitum to all animals except those whose feed intake was restricted to that of pair-mates fed another diet. Feed intake measured daily and the animals were weighed at 2- or 3-day intervals.

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[1] This is paper no. 504 of the Journal Series of the Georgia Experiment Station, Experiment, Georgia.

[2] A portion of the work was presented at the meeting of the Federation of American Societies for Experimental Biology at Atlantic City, New Jersey, 1965.

[3] Speirs, M., W. H. James, L. J. Harper and R. W. Engel 1960 Nitrogen retention in 7- to 9-year-old girls. Abstracts, Fifth International Congress on Nutrition, Washington, D. C., p. 29.

[4] Obtained from the Charles River Laboratories, Brookline, Massachusetts.

The composition of the diets used in these experiments was as follows (in %): casein, 14.3; L-methionine, 0.2; dextrin, 40.0; cottonseed oil, 5.0; cellulose,[5] 1.5; salt mixture,[6] 4.0; vitamin mixture,[7] 2.5; sodium bicarbonate, 0.5; amino acids, variable; and sucrose, to make to 100.0. The variable portion of the diets (table 1) was formed by adding, at the expense of sucrose, arginine or nonessential amino acids, or both, in amounts to make the diet resemble a 30.5% gelatin diet with respect to the amino acids added. All optically active amino acids used in these studies were of the L-configuration. Originally, a diet containing 30.5% of gelatin supplemented with essential amino acids was included in the series; however, it was discontinued because weanling rats fed this diet developed severe diarrhea within 24 to 48 hours and gained little or no weight. The diets used in each study are shown in table 2.

At the termination of the experiment, the rats were decapitated, liver and kidneys were weighed, and sections of lungs, liver, and one kidney were preserved in formaldehyde for histological studies. Carcasses were dried at 65° and digested in concentrated sulfuric acid. Nitrogen content in samples of liver and the sulfuric acid digests of carcasses was determined by the macro-Kjeldahl procedure. Carcass nitrogen was determined also in representative animals killed at the beginning of the experiments.

Statistical evaluation of dietary effects was made by making comparisons between treatments replicated in the same experiments. For example, to study the effects of supplemental alanine on weight gains (table 2) experiments 2, 3, 4, treatments 1, 5, 6, and 8 were analyzed together; whereas, to test the effects of supplemental aspartic acid or serine on weight gains, experiments 3 and 4, treatments 1, 5, 6, 8, 9, and 10 were used in the analyses.

RESULTS

Growth. Growth data for 4 experiments are summarized in table 2. Supplementation of the basal diet with 7.1% of glycine caused a reduction in growth rate of about 20%. Growth depression of about the same magnitude was produced during

[5] Solka Floc BW 100, kindly supplied by Brown Company, Boston.

[6] Salt mixture P-H, (Phillips, P. H., and E. B. Hart, J. Biol. Chem., *109:* 657, 1935), purchased from Nutritional Biochemicals Corporation, Cleveland.

[7] Vitamins were dispersed in sucrose and powdered cellulose to supply, per kg of diet: 5 g of cellulose, 12,000 IU of vitamin A palmitate, 1,200 IU of vitamin D_2, and the following amounts of the vitamin B complex group: (in mg) thiamine, 5; riboflavin, 5; niacin, 10; Ca pantothenate, 20; pyridoxine, 3; folic acid, 0.2; *p*-aminobenzoic acid, 100; choline chloride, 1,000; biotin, 0.1; inositol, 100; and vitamin B_{12}, 0.01.

TABLE 1

Dietary variables

	Diets										
	1	2	3	4	5	6	7	8	9	10	11
					grams/kilogram of diet						
Non-variable components [1]	680.0	680.0	680.0	680.0	680.0	680.0	680.0	680.0	680.0	680.0	680.0
L-Arginine·HCl						20.0	20.0				
L-Alanine						23.0		23.0			
L-Aspartic acid						9.0			9.0		
L-Glutamic acid						1.5					1.5
Glycine		71.5			71.5	71.5	71.5	71.5	71.5	71.5	71.5
L-Hydroxyproline			40.0		40.0	40.0	40.0	40.0	40.0	40.0	40.0
L-Proline				30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0
L-Serine						2.0				2.0	
Sucrose	320.0	248.5	280.0	290.0	178.5	123.0	158.5	155.5	169.5	176.5	177.0

[1] The non-variable portion of the diets supplied 143 g of vitamin-free casein and 2.0 g of L-methionine/kg of diet. See text for other ingredients.

TABLE 2

Effect of supplemental nonessential amino acids and arginine on weight gains of weanling rats

Diet no.	Diet	Experiment [1,2]			
		1	2	3	4
		g/day	*g/day*	*g/day*	*g/day*
1	Basal	5.0 ± 1.5 [3]	5.7 ± 0.6	5.8 ± 0.6	6.1 ± 0.4
2	Basal + gly	4.4 ± 0.6	4.2 ± 0.4	4.7 ± 0.8	
3	Basal + hypro	4.5 ± 0.9	4.9 ± 1.1	5.8 ± 0.8	
4	Basal + pro	5.5 ± 1.3	5.2 ± 0.6	5.3 ± 1.4	
5	Basal + gly + hypro + pro	2.4 ± 0.6	3.3 ± 1.4	3.6 ± 0.4	2.9 ± 0.8
1	Intake limited to that of pair-mates fed diet 5				3.1 ± 0.9
6	Diet 5 + arg + ala + asp + ser + glu	4.3 ± 0.9	4.6 ± 0.9	5.6 ± 1.0	5.5 ± 0.7
7	Diet 5 + arg	2.9 ± 0.8	3.0 ± 0.3	3.3 ± 1.0	
8	Diet 5 + ala		3.7 ± 0.9	3.4 ± 1.6	3.5 ± 1.0
9	Diet 5 + asp			4.3 ± 0.7	4.6 ± 0.6
10	Diet 5 + ser			4.3 ± 0.8	3.9 ± 1.3
11	Diet 5 + glu				2.9 ± 0.1

[1] Experiments no. 1 and 3 had 4 rats/treatment and no. 2 and 4 had 5 rats/treatment.
[2] The duration of experiment 1 was 10 days and of experiments 2–4, 20 days.
[3] Diet mean ± SD.

the first 10 days of the studies by the addition of 4.0% of hydroxyproline, but the animals appeared to adjust to the supplemental hydroxyproline so that the growth inhibition at 20 days was less marked. A supplement of 3.0% of proline caused smaller and inconsistent effects on growth rate; however, the concomitant addition of glycine and the 2 imino acids (diet 5) resulted in much more severe growth inhibition than occurred as the result of supplements of either glycine or hydroxyproline alone.

The toxic effects of diet 5 were not altered significantly by supplements of 2.3% of alanine, 2.0% of arginine hydrochloride, or 0.15% of glutamic acid but were partially overcome by supplements of 0.9% of aspartic acid or 0.2% of serine or, more effectively, by a combination of these 5 amino acids. Of the individual amino acids added, aspartic acid appeared to be the most effective in preventing the toxic effects of diet 5. Although the differences between mean weight gains of rats fed diets 5 and 8 (diet 5 plus alanine) were not statistically significant, it appears that supplemental alanine may have had a beneficial effect in 2 experiments.

Feed efficiency. During the first 10 days there were significant differences between diet means in efficiency of feed utilization, with the groups gaining most slowly having the lowest gain-to-feed-intake ratio. This effect was not statistically significant by the end of the 20-day experiments. In one group in which the animals were fed diet 1 in amounts equal to those of pair-mates fed diet 5, the 20-day gain was only slightly superior for those animals fed diet 1 (3.1 vs. 2.9 g/day).

Nitrogen utilization. Nitrogen retention, estimated by assaying animals for total body nitrogen, indicated that supplemental nonessential amino acids greatly decreased efficiency of nitrogen utilization (table 3). In fact, when expressed as a percentage of the casein nitrogen consumed, nitrogen retention was slightly higher for the animals fed the basal diet than for those fed the basal diet supplemented with nonessential amino acids.

Organ sizes. Relative to body weight, the kidneys of all animals receiving supple-

TABLE 3

Effect of supplemental nonessential amino acids on nitrogen retention

Diet	No. of observations	*a* N intake from casein [1]	*b* Total N intake	*c* Increase in body N	*d* % of N intake retained	*e*
					100 *c/a*	100 *c/b*
		g/20 days	*g/20 days*	*g/20 days*		
1	14	5.44	5.44	3.37	62±6 [2]	62±6 [2]
2	8	4.40	7.18	2.50	57±4	35±3
3	9	4.71	5.67	2.80	59±3	49±3
4	8	4.72	5.55	2.67	57±6	48±5
5	12	3.39	6.82	1.90	56±5	28±3
6	14	4.52	11.71	2.55	56±5	22±2
7	9	3.20	7.22	1.77	55±7	25±3
8	8	3.64	7.93	2.10	58±4	26±2
9	7	3.90	8.00	2.14	55±4	27±2
10	4	4.12	8.35	2.28	55±8	27±4

[1] Includes nitrogen from supplemental methionine.
[2] Mean ± SD.

mental amino acids were larger than those of rats fed the basal diet, with the largest kidneys being those of the animals given supplemental arginine hydrochloride. On a per unit of body weight basis, the kidneys of animals fed diets 2 to 10 averaged 1.3, 1.3, 1.1, 1.6, 1.8, 2.1, 1.6, 1.6, and 1.6 times larger, respectively, than those of the animals fed diet 1. The magnitude of the increase in kidney size in rats fed the supplemental diets appeared to be roughly related to the amount of nitrogen added in the supplemental amino acids.

The diet effect on liver size, expressed in milligrams per kilogram of body weight, was not statistically significant. Large, fatty livers were noted in some animals in some of the groups receiving supplemental nonessential amino acids but these observations were not consistent from experiment to experiment.

DISCUSSION

It has been suggested that glycine and the imino acids compete for a common transport system in the renal tubules (4), in intestinal mucosa (5) and in free-living tumor cells (6), or at least that they share interrelations in transport not shared with other amino acids (7). The close association of glycine and the imino acids in transport mechanisms may have been partially responsible for the increased growth depression which occurred when they were added concomitantly (diet 5) in these studies.

In view of the role of arginine in the urea cycle, supplemental arginine would have been expected to decrease the toxic effects of diet 5 if the effects were due to high levels of blood ammonia and a relative deficiency of arginine (8). However, the basal diet contained about 0.5% of arginine which may have been sufficient to prevent this amino acid, even in the presence of high levels of blood ammonia, from being a rate-limiting factor in the urea cycle. Under these conditions, no benefit would be expected by the addition of more arginine.

Since glycine is readily converted into serine, and since the concentration of serine in the blood is usually elevated by the addition of dietary glycine (9, 10), the beneficial effect on growth rate of adding serine to diet 5 was unexpected. On the other hand, the key position of aspartic acid in many reactions involved in protein metabolism (11), especially those associated with the citric acid cycle, the urea cycle, and transamination, afforded many areas in which it might have functioned to decrease the toxicity of diet 5.

It is possible that the antagonism between glycine, the 2 imino acids, and other amino acids was caused by their association in some coupled metabolic reaction. An example of such a mechanism involving these amino acids occurs in strictly anaeorobic microorganisms such as *Clostridium sporogenes* which employ an intermolecular oxidation-reduction reaction

(Strickland reaction) to deaminate amino acids (12). Glycine, hydroxyproline, proline, and arginine act as hydrogen acceptors, whereas, alanine, glutamic acid, and serine act as hydrogen donors in this reaction (12, 13).

Undoubtedly the general unpalatability of the supplemental free amino acids was partially responsible for the decreased feed intake and weight gain of rats fed diet 5, but it is unlikely that palatability was the major factor since the addition of equally unpalatable amino acids to diet 5 (diets 6, 9, and 10) partially alleviated the growth-depressing effects of that diet.

High intakes of nonessential amino acids have been associated with decreased blood levels of essential amino acids (9, 10) and with increased dietary requirements for the essential amino acids for optimal growth (10). In this study supplemental nonessential amino acids may have decreased nitrogen retention by decreasing the availability of the essential amino acids at the cellular level as well as by causing antagonisms among the nonessential amino acids.

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Nutritional, Genetic and Morphological Studies of an Abnormal Cartilage Formation in Young Chicks [1]

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ABSTRACT Factors affecting the occurrence of an abnormal cartilage formation in the bones of young chicks have been studied. The condition occurs in chicks fed a purified diet and is prevented by the use of natural feed ingredients. The incidence of this abnormality can be increased by genetic selection. The abnormal cartilage formation is characterized by an accumulation of immature chondrocytes in the proximal metaphyses of the tibiotarsus and tarsometatarsus. The microscopic appearance is similar to the lesion observed with copper deficiency; however, dietary copper does not prevent it in chicks fed the purified diets. Deficiences of other nutritional factors known to affect bone formation did not produce a lesion similar to the abnormal cartilage formation. The purified diet used to produce the deformity was not improved by increased amounts of individual vitamins and minerals. No concentrated source of abnormal cartilage-preventing activity was observed in the natural feedstuffs studied. Thus, the nutritional factor(s) involved in the prevention of this condition is (are) unknown at the present time.

Many nutrients have been reported to influence bone formation in the chick. The bone abnormalities produced by a deficiency or toxicity of these nutrients fall into 2 general categories. One type is the rickets-like bone abnormality characterized by widening of the epiphyseal plate and soft, poorly calcified bones. A second is the perosis-type which is characterized by some or all of the following symptoms: enlargement of the hock joint, slipped tendon, shortened bones, twisted or crooked bone and narrowing of the epiphyseal plate, with relatively small changes in ash content of the bone.

During a study of the effect of manganese deficiency on the composition of the epiphyseal plate (1), an abnormal cartilage formation was observed in the chicks fed the purified control diet. This was of particular concern for the following reasons: (a) the abnormality occurred in chicks fed a diet thought to be adequate in all known nutrients; (b) the cartilage formation did not resemble that observed in the 2 types of bone abnormalities described above; and (c) despite the grossly abnormal cartilage formation, the chicks having this condition showed no obvious signs of leg weakness.

The present paper reports the results of experiments conducted to determine the factors responsible for the occurrence of this cartilage abnormality. Gross and histological characteristics of the abnormality are described and data are presented showing that the condition can be corrected by dietary means and the incidence altered by genetic selection.

Characteristics of the abnormality. The abnormal cartilage formation was observed only in the proximal ends of the tibiotarsus and tarsometatarsus of chicks fed the basal diet shown in table 1. No other bones in the chick have been observed to be affected. Afflicted chicks grew normally and showed no obvious signs of leg weakness. Thus, the occurrence of the abnormality can be determined only on autopsy or by x-ray examination. When affected bones were split longitudinally, a mass of opaque cartilage irregular in shape and size was observed below the epiphyseal plate, extending into the metaphysis. In some instances, it appeared as a uniform band across the width of the bone. The gross appearance of the abnormality is shown in figure 1.

In some ways, the gross appearance of the cartilage resembles that observed in

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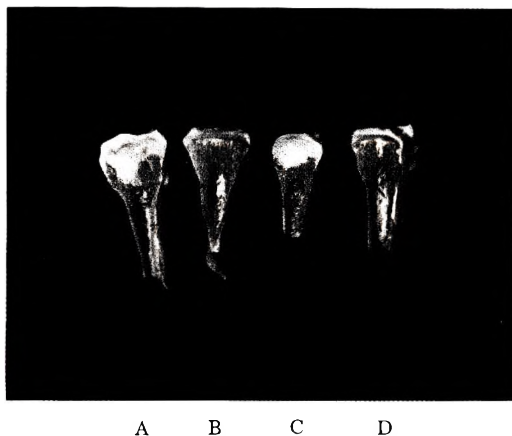
TABLE 1

Composition of basal diet

	%
Glucose	58.49
Isolated soybean protein	27.00
Corn oil	3.00
Cellulose	3.00
Vitamin mixture [1]	1.22
Mineral mixture [2]	6.29
Glycine	0.30
DL-Methionine	0.70
Protein (N × 6.25), %	24.86
Metabolizable energy, kcal/g	3.35

[1] Supplied the following per 100 g of diet: (in milligrams) inositol, 25.0; niacin, 5.0; Ca pantothenate, 2.0; pyridoxine·HCl, 0.45; folic acid, 0.40; menadione sodium bisulfite, 0.152; biotin, 0.02; α-tocopheryl acetate, 6.6; thiamine·HCl, 1.1; riboflavin, 1.1; choline chloride, 154; ethoxyquin, 12.5; and vitamin B_{12}, 2.0 μg; vitamin A, 540 IU, and vitamin D_3, 98 ICU.

[2] Supplied the following per 100 g of diet: (in grams) $CaHPO_4 \cdot 2H_2O$, 2.16; $CaCO_3$, 1.83; KH_2PO_4, 1.39; NaCl, 0.60; $MgSO_4$, 0.25; (in milligrams) $FeSO_4 \cdot 7H_2O$, 33.3; $MnSO_4 \cdot H_2O$, 33.3; KI, 0.26; $CuSO_4 \cdot 5H_2O$, 1.67; $CoCl_2 \cdot 6H_2O$, 0.16; $Na_2MoO_4 \cdot 2H_2O$, 0.83; and $ZnCO_3$, 11.5.



A B C D

Fig. 1 Bones A and C are from chicks having abnormal cartilage formation. Bones B and D are from chicks with normal bone development.

rickets resulting from vitamin D or calcium deficiency. However, this did not appear to be a truly rachitic condition since in an experiment in which bone ash was measured, the bone ash values of afflicted and nonafflicted chicks were 42.0 and 44.0% respectively. Attempts to detect this condition by means of blood analysis have also been unsuccessful. In these experiments, blood was obtained by heart puncture with a heparinized syringe and the plasma was assayed for calcium (2), phosphorus (3), magnesium (4) and acid and alkaline phosphatase activity.[2,3] Data presented in table 2 show that there were no differences in calcium, phosphorus or magnesium levels in the blood such as are associated with deficiencies of calcium, phosphorus, magnesium or vitamin D. Blood phosphatase levels were also similar. The body weight of chicks with or without the abnormality was the same in this experiment. Thus, the factor associated with the appearance of the abnormality does not appear to affect growth rate.

The bones of chicks showing the abnormality were also examined microscopically. The specimens taken for the histological studies were immediately fixed in cold 10% neutralized formalin, decalcified in Decal [3,4] and stained with hematoxylin-eosin (5). The photomicrographs of normal and abnormal bone are shown in figures 2–9. Examination of the epiphyseal plate indicates that the width of the plate and the sequence of cells is similar for the 2 bones (figs. 4 and 5). Examination of the metaphyseal area (fig. 7) shows that the tissue responsible for the opaque appearance of cartilage contains a mass of immature chondrocytes. Unlike the hypertrophic cells noted in the normal metaphysis (figs. 6 and 8) these chondrocytes have compact, dense-staining nuclei (figs. 7 and 9). With the alcian blue-PAS stain (5) the nuclei of the normal hypertrophic cartilage cells stain blue, whereas the nuclei of the abnormal cartilage cells stain deep pink. In the abnormal tissue there was little tunneling by blood vessels or formation of bony trabeculae as is usually observed in a comparable area of normal bone. The differences in the appearance of the tissue at the tip of a blood vessel are shown in figures 8 and 9.

In attempting to identify the possible factors involved in the occurrence of this condition we have examined the bone abnormalities associated with the following nutritional states: (a) deficiences of vitamins A and D, calcium, phosphorus, magnesium, manganese, zinc, choline,

[2] Technical Bulletin 104, Sigma Chemical Company, St. Louis.

[3] Trade names and company names are included for the benefit of the reader and do not infer endorsement or preferential treatment of the product listed by the U. S. Department of Agriculture.

[4] Product of Omega Chemical Corporation, New York.

TABLE 2

Effect of abnormal metaphyseal cartilage on body weight and composition of blood plasma [1]

Bone classification [1]	4-week weight [2]	Analysis of blood plasma [3]			Plasma phosphatase activity [3]	
		Calcium	Phosphorus	Magnesium	Alkaline phosphatase [4]	Acid phosphatase [5]
	g	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*		
Normal	540(245)	11.10 ± 0.54(19)	8.92 ± 0.60(19)	2.15 ± 0.21(19)	12.47 ± 6.7(20)	62.45 ± 16.6(20)
Abnormal	555(50)	11.36 ± 0.55(11)	8.74 ± 0.55(11)	2.14 ± 0.37(11)	11.82 ± 4.2(20)	57.71 ± 14.6(20)

[1] Chicks fed basal diet shown in table 1.
[2] Average of male and female. Figures in parentheses indicate number of chicks.
[3] Values shown are mean values with SE. Figures in parentheses indicate number of chicks used for determination.
[4] Expressed as milligrams of substrate hydrolyzed per 15 minutes per milliliter of plasma.
[5] Expressed as micrograms of substrate hydrolyzed per 15 minutes per milliliter of plasma.

biotin, folic acid, and niacin; (b) toxicities produced by excess amounts of vitamin A, β-aminoproprionitrile, magnesium plus fluoride and fluoride alone. With the exception of calcium and vitamin D deficiencies, none of the gross or histological lesions of the above conditions resemble those observed with the metaphyseal cartilage abnormality. Although the gross appearance of calcium or vitamin D deficiency and the cartilage abnormality are similar, there is little histological resemblance between the 2 conditions.

The possibility that this abnormality is a remnant of embryonic bone has also been considered. Below the epiphyseal plate in the one-day-old chick, a cartilage cone composed of mature chondrocytes which contained few invading blood vessels was found. Therefore, it would be possible to attribute the abnormal cartilage formation to the imperfect removal of this embryonic-type of cone. This does not appear to be the case since the cells of the embryonic cone resemble normal hypertrophic cartilage cells rather than those observed in the cartilage abnormality. Furthermore, studies on the effect of age on the occurrence of this syndrome do not support such a hypothesis. The incidence is higher in 4-week-old chicks than in 2-week-old chicks. The opposite would be anticipated if the cartilage abnormality were the result of the lack of destruction of the embryonic cartilage cone.

Carlton and Henderson [5] described the histological lesions which occurred as the result of copper deficiency in the young chick. Subsequently, copper deficiency has been produced in this laboratory for the purpose of comparison with the metaphyseal cartilage abnormality. The bone lesions of copper deficiency were similar to those obtained in these experiments. These lesions were also similar to those described in more detail by Carlton and Henderson (6). Although the histological appearance of the metaphyseal cartilage abnormality is similar to that in copper deficiency, afflicted chicks show no other symptoms of copper deficiency such as

[5] Carlton, W. W., and W. Henderson 1962 Histopathological lesions observed in the long bones of chickens fed a copper-deficient diet. Poultry Sci., *41*: 1634 (abstract).

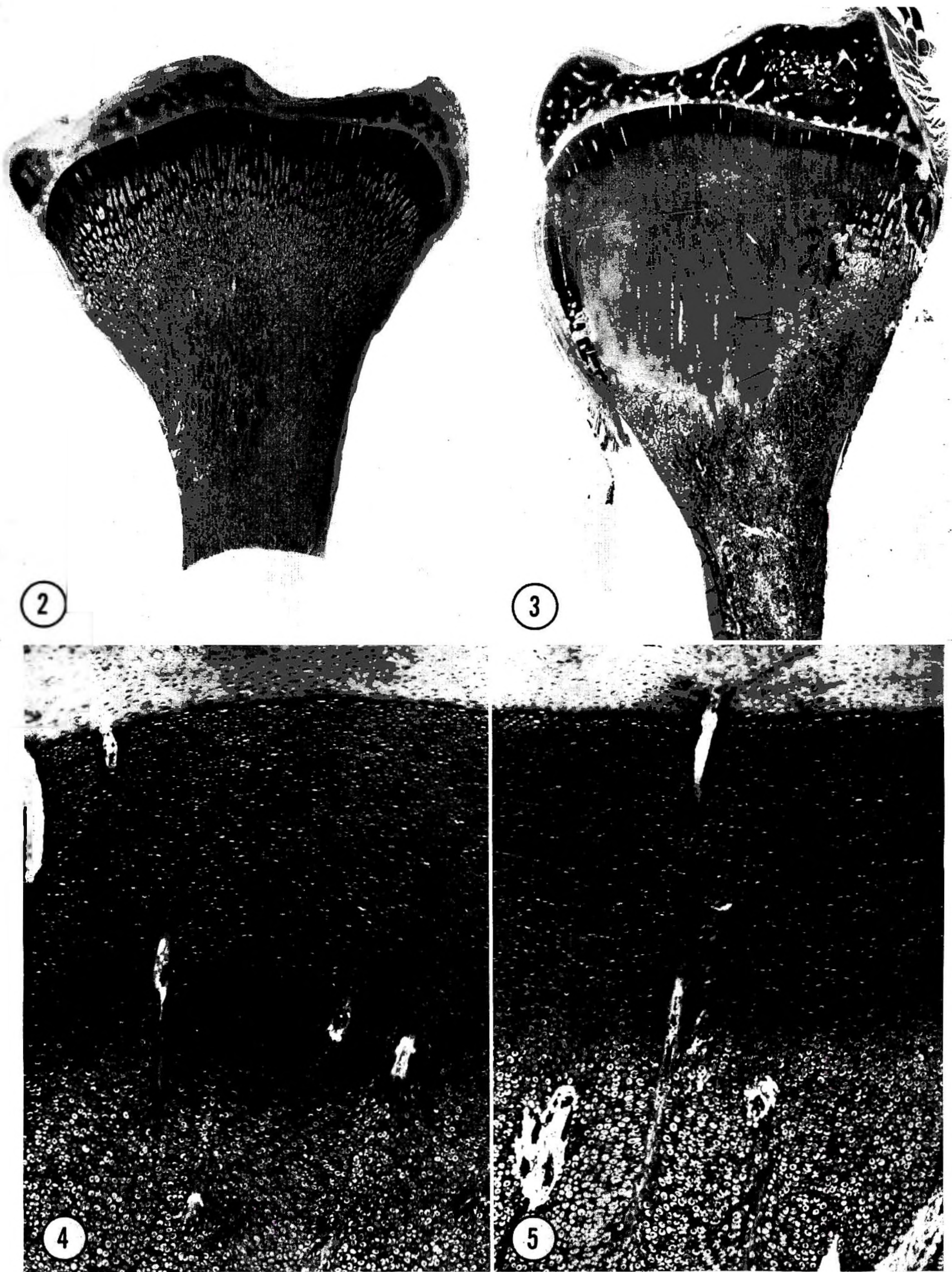


Fig. 2 Normal bone. Proximal end of the tibiotarsus. H & E. × 3.5.

Fig. 3 Abnormal bone. Proximal end of the tibiotarsus. H & E. × 3.5.

Fig. 4 Epiphyseal growth plate of normal bone. H & E. × 60.

Fig. 5 Epiphyseal growth plate of abnormal bone. H & E. × 60.

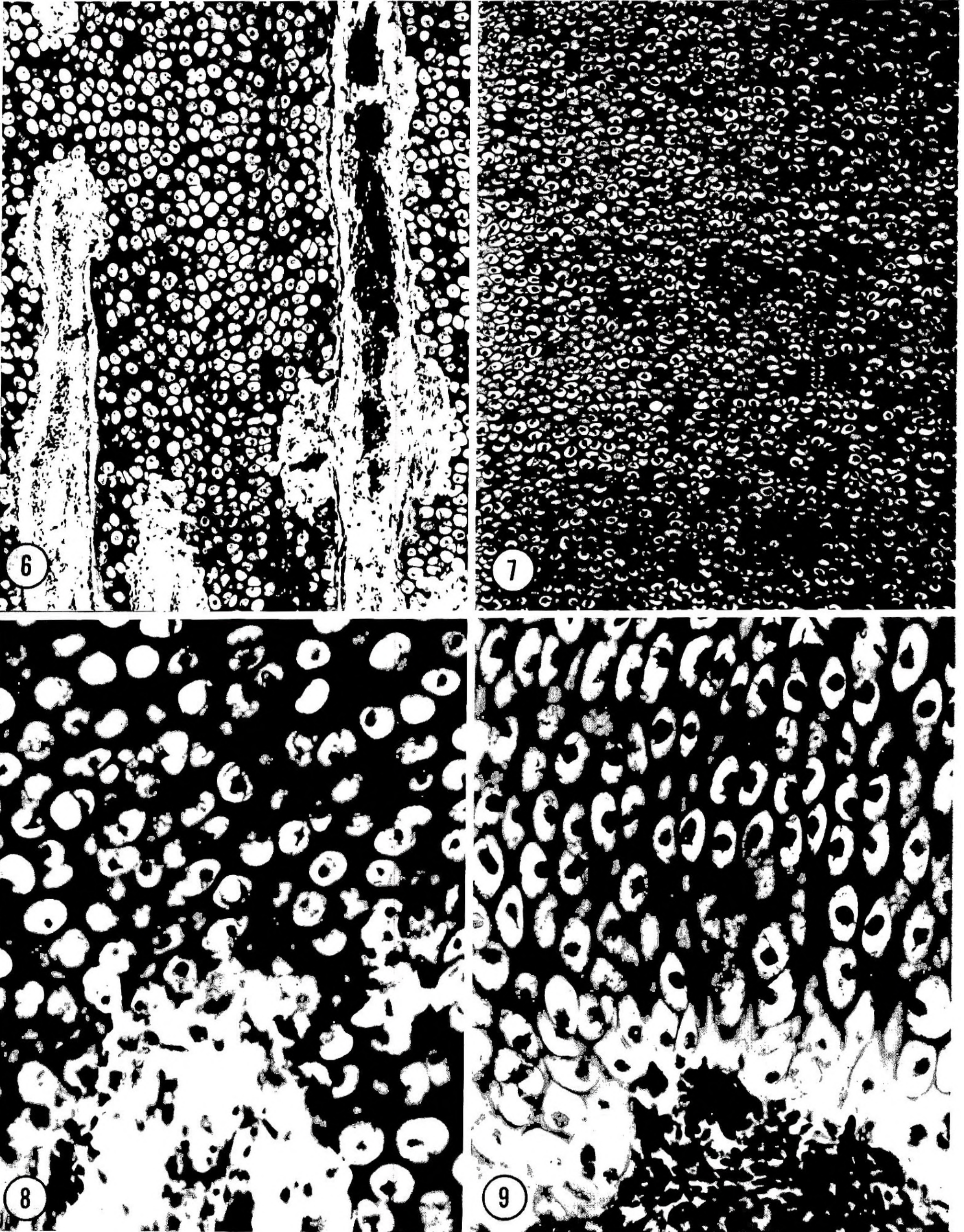


Fig. 6 Metaphysis of normal bone. Tunneling of the blood vessels through hypertrophic cartilage cells. H & E. × 120.

Fig. 7 Metaphysis of abnormal bone. Note absence of tunneling of blood vessels. H & E. × 120.

Fig. 8 Metaphysis of normal bone showing tip of blood vessel. H & E. × 450.

Fig. 9 Metaphysis of abnormal bone showing tip of blood vessel. H & E. × 450.

anemia, poor growth and aortic weakness (7, 8).

Genetic factors influencing the incidence of abnormal metaphyseal cartilage. Early investigations on the cartilage abnormality were hampered by the low rate (10 to 15%) of occurrence of this syndrome in the population of chicks studied. Therefore, it was necessary to develop procedures to increase the incidence. Genetic selection has proved to be an effective tool for this purpose.

Pedigreed progeny from matings of White Plymouth Rock females and Vantress males were fed the purified basal diet until 4 weeks of age, then killed and examined for the occurrence of abnormal metaphyseal cartilage. By selecting sire and dam combinations from progeny test data, 2 strains were developed from the original unselected population: one showing a high incidence (41%) and a second with a relatively low incidence (16%) of the abnormality. These data are shown in table 3. The incidence observed in the parent population is also included in this table for comparison, even though the data were not collected simultaneously with the data from the selected strains. There was a significant difference in the susceptibility of male and female chicks. During the past year 351 male chicks from the high incidence strain were fed the purified basal diet. The average incidence obtained in these experiments was 50% with a range of 33 to 70% for individual experiments. The genetic aspect of this condition is also indicated by the observation that no abnormality has been observed in

TABLE 3

Effect of genetic selection on the occurrence of abnormal metaphyseal cartilage

Progeny test	Incidence [1]		
	Male	Female	Both
	%	%	%
Unselected population	24	17	21(454) [2]
First generation			
High incidence strain	53	27	41(95) [3]
Low incidence strain	15	16	16(73)

[1] All chicks received diet shown in table 1.
[2] Figures in parentheses indicate number of chicks in treatment.
[3] Chi-square analysis indicates that the combined incidence of abnormal cartilage in males and females is significantly different ($P < 0.01$) between high incidence strain and low incidence strain.

2 strains of White Leghorn chicks, whereas in four other "meat-type" strains the incidence was from 7 to 25%.

The differences observed between male and female chicks suggested a possible relationship to sex hormones. In an experiment conducted to test such a possibility, male and female chicks were fed diets containing either testosterone proprionate or diethylstilbestrol. The levels fed were sufficient to alter secondary sex characteristics and gonad or uterine size. However, neither hormone had any significant effect upon the occurrence of the metaphyseal cartilage abnormality in male or female chicks.

Nutritional studies on the occurrence of abnormal metaphyseal cartilage. The appearance of the abnormality in 4-week-old chicks was apparently related to nutrition since studies showed that this condition did not occur in chicks fed a commercial chick starter. Data illustrating this observation are presented in table 4. Also presented are data showing the effects of a slightly toxic level of fluoride. The addition of this element to the basal diet increased the severity of the abnormality. This has been a consistent observation with genetically selected chicks. With unselected chicks, fluoride increases the incidence as well as the severity. However, the abnormality does not appear to be the direct result of fluoride toxicity since it occurs regularly in the absence of fluoride. The data in this table confirm the marked differences in susceptibility of male and female chicks.

Chicks from matings selected for high incidence were used in further experiments dealing with the nutritional aspects of the cartilage abnormality. One-day-old male chicks, placed in electrically heated battery brooders with raised wire floors, were supplied the experimental diets and tap water ad libitum. At the conclusion of the 4-week experimental period, the chicks were killed and examined for incidence and severity of the abnormality. Dietary additions were made at the expense of glucose except for corn, which was substituted for all of the glucose, and for soybean meal which replaced the isolated soybean protein.

