Invitation for Nominations for 1969 American Institute of Nutrition Awards

Nominations are requested for the 1969 annual awards administered by the American Institute of Nutrition to be presented at the next annual meeting. Nominations may be made by anyone, including members of the Nominating Committees and non-members of the Institute.

The following information must be submitted: (1) Name of the award for which the candidate is proposed. (2) *A brief convincing statement setting forth the basis for the nomination and, where appropriate, a selected bibliography which supports the nomination. Seconding or supporting letters are not to be submitted.* (3) Five copies of the nominating letter must be sent to the chairman of the appropriate nominating committee *before October 1, 1968*, to be considered for the 1969 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 during the banquet at the annual meeting.

1969 Borden Award in Nutrition

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

Former recipients of this award are:

1944 – E. V. McCollum
1945 – H. H. Mitchell
1946 – P. C. Jeans and Genevieve Stearns
1947 – L. A. Maynard
1948 – C. A. Cary
1949 – H. J. Deuel, Jr.
1950 – H. C. Sherman
1951 – P. György
1952 – M. Kleiber
1953 – H. H. Williams
1954 – A. F. Morgan and A. H. Smith
1955 – A. G. Hogan
1956 – F. M. Strong
1957 – no award
1958 – L. D. Wright
1959 – H. Steenbock
1960 – R. G. Hansen
1961 – K. Schwarz
1962 – H. A. Barker
1963 – Arthur L. Black
1964 – G. K. Davis
1965 – A. E. Harper
1966 – R. T. Holman
1967 – R. H. Barnes
1968 – C. L. Comar

NOMINATING COMMITTEE:

K. E. HARSHBARGER, *Chairman*
R. T. HOLMAN
R. G. HANSEN

Send nominations to:

K. E. HARSHBARGER
Department of Dairy Science
University of Illinois
Urbana, Illinois 61801

1969 Osborne and Mendel Award

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

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

Former recipients of this award are:

1949 – W. C. Rose
1950 – C. A. Elvehjem
1951 – E. E. Snell
1952 – Icie Macy Hoobler
1953 – V. du Vigneaud
1954 – L. A. Maynard
1955 – E. V. McCollum
1956 – A. G. Hogan
1957 – G. R. Cowgill
1958 – P. György
1959 – Grace A. Goldsmith
1960 – N. S. Scrimshaw
1961 – Max K. Horwitt
1962 – William J. Darby
1963 – James B. Allison
1964 – L. Emmett Holt, Jr.
1965 – D. M. Hegsted
1966 – H. H. Mitchell
1967 – Samuel Lepkovsky
1968 – C. H. Hill

NOMINATING COMMITTEE:

L. E. HOLT, JR., *Chairman*
M. K. HORWITT
E. L. R. STOKSTAD

Send nominations to:

L. E. HOLT, JR.
Professor of Pediatrics
New York University School of Medicine
550 First Avenue
New York, N. Y. 10016

1969 Mead Johnson Award for Research in Nutrition

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

Former recipients of this award are:

1939 – C. A. Elvehjem
1940 – W. H. Sebrell, Jr.
J. C. Keresztesy
J. R. Stevens
S. A. Harris
E. T. Stiller
K. Folkers
1941 – R. J. Williams
1942 – G. R. Cowgill
1943 – V. du Vigneaud
1944 – A. G. Hogan
1945 – D. W. Woolley
1946 – E. E. Snell
1947 – W. J. Darby
P. L. Day
E. L. R. Stokstad
1948 – F. Lipmann
1949 – Mary S. Shorb
K. Folkers
1950 – W. B. Castle
1951 – no award
1952 – H. E. Sauberlich
1964 – J. S. Dinning
1965 – J. G. Bieri
1966 – M. Daniel Lane
1967 – W. N. Pearson
1968 – H. F. DeLuca

NOMINATING COMMITTEE:

L. M. HENDERSON, *Chairman*
H. E. SAUBERLICH
H. P. BROQUIST

Send nominations to:

L. M. HENDERSON
Department of Biochemistry
University of Minnesota
St. Paul, Minnesota 55101

1969 Conrad A. Elvehjem Award for Public Service in Nutrition

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

Former recipients of this award are:

1966 – C. Glen King
1967 – J. B. Youmans
1968 – W. H. Sebrell, Jr.