TABLE 4

Effect of diet on the incidence of abnormal metaphyseal cartilage

Treatment	Males [1]		Females [1]		Both	
	Incidence	Severity index [2]	Incidence	Severity index [2]	Incidence	Severity index [2]
	%		%		%	
Basal diet [3]	46(58) [4]	1.67	18(66)	1.50	31	1.62
Basal + 0.04% fluoride	41(71)	2.48	27(51)	2.28	35	2.42
Chick starter [5]	0(62)	0	0(61)	0	0	0
Chick starter + 0.04% fluoride	0(63)	0	3(58)	1.0	1.6	1.0

[1] Chi-square analysis of pooled male vs. female incidence of abnormal cartilage in basal and basal + fluoride indicates they are significantly different ($P < 0.01$).
[2] Severity index: severe = 3.0, medium = 2.0, slight = 1.0. The sum of these values divided by the number of afflicted chicks equals the severity index.
[3] Composition of basal diet shown in table 1.
[4] Figures in parentheses indicate number of chicks.
[5] Nonmedicated chick starter purchased from Agway, Inc., Ithaca, New York. See text footnote 3.

One nutritional approach to the problem of abnormal cartilage has been to study components of the chick starter. To estimate the total abnormal cartilage-preventing activity of this diet, the effects of feeding varying amounts of chick starter were investigated. In this experiment, chick starter was mixed with the basal diet to give the desired proportions. The results, presented in table 5, indicate that between 50 and 100% chick starter is needed for complete prevention of the abnormality.

To determine whether the abnormal cartilage-preventing activity was concentrated in a specific feedstuff, ingredients which are normally used in chick rations of this type were also tested. In the testing procedure, ingredients were added to the purified basal diet in amounts comparable to those used in chick-starting rations. The ingredients tested were as follows: ground yellow corn, 50% protein soybean meal, alfalfa leaf meal, fish meal, meat scraps, dicalcium phosphate, ground limestone, animal fat, fermentation product, dried distillers solubles and brewer's yeast. Of the ingredients tested, only the corn and soybean meal had any appreciable abnormal cartilage-preventing properties. The residue produced by ashing the chick starter at 550° was ineffective when included in the purified diet. A diet composed of corn and soybean meal supplemented with minerals and vitamins would completely prevent the occurrence of the cartilage abnormality. Data from this experiment, presented in table 6, show that methanol extraction of the corn and soybean meal removed little activity from these ingredients.

The second nutritional approach was the study of the adequacy of the purified diet. This was accomplished by increasing the levels of many of the individual nutri-

TABLE 5

Effect of varying amounts of chick starter on the incidence of abnormal metaphyseal cartilage

Basal diet [1]	Chick starter [2]	Incidence	Severity index [3]
%	%	%	
100	0	60(20) [4]	1.47
75	25	33(21)	2.35
50	50	14(21)	1.00
0	100	0(20)	0

[1] Composition of basal diet shown in table 1.
[2] Nonmedicated chick starter purchased from Agway, Inc., Ithaca, New York. See text footnote 3.
[3] See footnote 2, table 4.
[4] Figures in parentheses represent the number of male chicks.

TABLE 6

Effect of corn and soybean meal on the incidence of abnormal metaphyseal cartilage

Treatment	Incidence	Severity index
	%	
Basal diet [1]	58(24) [2]	1.50 [3]
Corn-soybean meal [4]	0(24)	—
Corn-soybean meal, methanol extracted	9(23)	1.00

[1] Composition of basal diet shown in table 1.
[2] Figures in parentheses represent the number of male chicks.
[3] See footnote 2, table 4.
[4] Diet had following composition: (in g/100 g feed) ground yellow corn, 59.2; 50% soybean meal, 32.0; DL-methionine, 0.2; animal fat, 1.0; with the mineral mixture, vitamin mixture and antioxidant added at the same level as used in the basal diet.

ents present in this diet. The mineral elements tested were Ca, P, Mg, Fe, Cu, Zn and Se. The vitamin content of the diet was tested by increasing and decreasing the levels of vitamin mixture. Individual vitamins studied were: vitamin D_3, vitamin D_2, niacin, pyridoxine and inositol. None of the above variations in nutrient content affected the occurrence of the cartilage abnormality.

The occurrence of the abnormality does not appear to be related to the presence of the isolated soybean protein. Similar results were obtained when the soybean protein used in the purified diet was washed or replaced by crude casein or vitamin-free casein supplemented with the appropriate amino acids.

Supplementary copper was studied, since the histological lesions of copper deficiency are similar to those observed in these experiments. Addition of copper in levels up to 50 mg/kg of diet failed to influence the occurrence of this abnormality. Similar results were obtained with a combination of iron and copper. Chicks with abnormal metaphyseal cartilage show none of the other symptoms of copper deficiency such as reduced growth rate, low blood hemoglobin or aortic weakness. Thus, this abnormal cartilage formation does not appear to be the result of a simple copper deficiency.

DISCUSSION

These studies describe many factors affecting an atypical cartilage formation in young chicks. In many ways, the appearance of the condition fits the classical picture of a nutritional deficiency. The abnormality can be produced by feeding a purified diet and prevented by feeding a diet composed of natural ingredients. In other respects, however, the abnormal cartilage is a unique condition, since growth rate of the chick is not impaired and the only manifestation of the deficiency observed has been in the cartilage at the proximal end of the tibiotarsus and tarsal metatarsus. No evidence of a systemic defect in metabolism and no changes in blood components normally used as indicators of abnormal bone metabolism have been observed.

The lack of other systemic effects observed when the abnormal cartilage is present could be an indication that only a marginal "deficiency" of the dietary factor or factors is involved, and if some means of producing greater deficiency could be found, other manifestations might appear.

Possibly this condition is not the result of a true deficiency, but rather the result of a peculiar balance or combination of nutrients that results in changes in the metabolism of the affected cartilage. Although it is tempting to postulate the existence of an unidentified nutritional factor affecting bone formation, some further investigations are necessary to show that certain combinations of known nutrients are not responsible for the dietary effects observed.

Chicks having this grossly abnormal cartilage formation show no obvious sign of leg weakness. To date, studies have been confined to 4-week-old chicks. Thus, little is known about the consequences of this condition in older chicks. It is possible that this syndrome would manifest itself as some type of leg weakness if chicks were maintained with purified diets for longer periods of time.

The susceptibility of chicks to this condition appears to be genetically controlled. We have not noted abnormal cartilage in White Leghorn chicks fed the purified diet that produced the condition in White Plymouth Rocks. Selection for increased incidence has been successful and has been a useful tool in making dietary studies possible.

Although we have no evidence on the metabolic defect involved, the abnormal cartilage condition we have reported in this paper does have a nutritional origin, can be affected by genetic selection, and does not appear to be related to any of the known dietary factors affecting bone formation.

ACKNOWLEDGMENT

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Effect of Soybean Trypsin Inhibitor and Penicillin on Cystine Biosynthesis in the Pancreas and its Transport as Exocrine Protein Secretion in the Intestinal Tract of the Rat [1]

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ABSTRACT Rats were given, by intraperitoneal injection, a tracer dose of methionine-S^{35}. At intervals following this, cystine-S^{35} was determined by cysteic acid isolation. Protein-bound and non-protein cystine-S^{35} separations were made with trichloroacetic acid. Very rapid and extensive conversion of methionine-S^{35} to cystine-S^{35} was found in the pancreas. This conversion was increased by the prior administration by stomach tube of a single dose of crystalline soybean trypsin inhibitor. The cystine-S^{35} formed in the pancreas was observed subsequently in relatively high concentration in the small intestine. This phenomenon is interpreted to reflect the synthesis of pancreatic exocrine protein which is secreted into the intestinal tract. The amount of cystine-S^{35} in the pancreas was not altered by including penicillin in the diet, but there appeared to be a marked protection of protein-bound cystine-S^{35} in the small intestine and consequently a decrease in the amount of non-protein cystine-S^{35} in the small intestinal contents. This apparent protection of cystine-S^{35} protein from hydrolytic degradation by penicillin was reflected in an increase in the amount of protein-bound cystine-S^{35} in the large intestine which corresponded to earlier reported increase in trypsin activity of feces of rats fed penicillin and unheated soybeans. It is believed that this effect of penicillin forms the basis by which beneficial effects on growth are obtained when diets containing unheated soybeans and penicillin are fed.

A recent report from this laboratory has shown that orally administered soybean trypsin inhibitor increases the oxidation of methionine-2-C^{14} to $C^{14}O_2$ in the rat (1). Indirect evidence was presented that this effect of trypsin inhibitor probably reflected an increase in the biosynthesis of cystine. Since biosynthesis of cystine involves a complex series of reactions with the sulfur being the only part of the original methionine to be transferred to the new cystine molecule, C^{14} tagging procedures do not provide satisfactory proof of this transformation. The present study is an extension of the earlier investigation in which a more direct procedure utilizing methionine-S^{35} has been employed.

METHODS

Male, Holtzman rats that had been fed from approximately weaning age with a purified diet containing either raw ground soybeans or casein as the source of protein, were used at approximately 150 g body weight in these studies. The composition of the basal diets is shown in table 1. L-Methionine-S^{35} at a dosage of 0.05 mc as the tracer substance, was injected intraperitoneally into rats that had been fasting for 16 hours. At stated intervals following this injection, tissues such as pancreas, intestinal contents, intestinal mucosa, liver and blood sera were obtained. The samples were oxidized with performic acid in the refrigerator over night (16 hours). At the end of the oxidation period the reagent was removed in a flash evaporator. After dryness was achieved by placing the flask in a vacuum dessicator containing NaOH, 25 ml of 6 N HCl were added and the sample was autoclaved for 24 hours at 120°. The hydrolysate was filtered through a sintered glass funnel. The HCl was removed in the flash evaporator, taken to complete dryness in a vacuum dessicator over NaOH and the residue taken up with distilled water. The hydroly-

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TABLE 1

Composition of semipurified basal diets

Major components	Casein diet	Unheated soybean diet
	%	%
Casein	15.0	—
Soybean	—	25.0
Glucose monohydrate [1]	62.7	52.7
Salt mixture [2]	4.0	4.0
B vitamins	2.0	2.0
Fat-soluble vitamins	1.0	1.0
Hydrogenated vegetable oil [3]	15.0	15.0
Choline dehydrogen citrate	0.3	0.3
Total	100.0	100.0

B vitamins in 2.0 g glucose monohydrate

	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 Company, Argo, Illinois.
[2] Hubbell, R. B., L. B. Mendel and A. J. Wakeman 1937 A new salt mixture for use in experimental diets. J. Nutrition, *14*: 273.
[3] Primex, Procter and Gamble Company, Cincinnati.

sate was then placed on a column of Dowex 50–4 X resin H^+ form and eluted with distilled H_2O. The water was again removed in the flash evaporator and the residue was taken up in 1 ml of distilled water. The cysteic acid in the solution was then isolated by paper electrophoresis and determined quantitatively with ninhydrin. The S^{35} was determined in the paper by liquid scintillation spectrometer. This combines the essential features of the procedures of Schram et al. (2) and Diehl (3).

In the first set of experiments rats were fed the casein basal diet. Half of the animals were given a single dose of 50 mg of soybean trypsin inhibitor (Kunitz-type) by stomach tube 2 hours prior to the intraperitoneal administration of L-methionine-S^{35}. At intervals of 1, 2, 3 and 6 hours following the administration of the tracer methionine, one rat from each group was anesthetized with ether and blood was drawn to exanguinate the animal. The entire pancreas and liver were dissected free from other tissues. The contents of the small intestine were otbained by washing the intact small intestine with saline. After the contents had thus been removed, the intestinal mucosa was separated by gently scraping the outside of the intestinal wall with the edge of a microscope slide and collecting the mucosal tissue that was extruded from an open end of the intestine. In the second set of experiments, the rats had been fed a raw soybean diet either with or without 100 mg pencillin/100 g of diet. The animals were administered the tracer dose of L-methionine-S^{35} by intraperitoneal injection and at intervals of 2, 3, 6 and 16 hours, single rats were killed and the pancreas, the entire contents of the small intestine, intestinal mucosa and the entire contents of the large intestine (cecum plus colon) were collected. In this set of experiments, an aliquot was taken for measurement of total counts (see above) and a second aliquot was homogenized with 10% trichloroacetic acid and precipitated with alcohol. The protein residue was washed once with absolute alcohol, 3 times with ethanol and ether (3:1) and twice with ether, each followed by centrifuging. The residues were oxidized with performic acid and the amount of cysteic acid and its radioactivity were determined as before. Total counts in the TCA-soluble fraction were calculated by subtraction of TCA-insoluble counts from the total counts for the given tissue.

RESULTS

In figure 1 are shown the curves for the specific activity of cystine-S^{35} (counts/minute/mg cystine) for the rats in the first series of experiments in which the casein diet had been fed prior to the administration by stomach tube of a single dose of crystalline soybean trypsin inhibitor. These curves show that the specific activity of the cystine of the pancreas is higher than that of the other tissues that were sampled, with the contents of the small intestine showing the next highest activity.

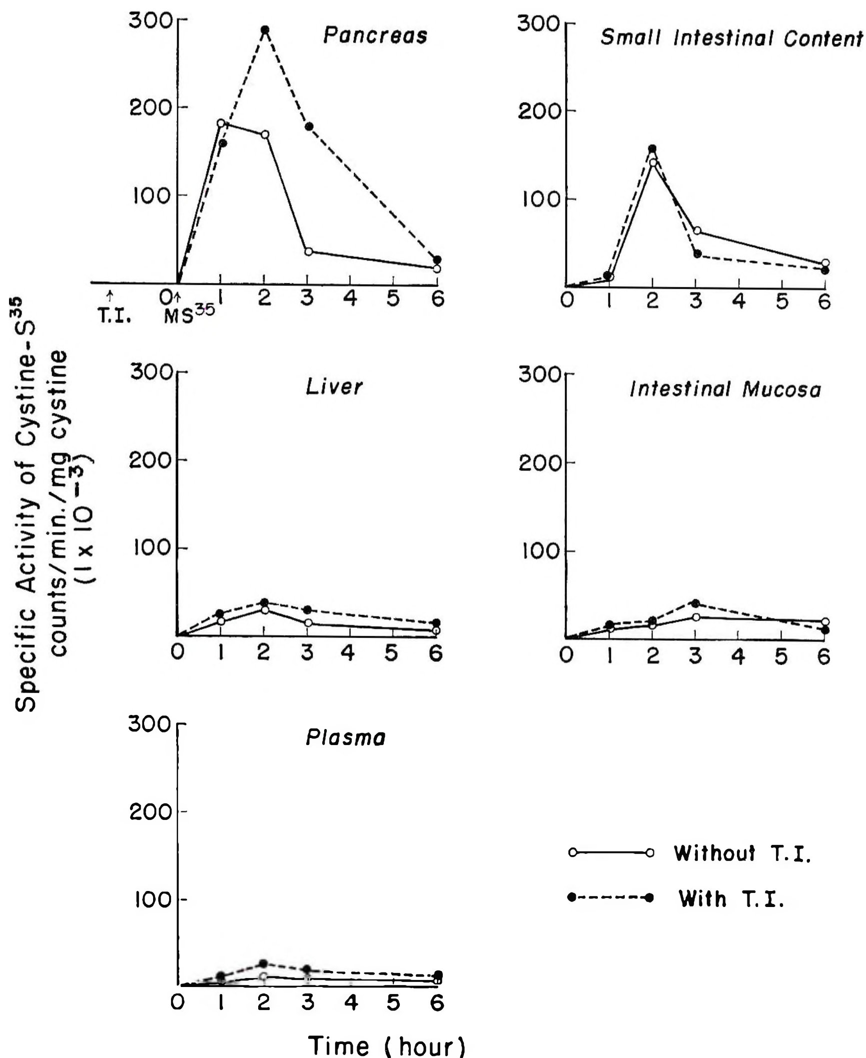

Fig. 1 Cystine-S^{35} specific activity (c/m/mg cystine) of tissues following the intraperitoneal administration of methionine-S^{35}. Solid line, individual rats that had not received trypsin inhibitor. Broken line, rats given 50 mg crystalline trypsin inhibitor 1 hour prior to the tracer methionine-S^{35}.

Biosynthesis of cystine from the sulfur of methionine probably takes place in most tissues of the body but it is evident from these curves that the pancreas is particularly active. The intestinal contents have such high specific activity that it is concluded that the newly formed cystine in the small intestine could not have been derived from the blood or by any other mechanism of passage through the intestinal mucosa to the lumen of the intestine, nor could it have been derived from the liver via passage with the bile. It is concluded that the cystine-containing S^{35} in the contents of the small intestine must have been derived from the pancreas. It is further evident that more cystine-containing S^{35} was formed in the pancreas 2 and 3 hours following the administration of the tracer dose of methionine-S^{35} in those rats that had received trypsin inhibitor. There were also higher specific activities of crystine in

liver, intestinal mucosa and plasma of the rats receiving trypsin inhibitor, and while these differences appear to be real, particularly in the case of liver and plasma, the absolute levels are far below the level for the pancreas. There were no differences in the specific activity of the small intestinal contents that were consistently observed at the different time intervals.

In the second set of experiments, rats were fed a diet containing unheated soybeans for 2 weeks and one-half of the animals were provided penicillin at a level of 100 mg/100 g of diet. At intervals of 2, 3, 6 and 16 hours after intraperitoneal administration of a tracer dose of L-methionine-S^{35}, individual rats were killed and the pancreas, small intestine contents, intestinal mucosa and large intestine contents were collected. In this study the separation of trichloroacetic acid-insoluble and -soluble fractions were obtained so that cystine-S^{35} in these tissues could be expressed as being protein-bound or non-protein. The specific activities of the cystine-S^{35} isolated in these rats are shown in figure 2. As in figure 1, the cystine-S^{35} in the small intestine contents must have been derived from the pancreas. It appears that there was good equilibrium between protein-bound and non-protein S^{35} in the pancreas and intestinal mucosa over the course of the study, but an extremely high non-protein cystine-S^{35} specific activity was observed in the intestinal contents of the rats that did not receive penicillin in the diet. No explanation is given for this very high specific activity at this time but it is believed that

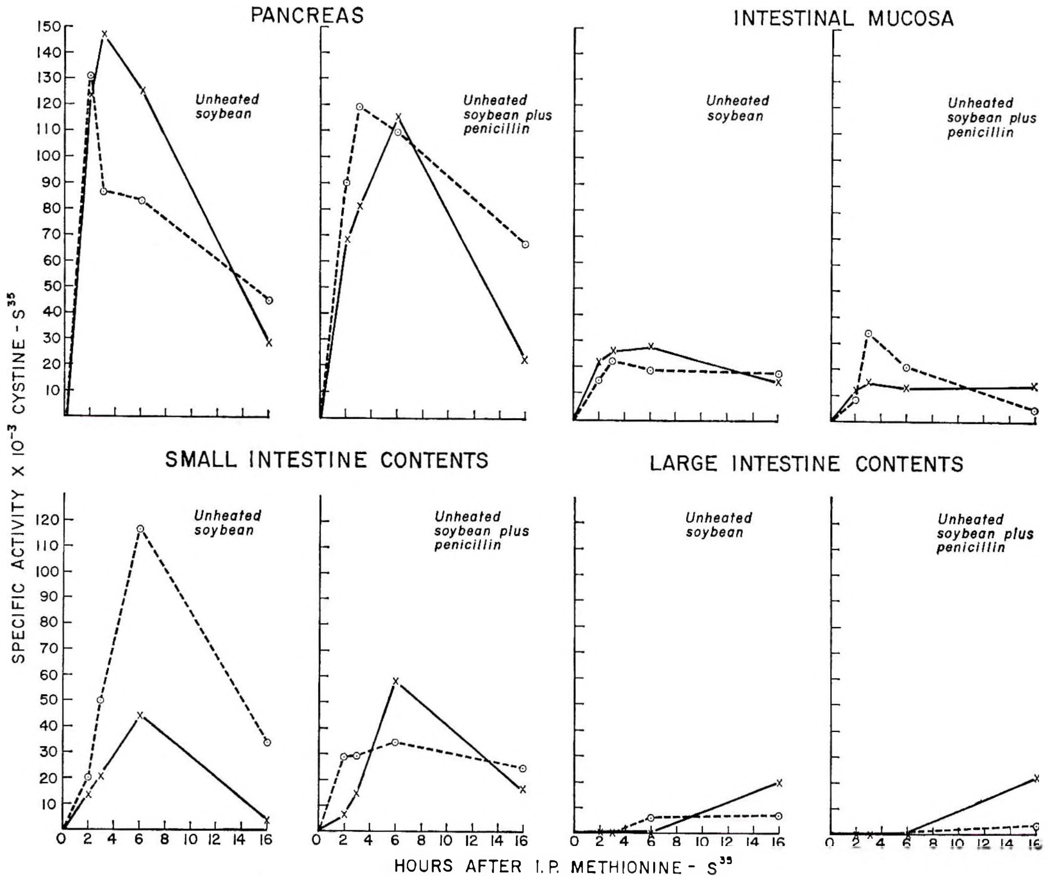

Fig. 2 Cysine-S^{35} specific activity (c/m/mg cystine) of tissues following the intraperitoneal administration of methionine-S^{35}. Solid line, TCA-insoluble; broken line, TCA-soluble cystine.

this cannot be an observational error since 3 determinations at different time intervals, involving different animals, all showed an elevated specific activity of the non-protein cystine-S^{35}. It would seem logical that in the small intestine contents the specific activity of cystine-S^{35} might decrease with time as a consequence of absorption of material that has passed from the pancreas into the small intestine and the replacement of the free cystine with non-radioactive S^{35} from other protein secretions and epithelial desquamation. This does appear to be the case. With large intestine contents at the 16-hour interval in rats both with and without penicillin there was an increase in the protein-bound cystine-S^{35} in excess of the S^{35}-specific activity of the non-protein fraction. The results in figure 2 are in terms of specific activity and therefore give an indication of rates of formation of new cystine relative to the total cystine content of the tissues, but this expression does not give any indication of the total amount of new cystine that has been formed. For a consideration of the total quantity of newly formed cystine in the different tissues, the graphical presentations in figure 3 are given. In the previous figures, the radioactivity on the ordinates has been shown on the same scale for the different tissues. In this set of 3 graphs, different scales have been used; the pancreas radioactivity being shown with ordinates that are 10 times those used for the small intestine contents and large intestine contents. In this set of graphs, the radioactivity of the cystine-S^{35} is expressed as total counts per minute per total organ or in other words, the total for the entire pancreas for each rat, the total for the small intestine contents from the pylorus to the cecum, and lastly, the total large intestine contents, including the cecum and colon. On the basis of this calculation, the total cystine-S^{35} in the non-protein fraction in the pancreas is about one-half to one-third the protein-bound form over the peak of activity which occurs from the second to the sixth hour. No difference is discernible as a result of the feeding of penicillin in the diet. The most outstanding effect of penicillin in the diet was observed in the contents of the gastrointestinal tract. In

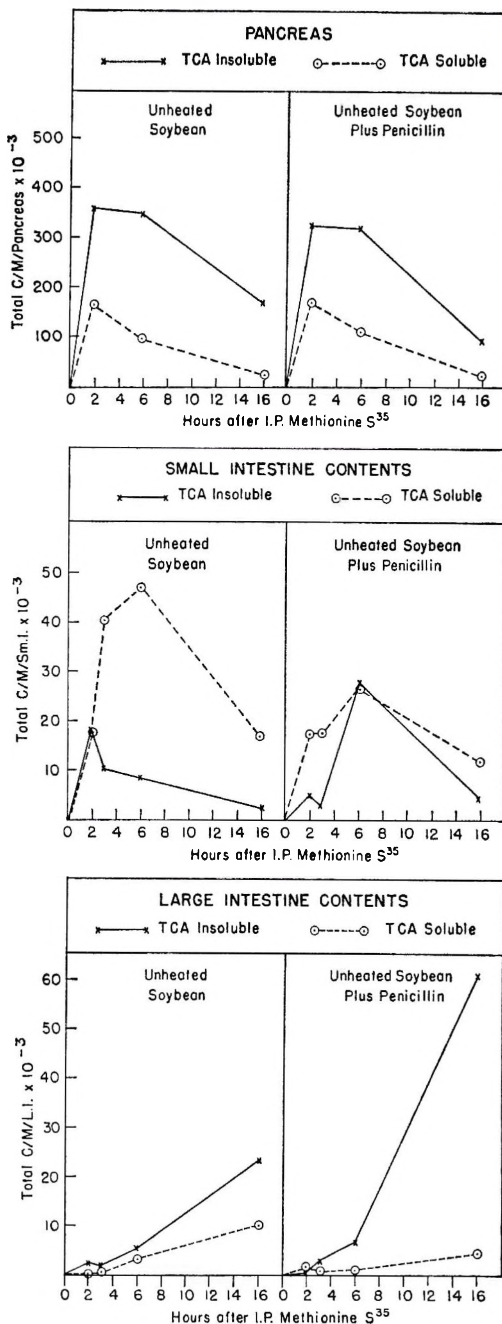

Fig. 3 (upper) Total cystine-S^{35} counts in the entire pancreas, (middle) the contents of the entire small intestine, and (lower) the contents of the entire large intestine including cecum and colon.

the contents of the small intestine the total amount of cystine-S^{35} in the non-protein form was two or more times that in the protein-bound form over the peak of activity which occurred during the second to the sixth hour. This is a reversal of the relationship that was observed in the pancreas, which is being considered as the precursor of the S^{35}-cystine in the intestinal contents. In the rats with penicillin added to the diet, the total quantity of cystine-S^{35} appears to be essentially equal in the protein and non-protein forms. In addition, there is some suggestion of a total increase in the protein-bound cystine-S^{35} of the intestinal contents of the rats receiving penicillin over that of the rats not receiving penicillin. In the large intestine contents there is again a striking difference in the distribution of cystine-S^{35} between protein and non-protein forms in the rats with or without penicillin. Without penicillin, at the end of 16 hours, which incidentally reflects approximately a complete passage time through the intestinal tract of the rat, there was a moderate increase in both non-protein and protein fractions but the protein-bound cystine-S^{35} was more than twice the total quantity of the non-protein form. In the rats fed penicillin in the diet, there appeared to be a smaller increase in the non-protein cystine-S^{35} but the protein form was many fold higher than the non-protein form and was approximately 3 times the total quantity of cystine-S^{35} found in the protein form of the rats without penicillin.

DISCUSSION

The results that have been presented here indicate that the pancreas is a very active organ in the biosynthesis of cystine from methionine and incorporation of this cystine into protein. It appears that most of the newly synthesized protein is mobile and can pass out of the pancreas into the small intestine, and it is concluded that this represents the normal exocrine protein secretion of the pancreas. The cystine content of proteolytic enzymes in the pancreas is extremely high (4), there being very few examples of proteins containing as high a concentration of cystine. Since the rate of synthesis of cystine in the pancreas is increased by trypsin inhibitor, it is assumed that this is a reflection of the increased secretion of enzymes that other workers (5, 6) have shown to occur. The majority of the newly formed cystine is in protein form but an extremely large quantity of non-protein cystine appears to be secreted from the pancreas into the small intestine. In the small intestine, it appears that there is a rather rapid hydrolysis of the protein that has been derived from the pancreas wtih the release of cystine-S^{35} in a non-protein form. This is inhibited by penicillin and it is therefore concluded that the penicillin has exerted its effect by preventing the formation of bacterial proteases, and it is largely the bacterial proteases which have been responsible for the hydrolysis of protein secreted in the pancreas. This conclusion is given strong support by the observation of Borgström et al. (7) that the inactivation of trypsin and invertase in the intestinal tract is due primarily to the microflora. A large increase in the fecal content of these pancreatic enzymes was observed in germfree rats.

The protection of the pancreatic secretion by penicillin may have resulted in a larger total quantity of intact pancreatic enzyme that passes into the large intestine. The very large differences in the amount of cystine-S^{35} in the large intestine as a consequence of the administration of penicillin is believed to be a reflection of an action that has taken place in the small intestine. Evidence has been presented that there is a protection of protein by penicillin in the small intestine, and it is further assumed that any penicillin ingested in the diet would be destroyed or absorbed before it could reach the large intestine. Even though the influence of penicillin is observed in the large intestine, it is concluded that the major, if not the total, effect in protecting pancreatic enzyme secretions against bacterial proteolysis has been in the small intestine. The protein-bound cystine-S^{35} in the large intestine at 16 hours, of the animals without penicillin relative to those that had received penicillin, is in a ratio of 1 to 3. The contents of the large intestine at 16 hours represents approximately the residue resulting from one complete passage from the pancreas to the anus in the rat. It is interesting, therefore, to compare the pres-

ent results with the activity of trypsin found in the feces of rats that have been given a similar diet containing unheated soybeans, either with or without penicillin, from another study which shows a threefold increase with penicillin (8). This is the same ratio found for protein-bound S^{35} in the large intestine of rats either with or without penicillin.

The general conclusions drawn from this study are that the ingestion of unheated soybeans or, more specifically, the oral administration of crystalline soybean trypsin inhibitor, causes an increase in the conversion of methionine-S^{35} to cystine-S^{35} in the pancreas. This increase may be observed in other tissues of the body but appears to be most marked in the pancreas. The newly formed cystine-S^{35} in the pancreas is largely in the form of protein exocrine secretions. This process of protein biosynthesis or conversion of methionine to cystine in the pancreas does not appear to be affected by the presence of penicillin in the diet. The protein secretions of the pancreas enter the small intestine where bacterial proteases cause extensive hydrolysis. This hydrolysis of proteins derived from the pancreas can be inhibited to a major extent by the inclusion of the antibiotic, penicillin, in the diet. The intact protein derived from the pancreas passes down the small intestine and enters the large intestine. It is believed that digestion of this protein in the large intestine is minimal and that most of the enzyme protein from the pancreas that enters the large intestine can be excreted in the feces in a biologically active form. Carroll et al. (9) reported that the cystine content of the small intestine increased when an antibiotic was fed with raw soybeans. They interpreted the antibiotic effect as protecting the cystine itself from bacterial degradation. This may be true to some extent but in view of the results presented here, together with earlier evidence that antibiotics increased protein-bound cystine excretion in the feces, the amino acid protection effect must be small.

Combining this information with that in 2 earlier publications in which it was shown, (a) that penicillin increased the amount of tryptic activity in the feces, and (b) that the growth-stimulating effect of penicillin was abolished if coprophagy was completely prevented (8, 10), leads to the following hypothesis. Active proteins from the pancreas can be excreted in the feces, particularly if there is a protection of these proteins in the gastrointestinal tract by the administration of an antibiotic. Some component of these active proteins in the feces has growth-promoting effects if ingested by a rat receiving unheated soybeans in the diet. One possible agent that might have such properties is trypsin. In these laboratories a number of studies have been conducted in which trypsin, both as commercial crystalline trypsin and as commercial pancreatin, have been fed to rats. Many conditions such as the inclusion of an antibiotic, an antacid, or the concomitant feeding of feces, have been employed. There have been some minimal increases in growth rate resulting from certain of the experimental conditions in which trypsin has been fed, but in no case has a growth stimulation equivalent to that obtainable by the administration of penicillin to a conventional rat receiving unheated soybeans in the diet been obtained. This does not disprove the hypothesis that has been presented but it does provide evidence that the complete explanation of the beneficial effects of antibiotics added to the diet of rats receiving unheated soybeans has not as yet been obtained.

These conclusions are not in accord with the recent report of Goldberg and Guggenheim (11) who observed that pancreatic enzymes are diminished by raw soybeans and that antibiotics reduce this lowering effect. They have also reported an increase in tryptic activity in the small intestine when raw soybeans are fed and that this effect is abolished by penicillin. Their conclusion that antibiotics counteract the effect of raw soybeans by some direct action on pancreatic secretions is contrary to the interpretations given in the present study. Furthermore, they propose that antibiotics increase the nutritive value of raw soybean protein by preventing endogenous losses of nitrogen originating in the pancreas. In an earlier publication from the authors' laboratory (12) it was reported that antibiotics may not affect the

total nitrogen excretion in the feces, but penicillin actually increases the amount of pancreatic protein (trypsin) that reaches the feces. It is unfortunate that these major differences in interpretation which arise from marked differences in experimental approaches cannot be resolved.

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Rate of Respiratory Carbon-14 Dioxide Excretion after Injection of C^{14}-Amino Acids in Rats Fed Raw Soybean Meal [1]

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ABSTRACT Weanling rats were fed heated or raw soybean meal rations, and C^{14}-amino acids were administered by intraperitoneal injection. The rate of respiratory C^{14} dioxide excretion was followed. The rates noted were typical asymptotic plots except following the injection of threonine or valine in animals fed raw soybean. In the latter, the plots showed 2 distinctly different characteristics: 1) the initial rate of C^{14} dioxide excretion was lower and, 2) the rate increased markedly at 4 to 5 hours after injection. The hypothesis was presented that the raw soybean growth inhibitory factor(s) was acting as a block to threonine and valine catabolism and that its effect was dissipated some 4 to 5 hours or more after feeding as the animals did not have access to food during the collection period.

Animals fed raw soybean grow at a slower rate than controls fed autoclaved soybean. Numerous explanations have been proposed for the cause of this decreased growth rate. Reports from our laboratory and others (1–4) indicate that supplementing raw and heated soybean rations with methionine, threonine, and valine results in more comparable growth rates. The question then follows: is the amino acid supplement effective because of increased requirements for these amino acids because of 1) increased loss of these amino acids via digestive enzymes in the fecal excreta as suggested by Haines and Lyman (5), a suggestion based on the observed increased secretion of pancreatic enzymes following raw soybean feeding; or 2) increased requirements because of metabolic derangements following raw soybean ingestion.

The following experiments were designed to investigate this question. Amino acids labeled with C^{14} were injected into rats fed heated or raw soybean rations. The rate of respiratory excretion of C^{14} dioxide was then followed. If threonine and valine were lost via fecal excretion in animals fed the raw soybean rations, the rate of respiratory excretion of labeled carbon dioxide should be less in the raw soybean ration than with the heated soybean ration. However, if threonine and valine were required in larger amounts because of metabolic derangements, respiratory excretion of labeled carbon dioxide might be expected to be increased in animals fed the raw soybean.

PROCEDURE AND RESULTS

Weanling rats of the Holtzman strain were fed rations containing 25% of heated or raw soybean meal prepared as described in an earlier publication (6). The housing area was maintained at 25 to 30° (4). The rations included 0.6% of DL-methionine except when labeled methionine or cystine were the subject of study. A 0.5-ml injection of 9.2 μc of labeled amino acid, 11 to 630 μg depending on the specific activity as listed in table 1, was administered intraperitoneally at approximately 8 AM. The animals were placed in a glass chamber and carbon dioxide was collected in hourly aliquots by bubbling through sodium hydroxide. Food was available to the animal prior to the injection but not during the collection period. Barium carbonate plates were prepared from aliquots

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TABLE 1

Ratio of respiratory C^{14} dioxide excretion after injection of C^{14}-amino acid by rats fed raw versus heated soybean rations

Amino acid		No. of rats	Ratio of counts, raw/heated	
Injected	Specific activity		Hours 1–4	Hours 5–8
	mc/mmole			
L-Arginine-U-C^{14}	89	4/4	1.16(0.87–1.33) [1]	1.25(0.89–1.49)
DL-Cystine-1-C^{14}	7.5	4/4	0.92(0.78–1.12)	1.01(0.87–1.25)
L-Leucine-U-C^{14}	110	4/4	0.98(0.82–1.23)	1.16(0.92–1.23)
L-Methionine-U-C^{14}	74	4/4	1.09(0.98–1.21)	0.94(0.85–1.12)
L-Phenylalanine-U-C^{14}	140	2/2	0.87(0.82–0.91)	1.02(0.91–1.13)
DL-Phenylalanine-3-C^{14}	2.4	2/2	0.93(0.85–1.01)	1.03(0.89–1.19)
L-Threonine-U-C^{14}	100	4/4	0.55(0.45–0.63)	1.79(1.29–2.42)
L-Valine-U-C^{14}	105	2/2	0.74(0.68–0.82)	2.26(2.23–2.29)
L-Valine-1-C^{14}	7.9	4/4	0.73(0.67–0.83)	2.24(1.96–2.61)

[1] Numbers in parentheses indicate range.

of the alkaline carbonate solutions and counted at infinite thickness with an end-window Geiger-Miller tube.

The amino acids used for injection are listed in table 1 with their specific activities according to the distributor's statement. The rate of radioactive carbon dioxide excretion following injection of labeled amino acid traced a typical asymptotic curve for all animals fed the heated soybean rations and likewise with raw soybean feedings except following threonine or valine injection. Such typical plots are shown in figure 1 following L-phenylalanine-U-C^{14} injection in rats fed either heated or raw soybean rations and following L-valine-U-C^{14} with heated soybean feeding. Atypical plots were noted following injection of valine or threonine in those rats fed the raw soybean ration as illustrated for L-valine-U-C^{14} in figure 1. These latter plots showed an initially lower rate of radioactive excretion for the first 3 to 4 hours. Beginning at the fourth or fifth hour, the rate increased for several hours followed by a later decrease.

Comparisons of the rates of excretion of radioactive carbon dioxide by animals fed the raw versus the heated soybean rations are shown in table 1. These comparisons are presented as the ratio of sum of counts from the collections of the first 4 hours and the second 4 hours. These values approximate a ratio of one in all cases except for threonine and valine. Furthermore, the ratios during the second 4-hour period are similar though slightly higher than the

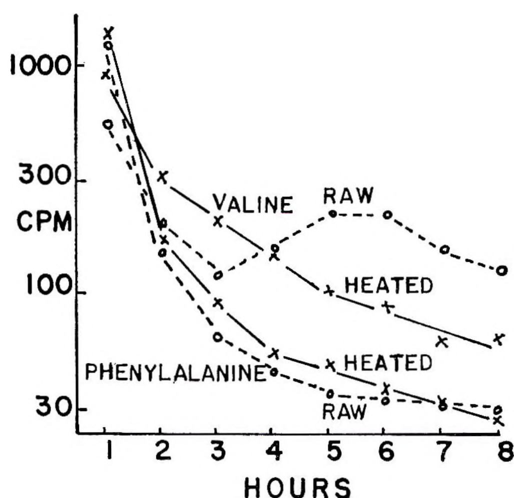

Fig. 1 Radioactive counts of respiratory carbon dioxide (barium carbonate plates) from rats fed raw or heated soybean rations after injection of L-valine-U-C^{14} or L-phenylalanine-U-C^{14}. Each curve traces the counts from one animal.

ratios for the first 4-hour period. However, following threonine or valine injection, the rate of radioactive carbon dioxide excretion by fats fed the raw soybean rations is distinctly less during the first 4 hours than from those fed heated soybeans, ratios of 0.55 to 0.74. But most strikingly, the comparative rate during the second 4 hours is much greater, ratios of 1.79 to 2.26, that is, a threefold increase in the ratio of excretion of label by those fed the raw over the heated soybean during the second 4-hour period following injection of threonine or valine.

DISCUSSION

An increase in the rate of respiratory C^{14} dioxide excretion some 4 to 5 hours after administration of radioactive threonine or valine to animals fed raw soybean meal is a distinctly different observation than has been noted with radioactive amino acids in several recent studies (7, 8). For one thing, this observation strongly suggests the specific involvement of these 2 amino acids in the raw soybean problem. The possibility exists that other amino acids not tested in these experiments might give similar observations.

Among the hypothetical explanations for this later increase, several suggestions occur in a somewhat sequential order. The absorption of threonine and valine from the peritoneal cavity might be delayed; 2) the catabolism of these 2 amino acids might be partially blocked or delayed by a metabolic inhibitor which could be the so-called soybean growth inhibitor; and 3) the threonine and valine or their metabolic products could be released from some bound or stored form (protein, digestive enzymes, glycogen, fat, etc.) at this later time and catabolized. This last suggestion appears most unlikely since similar observations should have been noted with each of the amino acids studied, not with only threonine and valine.

The second of the possibilities, namely, a metabolic block, is presently more acceptable and appears quite logical. Since the animals did not have access to food during the carbon dioxide collection period, it could be postulated that the effectiveness of the metabolic blocking agent arising from the raw soybean meal had dissipated some 4 to 5 hours or more after the last intake of raw soybean meal. Prior to this time, the metabolism of threonine and valine would be reduced both via catabolism as well as in protein synthesis for growth. The favorable effect of supplementary threonine and valine on the growth of rats fed raw soybean diets would constitute a mass action effect of overcoming this block and thereby improving the growth rate.

If the deleterious agent of raw soybeans, the so-called soybean growth inhibitor, is acting as a specific metabolic block towards threonine and valine, certain consequences should follow. Several of these are presently the subject of investigation, namely an increase in plasma threonine and valine, a decrease in activity of threonine catabolizing enzymes, and a decrease in activity of the threonine-activating enzyme.

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Effect of Protein and of Free L-Methionine Intake on Amino Acid Excretion by Human Subjects [1]

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ABSTRACT The ability of the adult human being to cope with a broad range of amino acid intake and even an excessive intake of one of the essential amino acids, methionine, without undue metabolic stress as judged by N balance, urinary S partition studies, and urinary amino acid excretion is illustrated in the study. Human subjects were fed at 2 levels of the same protein; in addition each level of protein intake was supplemented with 9 g of free L-methionine daily for 4 days. Between 78 and 93% of the methionine-S was excreted as inorganic sulfate S; the subjects were always in N equilibrium or positive balance. Methionine, cystathionine, and histidine content of the urine increased when the methionine supplement was fed. Glycine content decreased. Little effect on the excretion of 3-methylhistidine, 1-methylhistidine, and anserine was noted. Doubling the intake of protein caused a twofold increase in excretion of α-aminoadipic acid and cystathionine; no such relationship was noted in the other 25 ninhydrin-reacting substances determined in the urine by ion-exchange chromatography. The fasting plasma aminogram was altered when the protein intake was increased.

The amounts of the various amino acids in the urine of man, in general, tend to be relatively independent of level of protein intake (1). A possible exception is 1-methylhistidine (1), although recent work by Block and co-workers (2) indicates that the amount of this amino acid present in urine is influenced by source of protein ingested rather than by amount.

The amino acid content of urine from human adults has been determined by a number of investigators using chemical, microbiological, and chromatographic methods. In some studies the diets of the subjects were controlled, but the methods of analysis were of a biological nature (3–5); in other studies, ion-exchange chromatographic techniques were used, but the diets of the subjects were not controlled (6, 7). Stein et al. (1), using ion-exchange chromatography to determine amino acid content, have published values on one subject maintained with a controlled dietary regimen, but no details of the diet are given.

The influence of oral ingestion of free amino acids on the excretion of amino acids has been studied in various metabolic disorders such as phenylketonuria, hyperhistidinemia, cystinuria, and others (8), but data from normal human adults are lacking.

In the following investigation, ion-exchange chromatography was used to study the influence of intake of 2 levels of the same protein, without and with the addition of an essential amino acid (free L-methionine), on amino acid excretion by adult human subjects. Plasma amino acid content, nitrogen balance, and urinary sulfur partition studies were made.

EXPERIMENTAL

Subjects. Two healthy adults, K (female, age, 24; weight, 58 kg) and L (male, age, 21; weight, 78 kg) with normal liver and kidney function served as subjects. Throughout the study, they lived in a metabolic unit of the medical center.

Diets. The diets fed contained 54 and 104 g of protein daily (table 1). Milk accounted for 42 and 84 g of protein, respectively, in the 2 diets; bread for 8 and 16 g, respectively. The remaining 4 g of protein in each diet came from a variety of foods added for their caloric content. Free L-methionine, 9 g daily given in 3 equally divided doses, was dissolved in the orange juice during certain periods.

Plan of study. The subjects were fed the basic diet of 54 g of protein for a mini-

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TABLE 1
Composition of diets

	Protein level [1]	
	54	104
	g	*g*
Constants		
Milk	1200	2400
Bread	95	190
Orange juice	300	300
Canned pear	100	100
Canned peach	100	100
Coffee (instant)	2	2
Multivitamin supplement [2]	one daily	
Variables [3]		
Lettuce		
Salad oil		
Butter		
Jelly		
Cornstarch		
Sugar		
Soft drinks		
Plain hard candy		

[1] Calculated: Watt, B. K., and A. L. Merrill 1950 Composition of Foods. U.S.D.A. Handbook no. 8, U.S. Department of Agriculture, Washington, D. C.
[2] Atlavite, Brewer and Company, Inc., Worcester, Massachusetts.
[3] Amounts fed depended on the caloric needs of the subject.

mum of 7 days, followed by a 4-day period in which 9 g of methionine daily were added to the basic diet; the 104-g protein regimen was then instituted for a minimum of 7 days, after which 9 g of methionine daily were added to the basic 104-g protein diet for 4 days.

Collection and analysis of urine. Twenty-four hour urine collections were made throughout the study. Phenol and HCl were used as preservatives, and the samples kept frozen at −20° until analysis was performed. Samples from the last 3 days, each, of the 54- and 104-g protein diet periods and the 4 days for both periods of methionine feeding were prepared for amino acid analysis by the method of Stein (6), and analyzed for amino acid content by ion-exchange chromatography (9). Urinary sulfur partitions were determined by the technique of Folin (10); total sulfur was determined by the method of Denis-Benedict (11, 12). Nitrogen was determined by a modification of the Kjeldahl method (13).

Collection and analysis of feces. Fecal collections were pooled in periods of 4 days, using carmine markers, and frozen until they were analyzed for nitrogen content (13).

Collection and analysis of plasma. Blood was withdrawn from the cubital vein while the subject was in a fasting state on the morning after the end of the 54-g protein diet period, and again after the end of the 104-g protein diet period. Plasma was separated by centrifugation and prepared for analysis by the method of Stein and Moore (14). The content of the "acidic and neutral" amino acids was determined by ion-exchange chromatography (9).

Preparation and analysis of food. Milk was defatted by centrifugation and lyophilized. A weighed portion of the powder was placed in 6 N HCl in a Pyrex tube, the tube sealed, and heated for 24 hours at 120°. The HCl was removed under vacuum, the residue dissolved in a small amount of water, and transferred quantitatively to a volumetric flask with buffer (pH 2.2) (9). Bread was dried in an oven at 75° for 24 hours and ground to a fine powder. The powder was prepared for analysis in the same manner as was the milk powder. The amino acid content of the above preparations was determined by ion-exchange chromatography (9).

Nitrogen determinations were made on a measured portion of fluid milk, and a weighed portion of dried bread powder (13).

RESULTS

Protein level and amino acid excretion. Table 2 shows the daily intake of 20 of the amino acids and other ninhydrin-reacting substances by the subjects eating the 54- and 104-g protein diets; the excretion of 27 amino acids is expressed as milligrams per 24 hours, and where the intake is known, as percentage of intake.

α-Aminoadipic acid and cystathionine excretion by both subjects paralleled the level of protein intake. Subject K excreted 3.7 ± 0.1 and $8.4 \pm < 0.1$ mg, respectively, of the former amino acid when protein intake was increased from 54 to 104 g; subject L excreted 4.0 ± 0.5 and 8.6 ± 0.5 mg. Cystathionine excretion increased from 2.6 ± 0.3 to 5.7 ± 0.4 mg for subject K, and 1.9 ± 0.2 to 3.9 ± 1.6 mg for subject L, when the protein intake was doubled.

Excretion of the other amino acids by both subjects did not increase in proportion

TABLE 2

Dietary intake and urinary excretion of amino acids by human subjects ingesting the same protein at 2 levels

Amino acid	54 g Protein					104 g Protein				
	Intake	Subject K		Subject L		Intake	Subject K		Subject L	
		Excretion		Excretion			Excretion		Excretion	
	g	*mg*	%	*mg*	%	*g*	*mg*	%	*mg*	%
Ethanolamine	0.01	18.8 ± 0.7 [1]	188.0	23.0 ± 1.1	230.0	0.02	15.4 ± 2.3	77.0	26.3 ± 1.3	131.5
Lysine	3.13	31.8 ± 0.9	1.0	10.2 ± 3.0	0.3	6.26	57.0 ± 8.1	0.9	13.6 ± 2.5	0.2
1-Methylhistidine	0.00	3.2 ± 0.8		4.2 ± 1.4		0.00	3.4 ± 0.3		4.7 ± 1.2	
Histidine	1.04	101.8 ± 6.3	9.8	70.2 ± 3.4	6.8	2.08	138.4 ± 24.0	6.6	87.3 ± 11.2	4.2
3-Methylhistidine	0.00	25.9 ± 1.1		40.6 ± 2.3		0.00	23.9 ± 2.4		38.6 ± 3.2	
Anserine	0.00	5.8 ± 2.3		12.4 ± 3.3		0.00	11.8 ± 1.5		15.7 ± 8.0	
Carnosine	0.00	11.6 ± 2.3		13.1 ± 3.0		0.00	14.6 ± 0.9		14.4 ± 2.3	
Taurine		30.9 ± 17.8		94.6 ± 25.0			80.7 ± 8.4		99.8 ± 10.0	
Threonine	1.34	32.7 ± 5.5	2.4	19.8 ± 2.9	1.5	2.68	42.6 ± 2.7	1.6	22.7 ± 0.4	0.8
Serine	1.00	59.2 ± 7.1	5.9	42.1 ± 1.3	4.2	2.00	69.6 ± 3.1	3.5	36.7 ± 4.2	1.8
Asparagine + glutamine	3.25 [2]	142.3 ± 32.0		108.4 ± 4.7		6.50	180.8 ± 20.9		120.3 ± 14.1	
Glutamic acid	11.41	3.1 ± 0.4	1.0 [3]	4.2 ± 0.1	0.8	22.82	3.4 ± 0.4	0.6	4.1 ± 0.2	0.4
Glycine	1.04	315.6 ± 17.6	30.3	116.7 ± 8.5	11.2	2.08	278.0 ± 8.5	13.4	114.7 ± 7.4	5.5
Alanine	1.49	26.1 ± 5.4	1.8	28.9 ± 0.7	1.9	2.98	31.8 ± 0.2	1.1	28.9 ± 1.0	1.0
α-Aminoadipic acid		3.7 ± 0.1		4.0 ± 0.5			8.4 ± < 0.1		8.6 ± 0.5	
α-Amino-*n*-butyric acid		1.7 ± 0.1		2.1 ± 0.2			2.6 ± 0.1		3.0 ± 0.7	
Valine	2.82	9.4 ± 0.4	0.3	10.8 ± 1.0	0.4	5.64	12.5 ± 0.3	0.2	13.1 ± 0.1	0.2
Cystine	0.56 [4]	2.9 ± 0.6	0.5	3.0 ± 0.4	0.5	1.12	3.8 ± 0.3	0.3	3.0 ± 0.6	0.3
Cystathionine		2.6 ± 0.3		1.9 ± 0.2			5.7 ± 0.4		3.9 ± 1.6	
Methionine	1.13	2.6 ± 0.2	0.2	2.3 ± 0.1	0.2	2.26	4.1 ± < 0.1	0.2	3.5 ± 1.5	0.2
Isoleucine	2.44	2.4 ± < 0.1	0.1	2.4 ± 0.2	0.1	4.88	3.2 ± 0.4	0.1	5.5 ± 1.6	0.1
Leucine	4.88	4.4 ± 0.5	0.1	4.1 ± 0.1	0.1	9.76	6.7 ± 0.2	0.2	7.5 ± 1.6	0.1
Tyrosine	1.35	11.1 ± 0.7	0.8	24.8 ± 2.0	1.8	2.70	20.5 ± 1.3	0.8	32.0 ± 1.4	1.2
Phenylalanine	2.27	6.6 ± 0.1	0.3	14.3 ± 1.0	0.6	4.54	10.1 ± 0.4	0.2	12.1 ± 1.2	0.3
β-Alanine		2.0 ± 0.8		1.4 ± 0.4			3.1 ± 0.6		< 0.2 [5]	
β-Aminoisobutyric acid		4.1 ± 0.7		1.1 ± 0.3			7.2 ± 0.5		3.0 ± 1.3	
3,4 DOPA		0.8 ± 0.7		1.3 ± 1.0			1.5 ± 0.9		2.3 ± 0.3	

[1] Mean ± SD for three, 24-hour collections.

[2] Aspartic acid intake.

[3] Percentage excretion calculated as follows: $\frac{\text{mg asparagine + glutamine + glutamic acid excreted}}{\text{mg aspartic acid + glutamic acid ingested}} \times 100$.

[4] Intake calculated: Orr, M. L., and B. K. Watt 1957 Amino Acid content of foods, Home Economic Research Report no. 4, U.S. Department of Agriculture, Washington, D. C.

[5] Value for one 24-hour collection.

to protein intake. In general, however, doubling the protein intake resulted in increased excretion, by at least one of the subjects, of the following 17 amino acids: lysine, histidine, anserine, taurine, threonine, serine, asparagine + glutamine, α-aminobutyric acid, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, β-alanine, β-aminoisobutyric acid, and 3,4 DOPA. The other 8 amino acids tended to be excreted in amounts constant for each subject regardless of level of protein intake (table 2).

Effect of free L-methionine on amino acid excretion. The excretion of 27 amino acids and other ninhydrin-reacting substances by the subjects after the addition of 9 g of free L-methionine daily for 4 days to both the 54- and 104-g protein diets is expressed as milligrams per 24 hours and, where possible, as percentage of intake in table 3. Absolute amounts excreted, rather than changes in amounts excreted, are shown so that comparisons can be made directly with the values in table 2.

Of all the amino acids, methionine showed the greatest increase in excretion when free methionine was fed. Excretion by subjects K and L was increased 98.5 and 42.7 mg, respectively, when 9 g of the amino acid were added to the 54-g protein diet; and 91.1 and 44.5 mg, respectively, when it was added to the 104-g protein diet. Cystathionine excretion also increased, the amount of increase was approximately the same regardless of protein intake. The excretion of cystine was decreased when free L-methionine was added to the basic diets except for subject L who showed an average increase in excretion of 0.1 mg when given the 104-g protein diet plus methionine. Taurine, another sulfur-containing compound, increased in excretion when methionine was added to the 54-g protein diet, but decreased when methionine was added to the basic 104-g protein diet.

Histidine content of the urine from both subjects was increased when free L-methionine was added to the diets; the amount increased from 101.8 ± 6.3 to 118.6 ± 31.1, and 70.2 ± 3.4 to 79.2 ± 10.2 mg for subjects K and L, respectively, while they were ingesting the 54-g protein diet plus methionine. When methionine was added to the 104-g protein diet, histidine excretion increased from 138.4 ± 24.0 to 193.0 ± 22.9 mg for subject K, and from 87.3 ± 11.2 to 129.7 ± 26.3 mg for L (tables 2, 3).

Increases of less magnitude were shown by ethanolamine, 3-methylhistidine, α-aminobutyric acid, and β-alanine.

Glycine content of the urine was decreased from 315.6 ± 17.6 to 185.9 ± 18.5 mg for subject K, and from 116.7 ± 8.5 to 87.6 ± 12.4 mg for subject L when methionine was added to the 54-g protein diet; adding free methionine to the 104-g protein diet resulted in a decrease in glycine content of the urine from 278.0 ± 8.5 to 268.2 ± 17.2 mg for subject K, and from 114.7 ± 7.4 to 92.6 ± 33.8 mg for subject L (tables 2, 3).

The excretion of the other amino acids was either not affected or any change was equivocal (the 2 subjects did not respond alike) when free L-methionine was added to the basic diets.

Urinary sulfur. The increase in urinary content of total sulfur, total sulfate S, and inorganic sulfate S after the addition of 9 g of free L-methionine to the basic 54- and 104-g protein diets is shown in table 4. The values are expressed as milligrams per 24 hours, percentage of total S, and percentage of methionine-S excreted.

The methionine given supplied 1935 mg of S daily. Subject K excreted 1822 ± 182 (94%) and 1582 ± 144 mg (80%), respectively, of this supplementary S when the 54- and 104-g diets were fed; subject L excreted 1540 ± 103 (82%) and 1578 ± 98 mg (82%).

Total sulfate S accounted for 97 to 101% of the total S excreted, and 77 to 93% of the ingested methionine-S appearing in the urine.

Between 98 and 99% of the total S was in the oxidized form or inorganic sulfate S fraction of the urine. This fraction also accounted for between 78 and 93% of the administered methionine-S.

Subject K appeared to metabolize the methionine-S more efficiently when she was ingesting the 54-g protein diet plus methionine than when she received the 104-g protein diet plus methionine. No such difference was shown by subject L.

TABLE 3

Urinary excretion of amino acids by human subjects ingesting the same protein at 2 levels supplemented with free L-methionine [1]

Amino acid	54 g Protein + free L-methionine				104 g Protein + free L-methionine			
	Subject K		Subject L		Subject K		Subject L	
	Excretion		Excretion		Excretion		Excretion	
	mg	%	*mg*	%	*mg*	%	*mg*	%
Ethanolamine	19.8 ± 0.5 [2]	198.0	23.3 ± 3.8	233.0	21.6 ± 4.6	108.0	29.3 ± 1.6	146.5
Lysine	26.7 ± 7.3	0.8	14.2 ± 5.3	0.4	50.4 ± 4.2	0.8	14.2 ± 6.6	0.2
1-Methylhistidine	3.5 ± 1.1	> 35.0	3.9 ± 0.2	> 39.0	3.4 ± 0.5	34.0	4.0 ± 0.5	40.0
Histidine	118.6 ± 31.1	11.4	79.2 ± 10.2	7.6	193.0 ± 22.9	9.3	129.7 ± 26.3	6.2
3-Methylhistidine	28.2 ± 1.1		46.0 ± 2.4		25.6 ± 2.1		50.2 ± 7.9	
Anserine	7.5 ± 1.5		8.0 ± 3.3		10.7 ± 2.4		24.0 ± 4.5	
Carnosine	12.4 ± 2.3		10.2 ± 4.0		12.2 ± 3.0		14.1 ± 4.9	
Taurine	34.8 ± 14.0		101.8 ± 28.2		67.8 ± 1.4		91.2 ± 5.2	
Threonine	19.2 ± 2.4	1.4	16.1 ± 2.0	1.2	41.8 ± 5.0	1.6	24.4 ± 1.4	0.9
Serine	53.5 ± 4.1	5.4	31.1 ± 0.6	3.1	68.8 ± 6.8	3.4	43.8 ± 6.3	2.2
Asparagine + glutamine	136.5 ± 14.9		103.3 ± 9.5		167.4 ± 14.8		125.9 ± 9.9	
Glutamic acid	2.2 ± 0.6	0.9 [3]	3.5 ± 0.2	0.7	2.6 ± 0.3	0.6	3.6 ± 0.9	0.4
Glycine	185.9 ± 18.5	17.9	87.6 ± 12.4	8.4	268.2 ± 17.2	12.9	92.6 ± 33.8	4.4
Alanine	17.2 ± 2.9	1.2	21.8 ± 1.9	1.5	28.7 ± 2.2	1.0	30.1 ± 2.5	1.0
α-Aminoadipic acid	3.4 ± 0.3		3.4 ± 0.3		8.3 ± 0.4		9.5 ± 0.6	
α-Amino-*n*-butyric acid	4.3 ± 0.9		4.3 ± 0.8		4.4 ± 1.0		8.2 ± 1.4	
Valine	10.0 ± 0.4	0.4	3.9 ± 0.2	0.1	7.6 ± 3.9	0.1	7.0 ± 0.6	0.1
Cystine	2.3 ± 0.5	0.4	1.7 ± 0.3	0.3	3.4 ± 0.5	0.3	3.1 ± 0.5	0.3
Cystathionine	22.5 ± 4.5		6.7 ± 0.9		23.6 ± 2.8		8.3 ± 3.1	
Methionine	101.1 ± 28.0	1.0	45.0 ± 15.5	0.4	95.2 ± 21.2	0.8	48.0 ± 16.0	0.4
Isoleucine	1.8 ± 0.3	0.1	3.7 ± 0.2	0.2	2.9 ± 0.8	< 0.1	5.2 ± 0.5	0.1
Leucine	4.1 ± 1.0	0.1	4.0 ± 0.7	0.1	5.7 ± 1.0	< 0.1	5.9 ± 3.2	0.1
Tyrosine	9.3 ± < 0.1	0.7	17.4 ± 1.2	1.3	17.2 ± 1.9	0.6	29.5 ± 1.2	1.1
Phenylalanine	5.9 ± 0.3	0.2	10.2 ± 0.8	0.4	8.3 ± 0.5	0.2	13.7 ± 0.4	0.3
β-Alanine	3.2 ± 0.6		4.4 ± 2.0		4.4 ± 1.2		4.2 ± 0.4	
β-Aminoisobutyric acid	2.7 ± 0.7		0.6 ± 0.3		5.8 ± 0.5		1.5 ± 0.3	
3,4 DOPA	< 0.3 [4]		0.5 ± 0.1		1.0 ± 0.6		0.9 ± 0.1	

[1] Amino acid intake was the same as shown in table 1 for the 54-g and 104-g protein diets except that 9 g of free L-methionine (three 3-g doses) were fed daily for the 4-day period.

[2] Mean ± SD for four 24-hour collections.

[3] See footnote 3, table 2.

[4] See footnote 5, table 2.

Nitrogen balance. Nitrogen balance for the subjects when they were ingesting the 54- and 104-g protein diets without and with methionine supplementation is shown in table 5. Both subjects were in positive balance or in equilibrium when they received the basic diets. The methionine supplement had no consistent effect on nitrogen balance regardless of level of protein intake.

Plasma amino acids. In table 6, the amounts of 19 "acidic and neutral" amino acids in the plasma of the fasting subjects at the end of the 54- and 104-g protein diet periods are shown.

The amounts of threonine, proline, valine, cystine, isoleucine, leucine, and tyrosine were increased in the plasma of both subjects when the higher protein diet was fed. Proline content increased 63 and 35%

TABLE 4

Partition of urinary sulfur after oral administration of free L-methionine to subjects eating the same protein at 2 levels [1]

Urinary S partition	54 g Protein + methionine		104 g Protein + methionine	
	Subject K	Subject L	Subject K	Subject L
Total S				
mg/24 hours	1822 ± 182 [2]	1540 ± 103	1582 ± 144	1578 ± 98
% methionine-S	94	82	80	82
Total sulfate S				
mg/24 hours	1789 ± 164	1496 ± 113	1570 ± 412	1592 ± 70
% total S	98	97	99	101
% methionine-S	93	77	81	82
Inorganic sulfate S				
mg/24 hours	1786 ± 167	1503 ± 118	1555 ± 148	1564 ± 66
% total S	98	98	98	99
% methionine-S	93	78	80	81

[1] Free L-methionine fed in 3-g doses, 3 times daily for 4 days (1935 mg methionine-S daily).
[2] The mean value for each S partition excreted during the feeding of the basic diet was subtracted from the daily values determined during the 4 day period of methionine supplementation; the mean ± SD of the resultant values is shown.

TABLE 5

Nitrogen excretion by human subjects fed 2 levels of the same protein and free L-methionine

Diet	Intake	Excretion	Subjects K	Subjects L
	g/24 hours		g/24 hours	g/24 hours
54-g Protein	8.55	urine	7.00 ± 0.5 [1]	7.90 ± 0.9
		fecal	0.99	0.56
		total	7.99	8.46
		balance	0.56	0.09
104-g Protein	17.10	urine	14.37 ± 0.7	12.58 ± 0.7
		fecal	1.70	1.25
		total	16.07	13.83
		balance	1.03	3.27
54-g Protein + methionine [2]	9.39	urine	8.67 ± 0.5	7.71 ± 0.5
		fecal	0.95	1.17
		total	9.62	8.88
		balance	−0.23	0.51
104-g Protein + methionine	17.94	urine	14.50 ± 0.4	15.63 ± 1.5
		fecal	1.70	1.25
		total	16.20	16.88
		balance	1.74	1.06

[1] Mean ± SD for minimum of 4 days.
[2] Free L-methionine fed in 3-g doses, 3 times daily for 4 days

TABLE 6

Amino acids in plasma from subjects eating the same protein at 2 levels [1]

Amino acid	54 g Protein		104 g Protein	
	Subject K	Subject L	Subject K	Subject L
	mg/100 ml	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
Taurine	1.31	1.35	1.09	1.43
Aspartic acid	0.12	0.24	0.14	0.14
Threonine	1.53	1.55	1.89	1.61
Serine	1.27	1.59	1.37	1.34
Asparagine + glutamine	7.50	11.13	5.43	9.70
Proline	1.80	2.25	2.93	3.04
Glutamic acid	0.63	2.14	4.87	0.85
Citrulline	0.50	0.45	0.78	0.45
Glycine	1.55	1.91	1.45	1.66
Alanine	3.24	3.68	3.34	3.03
Valine	2.34	2.62	3.02	2.71
Cystine	0.33	0.37	0.46	0.55
Cystathionine	< 0.01	< 0.01	< 0.01	< 0.01
Methionine	0.32	0.36	0.25	0.34
Isoleucine	0.62	0.79	0.73	0.94
Leucine	1.20	1.49	1.56	1.77
Tyrosine	1.11	0.98	1.60	1.16
Phenylalanine	0.83	0.97	0.97	0.92
β-Alanine	0.09	0.14	0.09	0.12

[1] Plasma from blood withdrawn after subject fasted overnight.

for subjects K and L, respectively; cystine content increased 39 and 48%. The other increases were of less magnitude. On the other hand, the amounts of asparagine plus glutamine, glycine, and methionine were decreased in the plasma of both subjects when the protein intake was increased. The amounts of the other 9 amino acids either remained relatively constant or the response of the 2 subjects was not alike (table 6).

DISCUSSION

The ability of the adult human being to cope with a broad range of amino acid intake and even an excessive intake of one of the essential amino acids without undue metabolic stress as judged by nitrogen balance, urinary sulfur partition studies, and urinary amino acid excretion is illustrated in the present investigation.

Addition of 9 g of free L-methionine daily for 4 days to diets containing 54 and 104 g of protein increased the methionine intake of the subjects approximately nine- and fivefold, respectively, without altering the intake of the other amino acids. The ability of the subjects to metabolize the added methionine normally is indicated by the fact that between 78 and 93% of the additional methionine-S was oxidized and appeared in the urine as inorganic sulfate S. The subjects also remained in positive nitrogen balance or in equilibrium (as defined by Leverton et al. (15)) when the methionine supplement was added to both levels of protein intake.

Methionine content of the urine was increased during the period of free L-methionine ingestion; however, the excretion of free urinary methionine did not exceed 1% of the ingested methionine. Excretion of cystathionine, one of the products of methionine metabolism, increased when free methionine was fed. Analysis of the fasting plasma of the subjects for free cystathionine indicated less than 0.1 mg/100 ml present, and it appears that this substance is cleared rapidly from the blood.

Cystine content of urine either remained approximately the same or decreased during the period of free methionine ingestion. Cystine occurs after cystathionine in the cycle of methionine metabolism, and if the excess cystathionine formed is excreted, increased cystine formation and subsequent increased excretion because of methionine feeding would not be expected.

The amount of free L-methionine fed caused only a slight increase in excretion of 3-methylhistidine and no increase in

1-methylhistidine and anserine (β-alanyl-1-methylhistidine). Earlier, Block et al. (16) showed that oral L-histidine, free base, given to human adults had no consistent effect on the urinary content of 1-methylhistidine and 3-methylhistidine, whereas anserine excretion was decreased. These 2 studies indicate that the methyl derivatives of histidine, and the compound anserine do not arise directly in the human adult from the feeding of methionine or histidine, per se.

Histidine excretion by both subjects was consistently increased when free methionine was added to the diet. The significance of this is not known.

Glycine excretion by the subjects was decreased when free L-methionine was fed, and it is possible that glycine may have acted as a methyl group acceptor, and hence the excretion was decreased. No sarcosine (methylglycine) was observed in the urines, but possibly the methylglycine was further metabolized to choline, which was not determined in this study.

Although 27 amino acids and other ninhydrin-reacting substances were determined in the urine, glycine, asparagine plus glutamine, histidine, and methionine or taurine accounted for from 53 to 65% of the total content of amino acids in the urine when either the basic diets or the basic diets plus methionine were fed.

Only α-aminoadipic acid and cystathionine excretion paralleled protein intake in the present study, in which the sources of protein were milk and bread. Boulanger and Biserte (17) first detected α-aminoadipic acid in human urine using paper chromatography, and Stein (6) confirmed its presence using ion-exchange chromatography. No indications as to its source and importance are given, although it is an intermediate in the metabolic pathway of lysine (18). Cystathionine is an intermediate in the metabolism of methionine to cystine.

Stein and co-workers (1), using ion-exchange chromatography, determined the urinary excretion of amino acids by an individual fed, successively, diets containing 15, 70, and 200 g of protein, and reported that 1-methylhistidine was the only amino acid whose excretion reflected in a roughly linear fashion the level of protein in the diet. That this was probably due to the source of protein fed was shown by Block et al. (2) who gave human subjects diets containing beef or chicken, or milk and bread as the principal sources of protein. Only the subjects receiving beef and chicken excreted 1-methylhistidine roughly parallel to its intake; the subjects receiving the milk and bread diets did not respond to increased intakes of the proteins with increased excretions of 1-methylhistidine.