NOMINATING COMMITTEE:

R. E. SHANK, *Chairman*
OLAF MICKELSEN
O. C. JOHNSON

Send nominations to:

R. E. SHANK
Department of Preventive Medicine
Washington University School of Medicine
4550 Scott Avenue
St. Louis, Missouri 63110

Invitation for Nominations for 1969 American Institute of Nutrition Fellows

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

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

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

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

Fellows Committee:

AGNES F. MORGAN, *Chairman*
RICHARD M. FORBES
T. H. JUKES
L. A. MAYNARD
A. E. MORRISON

Send nominations to:

AGNES F. MORGAN
Department of Nutritional Sciences
University of California
Berkeley, California 94720

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

Georgian Adams (1967)
Herman J. Almquist (1968)
J. B Brown (1964)
Thorne M. Carpenter (1958)
George R. Cowgill (1958)
Earle W. Crampton (1967)
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)
L. Emmett Holt, Jr. (1967)
Icie Macy Hoobler (1960)
Paul E. Howe (1960)
J. S. Hughes (1962)
C. Glen King (1963)
Max Kleiber (1966)
Samuel Lepkovsky (1966)
Leonard A. Maynard (1960)
Elmer V. McCollum (1958)
Harold H. Mitchell (1958)
Agnes Fay Morgan (1959)
John R. Murlin (1958)
Leo C. Norris (1963)
Helen T. Parsons (1961)
Paul H. Phillips (1968)
Lydia J. Roberts (1962)
William C. Rose (1959)
W. D. Salmon (1962)
W. H. Sebrell, Jr. (1968)
Arthur H. Smith (1961)
Genevieve Stearns (1965)
Harry Steenbock (1958)
Hazel K. Stiebeling (1964)
Raymond W. Swift (1965)
Robert R. Williams (1958)
John B. Youmans (1966)

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

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

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

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

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

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:

R. W. ENGEL, *Chairman*
L. C. NORRIS
A. E. SCHAEFER

Send nominations to:

R. W. ENGEL
Associate Dean for Research
Virginia Polytechnic Institute
Blacksburg, Virginia 24061

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
Dame Harriette Chick
F. W. A. Clements
Hans D. Cremer
Sir David P. Cuthbertson
Herbert M. Evans
Karl Guggenheim
Joachim Kühnau
Joseph Masek
Toshio Oiso
H. A. P. C. Oomen
Lord John Boyd Orr
Conrado R. Pascual
V. N. Patwardhan
Sir Rudolph A. Peters
B. S. Platt
Juan Salcedo
Emile F. Terroine
Jean Tremolieres
Eric John Underwood
Artturi I. Virtanen

Experimental Amyloidosis in Mice: Effect of high and low protein diets

WILLIAM T. WEST [1]

Department of Anatomy, Downstate Medical Center, State University of New York, Brooklyn, New York

ABSTRACT Results of past studies concerning the relation of high and low protein diets to the development of sodium caseinate-induced amyloidosis in mice have been somewhat conflicting. A study was therefore made to reanalyze these factors using the more chemically defined diets now available. C3HeB/FeJ male mice were fed the experimental diet when 9 weeks of age; 2 weeks later caseinate injections were initiated and were given 5 times a week for 10 weeks. All animals were weighed weekly and spleen and liver weights were taken at autopsy. The proportion of amyloid deposits in sections of the spleen and liver was determined by the Chalkley method for the quantitative morphologic analysis of tissues. Neither the high casein nor low casein diets prevented the development of amyloidosis. The results suggest that the high casein diet tends to favor the development of amyloid disease. Although the low casein diet tended to inhibit the development of amyloid disease, the results were inconclusive. Morphologically, these effects were observed primarily in the liver. Organ weight data and survival of the injected animals suggest that these effects pertain to the general picture of amyloid disease in these animals.