Holt and co-workers (19) reported that in kwashiorkor, the plasma aminogram is altered in even the milder stages of the disease; most of the essential amino acids, and in particular the content of valine, leucine, and isoleucine, are decreased as is tyrosine; whereas glycine, aspartic acid, histidine, and many of the other nonessential amino acids are increased.

In the present study when the protein intake of the subjects was increased from 54 to 104 g, the plasma concentrations of valine, leucine, isoleucine, and tyrosine increased, and those of asparagine plus glutamine, and glycine decreased. This is a reverse of the pattern noted in kwashiorkor, because the present subjects went from a lower to a higher protein intake. The amino acid content of the plasma from the kwashiorkor patients who were eating inadequate diets was compared with plasma values for subjects eating adequate diets.

The 54-g protein diet contains all of the 8 amino acids essential for nitrogen equilibrium in man at a level equal to or greater than the "minimum" proposed by Rose (20), and was adequate to maintain positive nitrogen balance or equilibrium in the subjects.

The effect of level of protein intake on the concentration of amino acids in plasma from adult human subjects would seem to warrant further investigation.

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Effect of Intermittent Food Restriction on Growth, Food Utilization and Body Composition of the Rat[1]

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ABSTRACT Weanling rats were fed a high carbohydrate diet ad libitum for 3 days alternating with 3 days during which food was restricted to that required for weight maintenance. Growth rates, food consumption and efficiency of food utilization were compared with that of controls fed the same diet ad libitum. Rates of weight gain were the same for both groups. Experimental animals' total food intake was significantly less than that of control animals at all ages studied. Experimental rats contained significantly less protein and more fat than controls after 60 and 120 days. The differences in body composition were no longer present after 180 days of feeding. During initial periods of rapid weight gain (25 to 85 days) a significantly greater fraction of ingested calories was retained. In animals started on the intermittent feeding program at 55 rather than 25 days, experimental animals reduced food intake but no changes in body composition developed. When a diet high in fat was substituted for high carbohydrate diet, experimental animals at 60 days showed reduced protein without change in body fat content. Food consumption of intermittently fed animals fed the high fat diet was also significantly reduced.

The purposes of this investigation were to determine the effect of one pattern of intermittent food intake on growth, food utilization, and body composition of rapidly growing young rats. Many studies (1–3) have shown that the pattern of food ingestion profoundly influences the whole body economy. Older, slowly gaining rats fed by stomach tube or allowed access to food only 1 or 2 hours per day develop, in contrast with animals with food constantly available, gastric hypertrophy (4, 5), increased intestinal absorptive capacity (4), increased lipogenesis (4–6), decreased thyroid activity (7), and increased accumulation of body fat (2, 3, 8).

The present studies are an extension of earlier ones from this laboratory (9, 10). It was observed that infant rats weaned to an adequate diet high in glucose developed a transient 20% elevation in energy metabolism. By contrast, animals fed the same diet in insufficient amounts to promote growth for 3 to 6 days before food was allowed ad libitum, did not develop an increase in oxygen consumption. During the first several days that full feed was available, the latter group also retained a greater fraction of the ingested calories as fat than animals weaned directly to ad libitum feeding. This alteration in metabolism and body fat accumulation following a 3-day period of caloric restriction interposed between weaning and full feeding was transient, and differences in oxygen consumption and body composition could not be detected 14 days later.

The similarities in these transient body composition changes to those observed in force-fed animals led to the studies reported here. Weanling rats were maintained with this type of intermittent intake of a high carbohydrate diet for prolonged periods of time and body composition was determined at 60-day intervals. The feeding pattern used in this investigation differs from others, in that the food availability was cycled over a 6-day rather than a 1-day period and was started at weaning rather than later when the animals were older and growing less rapidly. The persistence of the changes observed was assessed by maintaining some animals as long as 180 days. To determine the effects of the non-protein composition

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of the diet on changes observed with intermittent feeding, groups of weanling animals were fed a diet high in fat intermittently or continuously for 60 days before killing. The effect of age on body composition and food utilization changes observed in animals started on intermittent feeding at weaning was compared with that observed in animals fed the high carbohydrate diet starting at 55 rather than 25 days of age.

METHODS

Weanling, Holtzman-strain rats were caged individually in a room maintained at 25 to 27°. Water was continuously available to both control and experimental animals. Two diets were used, one high in carbohydrate, and the second, high in fat. The proportions of protein and salt mixture in the high fat diet were increased to maintain their ratio to the caloric content approximately equivalent to that of the diet high in carbohydrate. The high carbohydrate diet contained the following: dextrose, 60% ; fat,[3] 15% ; casein, 21% ; Wesson salt mixture (11), 4% ; plus a complete vitamin supplement.[4] The diet high in fat contained: dextrose, 15% ; fat,[5] 52% ; casein 27% ; salts (11), 6% ; and the vitamin supplement.[6]

Control animals were fed one of the diets ad libitum; experimental rats were alternated between 3 days with only sufficient food to maintain body weight but not to permit growth, and 3 days of ad libitum intake. This feeding pattern was maintained until the animals were killed. Food intakes and body weights were measured at 3-day intervals. All experimental animals readily adapted to the cyclic availability of food. The occasional deaths that occurred were due to infection, usually pulmonic. The amount of food required for weight maintenance was determined empirically and, for the high carbohydrate diet, increased from 4 g/day at weaning to 10 g/day after one month; proportionately smaller amounts of high fat diet were required.

At indicated times animals were killed by overdosage with ether, the gastrointestinal contents were removed and the carcass dried by lyophilization. The total body water was calculated as the difference between body weight following removal of gastrointestinal contents and the dry carcass weight. The entire dry carcass was pulverized in a Waring Blendor with acetone. Acetone was not discarded but removed by evaporation. Mineral content was determined as the residual weight after ashing aliquots of dry powder at 500° for 96 hours; carcass carbohydrate was not measured. Protein was computed as 6.25 times nitrogen determined by micro-Kjehldal analysis. The use of this conversion factor (6.25) is not universally applicable but differences in final results would not be significantly modified if precise values for individual proteins were used. Fat was extracted from aliquots of dry carcass in a Soxhlet apparatus with methanol-chloroform (1:1) for 4 hours. Solvent was removed by evaporation and the petroleum ether-chloroform (6:1) soluble fraction was weighed. In one portion of the study, body fat was estimated from body weight, total body water and the known uniformity of the water content of the fat-free tissue (12, 13). The fraction of water in the fat-free carcass used for this computation was obtained from direct analysis of Holtzman-strain rats in this and other studies in this laboratory (table 1).

The efficiency of food utilization was calculated as the fraction of ingested calories retained. Values of 4, 4 and 9 kcal/g for carbohydrate, protein and fat, respectively, were used and values computed

TABLE 1

Percentage of water in fat-free body of Holtzman-strain albino rats

Age [1]	No. animals	Body wt	Fat-free body water
days		*g*	%
21	13	50 ± 1 [2]	76.0 ± 0.4 [2]
45	14	175 ± 4	73.9 ± 0.4
85	20	310 ± 7	71.8 ± 0.3
145	20	380 ± 8	70.3 ± 0.3
205	14	445 ± 10	68.1 ± 0.8

[1] Age from birth.
[2] Value ± SE.

[3] Crisco, Procter and Gamble, Cincinnati, Ohio.
[4] Each kilogram of the high carbohydrate diet contained: vitamin A, 25,000 units; vitamin D, 3,000 units; α-tocopherol, 150 mg; inositol, 150 mg; choline chloride, 2.25 g; riboflavin, 30 mg; menadione, 68 mg; p-aminobenzoic acid, 150 mg; niacin, 130 mg; pyridoxine·HCl, 30 mg; thiamine·HCl, 30 mg; Ca pantothenate, 90 mg; biotin, 0.60 mg; folic acid, 2.50 mg; vitamin B_{12}, 0.40 mg; amounts in diet high in fat were 150% of these values.
[5] See footnote 3.
[6] See footnote 4.

from food composition and intake and body composition. Modification of these approximations of caloric content for specific nutrients would not significantly modify results.

Protein utilization was expressed as the percentage of ingested protein that was retained and as milligrams of nitrogen retained per kilocalorie consumed.

RESULTS

1. *Responses in 25-day-old animals fed high carbohydrate diets.* Experimental and control animals fed the 60% carbohydrate diets gained body weight at equal rates (table 2). During periods of unrestricted intake, experimental rats increased the amount of food ingested approximately threefold over that allowed during weight maintenance periods but the total intake was insufficient to equal that of control group (table 3). Total food ingested was distributed relatively uniformly throughout the 3-day period of ad libitum feeding. The total wet weight of stomach and intestinal tract was not increased by this pattern of intermittent feeding. Carcass gains of experimental animals were thereby achieved with food intakes significantly less than those of the control animals.

During the first 60 days of feeding, when rate of growth was rapid, the efficiency of food utilization was significantly greater in intermittently fed rats (table 3). After 60 days the fraction of ingested calories retained was less than 10% of that ingested and was not significantly different in the 2 groups (table 3).

Body composition of the 2 groups of animals was significantly different after 60 and 120 days of feeding (table 2). Experimental animals accumulated more fat and less protein than the controls. These differences were no longer apparent after 180 days (table 2). No differences in mineral content developed at any age studied (table 2).

Efficiencies of protein utilization were the same for both control and experimental animals, measured either as percentage of ingested protein retained or as milligrams of nitrogen retained per kilocalorie ingested (table 3).

2. *Responses of 25-day-old animals fed high fat diet.* Animals fed the diet high in fat developed some of the changes, but

TABLE 2

Body composition of control and experimental animals

Duration of feeding	Body wt	No. animals	Protein	Fat	Ash
	g		*g*	*g*	*g*
	Animals fed high carbohydrate diet from weaning				
60 days					
Control	305 ± 5 [1]	10	61.2 ± 1.2 [1,2]	43.6 ± 2.3 [3]	10.9 ± 0.3
Experimental	320 ± 8	10	55.8 ± 1.0	56.3 ± 5.0	9.9 ± 0.3
120 days					
Control	383 ± 8	10	80.0 ± 1.9 [2]	70.6 ± 4.0 [2]	13.8 ± 0.3
Experimental	376 ± 9	10	72.2 ± 0.8	90.0 ± 5.2	12.6 ± 0.4
180 days					
Control	459 ± 12	7	88.9 ± 3.8	92.6 ± 4.0	16.3 ± 0.9
Experimental	430 ± 7	7	81.8 ± 2.4	97.0 ± 6.6	16.0 ± 0.6
	Animals fed high CHO diet from 55 days of age				
60 days					
Control	411 ± 10	9	80.8 ± 3.4	45.5 ± 4.5 [4]	—
Experimental	384 ± 4	9	82.1 ± 3.0	52.6 ± 2.5	—
	Animals fed high fat diet from weaning				
60 days					
Control	340 ± 9	8	69.3 ± 3.2 [2]	63.2 ± 4.0	9.3 ± 0.4
Experimental	313 ± 8	8	55.4 ± 3.2	52.2 ± 5.2	10.4 ± 0.3

[1] Value ± SE.
[2] Control and experimental value significantly different ($P < 0.02$).
[3] Control and experimental value different ($P < 0.05$).
[4] Fat content computed from total body water and dry solids.

TABLE 3

Efficiency of food and protein utilization

Duration of feeding	No. animals	Food intake	Food efficiency [1]	Protein retained [2]	Nitrogen retained/ kcal ingested
		g		%	*mg*
		Animals fed high CHO diet from weaning			
25 to 85 days					
Control	10	946 ± 40 [3,4]	14.8 ± 0.9 [4]	27.4 ± 0.8	2.01 ± 0.08
Experimental	10	788 ± 10	18.9 ± 1.0	29.6 ± 2.2	2.13 ± 0.06
85 to 145 days					
Control	10	982 ± 20 [4]	7.0 ± 0.6	7.14 ± 1.0	0.60 ± 0.06
Experimental	10	856 ± 16	9.2 ± 1.0	9.30 ± 0.5	0.69 ± 0.04
		Animals fed high CHO diet from age 55 days			
55 to 115 days					
Control	9	912 ± 9 [4]	11.3 ± 0.8	23.8 ± 0.9	1.72 ± 0.11
Experimental	9	716 ± 12	14.3 ± 0.7	25.5 ± 1.5	1.80 ± 0.12
		Animals fed high fat diet from weaning			
25 to 85 days					
Control	8	606 ± 22 [4]	21.7 ± 0.9 [5]	39.0 ± 2.0 [6]	2.61 ± 0.13 [6]
Experimental	8	532 ± 10	20.7 ± 0.7	35.4 ± 1.0	2.37 ± 0.06

[1] Fraction of ingested calories retained × 100. Computed from food intake and composition and carcass analysis.
[2] Grams protein retained/grams ingested × 100.
[3] Value ± SE.
[4] Control and experimental value significantly different ($P < 0.02$).
[5] Values significantly greater than corresponding value for control weanling animals fed high carbohydrate diet for comparable interval ($P < 0.02$).
[6] Values greater than value for animals fed high carbohydrate diet for comparable interval ($P < 0.05$).

not all of those observed in animals fed the high carbohydrate diet. Although fat content of control and intermittently fed experimental animals was not different after 60 days, animals fed continuously contained significantly greater amounts of protein than experimental animals (table 2).

Intermittently fed animals ingested significantly less food than control rats but, unlike the results observed in rats fed a diet high in carbohydrate, feed efficiencies of control and experimental animals were the same (table 3). Food efficiency of animals of both groups fed the diet high in fat was greater than that of control but not experimental animals fed the high carbohydrate diet (table 3).

As observed with the high carbohydrate diet, the feeding of the high fat diet produced no differences between control and experimental animals in the efficiency of protein utilization; but the values were significantly greater than those of animals fed the high carbohydrate diet (table 3).

3. *Responses of 55-day-old animals to intermittent feeding of high carbohydrate diet.* After 60 days of feeding, there were no differences in body composition between control and experimental animals started on intermittent feeding after the initial phase of rapid growth. The older animals also reduced intake significantly below that of control animals (table 3); differences were significant from the start of the feeding program.

As had been previously observed in weanling animals, the fraction of ingested nitrogen retained and the milligrams of N retained per kilocalorie were the same for control and experimental animals (table 3).

DISCUSSION

The changes of composition produced by modifying feeding pattern in the manner described in these studies bears a resemblance to those described for force-fed animals. Our results suggest, however, that the conditions are somewhat different in the 2 circumstances. The feeding pattern used in these studies probably produced less dietary stress, particularly in older slowly growing rats, than that of force

feeding. Even on the first day of the periods of ad libitum feeding, intake of experimental rats was not excessive, and animals did not develop the gastrointestinal hypertrophy reported to develop in animals fed only 1 to 2 hours per day (4, 5).

Nitrogen retention of animals used in these studies, whether computed as milligrams of N stored per kilocalorie ingested or as the percentage of the ingested protein that was retained, was the same in control and experimental groups. The proportion of protein retained was modified by fat and carbohydrate content of the diet and age of animals, but not by the feeding pattern. The observation that protein content is reduced in intermittently fed animals may reflect the lesser dietary intake of experimental animals and the known relationship between protein storage and energy intake (14, 15).

The mechanism whereby protein accumulation is reduced in intermittent feeding over a one-day cycle appears mediated in a different fashion than that observed in this study. When force-fed animals were pair-fed with animals having food continuously available, protein retention was significantly reduced by intermittent feeding (8). The control animals on the same food intake must have retained a greater fraction of ingested protein and the ratio of nitrogen retained to calories ingested must have been larger than that of experimental animals.

Control and experimental animals fed the 52% fat diet retained significantly more nitrogen per kilocalorie than animals fed the high carbohydrate diet. The reasons for the lower retention by animals fed the high glucose as compared with the high fat diet are not clear. Water intakes of these animals were not measured and the effect may be related to the differences in the osmotic effects of the 2 diets (16, 17).

Fat storage was increased by intermittent feeding only in weanling animals fed the diet high in carbohydrate. That larger animals in this study did not exhibit this change that is always observed in older force-fed animals may reflect the possibility that the stress of a 6-day rather than a one-day cycle are different. That protein but not fat accumulation developed in weanling animals fed the diet high in fat suggests that these 2 processes, decreased protein and increased fat accumulation, proceeded independently of each other.

The 6-day cycle of intermittent feeding permits normal weight gains with a reduced caloric intake. In most studies in which food intakes of force-fed and ad libitum-fed older animals are reported, rats trained to eat their total daily caloric intake in a relatively short interval of time generally reduced their caloric intake and showed a concomitant reduction in growth rate. Okey et al. (18) reported that rats permitted access to food for 2 hours per day ate less than control animals with food available ad libitum and weight gains were approximately proportional to intake. Hollifield and Parson (6) observed that rats fed only 2 hours per day gained significantly more weight and consumed 5 to 15% more feed than controls. The 5 animals fed ad libitum in that study weighed less than 300 g at approximately 15 weeks of age.

In most studies of modifications of feeding pattern, intermittent intakes have been maintained for only approximately 10 weeks. At this time, animals fed intermittently contain significantly more fat and less protein than control animals. In the present studies when diets high in carbohydrate were fed from weaning, similar differences were present at 17, but not at 23 weeks. It is possible that adaptation to intermittent feeding might also develop in animals fed over a 24-hour cycle if animals were maintained for a prolonged period of time.

No estimates of resting metabolism or total activity were carried out. To account for equal rates of weight gain of control and experimental animals, one or both pathways of energy expenditure must have been reduced in the animals subjected to the cyclic feeding pattern.

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Lipid Metabolism of Puppies as Affected by Kind and Amount of Fat and of Dietary Carbohydrate [1]

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ABSTRACT Feeding 37 young growing puppies diets similar in composition to those fed infants demonstrated that sucrose, Dextri-Maltose, and corn syrup are equally suitable as types of dietary carbohydrate. No significant differences were observed in rate of growth or response of blood sugar levels or serum levels of protein and total fatty acids between groups of animals fed these sugars as the major source of carbohydrate. Cholesterol levels were lowest when sucrose was fed in diets containing corn or hydrogenated coconut oil. Lactose was not acceptable as the sole source of carbohydrate for the young puppy. This may be attributed to a deficiency of the enzyme lactase in the intestinal mucosa of the puppy. Rate of growth and serum levels of unsaturated fatty acids in these animals differed from those fed sucrose, Dextri-Maltose, or corn syrup when the diet was low in linoleic acid. Differences in serum unsaturated fatty acids between puppies fed lactose and those fed sucrose, Dextri-Maltose, or corn syrup were very slight when corn oil diets were fed. It is concluded that during the period of rapid growth dietary fat affects the level and composition of serum lipids to a greater extent than does dietary carbohydrate.

Intense interest in the possible role of diet in development of aortic atheromatous lesions in young subjects has prompted considerable study of effects of various dietary components on the lipid fractions in blood and tissues. Studies with experimental animals (1–5) and with adult human subjects [2] have suggested the possibility of a relationship between type of dietary carbohydrate and lipid metabolism. Carbohydrate studies with young human subjects have been concerned primarily with the absorption of different types of carbohydrate in relation to blood sugar levels and urinary excretion after the administration of test doses of carbohydrate (6, 7). Scant attention has been given to the effect of type of carbohydrate on the metabolism of other nutrients in the infant's diet. Since linoleic acid has been demonstrated to be an essential component of fat for young growing infants (8, 9) as well as for experimental animals, the present study with puppies was undertaken to determine the physiologic response of 4 types of carbohydrate on fat metabolism during growth (a) when the diet is deficient in fat, (b) when the dietary fat is deficient in linoleic acid, and (c) when the dietary fat is high in linoleic acid content.

The young puppy was chosen as the experimental animal because it has been found to react to diets deficient in linoleic acid in a manner similar to young infants fed diets deficient in this nutrient. Changes in composition of blood serum lipids as well as gross and histologic alterations in skin occur in babies and puppies under these dietary conditions (9–12).

MATERIALS AND METHODS

Experimental diets. Twelve experimental diets were prepared to simulate infant formulas. There were 4 low fat basal diets which were considered fully adequate in calories, protein, vitamins, and minerals. Each of these 4 diets contained a different type of carbohydrate commonly used in infant feeding, i.e., β-lactose,[3] sucrose,

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[2] Wells, W. W., and S. C. Andersen 1962 The effect of dietary lactose on the serum cholesterol level of human subjects. Federation Proc., *21:* 100 (abstract).

[3] Beta-Lactose, The Borden Company, New York.

Dextri-Maltose[4] and corn syrup.[5] Four diets similar to those low in fat were prepared by isocaloric substitution of 30% of the carbohydrate calories by a hydrogenated coconut oil product[6] which was low in linoleic acid. Four additional diets, similar to those low in fat, were prepared by isocaloric substitutions of 30% of the carbohydrate calories with corn oil.[7] The caloric distribution of the diets low in fat and those moderate in fat content are shown in table 1.

All diets were fed at normal caloric levels, 150 kcal/kg/day from weaning to 4 months of age; 125 kcal/kg/day from 4 to 6 months of age and 90 to 100 kcal/kg/day thereafter.

The fat was weighed or measured daily and mixed with a weighed amount of dry basal diet and the vitamin mixture just before the addition of sufficient water to make a palatable mixture. The animals were fed once daily and notations made when all the food was not consumed.

Experimental animals. To eliminate possible variations in response to diet due to differences in breed, only Beagles were used in the study. All animals were started on the study at the time of weaning (6 to 9 weeks of age) and were housed in separate metal cages in an air conditioned room maintained at 23.5°. Care was taken to see that they were free from intestinal parasites and immunized for distemper and canine hepatitis. The animals were weighed weekly before feeding.

Each of the 12 experimental diets was fed to 5 or more puppies. Most of the animals were maintained with more than one diet. The length of period that each diet was fed varied depending on the acceptability of the diet and the physical condition of the animal. The shortest period was one week for each of 2 dogs that were fed the low fat-lactose diet. Each animal was observed with respect to acceptance of the diet, rate of growth, gross and histologic condition of the skin. Metabolic response to the diets was determined by analysis of whole blood for reducing sugars and true glucose, blood serum for total protein and lipid components and urine for reducing sugars.

Chemical analyses. At the end of each dietary regimen, fasted blood specimens, 24-hour urine specimens and skin biopsies were obtained from each animal. Heparinized arterial blood was used for determination of total reducing sugars by the Somogyi method (13) and true glucose with glucose-oxidase (14). The total protein content of blood serum was determined with the Biuret reagent according to

[4] Mead Johnson and Company, Evansville, Indiana.
[5] Karo (light), Corn Products Company, New York.
[6] Cobee 92, The Drew Company, Bolton, New Jersey.
[7] Mazola, Corn Products Company, Argo, Illinois.

TABLE 1

Caloric distribution for experimental diets

Diets low in fat	% calories	Diets with fat	% calories
Protein		Protein	
Casein	10	Casein	10
Skim milk powder	10	Skim milk powder	10
Carbohydrate		Carbohydrate	
Skim milk powder	14	Skim milk powder	14
Experimental	65	Experimental	36
Fat		Fat	
Skim milk powder	1	Skim milk powder	1
		Experimental	29
Vitamins [1]		Vitamins [1]	
Salt-bone ash		Salt-bone-ash	
Cellulose [2]		Cellulose [2]	

[1] Vi-Penta no. 3 (Roche Laboratories, Nutley, New Jersey)/supplied daily: vitamin A, 2,000 IU; vitamin D, 160 IU; 0.4 mg each of thiamine, riboflavin, pyridoxine, niacin and Ca pantothenate; vitamin C, 20 mg; biotin, 12 μg. Vitamin E, 25 mg weekly as *d*-α-tocopheryl acetate.
[2] Cellu Flour, Chicago Dietetic Supply House, Chicago.

a modification of the Kingsley method (15). Blood serum lipid analyses included total cholesterol (16), total fatty acids (17) and silicic acid separation of the glyceride, cholesterol ester and phospholipid fractions (18). Fatty acids from each of the above fractions were methylated at 70° with 2% sulfuric acid in methanol. The methyl esters were chromatographed in a Beckman GC-2A instrument using 183-cm diethylene glycol succinate columns at 220° and helium as the carrier gas. Peaks were identified by comparison with retention times of known methyl esters.[8] The 5, 8, 11-eicosatrienoic acid was identified by its conformity to the theoretical retention time of an eicosatrienoic acid which appeared in serum from linoleic acid deficient animals and subsequently disappeared from serum of the animals after feeding linoleic acid. This retention time also conformed to that of 5, 8, 11-eicosatrienoic acid reported by Ackman (19). Peak areas were quantitated by measurement of peak heights and relative retention times. Correction factors were applied to compensate for differences in detector response to individual fatty acid components. Detector response was linear within a given instrument sensitivity setting and injected sample sizes were chosen to allow use of a single sensitivity setting. Urine specimens were examined for total reducing sugars by the Somogyi method. Determination of true glucose in urine was not possible because toluol or thymol were used as preservatives during collection of the urine. Identification of individual sugars in urine was made by an adaptation [9] of the paper chromatographic method of White and Hess (20). Histologic sections of skin biopsies taken from the dorsal surface of the thigh were stained with hematoxylin and eosin.

Statistical methods. Equality of variance for serum cholesterol and fatty acids (mg/100 ml) within each group of animals fed the low fat, hydrogenated coconut oil or corn oil diets was determined by Bartlett's test (21). Equality of means for serum cholesterol and fatty acids (mg/100 ml) within each of the above groups was determined by Student's *t* test (21).

RESULTS

Data are presented for 37 dogs from the time of weaning to approximately 9 months of age.

Acceptability of the diets and rate of growth. It became evident early in the study that the 4 carbohydrates used in the diets were not equally acceptable to weanling puppies. Lactose as the sole source of carbohydrate was not tolerated by the weanling puppy. This may be attributed to a deficiency of the enzyme lactase in the intestinal mucosa. Diarrhea and usually anorexia occurred in all young animals fed the lactose diets. Death occurred during the first 12 weeks of life in 6 of the 9 weanling puppies fed lactose diets deficient in linoleic acid, compared with one death each among those fed the sucrose, Dextri-Maltose, or corn syrup diets deficient in linoleic acid. Growth was poor for young puppies fed lactose diets but for dogs over 8 months of age, weight loss was not a consistent feature. However, diarrhea was a common symptom at all ages when lactose was the dietary carbohydrate irrespective of the amount or type of fat in the diet.

Comparison of groups of 5 or more animals fed the same diet demonstrated that sucrose, Dextri-Maltose and corn syrup were equally acceptable as a source of carbohydrate in the diet of young growing puppies. With diets having the same amount and kind of fat, there were no consistent trends in rate of growth which could be attributed to sucrose, Dextri-Maltose or corn syrup as the source of carbohydrate.

Under our experimental conditions, improvement in growth rate was noticeable for only 2 dogs when corn oil was substituted for 30% of the carbohydrate calories in the low fat diet or 30% of the calories as hydrogenated coconut oil.

Gross and histologic changes in skin. Gross evidence of linoleic acid deficiency occurred only after prolonged feeding of

[8] Applied Science Laboratories, Inc., State College, Pennsylvania.

[9] Modifications introduced were (a) removal of substances insoluble in 66% methanol; (b) desalting with an electric desalter (Res. Specialty Model 1930, Berkeley, California) before concentration of urine; (c) use of the mixture 2:2:1 butanol-pyridine-water as solvent in a one dimensional descending chromatograph.

either a diet low in fat or one containing hydrogenated coconut oil. An unkempt appearance with desquamation and slight infection of the ears were characteristic of 3 growing puppies fed the low fat diet for 18 weeks. Desquamation was generalized but less marked for 3 dogs fed hydrogenated coconut oil for a similar length of time. There were no signs of involvement of the paws. Effects were similar whether the carbohydrate was sucrose, Dextri-Maltose, or corn syrup. Because of the generally debilitating effects of lactose, it was not possible to feed young puppies the lactose diets deficient in linoleic acid for long enough periods to note development of deficiency signs. Furthermore, as demonstrated by Wiese et al. (22), the poor growth exhibited by these animals would tend to prevent the development of gross and histologic signs of linoleic acid deficiency.

In general, histologic changes paralleled the gross appearance of the skin. The degree of histologic alterations in the epidermis and dermis were related to the linoleic acid intake, the length of time the diet was fed and to the composition of the previous diet when more than one diet was fed to an animal. Increased density of the keratin layer, which is one of the early signs of linoleic acid deficiency, was evident when puppies were fed either a diet low in fat or one containing hydrogenated coconut oil from the time of weaning. Severe histologic changes in the stratum corneum with peg formation and extensive parakeratosis were not observed in skin sections from animals that had been fed diets deficient in linoleic acid. Histologically, skin from animals fed diets containing hydrogenated coconut oil appeared to differ from those fed the diets low in fat in the rate of development of the sebaceous glands. In the former animals these glands often were more numerous and moderately large even when there was no evidence of increased density of the keratin or thickening of the epidermis.

Skin from animals fed diets containing corn oil was essentially normal except in instances of incomplete recovery from a previous linoleic-deficient diet. Definite histologic changes which occur during recovery from the deficient state were evident for 5 animals.

Among the puppies that were growing normally, there were no histologic differences which could be attributed to the type of carbohydrate fed.

Total protein in blood serum. Total protein in blood serum was determined as an indication of normal protein metabolism in the animals. Table 2 shows the mean values for groups of 5 or more animals fed the same diet. The mean protein levels for puppies fed the fat-deficient lactose diet was lower than for any other

TABLE 2

Mean serum protein and reducing sugars in blood and urine in relation to dietary fat and dietary carbohydrate

No. dogs	Dietary CHO	Dietary Fat	Feeding period	Age	Protein	Blood Reducing sugar	Blood Glucose	Urinary reducing sugar
			weeks	*weeks*	*g/100 ml*	*mg/100 ml*	*mg/100 ml*	*g/24 hr*
6	Lactose	no	3.3	25.2	4.49	88	81	2.6
7	Sucrose	no	7.3	30.5	5.43	92	86	0.9
8	Dextri-Maltose [1]	no	8.4	29.7	5.25	90	84	0.4
6	Corn syrup	no	11.2	31.1	5.25	82	70	0.3
6	Lactose	HCO [2]	3.3	29.6	5.28	85	82	7.4
8	Sucrose	HCO	7.3	31.5	5.16	82	82	0.3
6	Dextri-Maltose	HCO	7.7	31.6	5.12	82	74	0.3
5	Corn syrup	HCO	10.4	26.5	4.95	93	91	0.4
6	Lactose	corn oil	6.9	23.0	5.16	96	85	2.6
12	Sucrose	corn oil	17.1	36.0	5.80	91	87	0.4
11	Dextri-Maltose	corn oil	14.5	38.9	5.50	88	83	0.2
7	Corn syrup	corn oil	15.3	31.7	5.51	90	83	0.2

[1] Mead Johnson and Company, Evansville, Indiana.
[2] Hydrogenated coconut oil product, Cobee 92, The Drew Company, Bolton, New Jersey.

group. All other levels for total protein in blood serum can be considered satisfactory. There was an insignificant trend for mean serum protein levels to be higher in animals fed sucrose than for those fed Dextri-Maltose, or corn syrup. Serum protein levels of the animals that received these 3 carbohydrates with corn oil were slightly higher than of the animals fed the low fat or hydrogenated coconut oil diets. Because the dogs fed the lactose diets had diarrhea and some anorexia, they were always maintained with these diets for a shorter time than with the sucrose, Dextri-Maltose, or corn syrup diets.

Reducing sugars in whole blood. Total reducing sugars and true glucose were determined on arterial blood after an overnight fast. Table 2 shows the mean values for groups of 5 or more animals fed the 12 experimental diets. Mean values for blood sugar were considered normal for all animals. Trends with type of carbohydrate or dietary fat were not evident. Differences between total reducing sugars and true glucose often are considered to be due to galactose. However, Haworth and Ford (6) did not identify galactose by paper chromatography in the blood of infants fed lactose diets. They concluded that galactose was not present in amounts greater than 10 to 20 mg/100 ml of blood. With 2 exceptions differences between total reducing sugars and glucose were less than 10 mg/100 ml of blood for all groups of puppies fed the 4 types of carbohydrate. No attempt was made to identify sugars other than glucose in the blood.

Young dogs fed the lactose diets showed normal fasting blood sugar levels even though absorption of lactose appeared to be poor as evidenced by diarrhea. With adult dogs, Bennett and Coon[10] have demonstrated normal fasting blood sugar levels but lower glucose absorption curves following the ingestion of lactose than when sucrose, Dextri-Maltose, or corn syrup was the source of dietary carbohydrate. Similar absorption data have been reported for infants with an intolerance to lactose (23).

Sugars in urine. Table 2 shows a summary of mean values for total reducing sugars in urine as determined by the Somogyi method. Interpretation of the high values observed for animals that received diets containing lactose is not possible because of diarrheal contamination. Mean values for reducing sugars in urine of dogs receiving sucrose, Dextri-Maltose, and corn syrup were low, although sugars were present in all urines.

Individual sugars in urine were identified by means of paper chromatography. Because of persistent diarrhea in all animals receiving a lactose diet, no estimate could be made of the excretion of sugars by these animals. The sensitivity of the chromatographic method used for detection of urinary sugars for the dogs fed diets containing sucrose, Dextri-Maltose, or corn syrup was of the order of 2.5 mg/100 ml original urine for lactose, galactose and glucose and 10 mg/100 ml original urine for maltose, sucrose and fructose. When the major dietary carbohydrate was sucrose, 4 sugars were identified in most of the urine samples, i.e., lactose, sucrose, galactose and glucose. Fructose appeared in approximately 35% of these samples. When Dextri-Maltose was fed, lactose appeared in nearly all urines and galactose in 45% of the specimens. When corn syrup was fed, lactose and sucrose were identified in most of the urine samples, galactose in 50% and fructose in approximately 25% of the samples. The identification of lactose and galactose in most urine specimens regardless of the type of carbohydrate fed, may be attributed to the ingestion of 14% of the calories as lactose derived from the skim milk in all diets.

Maltose was not identified in any urine.

The chromatographic identification of lactose, sucrose and glucose in urine of dogs fed our experimental diets is in agreement with the observations of Haworth (7) with infants and children who were fed test doses of lactose, sucrose and maltose. The latter disaccharide was not noted in blood or urine from children fed this sugar.

Blood serum lipids. Figure 1 illustrates the mean serum levels for total cholesterol, total fatty acids and fatty acids in the glyceride, cholesteryl ester, and phospholipid fractions for groups of 5 or more dogs fed the same diet.

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[10] Unpublished data.

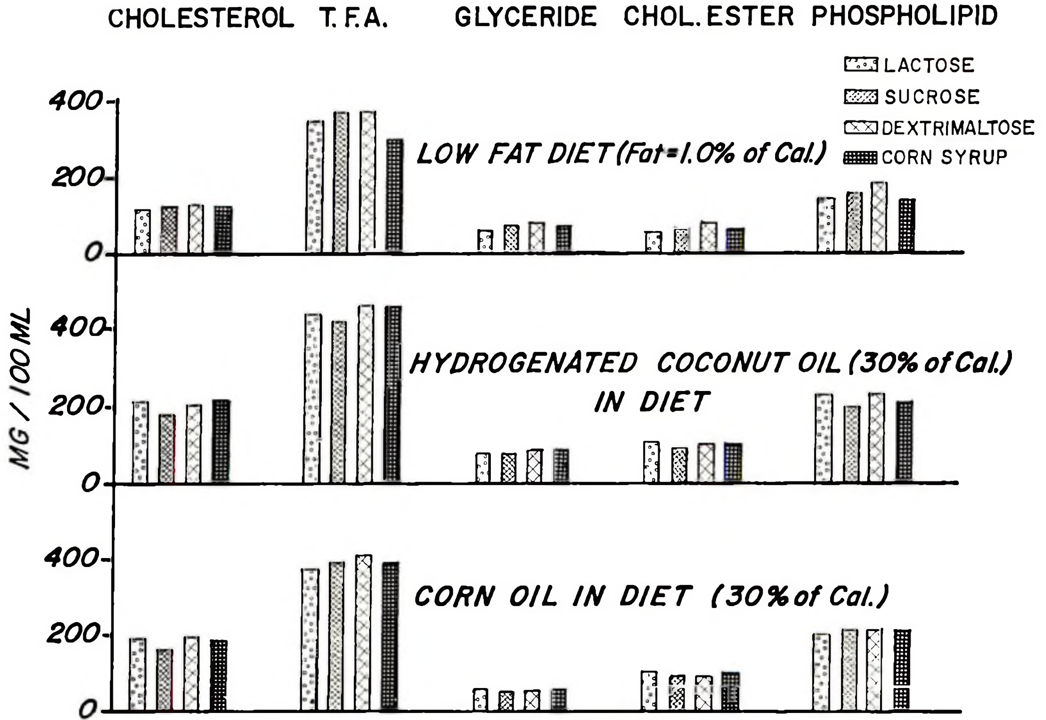

Fig. 1 Total cholesterol, total fatty acids and fatty acids in the glyceride, cholesteryl ester and phospholipid fractions of serum.

Differences in serum levels for these lipid components in relation to dietary fat were as follows:

1. Total cholesterol and total fatty acids were lowest for all dietary sugars when the low fat diets were consumed.
2. Glycerides were lowest when corn oil was fed.
3. Cholesteryl esters were lowest when low fat diets were consumed.
4. Phospholipids constituted the major lipid fraction in dog serum and were lowest when low fat diets were fed.

Except for diets containing sucrose, there were no consistent trends in serum levels for lipid components which were related to the type of dietary carbohydrate. For diets containing corn or hydrogenated coconut oil, serum cholesterol levels were lowest for animals fed the sucrose diets.

Statistical comparisons were made for mean serum levels of cholesterol, total fatty acids and fatty acids from the glyceride, cholesteryl ester and phospholipid fractions among the groups fed diets low in fat, those fed hydrogenated coconut oil and those fed corn oil irrespective of the type of carbohydrate. Using Student's t test as a measure of significance, the following differences between groups were noted:

Total cholesterol:	
low fat < hydrogenated coconut oil	$P < 0.01$
low fat < corn oil	$P < 0.01$
corn oil < hydrogenated coconut oil	$P > 0.05$
Total fatty acids:	
low fat < hydrogenated coconut oil	$P < 0.01$
low fat < corn oil	$P < 0.01$
corn oil < hydrogenated coconut oil	$P > 0.05$
Glyceride fatty acids:	
low fat < hydrogenated coconut oil	$P < 0.01$
corn oil < low fat	$P < 0.01$
corn oil < hydrogenated coconut oil	$P < 0.01$
Cholesteryl ester fatty acids:	
low fat < hydrogenated coconut oil	$P < 0.01$
low fat < corn oil	$P < 0.01$
hydrogenated coconut oil < corn oil	$P > 0.05$
Phospholipid fatty acids:	
low fat < hydrogenated coconut oil	$P < 0.01$
low fat < corn oil	$P < 0.01$
corn oil < hydrogenated coconut oil	$P > 0.05$

Mean levels for saturated and unsaturated fatty acids in serum for each of the

12 experimental diets are illustrated in figures 2, 3 and 4.

When the diet was low in fat (fig. 2) there were essentially no differences in mean serum levels for saturated fatty acids among the 4 types of carbohydrate in the total; glyceride; cholesteryl ester; or phospholipid fractions. However, phospholipids had the highest and cholesteryl esters the lowest percentage of saturated fatty acids. Total fatty acids were made up of approximately equal amounts of palmitic and stearic acids; glycerides and cholesteryl esters had predominantly palmitic acid and phospholipids had stearic acid as the major saturated fatty acid.

In all fractions of serum (total, glycerides, cholesteryl esters and phospholipids) similar trends with the 4 carbohydrates were noted in the unsaturated fatty acid serum levels. Monoene and the trienoic acid characteristic of fat deficiency tended to be lowest in the animals fed lactose and highest in the animals fed Dextri-Maltose and corn syrup. Linoleic and arachidonic acid levels were higher in the puppies fed the lactose diet than those fed the sucrose, Dextri-Maltose or corn syrup diets. In view of the effects of caloric intake on growth and unsaturated fatty acid levels in young dogs (22) it appears reasonable to assume the differences in serum unsaturated fatty acids between the dogs fed lactose and the other 3 carbohydrates were related to differences in degree of absorption and utilization of the carbohydrate calories which in turn were reflected in the growth rate of the young puppies.

Figure 3 illustrates mean serum levels for the saturated and unsaturated fatty acids when the diet contained hydrogenated coconut oil. Trends with type of carbohydrate were similar to those observed for diets low in fat.

Figure 4 illustrates mean levels for saturated and unsaturated fatty acids when the diet contained corn oil. Linoleic and arachidonic acids showed higher serum levels than when the diet was deficient in linoleic acid. No 5, 8, 11-eicosatrienoic

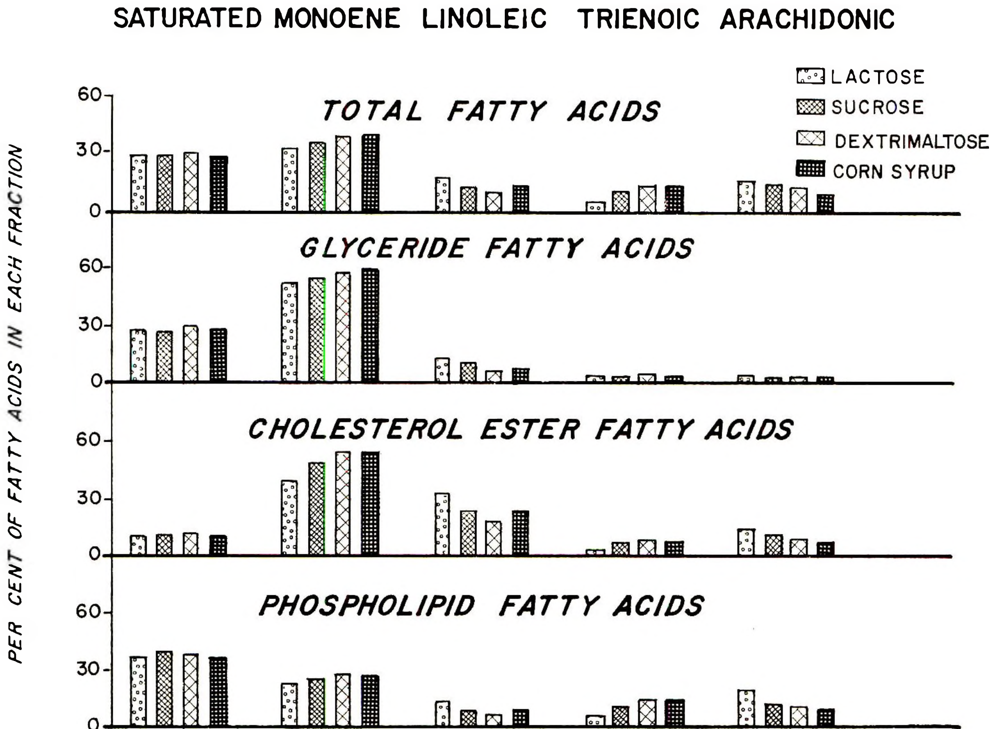

Fig. 2 Low fat diet; saturated and unsaturated fatty acids in the total fatty acids, glyceride, cholesteryl ester and phospholipid fatty acids in serum.

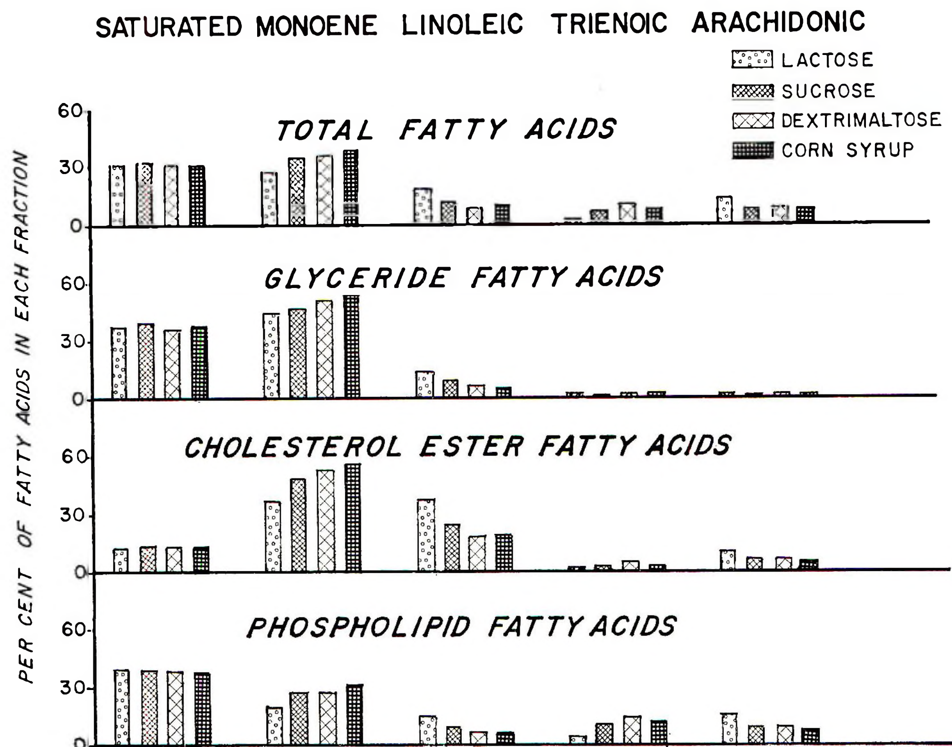

Fig. 3 Hydrogenated coconut oil diet; saturated and unsaturated fatty acids in the total fatty acids glyceride, cholesteryl ester and phospholipid fatty acids in serum.

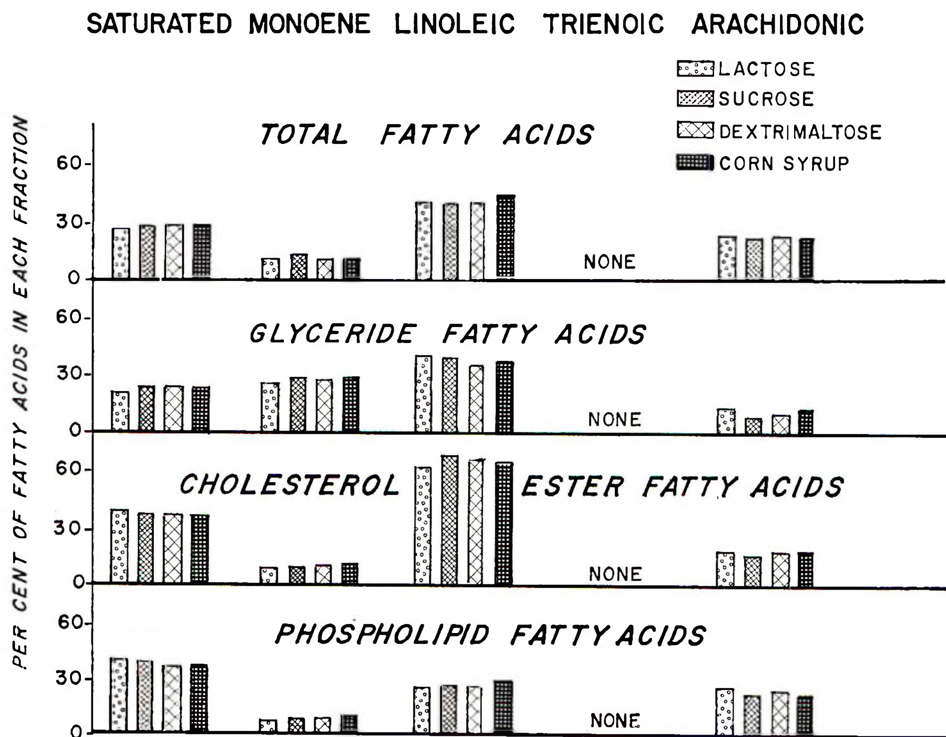

Fig. 4 Corn oil diet; saturated and unsaturated fatty acids in the total fatty acids, glyceride, cholesteryl ester and phospholipid fatty acids in serum.

acid was observed in serum when corn oil was fed. Trends with the type of carbohydrate were much less evident than when the diets were low in linoleic acid content.

DISCUSSION

Effects of dietary carbohydrate on the serum lipids of young puppies during the period of rapid growth were less dramatic than the effects of dietary fat. With the exception of total cholesterol, there were no consistent trends in the level of lipid components of blood serum which were characteristic for the kind of carbohydrate fed. Cholesterol levels were lower when sucrose was fed with corn or hydrogenated coconut oil than when the dietary carbohydrate was lactose, Dextri-Maltose or corn syrup. Similar effects have been reported by Wells and Andersen[11] for young human adults whose serum cholesterol levels were lower after sucrose than after lactose feeding. Macdonald and Braithwaite (24) observed lower serum levels for glycerides for adult subjects after a raw starch diet than after a sucrose diet. However, when receiving the same amount and kind of fat there were no significant differences in serum glycerides among the 4 types of carbohydrate fed to our young dogs.

When the diet was deficient in linoleic acid, retardation in growth and persistent diarrhea were prominent features for young puppies fed the lactose diets. Failure to thrive was reflected in the serum levels for linoleic, arachidonic and trienoic acids. These metabolic effects in young puppies fed lactose as the sole source of carbohydrate are attributed to poor absorption of lactose as a source of calories for growth. It is well known that the human infant is normally provided with the necessary enzyme system for absorption and utilization of the high lactose content of human milk. Hence metabolic effects of dietary lactose observed for the young puppy would not be applicable to the young infant unless he has hereditary lactase deficiency only recently appreciated clinically (23, 25–27).

It has been demonstrated by Carroll (28, 29) that responses to dietary carbohydrate by enzyme systems in livers of rats are modified by the type of dietary fat. Effects of dietary glucose, sucrose and rice starch on the cholesterol and fat content in the livers appeared to be a function of the type of fat fed. In our young puppies metabolic response to sucrose, Dextri-Maltose, or corn syrup did not show consistent trends when the intake of fat was the same and only differed when lactose was fed. However, irrespective of the type of carbohydrate, lipid components of blood serum differed significantly among the groups fed the low fat, the hydrogenated coconut oil and the corn oil diets. It is concluded from these data that in the young growing puppy, dietary fat affects the level and composition of serum lipids to a greater extent than does the dietary carbohydrate.

ACKNOWLEDGMENTS

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[11] See footnote 2.

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Protein Utilization in Ruminants

I. BLOOD UREA NITROGEN AS AFFECTED BY PROTEIN INTAKE [1,2]

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ABSTRACT Three experiments were conducted with growing-finishing lambs to determine whether blood urea nitrogen was associated with protein intake. A close relationship was found ($r = 0.986$) indicating that this blood nitrogen constituent could be quantitated with protein intake. An equation for the determination of digestible protein required by lambs is also presented. Approximately 21 g of digestible protein were required for each 1000 kcal of digestible energy consumed.

Protein adequacy in farm animals is generally assessed using one or more production factors as major criteria (1). This work has permitted protein requirements to be estimated in various animals, but when results from several experiments are reviewed, these estimates are not precise, probably because the measures used respond to many factors and are themselves an integration of a general physiological response. Criteria based on specific nitrogen components in the body may be more accurate measures of adequacy.

In the ruminant, nitrogen is absorbed from 2 major sites, namely the rumen-reticulum and the small intestine. Lewis (2) has shown that the quality of this absorbed nitrogen (including ammonia N) is reflected in the level of circulating blood urea nitrogen (BUN). Quality of protein fed to baby pigs was also shown to be reflected in BUN levels by Puchal et al. (3). The BUN in lambs was altered by feeding various carbohydrate and protein sources [3] and BUN in cattle was affected by dietary levels of protein and energy.[4] In humans, increasing protein intake increased serum urea concentration (4).

Therefore, it seemed desirable to see whether BUN levels could be quantified with dietary protein intake. If this were possible, a new dimension could be added to studies of protein nutrition through an evaluation of the relative amounts of protein being catabolized from changes in BUN.

METHODS

The first experiment indicated that BUN was related to the protein intake of growing wether lambs.[5] A second experiment consisted of group feeding 6 lots of wether lambs a finishing ration with varying levels of protein. These complete-mixed rations were fed ad libitum. The different protein levels (9.2, 11.5, 13.1, 16.5 and 22.0%) were achieved by varying the proportions of soybean meal and corn. One lot of lambs was fed a similar ration containing 14.4% protein in which urea instead of soybean meal was used to supply 47% of the total nitrogen in the ration.

Blood samples were withdrawn (1 PM) for BUN determination on various days during the course of both experiments.

From the preliminary information obtained in these 2 experiments, a third experiment was designed to study the influence of protein intake upon protein utilization and BUN. A extra-period Latin-square change-over design (5) was used

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[1] Contribution from the Missouri Agricultural Experiment Station. Journal Series no. 2816. Approved by the Director.

[2] This study was supported in part by a grant from the National Feed Ingredients Association and is part of Regional Project NC-63.

[3] Preston, R. L., L. H. Breuer and W. H. Pfander 1961 Blood urea and rumen ammonia in sheep as affected by level and source of carbohydrate and protein. J. Animal Sci., 20: 947 (abstract).

[4] Preston, R. L., L. H. Breuer and G. B. Thompson 1961 Blood urea in cattle as affected by energy, protein and stilbestrol. J. Animal Sci., 20: 977 (abstract).

[5] Preston, R. L., and W. H. Pfander 1963 Technique for evaluating protein adequacy in lambs. J. Animal Sci., 22: 844 (abstract).

with 8 lambs. This type of design tests treatment effects and also carry-over treatment effects during the subsequent period. Four levels of protein (6.2, 8.0, 11.7 and 13.5%) were fed ad libitum in complete mixed rations (table 1). The chemical composition of these rations is shown in table 2. The lambs (7 wethers and one ewe) weighed 36.9 ± 0.9 kg at the start of the experiment (December 19, 1963). Each period was 21 days in length at which time the lambs were changed to another treatment according to the balanced single-square sequence for 4 treatments (5). Body weight change and feed consumption were measured during each period. Blood samples were obtained (1 PM) on day 19 and 21 of each period for BUN determination. This portion of the experiment lasted for 105 days. After this time, the lambs continued on the same treatments fed during the last period for a 7-day digestion trial.

TABLE 1

Ingredient composition of the rations

	Dietary treatment			
	1	2	3	4
	kg	*kg*	*kg*	*kg*
Cottonseed hulls	50.0	50.0	50.0	50.0
Cane molasses	8.0	8.0	8.0	8.0
Corn, steamed-cracked	41.1	36.1	26.1	21.1
Soybean meal [1]	—	5.0	15.0	20.0
Dicalcium phosphate [2]	0.4	0.4	0.4	0.4
Limestone	0.1	0.1	0.1	0.1
Salt, iodized	0.4	0.4	0.4	0.4
	mg	*mg*	*mg*	*mg*
$CoCl_2 \cdot 6H_2O$	55	55	55	55
Vitamin A palmitate	30	30	30	30

[1] 44% crude protein guarantee.
[2] Supplied by International Minerals and Chemical Corporation, Skokie, Illinois.

TABLE 2

Chemical composition of the rations [1]

	Dietary treatment			
	1	2	3	4
	%	%	%	%
Moisture	9.3	9.2	9.1	9.1
Crude fat	1.94	1.78	1.47	1.32
Crude fiber	22.2	22.4	22.9	23.2
Crude protein [2]	6.2	8.0	11.7	13.5
Ash	3.07	3.29	3.73	3.95
Calcium	0.26	0.27	0.30	0.32
Phosphorus	0.20	0.21	0.24	0.25

[1] Chemical analyses performed by Missouri Agricultural Experiment Station Chemical Laboratory. Results of 2 independent samplings of each ration.
[2] N × 6.25.

Blood urea nitrogen was determined by hydrolyzing the urea in whole blood with urease [6] followed by aeration of the resulting ammonia (6). Koch urease solution [7] was diluted (1:25) with phosphate buffer [8] (pH 7.0) on the day determinations were made. This buffered urease (0.9 ml) was added to whole blood (0.1 ml) in a 30-ml serum bottle. Blanks (distilled water) and urea standards were prepared similarly. The bottles were stoppered and placed in a water bath at 39° for 15 minutes. Saturated K_2CO_3 (1 ml) was then added to each bottle (using a syringe) and the bottle quickly stoppered with an acid receiving rod coated with 1 N H_2SO_4. The bottle was aerated (15 minutes) on a rotating wheel. The acid receiving rod was then rinsed with Nessler's reagent and the resulting color read at 415 mμ. Triplicate analyses were performed. Duplicate values were used if they agreed within ± 2.5%, or triplicate values if within ± 3%. This procedure includes ammonia N, which was not determined. Previous studies indicate that ammonia N would not account for more than 4% of the BUN under the conditions of these experiments.

Statistical evaluation of the data was by analysis of variance and correlation analysis (7).

RESULTS

Gain and feed consumption were not significantly affected ($P > 0.05$) by the level of protein fed in the first 2 experiments. Average daily gain, feed consumption, and length of experiment were as follows for experiments 1 and 2, respectively: 155 g, 1650 g and 90 days; 260 g, 1810 g and 63 days. In both experiments, the standard error for daily gain was near 17 g.

In the third experiment, daily gain was increased significantly ($P < 0.005$) with increasing protein level (table 3). Maximal daily gain appears to occur first at

[6] Breuer, L. H. 1962 Ruminant nitrogen, sodium and potassium metabolism and alterations caused by diethylstilbestrol. M. S. Thesis, University of Missouri.
[7] Obtained from Fisher Scientific Company, New York, stock no. SoU26.
[8] Colowick, S. P., and N. O. Kaplan 1955 Methods of Enzymology, vol. 1. Academic Press, Inc., New York, p. 143.

9.5% protein (fig. 1). The protein level fed during the previous period did not affect the gain during the subsequent period ($P > 0.25$). Daily feed intake was increased with increasing protein level ($P = 0.05$), and it appeared that lambs previously fed at the 6.2% protein level consumed less feed during the subsequent period ($P = 0.15$). This was no doubt due to their comparatively lighter body weight since when feed consumption was expressed per unit weight$^{0.75}$, there was no carry-over effect ($P > 0.25$). On this basis, feed consumption was increased ($P =$

TABLE 3

Influence of protein level upon gain, feed intake and ration digestibility

	Protein, g/100 g feed				
	6.2	8.0	11.7	13.5	SE
Criterion:					
Daily gain, g	61	168	251	251	18
Daily feed intake, g	1700	1800	1920	1890	45
Daily feed intake, g/$W^{0.75}$	94	103	107	107	3
Gain, g/kg feed [1]	36	93	131	133	—
Digestibility coefficient, % :					
Energy	48.0	55.9	62.4	62.4	3.8
Protein	36.0 [2]	47.7	56.6	65.9	2.6

[1] Average and not individual values were used.
[2] One individual omitted.

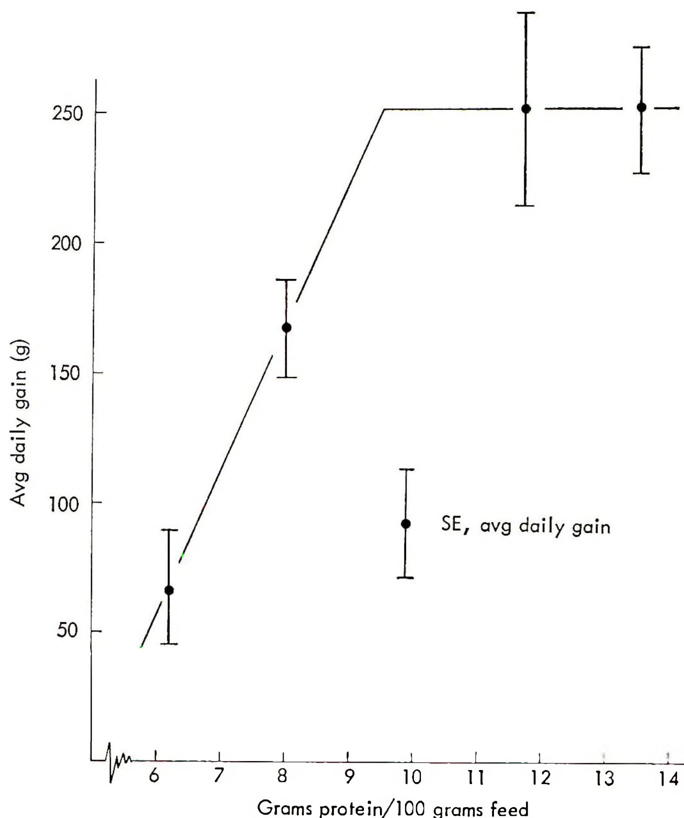

Fig. 1 Response in daily gain to increasing protein concentration in the ration.

0.02) with increasing protein level. Feed efficiency was also improved with increasing protein level.

Energy digestibility increased with increasing protein levels, the 2 lower protein levels giving significantly lower energy digestibility coefficients ($P = 0.05$). The digestible energy content of the rations therefore increased with increasing protein level (1.85, 2.15, 2.41 and 2.41 kcal/g, respectively). The estimated net energy value of the rations, using the equation of Garrett et al. (8), also increased with increasing protein levels (0.80, 0.99, 1.16 and 1.15 kcal/g, respectively). The availability of digestible energy for net energy purposes was therefore 43, 46, 48 and 48%, respectively, indicating greater heat increment in those rations that were deficient in protein (9).

Protein digestibility increased ($P < 0.01$) with increasing protein levels as would be expected. Protein digestibility was closely correlated (r = 0.98) with the

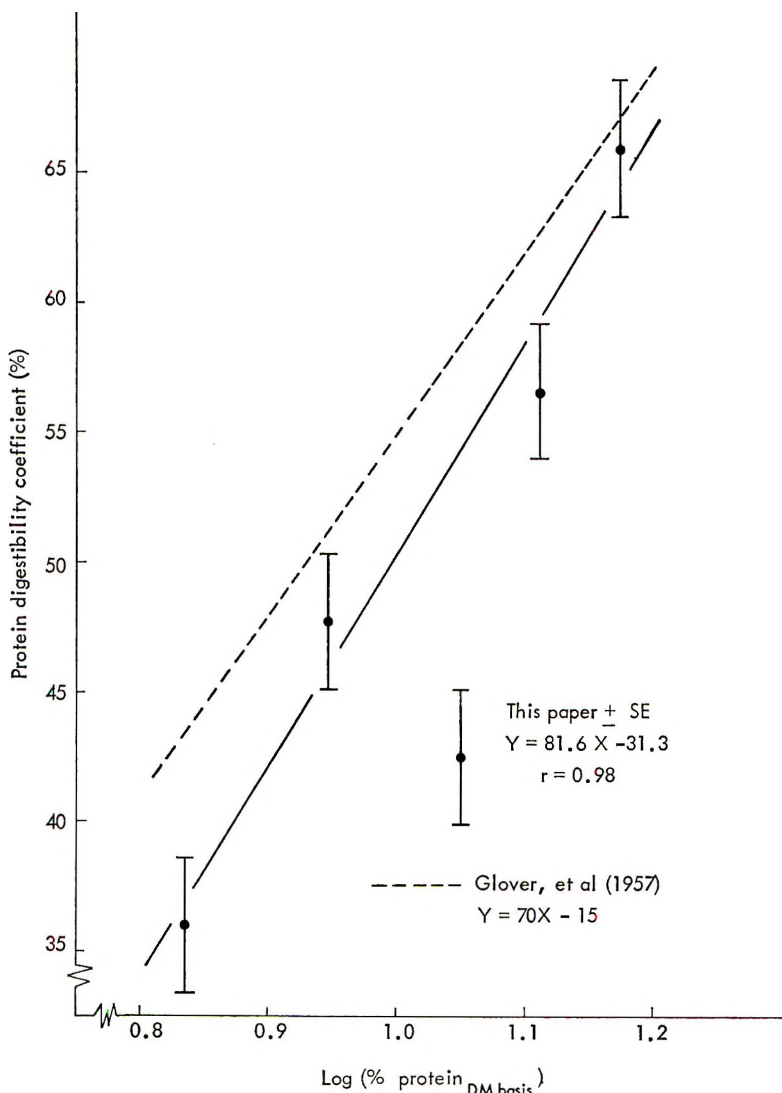

Fig. 2 Protein digestibility as affected by protein concentration in the ration.

logarithm of per cent protein (fig. 2). Glover et al. (10) summarized existing protein digestibility data and found slightly higher coefficients than reported here (fig. 2).

Using the protein digestibility coefficients determined in this experiment, protein composition of the rations, feed intake, body weight and gain, and considering Armsby's maintenance digestible protein requirement (11), it is possible to construct a graph (fig. 3) showing body weight gain as a function of digestible protein intake. Using the increasing portion of the curve, the digestible protein required can be described by the equation:

$$DP = 1.54\ W^{0.75}\ (1 + 0.01\ G)$$

where DP is the amount of digestible protein required (g), W is the body weight (kg) and G is the daily gain (g).

In all 3 experiments, increasing protein concentration in the ration markedly increased ($P < 0.005$) BUN levels (table 4). In experiment 1 where 2 energy concentrations were fed, the higher energy ration appeared to give a lower BUN value. This was no doubt due to a lower daily feed consumption (1530 g) com-

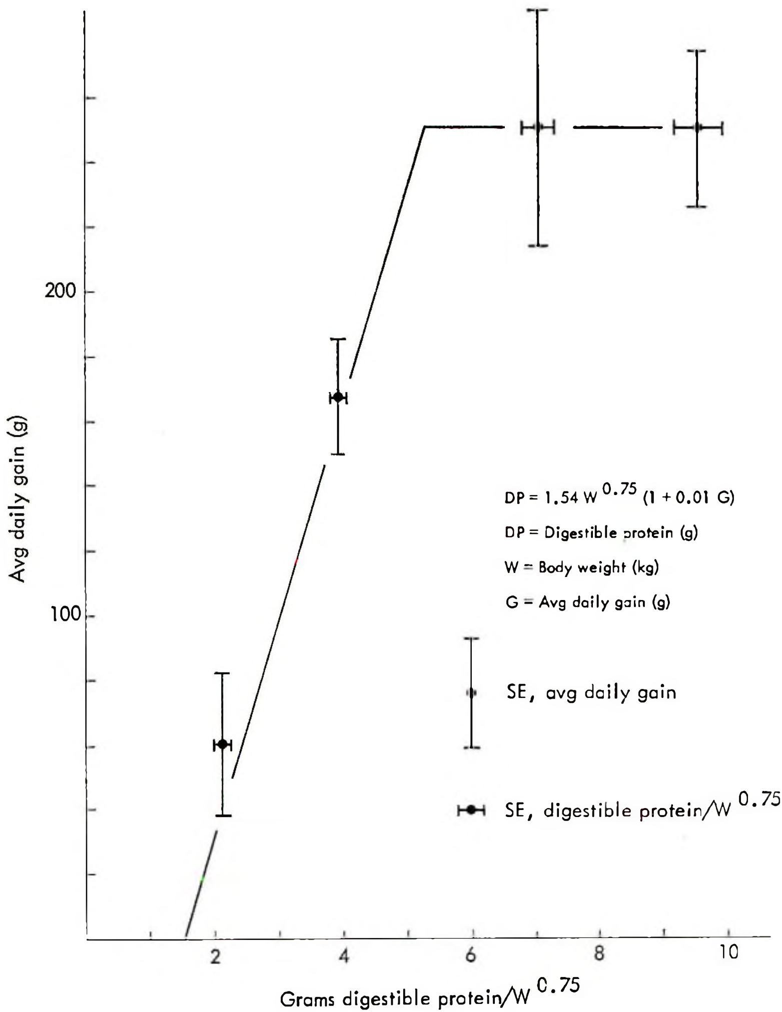

Fig. 3 Response in daily gain to increasing digestible protein intake.

TABLE 4

Blood urea nitrogen (BUN) as affected by dietary protein and energy

Estimated net energy [1]	Protein [2]	BUN
kcal/g	%	*mg/100 ml*
	Experiment 1	
1.17	11.0	10.2 ± 0.4 [3]
1.17	14.2	23.6 ± 0.6
1.17	18.6	29.5 ± 1.7
1.43	10.8	7.9 ± 0.4
1.43	14.9	17.7 ± 0.4
1.43	18.7	25.3 ± 0.5
	Experiment 2	
1.38	9.2	7.8 ± 0.4
1.38	11.5	14.4 ± 0.5
1.38	13.1	19.4 ± 0.7
1.38	16.5	25.8 ± 0.9
1.38	22.0	32.4 ± 2.1
1.38	14.4 [4]	27.3 ± 1.3
	Experiment 3	
1.15	6.2	2.5 ± 0.3
1.15	8.0	3.5 ± 0.3
1.15	11.7	10.0 ± 0.6
1.15	13.5	14.5 ± 0.8

[1] Morrison, F. B. 1956 Feeds and Feeding, ed. 22. Morrison Publishing Company, Ithaca, New York.
[2] N × 6.25.
[3] Average ± SE.
[4] Supplemental nitrogen from urea.

pared with the lower energy ration (1760 g) resulting in a lower total intake of protein.