That diet can affect the development of experimental amyloidosis in mice has been established (1–4). However, the results of the various studies have been somewhat conflicting. Ku and Simon (1) reported that a low protein diet reduced the percentage of mice in which amyloidosis could be induced by caseinate injections, but found no specific effect in animals fed a high protein diet. Jaffe (4) observed that feeding a diet composed entirely of dried beef heart inhibited or delayed the onset of experimental amyloid disease. Grayzel et al. (2) reported that animals fed an "adequate" diet and a diet supplemented with powdered whole liver showed a delayed onset of caseinate induced amyloidosis as compared with animals fed a high protein diet (60% protein).

Unfortunately, these dietary studies cannot be strictly compared since they varied widely in the natural and semipurified constituents of which the various diets were composed. Thus, the question of the relation of nutrition to the development of experimental amyloidosis is unresolved. The present paper reports the effects of diets high and low in a single protein, casein, as compared with a commercial stock diet on the development of caseinate induced amyloidosis in mice.

MATERIALS AND METHODS

Ninety strain C3HeB/FeJ male mice, obtained from the Jackson Laboratory, Bar Harbor, Maine, were used. The animals were assigned to the various dietary groups by means of random numbers.

Diets. The stock or "normal" reference diet was a commercial stock diet,[2] having the following composition: wheat germ meal, ground whole wheat, dried skim milk, dehulled 50% soybean meal, corn oil, brewer's yeast, stabilized vitamin A and D, salt and ferric citrate; protein, minimum 19.0%, fat, minimum 7.5%, fiber, maximum 2.0%, and N.F.E., minimum 52.0%. The high casein and low casein diets were commercial purified diets.[3] The high casein diet had the following composition: (in percent) "vitamin-free" casein, 64; sucrose, 22; corn oil, 8; brewer's yeast USP, 2; salt mixture USP II, 4; and vitamin mixture. The low casein diet had the following composition: (in percent) casein, 8; starch, 78; corn oil, 10; salt mixture USP XIV, 4; and vitamin mix-

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[1] This investigation was supported by Public Health Service Research Grant no. AM-08648-03 from the National Institute of Arthritis and Metabolic Diseases.

[2] Old Guilford Mouse and Rat Breeder Pellets, Emory Morse Company, Guilford, Connecticut.

[3] Obtained from Nutritional Biochemicals Corporation, Cleveland.

ture. The content of vitamins[4] per 45.5 kg of diet in the high and low casein diets was: vitamin A conc (as the acetate, 200,000 units/g) 4.5 g; vitamin D conc (as calciferol, 400,000 units/g), 0.25 g; α-tocopherol, 5.0 g; ascorbic acid, 45.0 g; inositol, 5.0 g; choline chloride, 75.0 g; menadione, 2.25 g; *p*-aminobenzoic acid, 5.0 g; niacin, 4.5 g; riboflavin, 1.0 g; pyridoxine·HCl, 1.0 g; thiamine·HCl, 1.0 g; Ca pantothenate, 3.0 g; biotin, 20 mg; folic acid, 90 mg and vitamin B_{12}, 1.35 mg.

Casein. The casein solution was prepared as follows: 10 g of casein[5] were dissolved with slight heating in 200 ml of 0.25% NaOH. This solution was dialyzed in the cold against distilled water for 48 hours to remove excess NaOH. After dialysis the casein solution was reduced to 100 ml final volume by rapid evaporation, which resulted in a 10% solution of casein. The casein solution was stored at zero to 4° and made up fresh weekly.

Different batches of the casein used varied greatly in their solubility, and their solubility in aqueous solutions depended to a great extent upon the pH of the solvent. Several batches of the casein were tested for solubility in $NaHCO_3$ and NaOH and that which gave the clearest solution was chosen for this and future studies (control no. 9393).