When the BUN response observed in the third experiment was plotted in a manner similar to figure 1, a marked increase in BUN appeared to take place above 9.5 g protein/100 g feed. This point is similar to the one observed for daily gain (fig. 1). Lambs fed the 6.4% protein level during the previous period had lower BUN values during the subsequent period ($P < 0.005$). Thus, more than 19 to 21 days were required for BUN to stabilize following the feeding of a protein-deficient ration.

Combining the BUN data from all 3 experiments, it appears that this blood constituent can be quantified with protein intake per unit weight$^{0.75}$ (fig. 4). This curve has an indicated minimum and maximum of 2.7 and 32.9 mg urea N/100 ml blood, respectively. The relation between BUN and digestible protein intake (exp. 3) is similar, being displaced to the left in proportion to the protein digestibility coefficient.

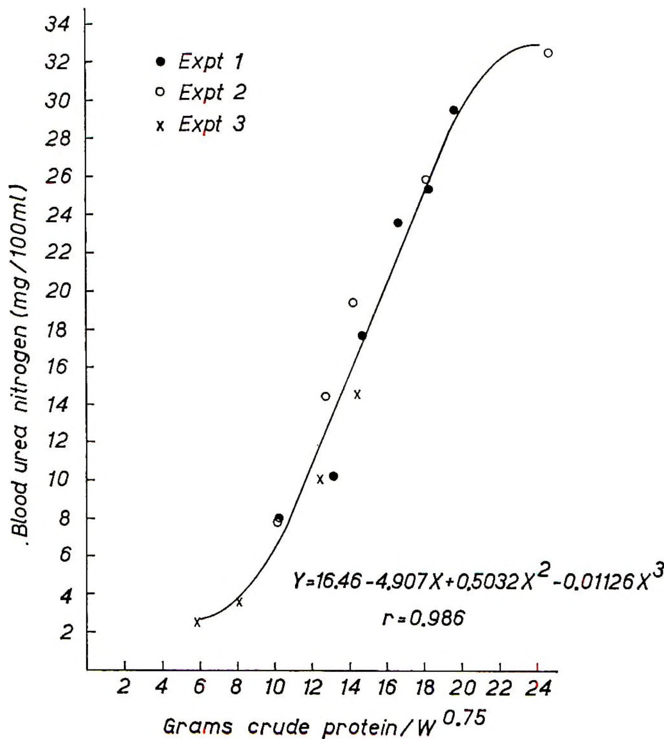

Fig. 4 Blood urea nitrogen as affected by increasing protein intake.

DISCUSSION

The results presented indicate that variation in the protein intake of the growing lamb can result in BUN ranging from 2.7 to 32.9 mg/100 ml. The relation is quite good ($r = 0.986$) and it is proposed that the protein status of the lamb can be at least partially assessed by the concentration of this blood constituent. In terms of protein adequacy, a BUN in excess of 10 mg/100 ml would indicate adequate protein intake with the type of rations fed in these experiments.

It is important to recall the conditions of this work as they relate to BUN changes. First, adequate feed (energy) intake for rapid body weight gain was present throughout (except when protein deficiency restricted feed intake in experiment 3). Limiting energy intake would no doubt upset the relationship presented. Secondly, although different roughage sources were used, variations in protein level were made by varying in the proportions of soybean meal and corn. Other protein sources (protein quality) may result in different curves, although a general similarity in shape would be expected. In the second exepriment, urea was used as a supplemental source of "protein" (47% of total protein) in one group of lambs. Their average BUN was 27.3 mg/100 ml and they consumed 16 g protein/$W^{0.75}$. This BUN is higher than the curve obtained with soybean meal and corn (fig. 4). This may be related to a lower biological value of urea when fed at this level (12, 13).

The equation presented expressing the digestible protein requirement of growing lambs as a function of weight and gain yields figures lower than those listed by the NRC (14). This is contrary to what was expected (1) and therefore the constants indicated in the equation require further study before being used in practice. The major difference is probably in the maintenance requirement shown by the equation (1.54 g/$W^{0.75}$) compared with Armsby's estimate (2.78 g/$W^{0.75}$). Values lower than Armsby's have been suggested (15, 16).

Plotting daily gain (exp. 3) as a function of the actual ratio of digestible protein to digestible energy content of the ration fed reveals that maximal gain would result when the ratio is 22 g digestible protein/1000 kcal digestible energy. This is similar to that calculated for cattle (21–27) [9] and may well represent the optimal ratio for growing-finishing sheep and cattle.

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Effect of Type of Carbohydrate on Energy Metabolism and Body Composition of Rats Fed Low Protein Diets [1]

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ABSTRACT The effects of using dextrin (autoclaved cornstarch) instead of sucrose in low protein diets were investigated in growing rats. Two experiments involving metabolism studies were carried out, and differences in body composition and energy balance were evaluated. Since rats that were fed dextrin had greater food and caloric intakes than those fed sucrose, the former animals were able to maintain equivalent growth rates at lower protein levels in the diet, but retained a greater proportion of the calorie intake as fat. At the same mean body weight, the rats fed dextrin retained significantly more fat and energy in the carcass, and less nitrogen and water than those fed sucrose. At the same protein level, the use of dextrin in the diet caused an increase in the proportion of metabolized energy which was retained in the carcass; but when equivalent growth rates were obtained by lowering the protein content of the dextrin diet, the same proportion of metabolized energy was retained with each dietary treatment. However, the actual values for metabolized energy and for energy retained were higher at the same body weight for the rats fed the low protein-dextrin diet than for those fed the higher protein-sucrose diet. It is concluded that the use of dextrin instead of sucrose as the dietary source of carbohydrate alters the mechanism of food intake control and also the subsequent utilization and metabolism of the ingested calories.

In the early amino acid and vitamin studies, it was noted that when carbohydrates with more complex molecular structures were used in deficient rations, it was more difficult to obtain deficiency symptoms than when a simple sugar such as sucrose was used. The effects of type of dietary carbohydrate have been extensively reviewed by Harper and Elvehjem (1), and it is generally accepted that most of the more complex carbohydrates give better growth at low protein levels than the simpler sugars.

The complex carbohydrate to which Harper and Elvehjem (1) refer most frequently is "dextrin." They stated that this product was obtained when cornstarch was autoclaved, dried and ground, and it gave a blue color with iodine. However, they did not distinguish between this product and commercial dextrin (which gives a red-violet color with iodine) when quoting the results obtained by some workers who used the latter product. Commercial dextrin gives a rate of growth intermediate between that of sucrose and the autoclaved cornstarch product at low levels of protein (2). In the present study, the term "dextrin" refers to the autoclaved cornstarch product, which is similar to that used by Harper and co-workers at Wisconsin (1–5, 13, 19).

The growth-stimulating effect of dextrin was most marked at low levels of protein in the diet (3–5). Equivalent growth rates were obtained when the protein level in the sucrose diets was increased by 2 to 3% above that in the dextrin diets. Accompanying the growth-stimulating effect of dextrin was a marked increase in the fat content of the carcass (5). Dextrin-fed rats had higher fat even when grown at equivalent rates to the sucrose-fed animals. Wiener and associates (5) were the first to evaluate the differences in body composition, but the energy aspect of this change in metabolism was not adequately studied.

In body composition studies, the interpretation of results becomes an important problem. In the past, workers have expressed a particular body component (e.g., fat, water, protein) as a percentage of the total body weight. However, the use of

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percentages or other ratios is not justified unless certain statistical considerations are fulfilled (6), and may be very misleading in group comparisons (7). Many workers in the field of body composition now accept the theory of "compositional homeostasis," which implies that there is a balance between the rates of deposition of the body components, independent of age, nutrition or any other factor except some parameter of body weight. This means that there is a specific curvilinear relationship between a particular body component and, for example, empty body weight. This relationship becomes linear if the logarithms of the variables are used. However, in the early stages of growth, the relationship is almost linear even when the actual values are used, and hence regression analysis is easily applied. The various relationships between the individual body components and body weight hold for a large population of a species. However, when a highly uniform sample is selected, the slope of these regressions may be significantly altered within the total biological variation by nutritional or other treatments. For example, if the slopes (or regression coefficients) of fat versus empty body weight are different for groups of rats receiving different dietary treatments, then it may be concluded that the treatment caused an alteration in the pattern of metabolism, leading to different rates of fat deposition. Also, from the regression equations obtained in one particular experiment, direct comparisons may be made in other experiments using similar treatments.

The above approach to the interpretation of body composition data was used in the present study to evaluate more precisely the differences in body composition when either dextrin or sucrose was the source of carbohydrate in low protein diets. Energy metabolism was also studied, since this aspect has not yet been evaluated, and since some differences would be expected if the pattern of metabolism is altered sufficiently to cause a greater fat deposition when the animals are at the same body weight.

EXPERIMENTAL

The first (experiment 1) was designed to examine more fully certain aspects of the data obtained by Wiener and associates (5). The 2 dietary carbohydrates used were sucrose and dextrin.[2] The protein levels were chosen from the graph presented by these workers for methionine-supplemented casein, indicating that equal growth could be obtained by using a level of 7% casein with dextrin (diet A), and 10% casein with sucrose (diet D). The composition of these diets is shown in table 1.

Male weanling Holtzman rats were fed a stock diet[3] until the mean body weight was 89 g. The rats were divided into 3 groups of 6 rats each with equal mean body weights. Two groups were placed in individual metabolism cages for an experimental period of 2 weeks, and a third group was killed at the beginning of the period to evaluate the original nitrogen content and energy status of the animals placed on the dietary treatments. Body weight gains were recorded each week; food intake measurements, and fecal and urinary collections were made daily for each rat. The urine samples were preserved with dilute suphuric acid, and neutralized later with 1 N sodium hydroxide. All samples were stored at − 10° before freeze-drying for 5 days.

At the end of 2 weeks, the remaining 2 groups of rats were killed with ether. All carcasses were prepared for freeze-drying by removal of the gastrointestinal contents. The material from the cecum and colon of the animals killed at the end of the experiment was weighed and added to the individual fecal samples, whereas that from the animals killed at the start of the experiment was pooled to give a value for the energy in the colon and cecum at this time. The material from the small intestine and stomach was weighed and pooled for each of the treated groups to determine the energy of the ingested food remaining in the tract. Empty body weights were obtained for each animal, and then the carcasses were frozen.

The carcasses were freeze-dried for 5 days. This reduced the moisture such that heating further at 70° under vacuum for

[2] Cornstarch was moistened with water and autoclaved at 120° for 3 hours, dried at 60° for 3 days and finely ground.

[3] Big Red Laboratory Animal Food, Agway Inc., Syracuse, New York.

TABLE 1

Composition of experimental diets

	Dextrin		Sucrose	
	A	B	C	D
	%	%	%	%
Casein	7.0	10.0	7.0	10.0
Carbohydrate	83.0	80.0	83.0	80.0
DL-Methionine	0.3	0.3	0.3	0.3
Choline chloride	0.2	0.2	0.2	0.2
Vitamin mixture [1]	0.5	0.5	0.5	0.5
Salt mix [2]	4.0	4.0	4.0	4.0
Corn oil	5.0	5.0	5.0	5.0
Analysis:				
Dry matter, % (exp. 1)	90.59			99.45
Dry matter, % (exp. 2)	91.24	92.77	99.17	99.00
Nitrogen, %, dry-matter basis (exp. 1)	1.349			1.504
Nitrogen, %, dry-matter basis (exp. 2)	1.206	1.576	1.144	1.442
Energy, kcal/g, dry-matter basis (exp. 1)	4.446			4.179
Energy, kcal/g, dry-matter basis (exp. 2)	4.315	4.371	4.156	4.196
Cal/N ratio, kcal/g N (exp. 1)	320			278
Cal/N ratio, kcal/g N (exp. 2)	358	277	363	291

[1] Vitamin mixture (water-soluble): (in grams) nicotinic acid, 9.9; Ca pantothenate, 6.6; riboflavin, 2.2; thiamine·HCl, 2.2; pyridoxine·HCl, 2.2; menadione, 0.5; folic acid, 0.198; biotin, 0.044; cyanocobalamin (0.1% vitamin B_{12} activity), 3.0; glucose monohydrate (Cerelose, Corn Products Co., Argo, Ill.), 473.1. Five grams of this mixture were fed per kilogram of diet. Water-insoluble vitamins: vitamin A, 700 IU/ml and vitamin E, 21 IU/ml in corn oil; 0.2 ml to 0.4 ml were given per week to each rat, depending on body size.

[2] Jones, J. H., and C. Foster 1942 A salt mixture for use with basal diets either low or high in phosphorus. J. Nutrition, 24: 245.

24 hours would not reduce the weight further. The dry carcasses were ground in a Wiley mill with approximately 4 times their weight of dry ice.[4] The carcass samples prepared in this manner were homogeneous and easily handled. A small amount of moisture was absorbed during the grinding process, however, and this was determined by drying a subsample in a vacuum oven at 70° for 24 hours.

Energy determinations were made on all feces, urine, diet and carcass samples in an adiabatic bomb calorimeter. Nitrogen was determined on the diet and the carcass samples by the macro-Kjeldahl method using copper sulphate as the catalyst, and fat was determined on the carcass samples only, by extraction for 24 hours with ethyl ether, after obtaining a dry sample in a forced draught oven at 100° for 24 hours.

The second experiment evolved from the the results of experiment 1, since there was some question whether the differences obtained were due to the protein levels or to the type of carbohydrate. Thus a factorial 2 × 2 arrangement was used to test the interaction between protein level and type of carbohydrate. The same 2 levels of protein (7 and 10% casein) as in experiment 1 were fed with both dextrin and sucrose (4 dietary treatments: table 1).

Fifty-six male weanling rats of the Holtzman strain were divided into 4 equal groups, each containing 14 rats. Each group was assigned to one of the dietary treatments as follows: group A received diet A (7% casein + dextrin); group B, diet B (10% casein + dextrin); group C, diet C (7% casein + sucrose); and group D, diet D (10% casein + sucrose).

Two rats from each group were selected at random to be killed on reaching one of the following weights: (in grams) 75, 85, 95, 105, 115, 125 and 135. Thus a wider body weight range (75 to 135 g) was covered than in experiment 1. However, collections of urine and feces were made for only 4 animals in each group, from which the daily urinary and fecal energy for the other animals in that group was calculated. Food intake measurements were made on all animals in each group. The 2 rats in each group that were killed at 75 g served as the controls in the calculations of energy and nitrogen balance.

[4] Unpublished method, A. Bensadoun, Department of Animal Husbandry, Cornell University.

All samples of urine, feces, diets and carcasses were treated and analyzed in the same way as in experiment 1, giving the carcass content of water, nitrogen, fat and energy, and the energy balance, for each animal.

For the statistical treatment of the results, the analysis of covariance was used in both experiments (8). Choice of the independent variable is generally regarded as arbitrary (7). It was felt that treatment may have affected several or all of the body components, so that the total body component was the most useful indicator, especially in young growing animals when this parameter accounts for most of the variation in the dependent variable. With an increase in weight (or age), fat becomes much more unpredictable, and other parameters may become more accurate, for example, fat-free wet weight (9). In this work, empty body weight was used, and in all cases the variation in mean square deviation was small.

The statistical analysis was applied to test the effect of the type of carbohydrate on body composition and energy metabolism, and to examine the interaction between the type of carbohydrate and the protein level. The significance of each regression was tested, and in only one instance was the use of regression analysis not justified. Differences in the slopes of the regressions were tested first, and if these differences were significant, then the values for the adjusted means obtained from the analysis of covariance became meaningless. These values are derived by assuming that the regression coefficients are similar, and then by adjusting the individual means along a common regression coefficient to the mean of the independent variable.

If the differences in the regression coefficients were not significant, however, analysis of covariance could then be used to test the differences in *elevation* of the adjusted means due to treatment.

RESULTS

Mean values for body weights, nitrogen retention and energy balance for experiment 1 are presented in table 2. The rats fed dextrin had slightly lower body weights than those fed sucrose, but the difference was not significant. Intakes of total diet, nitrogen and calories were not significantly different for the 2 groups, but the dextrin-fed group had greater excretory nitrogen and fecal energy than that fed sucrose.

TABLE 2

Approximate demonstration of growth, nitrogen and energy balance (exp. 1) [1]

	Group A		Group D	
	7% Casein + dextrin		10% Casein + sucrose	
	Mean	Range	Mean	Range
Body weight				
Initial, g	89.2	81–94	88.7	83–94
Final, g	120.3	112–131	127.7	118–144
Gain/2 weeks, g	31.1	25–37	39.0	31–55
Dry food intake, g	153	135–170	152	135–169
Nitrogen balance				
Intake, g	2.07	1.82–2.36	2.29	2.02–2.55
Retained, g	0.96	0.77–1.11	1.43	1.16–1.67**
Excreted, g	1.11	0.97–1.30	0.86	0.70–1.00**
Empty body weight, g	113.0	106–124	122.6	113–137
Energy balance				
Intake, kcal	679.4	598–776	633.0	560–704
Feces, kcal	29.9	26–33	18.5	15–22 **
Urine, kcal	7.6	6–10	15.2	7–36
Metabolized, kcal	641.9	562–733	599.3	530–668
Energy retained, kcal	100.5	78–124	101.1	70–164
Heat produced, kcal	541.4	472–620	498.2	460–531
Metabolized energy				
Retained in the carcass, %	15.6	13.0–18.8	16.6	11.6–24.6

** Significance level $P < 0.01$ by the simple range test (Snedecor, G. W. 1956 Statistical Methods. Iowa State College Press, Ames).

[1] Six rats/group.

The values for metabolized energy and for energy retention were determined from the energy balance data for 2-week period. The regression between these 2 variables was not significant; hence the proportion of metabolized energy retained as carcass energy was tested by group comparison and by the range test (8). However, there was little difference between the 2 groups (table 2).

The statistical analysis of the body component versus empty body weight in experiment 1 is shown in table 3. The slopes of the individual regressions were not different; hence the means for each component adjusted to the mean empty body weight have been presented. Group A (7% casein + dextrin) retained significantly more fat and total calories, and less nitrogen and water, than group D (10% casein + sucrose).

In experiment 2, the rats that received diet B attained the required body weights most rapidly. The rats fed diets A and D took approximately twice as long to reach the required weights as those fed diet B, whereas those fed diet C took 4 times as long. The analysis of regressions and the regression coefficients are presented in table 4.

When dextrin was fed at both protein levels, there was a significant increase in the overall rate of fat deposition with increasing empty body weight. The lower level of protein also caused a response, showing that some of the apparent effect of dextrin in experiment 1 was due to the lower protein level in the diet. Total carcass energy was similarly affected by the type of carbohydrate in the diet, but in this case the level of protein was without significant influence. Body water retention was opposite in the order of groups to that of fat, and dextrin caused significantly lower retention.

The *rate* of nitrogen retention was not affected by either the type of carbohydrate or the level of protein in the diet. Thus the differences in the elevation of the means adjusted to the mean empty body weight could be tested. It was found by the analysis of covariance that dextrin caused a significantly lower nitrogen retention at each level of protein in the diet ($P < 0.01$). The adjusted means for the

TABLE 3

Analysis of covariance and adjusted treatment means for body components vs. empty body weight (exp. 1)

Source of variation	df	Independent variable, empty body weight, g							
		Water, g		Nitrogen, g		Fat, g		Carcass energy, kcal	
		Coefficient	Mean square	Coefficient	Mean square	Coefficient	Mean square	Coefficient	Mean square
Group A (7% casein + dextrin)	4	0.554	1.398	0.0214	0.0090	0.235	1.555	3.075	129.2
Group D (10% casein + sucrose)	4	0.504	3.198	0.0255	0.0019	0.301	2.885	3.998	333.4
Within	8		2.298		0.0054		2.220		231.3
Regression coefficients (not significant)	1		0.540		0.0027		0.728		143.2
Common	9	0.525	2.103	0.0239	0.0051	0.276	2.054	3.649	221.5
Adjusted means	1		19.028*		0.4404**		41.682**		2252.0**
Total	10								
Adjusted means:									
Group A (7% casein + dextrin)		76.57		3.411		14.11		262.2	
Group D (10% casein + sucrose)		79.53		3.662		9.73		227.9	

* Significance level, $P < 0.05$.
** Significance level, $P < 0.01$.

TABLE 4

Analysis of regressions and regression coefficients for body components and energy metabolism (exp. 2)

Source of variation	Independent variable (X_1 = empty body weight, g) [1]								X_2 = Metabolized energy, kcal	
	Water, g		Nitrogen, g		Fat, g		Carcass energy, kcal		Energy retained, kcal	
	df	Mean square	df	Mean square	df	Mean square	df	Mean square	df	Mean square
Carbohydrate	1	8.95**	1	0.0320	1	18.84**	1	777.8*	1	1984**
Protein	1	3.20	1	0.0201	1	7.54*	1	330.9	1	3444**
Interaction	1	2.30	1	0.0029	1	4.35	1	36.6	1	603
Error	48	1.04	48	0.0103	48	1.88	48	109.5	40	261
Regression coefficients:										
Casein, 7% dextrin	0.547		0.0283		0.236		3.094		0.182	
Casein, 10% dextrin	0.592		0.0300		0.170		2.766		0.223	
Casein, 7% sucrose	0.608		0.0295		0.148		2.634		0.099	
Casein, 10% sucrose	0.612		0.0326		0.139		2.469		0.199	

* Significance level, $P < 0.05$.
** Significance level, $P < 0.01$.
[1] Since there were no differences in slopes of nitrogen vs. empty body weight, the treatment differences in adjusted means were tested. The effect of carbohydrate was significant at the level, $P < 0.01$. The adjusted means were A = 2.80 g, B = 2.80 g, C = 2.99 g, and D = 3.01 g.

dextrin diets were both 2.80 g nitrogen in the carcass for each level of protein, and in the sucrose diets, 2.99 and 3.01 g for 7 and 10% casein, respectively. Since the values for metabolized energy increased with increasing body weight, the proportion of energy retained in the carcass was also analyzed on a regression basis, with metabolized energy as the independent variable. The regression was highly significant in this experiment, and the individual regression coefficients were significantly affected by both the type of carbohydrate and the level of protein in the diet. Thus the use of dextrin and the higher level of protein each caused an increase in the proportion of metabolized energy which was retained as carcass energy. When equivalent growth rates were obtained, as in groups A and D, the proportion of metabolized energy retained in the carcass was the same, which indicates that these values are related to growth rate. However, the actual values for metabolized energy and for energy retained in the carcass were higher for the animals in group A than for those in group D, and hence the values for heat production were also higher, but still constituted the same proportion of metabolized energy in these 2 groups.

One point should be made here concerning the statistical results from both experiments. In the first experiment, the slopes were not different, probably due to the small range of the independent variable and to the proportionately larger deviations from the regressions in each group. Since the animals in the second experiment were killed over a much larger range of body weights, the individual regression coefficients were highly significant, and hence even small differences in slope could be detected easily.

DISCUSSION

The results will be discussed largely in relation to the most recent work carried out by Wiener and associates (5), since this is the first indication that there is a significant difference in total body composition arising from the 2 types of dietary carbohydrates.

It was found that the groups which had equivalent growth (i.e., those groups fed

diets A and D in both experiments) had the largest differences in fat deposition. The caloric intakes were similar for both of these dietary treatments, but the intake of nitrogen was slightly less for the groups fed diet A (7% casein + dextrin). The calorie-to-nitrogen ratio was higher with diet A (358 kcal/g nitrogen) than with diet D (7% casein + sucrose; 291 kcal/g nitrogen). Despite this unfavorable ratio (10) the groups fed diet A were able to consume much more than the group fed diet C with an equivalent calorie-to-nitrogen ratio (363 kcal/g nitrogen), and thus they could maintain growth with the groups fed at a more favorable ratio.

Meyer (11) has investigated the effect of calorie-to-protein ratios in the diet, and reported that the 30% substitution of cellulose for sucrose in a low protein-sucrose diet caused increased food intakes and lower fat deposition than when no substitution was made. He also observed a highly increased value for metabolic fecal nitrogen which caused increased nitrogen requirement of the animals fed the cellulose diets. That this was perhaps also the case when dextrin was fed, is shown particularly in table 2. Nitrogen excretion was obtained by subtraction only, but in all cases where dextrin was fed, this value was consistently higher than in the sucrose groups. This agrees with the results of nitrogen balance studies made by Wiener and associates (5). The more granular and insoluble properties of dextrin when prepared by autoclaving cornstarch may account for these differences.

Wiener and associates (5) suggested some interesting points in relation to the "efficiency" with which dextrin-fed animals utilize ingested calories. However, these workers did not mention any energy determinations made by calorimetry, and it must be assumed that all of their values were calculated. Nevertheless they stated that the dextrin-fed animals have lower efficiency of calorie retention (retained calories/ingested × 100) than those fed sucrose. Also these workers did not specify whether this reduced efficiency with dextrin occurred with equivalent growth or with similar protein levels. In experiment 2, the proportion of metabolized energy which was retained in the body was the same for the groups with equivalent growth; when the same level of protein was used, dextrin gave much higher efficiencies than sucrose.

Hartsook and Hershberger (12) observed that when the ratio of heat production to metabolized energy is plotted against the protein content of the diet, a shallow saucer-shaped curve is obtained. Thus this ratio decreases at low protein levels, maintains a constant figure at "optimum" levels, and increases again at very high protein levels. From the data given in their paper, this relationship appears to hold whether that portion of heat production due to basal metabolism is included or not, although Hartsook and Hershberger strongly recommended its subtraction from total heat production for several reasons. The ratio of heat production to metabolized energy (HP/ME) may be regarded as the complement of the ratio of energy retained to metabolized energy, which is presented for experiment 2 in table 4, since the sum of these 2 fractions equals unity. The values presented in this table are in that region of the curve where the ratio HP/ME is decreasing with a concomitant increase in dietary protein. However, these values indicate that, *at the same protein level*, the ratio HP/ME is decreased by the use of dextrin instead of sucrose as the dietary source of carbohydrate. It may be postulated therefore, that dextrin caused a decrease in the *elevation* of the curve presented by Hartsook and Hershberger (12). In this way, the discrepancies in the ratios at the same protein levels caused by the different carbohydrates may be explained.

The Wisconsin group favors the theory that sucrose or other simple sugars in the diet reduced appetite by their greater osmotic effect in the stomach (13). However, they do not attempt to explain why this difference in food intake does not persist at much higher levels of protein, when the carbohydrate in the diet still constitutes more than 60% of the diet.

Tepperman and Tepperman (14) have observed increased lipogenesis in mice with certain hypothalamic lesions, and also in mice that were trained to eat the entire day's ration in 2 hours. These animals showed a very high capacity for convert-

ing non-fat precursors into fatty acids in both the liver and the epididymal fat pad. This situation also occurred in rats that were force-fed once per day (15). These and many other workers observed adaptive enzymatic changes, including increased activity of the direct oxidative pathway. Tepperman and Tepperman (14) also noted increased glucose absorptive rates in the intestines of trained animals, and they quote work which indicates that there is an increase in insulin-like activity in the blood of the intermittently over-fed animals. They conclude that adaptive hyperlipogenesis is the end result of a number of physiological and biochemical adjustments.

The effects of dietary carbohydrate on the food intake of the rats in these studies is an important aspect of the phenomena observed, if not its direct cause. Factors thought to be involved in regulation of food intake have been reviewed by Mayer (16), Kennedy (17) and Brobeck (18). These theories are not developed to the point where they account for a specific change in food intake.

The dextrin is removed from the gastrointestinal tract much more slowly than sucrose.[5] This clearly makes the carbohydrate available to the animal in a much different pattern. This may cause changes in the endocrine or metabolic responses that control food intake. It is not clear whether the increase in body fat is directly the result of increased food intake with a low protein diet or whether the endocrine or metabolic effects of the dextrin directly stimulate fat synthesis and have only a secondary effect on food intake. Any proposed mechanism for the effects studied in this paper must explain why an inadequate level of protein in the diet is essential to produce these effects.

It is also interesting to consider similarities and differences between animals fed dextrin and those that are intermittently overfed, either by forced feeding or training the animals to eat only once or twice per day. The net result in each case is an increase in fat synthesis. The Wisconsin group (2, 19) observed in their studies of liver fat that when sucrose was fed at the same level of protein as dextrin the latter caused a decrease in fat content. Later they discovered that this was almost entirely accounted for by the increased nitrogen intake (5). Tepperman and Tepperman (14) reported increased lipogenesis in the livers of their trained "meal-eating" rats, but noted no changes in liver fat content. They did observe increased glycogen storage in the liver however, which agrees with the results of Desai,[6] who determined the liver glycogen of rats treated in the same way as in experiment 1 of this study. The latter work has yet to be verified, and there may be an interaction with protein level. Despite these similarities, it is very difficult to reconcile the pattern of feeding of the intermittently over-fed animals with that of the animals fed dextrin, since it was observed that the pattern of feeding of rats with dextrin or sucrose diets was identical.[7]

It may be concluded that much more work is necessary to evaluate the site of differentiation between the utilization of dextrin and sucrose from the diet. This work should be closely connected with research in the regulatory mechanisms of food intake. The basic overall differences in body composition and energy metabolism due to the feeding of either sucrose or dextrin in low protein diets have by now been fairly well established.

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Biochemical Changes in Progressive Muscular Dystrophy

III. NUCLEIC ACID, PHOSPHORUS AND CREATINE METABOLISM IN THE MUSCLE, LIVER AND BRAIN OF RABBITS MAINTAINED WITH A CHOLINE-DEFICIENT DIET [1]

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ABSTRACT Rabbits maintained with a choline-deficient diet developed weakness in the skeletal muscle, lost body weight, and excreted more creatine and less creatinine in the urine. In the muscle of such severely affected animals a threefold increase in the concentration of DNA and RNA were observed. In the liver and brain, these concentrations decreased by 20 to 30%. The distribution pattern of phosphorus in the various acid-soluble and acid-insoluble fractions was also altered. Total phosphorus (TP), total inorganic phosphorus (TPi) and total acid-soluble phosphorus (TASP) of the muscle decreased by 28, 46 and 47%, respectively. Total acid-insoluble phosphorus (TAIP) did not show any change. In the liver, TP, Pi, TASP and TAIP all decreased. ATP and creatine were also reduced considerably in the affected muscles of such animals. In the liver and brain, creatine concentration was increased by 400 and 16%, respectively. An increase in ATP concentration was noted in the liver and brain of such animals. The present work illustrates the similarity between the various secondary biochemical changes that can be observed in the muscles of experimental animals with nutritional and hereditary dystrophy.

As earlier studies by Hove and Copeland had showed that vitamin E might be involved in the synthesis of acetylcholine from choline and acetate, and that acetylcholine is involved in the transmission of nerve impulse, these workers (1) investigated the possibility of producing muscular dystrophy in animals by a choline-deficient diet. In this study they observed that rabbits maintained with a choline-deficient diet develop weakness in the skeletal muscle. Abnormal urinary excretion of creatine and creatine was also observed (1), but resembled more closely that noted in human muscular dystrophy. The work carried out in our laboratory, as well as in the laboratories of other investigators, clearly demonstrates a similarity among many biochemical changes noted in the muscle and liver of animals with nutritional and hereditary dystrophy. Recently we showed a close parallelism in nucleic acid and protein metabolism in the muscle, liver, and brain of animals so affected (2, 3).[3] This similarity in biochemical changes prompted the present investigation of nucleic acid, protein and phosphorus metabolism in the muscle, liver and brain of experimental animals maintained with a choline-deficient diet.

METHODS AND MATERIALS

Twenty young white rabbits of New Zealand strain, weighing approximately 1000 g each, were maintained individually in metabolic cages and fed a choline-deficient diet containing: (in per cent) casein, 6; peanut meal (extracted by exhaustive continuous methanol extraction), 30; sucrose, 23.9; salt mixture W, 5;[4] lard, 19;

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[2] Holder of a Postdoctoral fellowship from the Muscular Dystrophy Association of Canada.

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[4] The salt mixture had the following percentage composition: calcium carbonate, 21; copper sulfate ($5H_2O$), 0.039; ferric phosphate, 1.47; manganous sulphate (anhyd), 0.02; magnesium sulphate (anhyd), 9.0; potassium aluminum sulphate, 0.009; potassium chloride, 12.0; potassium dihydrogen phosphate, 31.0; potassium iodide, 0.05; sodium chloride, 10.5; sodium fluoride, 0.057; tricalcium phosphate, 14.9; (obtained from Nutritional Biochemicals Corporation, Cleveland).

cod liver oil, 1; processed rice bran, 5;[5] L-cystine, 0.1; and vitamin premix, 5;[6] as recommended by Hove and Copeland (1). The body weight and the pattern of urinary excretion of creatine and creatinine were determined at an interval of 6 to 8 days initially and then every third day during the latter part of an 80-day period, in which the disease develops fully. The same choline-deficient diet was supplemented with 0.12% choline chloride and fed to a control group of 10 rabbits of approximately the same age and weight.

At regular intervals during the entire period of 80 days, 2 rabbits from each group (experimental and control) were decapitated. Roughly 1 to 2 g of skeletal muscle (hind leg), liver and brain of each animal were quickly removed, blotted between filter paper, freed of connective tissue, fat, etc., and chilled in ice. One gram of each of these tissues, muscle, liver and brain of normal and experimental animals, was separately homogenized in 15 ml of physiological saline at 0°. Detailed procedure for preparation of muscle, liver and brain homogenates has been described previously (2, 3).

An equal volume of 10% TCA was added to the homogenate. It was allowed to stand for 30 minutes at 0° and then centrifuged in a clinical centrifuge at 0°. The TCA extract thus obtained was used for the determination of acid-soluble phosphorus, (TASP), inorganic phosphorus (TPi),[7] 7-minute hydrolyzable phosphorus, and creatine. The residue was washed twice with cold 5% TCA, and then resuspended in 5 ml of 5% TCA. One part of this was used for the determination of acid-insoluble phosphorus (TAIP). The other part was heated at 90° for 15 minutes for the extraction of nucleic acids as nucleotides, nucleosides, etc. The supernatant fraction obtained after centrifugation was used for the determination of deoxyribose by Dische's diphenylamine reaction (4), and ribose by the orcinol reaction of Bial (5). The total phosphorus was determined directly from the hydrolysates of the mucle, liver or brain homogenate in 5 N sulphuric acid. Phosphorus was estimated according to the method of King (6), creatine by the diacetyl reaction (7), and 7-minute hydrolyzable phosphorus according to the method described in our previous paper (3).

RESULTS

Variation of DNA and RNA concentrations. A regular increase in DNA and RNA concentration in the muscle and a steady decrease in concentration in the liver and brain of the animals with progressive development of the disease are shown in table 1. A threefold increase in DNA and RNA concentration was observed in the skeletal muscle of severely affected animals. In the 45 to 50% diseased condition,[8] the concentration of DNA and RNA in the muscle increased by approximately 100 to 125%. The changes in nucleic acid concentration as shown in table 1 appear to follow the pattern observed previously in the skeletal muscle of vitamin E-deficient animals and those with hereditary dystrophy (2). In the liver and brain of these animals the concentration of these 2 nucleic acids also undergoes changes, but to a much lesser extent.

Changes in the concentration of total phosphorus, total inorganic phosphorus, total acid-soluble and acid-insoluble phosphorus. In table 2 is shown the variation in the concentration of total phosphorus (TP), total inorganic phosphorus, total acid-soluble and acid-insoluble phosphorus in the muscle, liver and brain of severely affected animals. In the muscle, the concentration of these changed considerably; TP, TPi and TASP decreased by 28, 46 and 47% respectively. No measurable change in the concentration of

[5] Processed rice bran was obtained from Fischer Scientific Company, Montreal, Canada.

[6] The vitamin premix contained pure vitamins added to the sucrose to give the following levels per g of diet: thiamine, riboflavin and pyridoxine, 3 μg each; Ca pantothenate, 17 μg; i-inositol, 200 μg; niacin, 30 μg; methyl 1,4-naphthoquinone, 0.3 μg.

[7] Total inorganic phosphate (TPi) represents the "phosphorus" that can be directly estimated in TCA extract and therefore includes the creatine "phosphorus," since we have observed that creatine phosphate (CP) undergoes complete hydrolysis at room temperature under the conditions of the experiment.

[8] Throughout the text, wherever 45 to 50% or 90 to 95% dystrophy is used, it is implied that the disease has actually developed in each individual animal to that extent. Percentage of dystrophy developed was calculated from the ratio of the excretion of creatine and creatinine in the urine. This was further confirmed on the basis of our observations that 100% dystrophic condition is reached by maintaining the rabbits with a choline-deficient diet for 80 days. One hundred per cent dystrophy has been arbitrarily chosen to indicate the stage of the diseased condition when the animal dies

TABLE 1

Variation of DNA and RNA in muscle, liver and brain of rabbits maintained with a choline-deficient diet [1]

Group [2]	Muscle		Liver		Brain	
	DNA	RNA	DNA	RNA	DNA	RNA
	mg/g wet tissue		*mg/g wet tissue*		*mg/g wet tissue*	
Normal	0.30 ± 0.06 [3]	0.38 ± 0.08	1.68 ± 0.18	3.33 ± 0.30	1.15 ± 0.10	0.55 ± 0.06
Dystrophic (45–50%)	0.67 ± 0.10	0.84 ± 0.08	1.48 ± 0.12	3.05 ± 0.38	0.87 ± 0.05	0.49 ± 0.08
Dystrophic (90–95%)	1.00 ± 0.15	1.28 ± 0.12	1.39 ± 0.15	2.76 ± 0.20	0.80 ± 0.08	0.43 ± 0.05

[1] Nutritional dystrophy was produced in rabbits by maintaining them with a choline-deficient diet. The standard deviation was calculated from 5 sets of experiments. Each set consisted of 2 animals.

[2] In using the terms 45–50% and 90–95% dystrophic, it is implied that the disease had actually developed to that extent in each individual animal.

[3] SD.

TAIP in the affected muscle could be detected. In the liver of these animals, a decrease of 18, 17, 28 and 9% in the concentration of TP, TPi, TASP and TAIP was noted. In the brain, there was little change in TP; TPi and TASP decreased by 27 and 20% respectively; and TAIP showed a slight increase.

Changes in concentration of 7-minute hydrolyzable phosphorus and creatine. Among the tissues tested for creatine concentration, such as muscle, liver and brain, only in the muscle of severely affected animals was the concentration decreased by 33%. In the liver and brain, the concentration increased by 20 and 17%, re-16%, respectively. ATP [9] concentration was reduced by 44% in the muscle of the affected (90 to 95% diseased state) animals. In the liver and brain its concentration increased by 20 and 17%, respectively. These results are illustrated in table 3.

DISCUSSION

Our results on the variation of nucleic acid concentration in the muscle of rabbits maintained with a choline-deficient diet show a gradual shift in nucleic acid metabolism towards abnormality. DNA and RNA concentration in the muscle of rabbits maintained with a choline-deficient diet increased by 300%; in liver and brain the concentration of these decreased. Krupnick et al. (8) have recently suggested that the increase in DNA concentration in the muscle of vitamin E-deficient rabbits may indicate the ability of damaged muscle to regenerate. Since the variation of nucleic acid concentration in the skeletal muscle of choline-deficient rabbits follows the same pattern as noted in the muscles of vitamin E-deficient rabbits and mice with hereditary dystrophy, the observed increase

[9] The 7-minute hydrolyzable phosphorus represents the phosphorus obtained by subtracting the total inorganic phosphorus (which also included creatine phosphorus) from the "phosphorus" determined after boiling the TCA extract for 7 minutes at 100°. It represents all the tri-, di-, and monophosphates of 4-deoxyribose and ribose nucleotides, di- and triphosphates of pyridine nucleotides (DPN and TPN), IMP, etc. In skeletal muscle, ATP actually represents 90 to 92% of the 7-minute hydrolyzable phosphorus (12) and therefore in the text ATP concentration has been used loosely to represent 7-minute hydrolyzable phosphorus concentration in muscle. Since the percentage of ATP in 7-minute hydrolyzable phosphorus of liver and brain is less than 50%, we have used 7-minute hydrolyzable phosphorus instead of ATP to indicate the changes of all high energy phosphate compounds: DPN, TPN, etc.

TABLE 2

Distribution pattern of total phosphorus, total inorganic, total acid-soluble and total acid-insoluble phosphorus in muscle, liver and brain of rabbits maintained with a choline-deficient diet [1]

Tissue	Group	Total phosphorus	Total inorganic phosphorus	Total acid-soluble phosphorus	Total acid-insoluble phosphorus
		mg/g wet tissue	*mg/g wet tissue*	*mg/g wet tissue*	*mg/g wet tissue*
Muscle	normal	2.99 ± 0.20 [2]	1.63 ± 0.18	2.20 ± 0.27	0.72 ± 0.10
	dystrophic (90–95%)	2.15 ± 0.14	0.88 ± 0.20	1.38 ± 0.20	0.76 ± 0.08
	normal	4.28 ± 0.06	0.51 ± 0.05	2.31 ± 0.38	2.87 ± 0.15
Liver	dystrophic (90–95%)	4.18 ± 0.10	0.42 ± 0.03	1.65 ± 0.30	2.62 ± 0.10
	normal	4.18 ± 0.32	0.81 ± 0.15	1.32 ± 0.20	2.73 ± 0.20
Brain	dystrophic (90–95%)	4.18 ± 0.28	0.59 ± 0.10	1.05 ± 0.18	3.01 ± 0.28

[1] See table 1, footnotes 1 and 2 for experimental conditions.
[2] SD.

TABLE 3

Changes in ATP (muscle), 7-minute hydrolyzable phosphorus (liver, brain) and creatine concentration in muscle, liver and brain of rabbits maintained with a choline-deficient diet [1]

Tissue	Group	ATP (muscle) and 7-minute hydrolyzable phosphorus (liver, brain)	Creatine
		mg/g wet tissue	*mg/g wet tissue*
Muscle	normal	0.38 ± 0.08 [2]	3.33 ± 0.33
	dystrophic (90–95%)	0.21 ± 0.06	2.22 ± 0.38
Liver	normal	0.26 ± 0.07	0.25 ± 0.08
	dystrophic (90–95%)	0.31 ± 0.05	1.10 ± 0.18
Brain	normal	0.22 ± 0.04	2.44 ± 0.31
	dystrophic (90–95%)	0.26 ± 0.06	2.88 ± 0.40

[1] See table 1, footnotes 1 and 2 for experimental conditions.
[2] SD.

in the DNA concentration in the muscle of choline-deficient rabbits could be an indication of the ability of the degenerated muscle fibers to regenerate. This increase in DNA concentration may also be due to the infiltration of degenerated muscle fibers by macrophages. Recently such a possibility has been suggested by Weinstock et al. (9), who observed a significant increase in the DNAase II activity in the muscle of animals with nutritional and hereditary dystrophy. These authors concluded that most of the increase in DNAase II activity and DNA content could be due to infiltration, hence causing a block in the loss of DNA from dystrophic muscle. It was also suggested by these authors (9) that high levels of DNAase II in the dystrophic muscle is an indication of the ability of damaged muscle to regenerate. Walker (10) has recently shown that dystrophic muscle regenerates faster than normal muscle which confirms the hypothesis of Krupnick et al. (8) and Weinstock et al. (9). An increased rate of synthesis of DNA and RNA in the skeletal muscle of dystrophic animals can also explain the high concentration of these 2 nucleic acids in the muscle. Such observations have been noted by Srivastava and Sarkar [10] in their studies on nucleic acid metabolism in the muscle of rabbits and mice with nutritional and herditary dystrophy. These observations were in agreement with the work of other investigators (2, 8–10).

The numerous studies on all laboratory animals with nutritional and hereditary dystrophy show without exception an ab-

[10] Unpublished data, U. Srivastava and N. K. Sarkar.

normal phosphorus metabolism in the muscle. Such an abnormality is also observed in rabbits maintained with a choline-deficient diet. Total acid-insoluble phosphorus does not undergo any change in the muscle of choline-deficient rabbits. Many explanations can be provided for this anamoly. One of the most likely possibilities in our opinion appears to be that the proportion of phospholipid phosphorus in the acid-insoluble fraction is very low because of choline deficiency. This hypothesis is being tested in our laboratory. The ATP concentration in the muscle is reduced by 44%. This is important as any variation in ATP and creatine phosphate concentration reflects an abnormality in the process of muscle contraction and relaxation. Creatine concentration in the muscle is considerably decreased and always parallels the decrease of ATP in the muscle of these affected animals. This at least suggests that a sufficient amount of ATP in such muscles is not available for phosphorylation of creatine. A marked similarity exists in the ability of the liver to synthesize creatine in experimental animals with either hereditary dystrophy or dystrophy produced by vitamin E or choline deficiency. The increase in the liver creatine and decrease in muscle creatine appears to be a typical symptom manifested in experimental animals with hereditary or nutritional dystrophy.

The present work illustrates that the decrease in the content of organic phosphorus in the muscle and liver of rabbits maintained with a choline-deficient diet, corroborates observations of Kasuga (11), indicating that the organophosphorus materials are not available as a metabolic pool for the normal maintenance of the tissue. A close parallelism has been observed in animals with hereditary and nutritional dystrophy (induced either by vitamin E deficiency or choline deficiency) in that there is a decrease of creatine concentration in the muscle with an increase in the liver.

Although hereditary muscular dystrophy is a genetic disease, several of the biochemical abnormalities in the muscle and liver of mice with hereditary dystrophy follow the same pattern of changes normally observed in the muscle and liver of rabbits maintained with vitamin E-deficient diet. The results of the present study, dealing with the changes in nucleic acid, phosphorus and creatine metabolism in various tissues of animals maintained with a choline-deficient diet show a close similarity to that noted in muscle, liver and brain of animals with nutritional (vitamin E-deficient diet) and hereditary dystrophy.

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Cytochrome Oxidase in Mice and Rats Maintained with a Meat Diet

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ABSTRACT Young mice and rats were fed for 6 weeks either a meat diet offered unsupplemented or supplemented with copper, calcium and zinc or certain combinations of these, or a nutritionally adequate semipurified diet. Copper concentration of liver and cytochrome oxidase activity of liver and kidney were decreased in animals fed meat in comparison with those of controls. No close correlation was found between the activities of cytochrome oxidase of liver and kidney. Supplementation of meat with calcium or copper increased activity of cytochrome oxidase of liver, whereas zinc decreased it. Calcium and copper raised the level of liver copper and lowered that of liver zinc. In mice, but not in rats, reduction of copper and cytochrome oxidase in liver were accompanied by anemia. The addition of copper to liver homogenate from rats fed meat had no significant effect on the low cytochrome oxidase activity; cytochrome oxidase was not affected by the addition of zinc to homogenates of liver from rats fed meat plus copper. It is concluded that the decreased activity of liver cytochrome oxidase of mice and rats subsisting on meat results from an excess of zinc which is insufficiently counteracted by copper and accentuated by a concomitant lack of calcium. The adverse effect of excess of zinc on erythrogenesis is not mediated through its action on cytochrome oxidase.

Anemia has been reported to develop in mice subsisting on a diet composed entirely of meat (1, 2). Further studies (3) have shown that the anemia results mainly from an excess of zinc which is insufficiently counteracted by the small amounts of copper present in meat. Dietary excess of zinc has been reported to induce signs of copper deficiency including anemia which can be prevented by adding copper to the diet (4–9). The zinc toxicity is further accentuated by the deficiency of calcium in meat (3), as calcium interferes with its intestinal absorption (10–12). Rats, in contrast with mice, are rather insensitive to the anemia-producing effect of zinc on the hematopoietic system (3).

Copper deficiency diminishes the activity of cytochrome oxidase, particularly in liver (13). Zinc poisoning, which is accompanied by subnormal concentrations of copper in the liver (14, 15), leads to a lowering of cytochrome oxidase activity of the liver (15) and the heart (6, 14), which can be overcome by a dietary supplement of copper (6).

We have studied the effect of a meat diet on cytochrome oxidase activity of liver and kidney in mice and rats. The question whether the effect of meat supplemented by copper, zinc and calcium on cytochrome oxidase parallels its effect on hemoglobin level was of particular interest.

MATERIALS AND METHODS

Male mice and rats, 3 to 4 weeks old, were maintained with their experimental diets for 6 weeks.

The experimental diets consisted of either raw, minced beef muscle or of a semipurified diet: (in per cent) casein, 18; cornstarch, 73; vegetable oil, 5; and salts (USP XIII, no. 2),[1] 4. This diet was supplemented with the water-soluble vitamins as indicated previously (3). Each mouse received 50 IU vitamin A and 2 IU vitamin D twice weekly and each rat 100 IU vitamin A and 4 IU vitamin D. In some experiments the meat diet was supplemented with 2 mg of copper (as $CuSO_4 \cdot 5H_2O$), 100 mg of zinc (as $ZnCl_2$) and 3600 mg of calcium (as $CaCO_3$), respectively, per kg of meat. The indicated

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[1] Contained: (in per cent) calcium biphosphate, 13.58; calcium lactate, 32.70; ferric citrate, 2.97; magnesium sulfate, 13.70; potassium phosphate (dibasic), 23.98; sodium biphosphate, 8.72; and sodium chloride, 4.35.

amounts of copper and calcium were previously found to prevent anemia which develops in mice fed meat, whereas 100 mg of zinc/kg meat induces anemia in mice maintained with meat supplemented with 2 mg of copper/kg (3).

At the end of the experimental period the animals were killed. The livers and kidneys were removed, homogenized and cytochrome oxidase was manometrically determined as described by Umbreit et al. (16).

Chemical examinations. Hemoglobin was determined by the cyanmethemoglobin method (17). Copper was determined as suggested by the Society for Analytical Chemistry (18). Zinc was determined colorimetrically, as described by the Society for Analytical Chemistry (18). The following modifications, however, were adopted. Chloroform was used for extraction in place of carbon tetrachloride and the chloroform-dithizone solution was washed with alkalinized water (pH 10 to 11) to remove impurities and free dithizone. Color intensity of the zinc-dithizone was measured in a Klett photocolorimeter, using filter no. 50.

RESULTS

Table 1 presents data on the amount of calcium, copper and zinc in the diets. As animals eat for calories rather than for weight of food, the approximate concentrations of the minerals per 1000 kcal have been calculated. These indicate that meat is very low in calcium and contains large amounts of zinc.

The first series of experiments was conducted in mice. Five experimental groups were maintained with meat which was offered either unsupplemented or supplemented with copper, calcium and zinc or certain combinations of these, and one group was fed the semipurified diet. Table 2 presents the results.

Cytochrome oxidase in liver and kidney, copper content of liver, hemoglobin and weight of mice with the unsupplemented meat diet were much lower than in the controls. Supplementation with copper significantly increased all values; addition of calcium had the same effect, except on kidney cytochrome oxidase. Addition of zinc to copper supplemented meat depressed cytochrome oxidase, copper content of liver and hemoglobin, whereas addition of calcium to meat supplemented with both copper and zinc largely abolished the effect of zinc (except that on kidney cytochrome oxidase). It appears that cytochrome oxidase and the copper content of liver and hemoglobin level are roughly correlated. Apparently no such correlation exists for kidney cytochrome oxidase. The copper content of liver increased when meat was supplemented with copper or calcium and decreased when zinc was added, thus confirming previous reports (14, 15). Weight was lowest in mice subsisting on unsupplemented meat and highest in animals offered the semipurified diet or meat supplemented with calcium. Addition of zinc appeared to depress weight.

In the second series of experiments, 3 groups of rats were used. They were fed meat either unsupplemented or supplemented with copper or with calcium (table 3). Both supplements increased cytochrome oxidase of liver. Concentration of copper in liver was increased and that of zinc decreased. Hemoglobin and weight were not affected. Cytochrome oxidase of kidney was likewise not affected by supplementation with copper but increased when calcium was added to the diet.

Cytochrome oxidase and copper concentration of liver of rats respond to supple-

TABLE 1

Calcium, copper and zinc content in meat and in semipurified diet

Mineral	Meat		Semipurified diet	
	mg/kg	*mg/1000 kcal* [1]	*mg/kg*	*mg/1000 kcal* [1]
Calcium	95 ± 5.9 [2] (12)	80	4340 ± 11(4)	1100
Copper	1.29 ± 0.043(18)	1.0	3.21 ± 0.12(8)	0.8
Zinc	42.1 ± 0.74(16)	35	5.08 ± 0.053(7)	1.3

[1] Approximately.
[2] Results are expressed as averages ± SE of mean. Figures in parentheses indicate number of analyses.

TABLE 2

Cytochrome oxidase of liver and kidney, copper concentration of liver, hemoglobin and weight of mice maintained for 6 weeks with different diets

Group	Diet	Supplement			Cytochrome oxidase		Copper content of dry liver	Hemoglobin	Body wt
		Calcium	Copper	Zinc	Liver	Kidney			
		mg/kg	*mg/kg*	*mg/kg*	QO_2	QO_2	*mg/kg*	*g/100 ml*	*g*
1	Meat	—	–	—	110 ± 7.2 [1] (20)	169 ± 12.4 (22)	7.5 ± 0.96 (7)	4.6 ± 0.22 (24)	17.8 ± 0.67 (24)
2	Meat	—	2	—	232 ± 4.1 (20)	212 ± 2.8 (19)	11.9 ± 0.74 (21)	13.9 ± 0.51 (21)	22.6 ± 1.00 (22)
3	Meat	3600	–	—	200 ± 8.4 (11)	187 ± 9.0 (12)	18.4 ± 0.73 (9)	14.5 ± 0.51 (11)	24.7 ± 1.11 (12)
4	Meat	—	2	100	133 ± 14.4 (10)	170 ± 8.2 (10)	7.8 ± 1.55 (7)	7.0 ± 0.73 (10)	21.5 ± 1.20 (10)
5	Meat	3600	2	100	207 ± 15.2 (10)	201 ± 18.7 (10)	12.6 ± 1.47 (8)	13.4 ± 0.43 (10)	26.6 ± 0.73 (10)
6	Semi-purified	—	–	—	209 ± 13.3 (17)	258 ± 13.4 (18)	18.9 ± 1.78 (10)	14.3 ± 0.46 (21)	26.4 ± 1.51 (21)
t values [2]				1 vs. 2	7.8	3.6	3.7	16.9	4.0
				1 vs. 3	8.2		9.1	18.0	5.3
				2 vs. 4	5.0	6.2	2.4	7.8	
				4 vs. 5	3.6		2.2	7.5	3.6
				1 vs. 6	6.6	4.9	5.6	19.0	5.2
				2 vs. 6		3.4	3.1		2.1
				3 vs. 6		4.4			

[1] Results are expressed as averages ± SE of mean. Figures in parentheses indicate number of animals.
[2] Only significant t values ($P < 0.05$) are indicated.

TABLE 3

Cytochrome oxidase of liver and kidney, copper and zinc concentrations of liver, hemoglobin and weight of rats maintained for 6 weeks with meat

Group	Diet	Supplement: Calcium	Supplement: Copper	Cytochrome oxidase: Liver	Cytochrome oxidase: Kidney	Copper content of dry liver	Zinc content of dry liver	Hemoglobin	Body wt
		mg/kg	*mg/kg*	QO_2	QO_2	*mg/kg*	*mg/kg*	*g/100 ml*	*g*
1	Meat	—	—	74 ± 13.9 [1] (15)	112 ± 8.2 (6)	5.5 ± 0.57 (15)	110 ± 4.1 (10)	14.5 ± 0.58 (18)	152 ± 5.7 (18)
2	Meat	—	2	184 ± 8.1 (16)	119 ± 10.7 (5)	17.6 ± 0.72 (16)	81 ± 2.8 (10)	14.7 ± 0.30 (16)	138 ± 6.0 (16)
3	Meat	3600	—	206 ± 9.0 (8)	181 ± 10.1 (8)	15.6 ± 0.49 (8)	78 ± 6.3 (9)	13.9 ± 1.49 (8)	142 ± 4.8 (8)
t values [2]		1 vs. 2		6.9		13.2	5.9		
		1 vs. 3		8.0	4.3	8.6	4.3		
		2 vs. 3			4.1	2.3			

[1] Results are expressed as averages ± SE of mean. Figures in parentheses indicate number of animals.
[2] Only significant *t* values ($P < 0.05$) are indicated.

mentation of meat with copper and calcium as in mice but there is no parallel between cytochrome oxidase in liver and in kidney as has been observed in mice. It was again found that rats are resistant to the anemia producing effect of meat. This contrasts with the response of their liver cytochrome oxidase.

In the last experiments, copper and zinc were added to liver homogenates of rats and the effect of these on activity of cytochrome oxidase was studied. Nine rats were maintained with an unsupplemented meat diet and the liver cytochrome oxidase was measured. The QO_2 was found to be 101 (SE, 18.2). In a part of their livers cytochrome oxidase was measured after 2 μg of copper (as $CuSO_4$) had been added per gram of fresh liver. This supplement corresponds to the difference in copper content between the livers of rats fed unsupplemented and copper-supplemented meat. After adding copper the QO_2 increased by 6 to 107. The small difference proved to be statistically significant (SD of the difference, 6.3).

In another experiment 11 rats received the copper-supplemented meat diet. Liver QO_2 was 188 (SE, 10.0). Addition of 50 μg zinc (as $ZnCl_2$) per gram of fresh liver decreased the QO_2 to 186. This decrease was statistically insignificant (SD of the difference, 9.9).

DISCUSSION

Our experiments show that the activity of cytochrome oxidase and the concentration of copper are decreased in livers of mice and rats subsisting on meat. The decrease in mice, but not in rats, is accompanied by anemia. Both copper and calcium when added to meat increase the activity of cytochrome oxidase in liver but not necessarily in kidney. The increased activity of liver cytochrome oxidase is accompanied by an increase in the concentration of copper and, in rats, by a decrease of the level of zinc. In mice, supplementation of meat with zinc antagonizes the effects of copper on cytochrome oxidase and copper level of liver and on hemoglobin, and calcium the effects of zinc. These results confirm the well-known antagonism of zinc and copper (13) and of zinc and

calcium (21). They agree with the suggestion that calcium inhibits the intestinal absorption of zinc (11, 12, 21) and with our conclusion (3) that the effect of calcium on prevention of meat anemia in mice — confirmed by the data presented in this paper — resides on its hindering the entrance in the body of an excess of zinc through the intestinal wall.

The effects of copper, calcium and zinc on copper concentration of liver are accompanied by similar effects on cytochrome oxidase of the liver but not of the kidney. The activity of the enzyme in the kidney appears to respond less closely to the state of copper nutrition than to the enzyme level in the liver. Gallagher et al. (19) studied cytochrome oxidase in copper-depleted rats. Cytochrome oxidase in liver was severely impaired after 4 weeks, whereas the impairment was delayed in kidney and brain. The authors claim that the loss of activity of cytochrome oxidase containing a heme prosthetic group results from a failure of synthesis of the prosthetic group rather than of its protein. The fact that addition of copper to homogenates of liver with reduced activity of cytochrome oxidase results in a very small increase only, agrees with this conclusion (19, 20) and does not warrant the suggestion that it serves as activator of the enzyme. For the same reason it is impossible for zinc added to homogenate of livers with normal cytochrome oxidase to depress the activity of the enzyme. Zinc apparently lowers cytochrome oxidase when it is present at the time of formation of the enzyme.

The discrepancy between hemoglobin level, on the one hand, and liver copper and cytochrome oxidase, on the other, observed in rats, demonstrates that the adverse effect of zinc on hematopoiesis is not mediated through its inhibition of cytochrome oxidase. Similarly, there is no evidence that the role of copper on erythrogenesis is mediated through this particular function (13).

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Metabolic Patterns in Preadolescent Children

XIV. EXCRETION OF NIACIN OR TRYPTOPHAN METABOLITES BY GIRLS FED CONTROLLED DIETS SUPPLEMENTED WITH NICOTINAMIDE [1,2]

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ABSTRACT A metabolic study was carried out with twelve 7- to 9-year-old girls maintained with 5 controlled diets varying in niacin content from 10 to 31 mg/day. The adjustment diet and two of the four experimental diets were supplemented with crystalline nicotinamide in gelatin capsules. Urinary excretion of N^1-methyl-nicotinamide, N^1-methyl-2-pyridone-5-carboxamide, niacin, and quinolinic acid were measured during an adjustment period and during 6 consecutive 6-day experimental periods. Subjects fed a low-protein, low-riboflavin diet or a moderate-protein, adequate-riboflavin diet supplemented with 10.3 mg/day of nicotinamide excreted 8.9 and 8.2 mg more of niacin metabolites (NM) per day than did girls fed the same diets without supplemental niacin. The girls fed the low-protein, low-riboflavin diet supplemented with crystalline nicotinamide excreted more NM than predicted from a regression equation. Thus it is possible that the low protein or riboflavin content, or both, of this diet resulted in a lowered requirement for niacin by these subjects or interfered with their utilization of the added nicotinamide. Almost constant excretion of NM was attained after the girls were fed the controlled diets for only 6 days.

Moyer and associates (1) reported data from 3 metabolic studies on excretion of several niacin [3] metabolites by 7- to 9-year-old girls maintained with relatively constant diets of known composition. This report presents data from an additional study of the same series in which the diets contained no foods of animal origin and three of the five diets were supplemented with crystalline nicotinamide.

EXPERIMENTAL PROCEDURES

A detailed description of the study and information on the kinds and amounts of foods in each diet, together with data on the physical and biochemical status of each subject, are presented in Southern Cooperative Series Bulletin no. 94 (2).

The study was conducted with 12 healthy 7- to 9-year-old girls consuming controlled diets made up of plant products and supplemental nutrients. All subjects were given diet 13 during an adjustment period of 6 days. Plant protein was fed at 2 levels during six 6-day experimental periods (table 1); one group of 6 girls received about 22 g of protein (diets 9 and 10) and the other group of six received approximately 40 g of protein (diets 11 and 12). Diet 9 differed from diet 10, and diet 11 from diet 12, only in the supplemental nutrients supplied in capsules. The capsules containing the supplements were inadvertently transposed throughout all experimental periods and as a result the niacin and riboflavin content of the 4 diets was markedly different.

Analyses were made on food composites and urine composites collected during the 7 consecutive 6-day periods. Analyses of urine for N^1-methylnicotinamide, N^1-methyl-2-pyridone-5-carboxamide, niacin, and quinolinic acid content and of food for niacin content were carried out by the same methods used previously (1). Tryptophan content of the food was calculated

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[2] This study was a phase of the Southern Regional Research Project, Requirements and Utilization of Selected Nutrients by Preadolescent Children, supported in part by funds appropriated to the U. S. Department of Agriculture under the Research and Marketing Act of 1946, and the Hatch Act, as amended. The Human Nutrition Research Division of the Agricultural Research Service was a cooperator in this project.

[3] Niacin denotes the vitamin whether it is in the amide or free acid form.

from Home Economics Research Report no. 4 (3).

RESULTS AND DISCUSSION

Table 1 shows the average weight of the group of girls fed each diet and the average daily contents of nitrogen, energy, riboflavin, tryptophan and niacin in the diets. Foods of the adjustment diet furnished 9.0 mg of niacin and 479 mg of tryptophan daily. Of the experimental diets, foods of the low protein diet supplied 4.5 mg of niacin and 330 mg of tryptophan daily and those of the high protein diet furnished 10.9 mg of niacin and 608 mg of tryptophan daily. The adjustment diet was supplemented with 5.75 mg of nicotinamide/day and diets 9 and 11 were supplemented with 10.3 mg of nicotinamide/day.

The prestudy diets of the girls calculated from records of their self-selected food intake for 2 weeks indicated that the average daily intake of niacin was 12.8 (range 8.6 to 18.7) mg/day. These data do not include an estimate of niacin formed from dietary tryptophan.

The niacin and tryptophan content of each diet is expressed as niacin-equivalants (NE) in table 2. This table also contains the average values for urinary excretion of N^1-methylnicotinamide (N^1-Me), N^1-methyl-2-pyridone-5-carboxamide (pyridone), niacin and quinolinic acid by the subjects fed each diet. The sum of N^1-Me and pyridone, both expressed in terms of niacin, plus the niacin is shown as a combined value for niacin metabolites (NM) for each group.

TABLE 1

Average weight of girls and daily nitrogen, energy, riboflavin, niacin and tryptophan content of the diets

Diet no.[1]	Wt of subject	Average daily intake						
		Nitrogen	Energy	Riboflavin		Niacin		Tryptophan
		Food [2]	Food [2,3]	Food [4]	Supplement	Food	Supplement	Food [4]
	kg	*g*	*kcal*	*mg*	*mg*	*mg*	*mg*	*mg*
13	27.6	6.2	2206	0.5	0.8	9.0	5.8	479
9	26.6	3.6	2179	0.4	0.2	4.5	10.3	330
10	27.6	3.6	2220	0.4	0.0	4.5	0.0	330
11	30.0	6.4	2379	0.6	1.6	10.9	10.3	608
12	27.9	6.3	2262	0.6	1.4	10.9	0.0	608

[1] All 12 girls were fed diet 13 for the initial 6-day adjustment period. Each of the other diets was given to 3 girls for six 6-day periods.
[2] Small differences in the average values for nitrogen and energy intake between subjects fed diets 9 and 10 and between those fed diets 11 and 12 are due to additional foods given to 3 girls to maintain weight and to corrections for foods not eaten and for vomitus (2).
[3] Published with the permission of the Louisiana Agricultural Experiment Station.
[4] Values calculated from tables of food composition (3, 8).

TABLE 2

Intakes of niacin equivalents (NE) and urinary excretions of N^1-methylnicotinamide (N^1-Me), *pyridone, nicotinic acid and quinolinic acid*

Diet no.[1]	Average daily intake			Average daily urinary excretion					
	NE [2]	NE/1000 kcal	NE/kg body wt	N^1-Me	Pyridone	Niacin	Combined NM [3] Total	Combined NM [3] % of NE	Quinolinic acid
	mg	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*%*	*mg*
13	22.7	10.3	0.82	3.8	9.0	0.35	11.1	48	2.8
9	20.3	9.3	0.77	5.3	8.5	0.30	12.0	59	2.7
10	9.8	4.5	0.36	1.5	1.8	0.25	3.1	31	2.4
11	31.3	13.2	1.06	5.0	12.6	0.37	15.1	48	2.9
12	20.6	9.1	0.75	2.9	4.9	0.34	6.9	34	3.2

[1] All 12 girls were fed diet 13 for the initial 6-day adjustment period. Each of the other diets was given to 3 girls for six 6-day periods.
[2] Niacin equivalent indicates dietary niacin plus 1/60 dietary tryptophan.
[3] Combined niacin metabolite (NM) excretion indicates N^1-Me plus pyridone (both expressed in terms of nicotinic acid) plus nicotinic acid.

The mean and standard deviation of excretion of each of the 4 metabolites by each subject during the 6 experimental periods are shown in table 3.

Urinary excretion of NM in relation to NE intake. Subjects fed diets 13, 9, and 11 containing supplemental nicotinamide in gelatin capsules excreted more NM than subjects fed unsupplemented diets 10 and 12. The subjects were given diet 13 only during the initial 6-day adjustment period, and excretion of NM during this time was probably influenced by pre-experimental dietary regimens. Diets 9 and 11 were supplemented with 10.3 mg of nicotinamide, and the girls fed these diets excreted 8.9 and 8.2 mg more of NM than those fed the unsupplemented diets 10 and 12, respectively.

The regressions of urinary NM on NE-intake expressed as milligrams per day and as mg/1000 kcal were calculated from data in the previous studies (1), and approximately 70% of the variation in NM excretion was explained by variation in NE-intake in each equation. Excretion of NM by subjects in this study fed diets 9, 10, 11, and 12 were 122, 82, 94, and 70%, respectively, of predicted values based on NE-intake in milligrams per day and 133, 67, 120, and 78%, respectively, of predicted values based on NE-intake in mg NE/1000 kcal.

Diets 9 and 12 contained almost the same amount of NE but subjects fed diet 9 excreted about 5 mg/day more of NM than those fed diet 12. In addition to having about one-half of its NE in supplemental form, diet 9 contained less protein and much less riboflavin than diet 12.

These data indicate that the supplemental nicotinamide in these diets may not have been utilized in the same manner as the NE supplied by niacin and tryptophan in the foods, but the possibility that the low riboflavin or protein content, or both, of diet 9 interfered with utilization of niacin by the subjects fed this diet cannot be ruled out. The estimation that 60 mg of dietary tryptophan can be converted to 1 mg of niacin is based on the assumptions that supplemental niacin and tryptophan are metabolized in the same manner as are these nutrients supplied in foods (4, 5).

Excretion of N¹-Me and pyridone. In the previous studies (1) subjects fed 7 diets ranging in NE content from 14.5 to 25.5 mg/day excreted between 2.3 and 3.2 mg/day of N¹-Me. In this study the 3 subjects fed diet 10 excreted only 1.5 mg/day of N¹Me while the 6 subjects fed supplemented diets (9 and 11) excreted an average of 5.3 and 5.0 mg/day, respectively.

TABLE 3

Means and standard deviations of excretion of niacin-tryptophan metabolites by each girl fed controlled diets during six 6-day periods

Diet no.	Subject		N¹-Me [1]	Pyridone	Niacin	Quinolinic acid
	no.	wt				
		kg	*mg*	*mg*	*mg*	*mg*
9	42	23.6	5.5 ± 0.52 [2]	8.1 ± 0.31	0.30 ± 0.017	2.7 ± 0.14
	44	26.6	5.5 ± 0.69	7.8 ± 0.67	0.30 ± 0.031	2.8 ± 0.34
	50	29.8	4.8 ± 0.44	9.7 ± 0.59	0.31 ± 0.035	2.6 ± 0.20
10	40	25.3	1.4 ± 0.20	1.9 ± 0.70	0.24 ± 0.023	2.1 ± 0.12
	46	27.9	1.7 ± 0.47	2.0 ± 0.26	0.24 ± 0.017	2.5 ± 0.31
	49	29.6	1.5 ± 0.14	1.6 ± 0.87	0.26 ± 0.043	2.7 ± 0.58
11	47	25.4	4.7 ± 0.81	11.2 ± 0.79	0.36 ± 0.036	2.8 ± 0.34
	43	30.0	4.6 ± 0.75	12.1 ± 0.92	0.33 ± 0.019	2.8 ± 0.12
	41	34.6	5.6 ± 0.35	14.5 ± 1.10	0.42 ± 0.027	3.1 ± 0.26
12	39	24.9	2.8 ± 0.39	6.1 ± 0.83	0.32 ± 0.024 [3]	3.7 ± 0.07 [3]
	48	28.8	2.8 ± 0.21	3.9 ± 0.47	0.35 ± 0.023	2.6 ± 0.26
	45	30.0	3.2 ± 0.47	4.6 ± 0.85	0.34 ± 0.030	3.2 ± 0.13

[1] Indicates N¹-methylnicotinamide.
[2] Mean ± SD.
[3] Mean and SD for 4 periods only. During the last two periods this subject was given antibiotics which interfered with the microbiological assays for these two metabolites.

Excretion of pyridone ranged from 1.8 mg/day by subjects fed diet 10 to 12.6 mg/day by subjects fed diet 11. From the average excretion of pyridone by subjects fed 11 of the 12 diets in the 4 metabolic studies (1, 2) (omitting adjustment diet 13), the regression of pyridone (Py) excretion on NE-intake was calculated ($Py = 0.45\ NE - 2.41$, $r^2 = 0.66$). Subjects fed the supplemented diets 9 and 11 excreted more pyridone than was predicted by the equation.

The molar ratio of excretion of pyridone to N^1-Me was less than 2.0 for all subjects except those fed diet 11, for which the average ratio was 2.3. The average ratio for those fed both diets 9 and 12 was 1.6 and for those fed diet 10 was 1.1. In the previous studies (1) the lowest ratio found was 2.0 when subjects were fed a diet containing 14.5 mg NE/day. Using a semipurified diet containing foods from plants supplemented with amino acids, Vivian et al. (6) found the ratio of pyridone to N^1-Me to be 1.42 when the diet supplied 21 mg of NE. These values are similar to the ratio of urinary metabolites and NE intake of the subjects fed diets 9 and 12 in this study. In experimental pellagra, Goldsmith and associates (7) observed that at low levels of excretion about equal amounts of pyridone and N^1-Me were excreted. The low ratio of pyridone-to-N^1-Me excretion by the girls fed diet 10 probably indicates that the NE intake of 9.8 mg/day was no more than marginal. These girls were fed the low niacin diet for only 36 days and no evidence of vitamin deficiency was noted when they were examined by a pediatrician at the end of the study.

Excretion of niacin and quinolinic acid. Excretion of niacin by all subjects ranged from 0.2 to 0.4 mg/day regardless of intake. Low, nearly constant excretion of niacin has been reported in several studies.

Excretion of quinolinic acid was generally lower than that in the previous studies, especially for subjects fed diet 10. However, even at this low level of NE intake, quinolinic acid excretion was less variable than pyridone excretion and all 3 subjects fed this diet excreted more quinolinic acid than pyridone.

Variation in excretion of NM with constant diets. In studies with adults fed constant diets, Goldsmith et al. (7) and Horwitt et al. (4) noted that several weeks were required for excretion of niacin metabolites to become constant. The preadolescent girls fed the controlled diets in this study attained essentially constant levels of excretion in a few days, as indicated by the relatively low standard deviations of mean excretion of the metabolites by each subject during the 6 experimental periods (table 3). For example, the subjects fed diet 10 excreted 8.0, 2.8 and 1.7 mg of pyridone/day during the adjustment period and the first and second experimental periods, respectively. The overall mean for the 6 experimental periods was 1.8 mg/day, indicating that when the subjects were changed from a diet (no. 13) containing 22.7 mg NE/day to one supplying only 9.8 mg/day (no. 10), a nearly constant level of excretion was obtained within 6 days.

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Effect of Carbohydrates of Leguminous Seeds, Wheat and Potatoes on Serum Cholesterol Concentration in Man [1]

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ABSTRACT Two dietary comparisons were made to study the effect of natural carbohydrates on serum cholesterol concentration in man. In the first experiment, sucrose and gluten were substituted isocalorically for the carbohydrate and protein of bread and potatoes. The exchanges involved (as percentage of total energy intake) were 17 and 2% of carbohydrate and protein, respectively. In the second similar experiment, sucrose and soybean protein were substituted for leguminous seeds. The corresponding exchanges were 16 and 2% of total energy. The sucrose diet and the bread and potato diet caused identical serum cholesterol levels, whereas the diet containing leguminous seeds caused a serum cholesterol level lower by 9% than that of the sucrose diet. It is concluded that the substitution in the diet of the natural carbohydrates of leguminous seeds for sucrose produces a lowering of serum cholesterol concentration in man.

Total serum cholesterol concentration in man has been shown to be influenced in a predictable manner by changing the fatty acid composition of the diet (1–3).

We have previously reported that the nature of the dietary carbohydrate is also important with respect to the serum cholesterol level, apart from any influence of fat. A diet high in fruits, vegetables and legumes was shown to give lower serum cholesterol levels than a diet comparable in other respects, but providing 17% of the total calories from sucrose and milk carbohydrates in place of the carbohydrates from fruit and vegetables (4). It was also shown that a daily supplement of 15 g of pectin produces an average decrease of serum cholesterol of about 5%, but the same amount of cellulose failed to show any significant effect on serum cholesterol concentration (5). The lack of effect of cellulose has been confirmed by Prather (6) in young women. More recently we have shown that isocaloric substitution of glucose, sucrose and lactose for each other does not change the serum cholesterol concentration in man (7) and a similar observation has been reported by Shama'A and Al-Khalidi (8). The experiments to be reported here were planned to compare the effects on serum cholesterol of the carbohydrates of bread and potatoes with those of the carbohydrates of leguminous seeds, when they were isocalorically substituted for sucrose. In the first experiment (exp. S), sucrose was compared with the carbohydrates of a mixture of bread and potatoes; in the second experiment (exp. U) sucrose was compared with the carbohydrates of a mixture of leguminous seeds.

SUBJECTS, METHODS AND PROCEDURE

The subjects, as in previous experiments, were physically healthy middle-aged male mental patients long resident at the Hastings State Hospital, maintained under completely controlled conditions in a metabolic unit. In each experiment the men were maintained with a stardard diet for a 3-week stabilization period and then were assigned to dietary subgroups matched as to age, relative body weight, activity habits, psychiatric diagnosis and general level of serum cholesterol. Thereafter, for successive 3-week periods, the subgroups were assigned the experimental diets in a switch-back or reversal pattern designed to compensate for possible time trends. The design of the present experiments has been previously reported (5).

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The methods and procedures were the same as in previously reported experiments (1–4). Fasting blood samples were drawn from each man on 2 occasions at the end of each dietary period and the serum was analyzed for total cholesterol in duplicate by the modification of Anderson and Keys (9) of the method of Abell et al. (10).

Diets. The diets were composed of natural foods in a rotating series of 7 daily menus of 3 meals corresponding with ordinary American customs. All of the diets were similar in calories, fat and cholesterol content, but differed in the source of part of the carbohydrates and proteins.

The nutrient content of these diets was estimated from standard food tables. Individual differences in calorie requirements were observed during the pre-experimental stabilization periods and individual allowance was made by adjusting the amounts of sweets and bread. All servings were individually measured and rejections and plate waste were recorded. Constancy of calorie expenditure was maintained by a closely supervised regimen of exercise, rest and recreation. The subjects maintained constant body weight as in previous studies (5). Some of the subjects in experiment S received the experimental diets containing a daily supplement of 15 g of cellulose.[2] Experiment U was repeated with all the men receiving a daily supplement of 15 g of pectin.[3]

The average composition of the diets (amounts actually eaten) in experiments S and U, exclusive of the cellulose and pectin supplements, is presented in table 1.

TABLE 1

Average composition of the diets in experiments S and U;[1] *amounts eaten per man per day*

	Experiment	
	S	U
Calories, kcal	3060	3040
Protein, g	97	124
Total fat, g	138	138
Total carbohydrate, g	358	326
Cholesterol, mg	568	576
Fat calories as % of total calories:		
Total fat, kcal	40	40
Saturated,[2] kcal	20	20
Monoene, kcal	18	18
Polyene, kcal	2	2

[1] The cellulose and pectin supplements are not included.

[2] Saturated, monoene and polyene refer to glycerides of saturated, monoene and polyene fatty acids.

The dietary comparisons in these experiments involved the exchange of a part of the carbohydrates and of the proteins. In experiment S, the diet SS contained 128 g of sucrose and 15 g of gluten in place of 121 g of carbohydrates and 16 g of proteins from bread and potatoes contained in diet SB. These 2 diets were fed alternately to 12 men, while the other 13 men received the same diets supplemented with 15 g/day of cellulose. The carbohydrates and proteins exchanged in this experiment are described in table 2.

In experiment U, diet US contained 123 g of sucrose and 45 g of soybean protein [4] in place of 115 g of carbohydrate and

[2] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

[3] Pure citrus pectin, lot 444 H, donated by Sunkist Growers Company, Ontario, California.

[4] ADM C-1 Assay Protein, Archer-Daniels-Midland Company, Minneapolis.

TABLE 2

Comparison of carbohydrate and proteins exchanged in experiment S;[1] *amounts eaten per man per day*

Diet [2]	Carbohydrate			Protein		
	Sucrose	Bread and potatoes	Sum	Added gluten	Bread and potatoes	Sum
	g	*g*	*g*	*g*	*g*	*g*
S S	148	104	252	15	13	28
S B	20	225	245	—	29	29
Δ	128	121		15	16	
S S+C	163	112	275	15	15	30
S B+C	27	224	251	—	29	29
Δ	136	112		15	14	

[1] Carbohydrates are sucrose and starch from bread (2/3) and potatoes (1/3). Proteins are the added gluten and the proteins from bread and potatoes.

[2] S S = sucrose and gluten diet; S B = bread and potato diet; and C = cellulose supplement (15 g/day).

48 g of proteins from a mixture of equal parts of beans (*Phaseolus vulgaris*), lima beans (*Phaseolus lunatus*) and split peas (*Pisum sativum*) contained in diet UB. In this experiment the comparison of diets US and UB was made with all the men, and was repeated again with all the men using the same diets including a daily supplement of 15 g of pectin as described elsewhere (5). The carbohydrates and proteins exchanged in this experiment are described in table 3.

TABLE 3

Comparison of carbohydrate and proteins exchanged in experiment U; [1] *amounts eaten per man per day*

Diet [2]	Carbohydrate			Protein		
	Sucrose	Beans	Sum	Soy	Beans	Sum
	g	*g*	*g*	*g*	*g*	*g*
U S	139	3	142	45	—	45
U B	16	118	134	—	48	48
Δ	123	115		45	48	
U S+P	139	3	142	45	—	45
U B+P	16	118	134	—	48	48
Δ	123	115		45	48	

[1] Carbohydrates are sucrose and the carbohydrates from the leguminous seeds. Proteins are the added soybean protein and the proteins from the leguminous seeds.
[2] U S = sucrose and soybean protein diet; U B = bean diet; and P = pectin supplements (15 g/day).

TABLE 4

Total serum cholesterol in experiment S at the end of 3 weeks with each of the 4 diets and the serum cholesterol differences due to exchanging diets

Groups	R, T		Q, S	
No. of men	12		13	
Diet [1]	S S	S B	S S + C	S B + C
	mg/100 ml		*mg/100 ml*	
Serum cholesterol:				
Mean	214	210	230	225
Standard deviation	39.9	37.1	40.4	37.5
Δ Cholesterol: [2]				
Mean [3]	4		5	
Standard error of mean [3]	5.7		3.3	

[1] S S = sucrose diet; S B = bread and potato diet; S S + C = sucrose diet + cellulose (15 g/day); and S B + C = bread and potato diet + cellulose (15 g/day).
[2] Δ Cholesterol = serum cholesterol with the sucrose diet *minus* serum cholesterol with the bean diet.
[3] Calculated from the serum cholesterol differences, between the 2 dietary situations, of each man.

TABLE 5

Serum cholesterol for the 24 men in experiment U at the end of 3 weeks with each of the 4 diets and serum cholesterol differences due to exchanging diets

Diet [1]	Serum cholesterol	Δ Cholesterol [2]	P value
	mg/100 ml	*mg/100 ml*	
Sucrose U S	221 ± 36.5 [3]	Δ[3] 19 ± 5.2 [4]	0.0015
Bean U B	202 ± 33.6		
Sucrose U S+P	211 ± 35.3 [3]	Δ 18 ± 3.2 [4]	< 0.0001
Bean U B+P	193 ± 35.3		

[1] U S = sucrose diet; U B = bean diet; U S + P = sucrose diet + pectin (15 g/day); and U B + P = bean diet + pectin (15 g/day).
[2] Δ Cholesterol = serum cholesterol with the sucrose diet *minus* serum cholesterol with the bean diet.
[3] Mean ± SD.
[4] Mean ± SE; calculated from the serum cholesterol differences, between the 2 dietary situations, of each man.

RESULTS

The results of experiment S are presented in table 4. The serum cholesterol concentration was slightly higher with the sucrose diet than with the bread and potato diet, and a similar difference was observed when the comparison was made adding 15 g/day of cellulose. These differences were, however, not statistically significant.

The results of experiment U are presented in table 5. The mean serum cholesterol was higher with the sucrose diet than with the bean diet and a similar difference was observed when the comparison was repeated with the 24 men with the daily addition of 15 g of pectin. The mean differences were statistically highly significant.

DISCUSSION

The results of these experiments indicate that the substitution of carbohydrates from leguminous seeds for sucrose causes a significant decrease of serum cholesterol concentration, but no significant difference was observed when carbohydrates from bread and potatoes were substituted for sucrose. This result is in agreement with our previous observations about the effect on serum cholesterol concentration of diets containing carbohydrates from fruits, leafy vegetables and legumes in place of sucrose and milk sugar.

Other reports have confirmed the hypocholesterolemic effect of diets containing a high proportion of leguminous seeds. Mathur et al. (11) noted that people of low socioeconomic status in Agra (India), whose staple diet was chick peas (*Cicer arietinum*), had much lower serum cholesterol than those not consuming that diet. In the Netherlands, Groen et al. (12) observed that Trappist monks had lower serum cholesterol than Benedictines and noted that leguminous seeds are an important part of the diet of the former. In experiments with 20 volunteers, Luyken et al. (13) showed that introduction of 100 g of legumes (mainly brown beans) caused a mean serum cholesterol lowering of 12 mg/100 ml. Since the dietary exchange in our experiments involved not only an exchange of carbohydrates but also an exchange of proteins, the possibility must be considered that differences in the proteins of the diets might have played a role in the production of the serum cholesterol changes. The exchange of proteins in experiment U involved the substitution of 48 g of proteins from leguminous seeds (beans, lima beans and split peas) for 45 g of soybean protein. An examination of the amino acid composition of these proteins (14, 15) reveals that the greatest difference between diets was less than 0.5 g per man per day of any one of the essential amino acids or of cystine in a diet containing plenty of all the amino acids and 124 g of total proteins. On the other hand, previous experiments from this laboratory (16) and the recent work by Beveridge et al. (17) and by Connor et al. (18) have shown that marked changes in the protein content of the diet do not significantly modify the serum cholesterol concentration in man.

In 1958 Olson et al. (19) reported a significant decrease of serum cholesterol concentration in men changing from a diet high in protein to a diet containing only 25 g of protein/day, all from cereals and legumes. It is possible that the serum cholesterol change could have been due to gross protein deficiency; alternatively, the change in the carbohydrate character of the diet might have been responsible.

The cholesterol-depressing effect caused by the bean diet in our experiments could obviously be due to the presence in the beans of some unknown substance or substances having a hypocholesterolemic effect. In this respect, Mathur et al. (20) have reported that the cholesterol-depressing effect of *Cicer arietinum* in rats fed a diet containing cholesterol, and cholic acid, is also produced by the defatted seed and by its lipid extract.[5]

In the absence of evidence about the existence of a specific cholesterol-depressing substance in the beans, we postulate that the decrease of serum cholesterol concentration observed in experiment U was caused by the carbohydrate of the leguminous seeds when it was substituted for sucrose.

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[5] At the Asian-Pacific Congress of Cardiology in Kyoto, Japan, in May 1964 Mathur et al. reported results from 12 human volunteers in dietary experiments involving chick peas. With a diet high in butter fat the inclusion of chick peas apparently produced an average decrease of 16% (28 mg/100 ml) in the serum cholesterol level.

The fact that the substitution of wheat and potatoes for sucrose did not produce any significant change in serum cholesterol does not exclude the possibility that other starches may cause such a change as indicated by the work of Macdonald and Braithwaite using 500-g daily doses of raw maize starch (21). Finally, the effects of dietary carbohydrate on serum cholesterol concentration appear to be affected by the composition of the rest of the diet. Thus Winitz et al. (22) have reported that partial replacement of glucose with sucrose caused a significant elevation of serum cholesterol concentration in men consuming a chemical diet containing 2 g daily of ethyl linoleate as the only source of fat, and glucose as the only carbohydrate, providing about 90% of the total calories. On the other hand, we have observed that isocaloric substitution of sucrose for glucose (30% of total calories) caused no change of serum cholesterol concentration in men eating a diet made up of common foodstuffs (7).

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letters

EVALUATION OF THIAMINE ADEQUACY IN ADULT HUMANS

Ziporin et al. have recently reported two rather intriguing studies on the excretion of thiamine metabolites in urine (1, 2). There appeared in these papers information concerning the source of and the methodology for the assay of erythrocyte transketolase activity, and also some opinions on the interpretation of the assay results, which are worthy of comment.

1. On page 288 it was described that samples for transketolase determinations were "analyzed by the method of Wolfe et al. (3) as modified by Sauberlich and Bunce (4) . . . " It is noteworthy that Wolfe et al. did not present an assay for transketolase activity, but rather presented a clinical application of the assay of Brin et al. (5) to a type of human thiamine deficiency, Wernicke's encephalopathy. As a matter of record, the assay of Brin et al. (5) as applied by Wolfe et al. (3) employed intact erythrocytes, and glucose-2-C^{14} as substrate, and therefore the assay used by Sauberlich and Bunce (4) was *not* a direct modification of this procedure. Rather, the Sauberlich and Bunce (4) assay, which was described in the Burma report (4) was but a minor modification of the assay of Brin et al., which was presented at the Federation Meetings in 1959 and published in 1960 (6). This assay employed erythrocyte hemolysates rather than whole cells, and unlabeled ribose-5-phosphate for substrate rather than radioactive glucose. Both the disappearance of the ribose substrate and the appearance of hexose phosphate were used as parameters of thiamine deficiency in the assay, as well as the stimulation which resulted from adding thiamine pyrophosphate to the system which is now called the "TPP-effect." That the Sauberlich and Bunce (4) procedure was but a modification of the hemolysate procedure of Brin et al. (6) was stated in the Burma report (4) on page 19 by a direct reference. Their full assay was described on pages 163–166 of that report (2) and was an almost direct description of the original (5).

2. Also, I find disagreement with the interpretation on page 294: "It appears that the transketolase activity measures the amount of thiamine pyrophosphate in the red cell." This interpretation can be shown to be in conflict with the previous Burma report (4). For example on page 166, table 147, it was indicated that 74.3% of the subjects with values of urine thiamine in excess of 65 µg/g creatinine showed a TPP stimulation of less than 16%. We might take the liberty to reword this by stating that the apotransketolase enzyme in erythrocytes obtained from that group was at least 84% saturated with thiamine pyrophosphate. In other words, the TPP-effect did not measure the amount of TPP in the cells, but rather it estimated the proportion of apotransketolase enzyme which had inadequate TPP to carry out the transketolase reaction as measured. This is further supported by the fact that the addition of TPP to erythrocyte hemolysates will often raise activity levels up to, but not in excess of, normal activity levels. This demonstrates therefore, that once the apotransketolase protein is saturated with TPP, the *adding of additional TPP has no additional stimulatory effect* and this finding is actually inherent in the assay employed.

Letters may be considered for publication when the writer comments constructively concerning a paper that has been published in the *Journal of Nutrition.* Such letters will be subject to the usual editorial review and at the same time, the author or authors of the paper in question will be given the privilege of submitting a rebuttal. Final acceptance of Letters shall be the prerogative of the Editorial Board. (Letters should be as concise as possible and, in the future, not longer than 500 words including references cited.)

3. I have no issue with the statement on page 294: "The correlation in this study between the progressive development of a thiamine deficiency and the decreased activity of the enzyme transketolase is confirmed, thus supporting the validity of the transketolase measurement as an index of thiamine deficiency," (although I feel that the TPP-effect is the better index); however, the paragraph continues to describe that "as reported by Brin (7) a period of 13 days was required for the thiamine deficiency to manifest itself in a reduction of transketolase activity of rat erythrocytes," and continues that it took 12 days for Ziporin et al. (1) to recognize a biochemical change in urinary metabolites as it did us to recognize it in erythrocyte transketolase parameters (8). The discussion continues that Brin (7) employed a thiamine-free diet for the rats while for humans both they and we fed 0.2 g thiamine per day. Once again their reference was incorrect. In fact, our basic presentation (6) demonstrated a 25% reduction in erythrocyte transketolase activity in 5–7 days, and a 50% reduction in two weeks and this was essentially confirmed for heavy rats as well (8). This differentiation in time is important, as the rats continued to grow at a normal rate for about 10 days.

4. Lastly, on page 297, it was stated, following the mention of the use of blood transketolase activity to reflect thiamine status, that "While these methods have proved adequate for the detection of a developing and impending deficiency of thiamine, they were subject to error, since they depended, in the final analysis, on a correlation between the biochemical values and the appearance of clinical deficiency symptoms." This statement, too, is without basis in fact, because (a) reduced transketolase activity and an elevated TPP-effect appear before clinical signs are evident, and (b) the deviation from normal for each parameter becomes much greater as the deficiency becomes more severe. This we have shown previously (5–9). Also, it is important to recognize that whether an erythrocyte apotransketolase enzyme is, or is not, saturated, is a biochemical observation which is completely independent of clinical signs whether the latter be objective or subjective. There is no need to do a double-blind therapeutic trial in this case, because the assay, as designed (6) is an in vitro therapeutic trial. As a matter of fact we feel that it is this relationship, combined with the order of magnitude of the TPP-effect, which renders the transketolase assay uniquely useful in the objective evaluation of thiamine adequacy.

Whether the measurement of thiamine metabolites in urine as reported by Ziporin et al. (1, 2) is as useful or more useful than the TPP-effect in erythrocyte transketolase activity for the evaluation of thiamine adequacy, for individual determinations or for survey needs remains to be determined, of course.

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THE AUTHOR'S REPLY:

Dr. Brin's comments regarding our papers (1, 2), recently published in the Journal of Nutrition, are welcomed since errors or inaccuracies should not be permitted to go unchallenged. Furthermore, if Dr. Brin has misunderstood significant points in these papers, then perhaps these points were not properly stated and other readers might derive erroneous conclusions as did Dr. Brin. It is important, then, that these misunderstandings be corrected. The opportunity to clarify by further explanation and amplification is appreciated. In the interest of following Dr. Brin's comments, it may be best to direct the replies to paragraphs as he has numbered them.

Paragraph 1. Careful re-reading of the paper by Wolfe, Brin and Davidson (3) reveals that a method for the assay of transketolase is, in fact, described. This is almost a word-for-word copy of the paper by Brin et al. (4). Since both are the same, this point of criticism is not founded. However, Dr. Brin is correct in stating that the work reported by Bunce and Sauberlich (5) used the method reported by Brin et al. (6), the only difference being the elimination of hexose formation as part of the assay. This may not be considered significant.

Paragraph 2. Dr. Brin's criticism in this paragraph is not fully understood. For the interpretation quoted by Dr. Brin, it was assumed that the apoenzyme, transketolase (not "apotransketolase" as stated by Dr. Brin), requires thiamine pyrophosphate (TPP) as a cofactor in order to perform its specific catalytic functions. As thiamine deficiency progresses, the activity of this enzyme decreases because of an insufficient amount of TPP to saturate the available apoenzyme. (Horecker and Smyrniotis (7) have reported that 1 mole of transketolase protein contained 0.8 mole of TPP.) As thiamine repletion occurs and there is a return to control transketolase levels, it may be assumed that TPP is formed and is available to the transketolase protein in the hemolysate, thus forming the combination necessary for full activity. With this hypothesis, the statement that "transketolase activity measures the amount of thiamine pyrophosphate in the red cell" appears justified.

But further work by Brin et al. (6) revealed that the above explanation does not suffice for all the data he has accumulated regarding the transketolase activity measurements. This may explain why he shifts in his paragraph 2 from TPP saturation of transketolase enzyme to the TPP effect. This is an increase in transketolase activity when TPP is added to an hemolysate of thiamine pyrophosphate-deficient red blood cells. While both saturation and stimulation may be related, it is not necessarily true that they are, in fact, the same phenomenon.

In table 2, Brin et al. (6) report that the transketolase activity of thiamine-deficient rats, as measured by the appearance of hexose from pentose, decreases from 24–61% of the control as the period of deficiency increases from 7–20 days. While the control values are approximately 900–1,000 μg hexose/ml/hour, the 20-day deficient animals show activity of 371 μg hexose/ml/hour, a decrease of 61%. If to the hemolysate of red blood cells of these deficient animals one adds TPP, the value increases from 371 to 487 μg hexose /ml/hour, an increase of 31%. The transketolase activity does *not* reach the control levels even though a less-than-full stimulation in activity may be measured.

Since there are no experimental data to explain this finding, hypothetical considerations may be permitted: 1) During the period of developing thiamine deficiency there is a simultaneous decrease in the amount of transketolase protein in the red cell. Added TPP would therefore have no apoenzyme to which it may bind, 2) TPP must be bound to the transketolase before activity is restored. Added TPP may not be efficiently bound in an in vitro incubation period. Certainly, other relationships may be hypothesized. Thus, while saturation of the apoenzyme by the coenzyme may be necessary, stimulation may depend

on the amount of enzyme protein as well as the amount of cofactor available and its effective association with the protein. In view of these complexities, it is therefore reasonable to say at the present time, that which the data permit: that as TPP is not available the transketolase activity decreases, and therefore the latter activity is a measure of the amount of TPP in the red cell.

Paragraph 3. Re-examination of the statements contained in our papers (1, 2), now in question, reveals the exactness of the information contained therein, and the correctness of the reference which Dr. Brin criticizes. The work reported by Brin (8) using rats, reports the transketolase activities in 9 tissues as a function of various treatments. Considering only the comparison between controls fed adequate thiamine and those fed the thiamine-deficient diet, the data presented in figure 1 reveal that 6 of the 9 tissues of the deficient group have more than 75% of their control transketolase activity after 7 days of depletion, while after 13 days of depletion 7 of 9 tissues of this group have 50% or less transketolase activity than their thiamine-fed controls. In the absence of any definitive statement amply supported by valid data showing significance of differences, the conclusion that deficiency was achieved at 13 days is justified, assuming that 50% activity or less is direct evidence of deficiency. This will be discussed further under "paragraph 4." Since the work of Brin (8) reported new work, there was no necessity for quoting a "basic presentation" (6) which was not at all in question.

In our studies, the urinary excretion of thiamine was low at 12 days' depletion and was not detectable at 18 days' depletion. When coupled with the physical findings which showed some evidence of thiamine deficiency after 9 days' depletion, we have taken the 12-day depletion as the point at which an "effect was noted." Dr. Brin (9) demonstrates in his figure 4 (lower graph) that 10 days after being placed on a thiamine deficiency test, the TPP stimulation is approximately equal to zero day on the test, with intermediate days ranging as high as 15–16% TPP stimulation. After the 10th day, read as the 12th day, the TPP effect appears to rise consistently to a height of about 25–27% at the 18th or so day. Day 12 may reasonably be viewed as the beginning of the deficiency. These figures are approximations since they are interpolated from graphs. The correctness of the statements made in the paper in question is thereby validated.

The reference reporting that Brin fed his rats a "semi-purified diet . . . presumably containing no thiamine" (1) is supported by the statement from Brin (8) which says that the rats "were fed the purified diet devoid of added thiamine." In the human studies, we report that our subjects were fed a diet containing 0.11 to 0.18 mg thiamine/day. "The diet in Brin's human studies contained 0.19 mg of thiamine/day." The quote is, "The daily intake of thiamine to approximately 190 μg per day." (9) The incorrectness of the statement appearing in our paper is not evident.

Paragraph 4. The issue raised by Dr. Brin in his "paragraph 4" is most important, since it presents a perspective that was apparently not very clear, and deserves to be clarified.

The definition of a thiamine-deficient state in the human or laboratory animal has been defined by symptoms which develop after the subject has been placed on a thiamine-deficient diet for a period of time. Until the now-classic symptoms of beriberi are recognized, the patient must be judged normal, as far as thiamine symptoms are concerned. In developing a test for assessing thiamine status, using biochemical criteria, the chemist measures changes in a pertinent parameter. He must then decide what magnitude of change must occur before the patient is considered deficient. This can only be done by correlating changes in biochemical parameters with the clinical symptoms. Thus, using arbitrary figures: A change of 15% may be associated with sinus tachycardia at rest; a 25% change may be associated with nausea, until finally a 75% change may be associated with the above symptoms as well as insomnia, anorexia, irritability and so on to even more severe deficiency states. The biochemist

may then set up an index after a sufficient number of people have been tested for the validity of the index. Thus, a correlation is necessary between clinical symptoms and biochemical criteria. The clinical criteria, however, are not as exact as are the biochemical tests. Thus, we may expect errors in classifying patients, since the relatively exact measurement is related to a rather inexact estimation. These considerations led to the statement: "Since the appearance of these symptoms is often slow and gradual, it is difficult for the clinician to establish the point at which the patient is considered to be sufficient in thiamine and beyond which he is deficient. These difficulties have prevented the precise definition of the deficient state in terms of urine thiamine and blood transketolase" (2).

There appears to be no publication by Dr. Brin which sets up such an index relating transketolase activities and/or TPP effect to the extent of thiamine deficiency. The most recent publication by Dr. Brin which I have been able to find (10) and written for the medical profession, very carefully avoids telling the physician how much depression of transketolase activity or TPP stimulation is required to indicate a slight, moderate or severe deficiency. Instead, his five successive stages of thiamine deficiency are defined by clinical symptoms. It is interesting that the following definitions of transketolase activities may be excerpted from his description of the 5 stages of thiamine deficiency:

	Transketolase	*TPP*
Stage 1	not defined	not defined
Stage 2	reduced	positive
Stage 3	reduced further	larger effect
Stage 4	greatly reduced	not defined
Stage 5	not defined	not defined

There is good reason to believe that the clinician may find it difficult to use this test as an index of thiamine status in his patients.

To establish such an index, the Interdepartmental Committee on Nutrition for National Defense has set up the necessary correlation with numerical values designed to delineate the stages of deficiency. To be valid this index must be based on clinical data. They have set up (5) an "arbitrary range of values:

Up to 15% stimulation = acceptable
16–20% stimulation = low
Greater than 20% stimulation = deficient"

Within such a framework decisions are possible.

In contrast to the transketolase and urine thiamine which must be correlated to clinical symptoms, the metabolite levels do not require such a correlation. We maintain that a thiamine deficiency exists when the metabolite plus thiamine excretion is greater than the thiamine intake. Continued subsistence on such a regimen will ultimately lead to clinical deficiency symptoms. Such a perspective has application to many problem areas where nutritional deficiencies are suspected. Thus, where populations have lower intakes than that recommended by our National Research Council, there may still be many who do not manifest clinical symptoms of thiamine deficiency. A survey including a measure of thiamine intake, thiamine and metabolite excretion should reveal the manner in which these people utilize the thiamine, and the extent of negative balance per day. Such procedures are applicable to the sufficient, marginal and deficient intakes of thiamine.

The question must then be answered: How can the clinician establish thiamine status before the appearance of clinical symptoms? We have proposed that three biochemical tests be used to assess different parameters of thiamine metabolism. 1) Retention or load test to measure thiamine saturation of the tissues; 2) transketolase assay with or without TPP effect. The values for controls range around 1000 μg hexose formed from pentose/ml hemolysate/hour; 3) metabolite and thiamine excretion in the urine. These are the views expressed in the papers recently published. There appears to be no reason at the present time for changing these views.

At no point in either of the papers recently published was there an intimation that the question of thiamine metabolism and nutritional status was solved. It was only proposed that we now have new parameters for following thiamine metabolism, and these have led to a basis for

calculating the thiamine requirement of young adult males. It cannot be mere coincidence that the thiamine requirement thus calculated is so close in agreement with many excellent studies using various other criteria to assess this requirement. It is hoped that proper application of the available knowledge regarding thiamine will help in our continuing efforts toward eradicating misery by the use of science.

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