Procedure. Three groups of 30 animals each were used, twenty of which were injected with a casein solution, and ten of which were not injected. At 9 weeks of age, each group was fed one of the diets described: commercial pellet, high casein or low casein. Water and food were given ad libitum. Paired feeding studies were not conducted. The animals were weighed the day that the diets were initiated and every week thereafter.

Two weeks after the diets were initiated, injection of the casein solution was started and continued for 10 weeks. Each animal designated for injection was given 0.3 ml (30 mg) of the 10% casein solution subcutaneously 5 times a week for 10 weeks. Control animals received no injections. The injection sites were rotated daily.

At the termination of the experiment the animals were weighed, killed by cervical dislocation and the spleens and livers removed and weighed on a torsion balance. Samples of liver, spleen and both kidneys were fixed in neutral formalin for 48 hours, washed overnight in running tap water, dehydrated and embedded in paraffin[6] (56 to 58°). Each spleen, immediately after fixation, was cut transversely into quarters and so oriented at embedding that uniform samples of the whole spleen were available for histologic study.

The blocks were sectioned at 5 μ and all sections routinely stained with hematoxylin and eosin. Selected material from each group was also stained with crystal violet (5) at different pH levels, congo red (6), and thioflavin T (7).

The proportion of amyloid in the liver and spleen (hematoxylin and eosin-stained material) was determined by the Chalkley (8) method for the quantitative morphologic analysis of tissues. Briefly, this method is based on the concept that a point moving randomly through a tissue or organ will impinge upon each component of that tissue or organ in proportion to the volume occupied by each component. It was felt that this method would give more objective quantitation of the amyloid in an organ than the rather subjective quantitative methods based on visual estimates of amyloid deposits compared with arbitrary standards.

Body weights, organ weights and the data obtained from the Chalkley method for quantitative morphologic analysis of tissues (amyloid quantitation) were analyzed by one-way analysis of variances; where significant differences were found, Tukey's honest test was used to determine the nature of the significance. Survival data were tested for significance by the chi-square test with Yate's correction.[7] Statistical comparisons of body weights and organ weights were made within the control groups and within the injected

[4] Vitamin Diet Fortification Mixture, Nutritional Biochemicals Corporation.

[5] Hammersten Quality Casein (control no. 9393), Nutritional Biochemicals Corporation.

[6] Peel-A-Way Embedding Paraffin, regular, Lipshaw Manufacturing Company, Detroit.

[7] Wollman, A. L. 1967 Studies on the site of action of the anti-androgen cyproterone acetate and on the range of androgenic compounds against which this anti-androgen is effective. Thesis, Appendix, pp. 132–141.

groups of animals, but not between these groups.

RESULTS

Control animals

All control (uninjected) animals remained healthy in appearance throughout the experiment. At autopsy no evidence of infections or other pathologic processes were observed. Histologically, no overt abnormal changes were noted in the spleens, livers or kidneys of these animals. However, there appeared to be fewer megakaryocytes in the spleens of the animals fed low casein than in those fed the commercial pellet and high casein diets. The organs examined were free of amyloid deposits. All animals survived to termination of the experiment.

Growth and weight data. Figure 1 shows the growth curves of the control animals. All animals gained weight during the experiment. However, the animals fed the powdered high and low casein diets showed an initial weight loss during the first week of the diets and did not gain as much during the course of the experiment as the animals fed the commercial pelleted food. During the ninth week the high casein animals showed a sudden weight loss which cannot be fully explained; it is possible that this sudden loss may have been due to an inadvertent temporary insufficiency of food presented to the animals during this week.

At termination of the experiment the body weights of animals fed the high and low casein diets were significantly lower than the body weights of the animals fed the commercial pelleted food (table 1), but were not significantly different from each other.

The mean weight of spleens from animals fed the low casein was significantly lower than the mean spleen weights from the animals fed the commercial pellets and the high casein diet; the mean spleen weights of the latter 2 groups were not significantly different (table 1).

The mean liver weights from the 3 dietary groups were all significantly different, being highest in the animals fed the

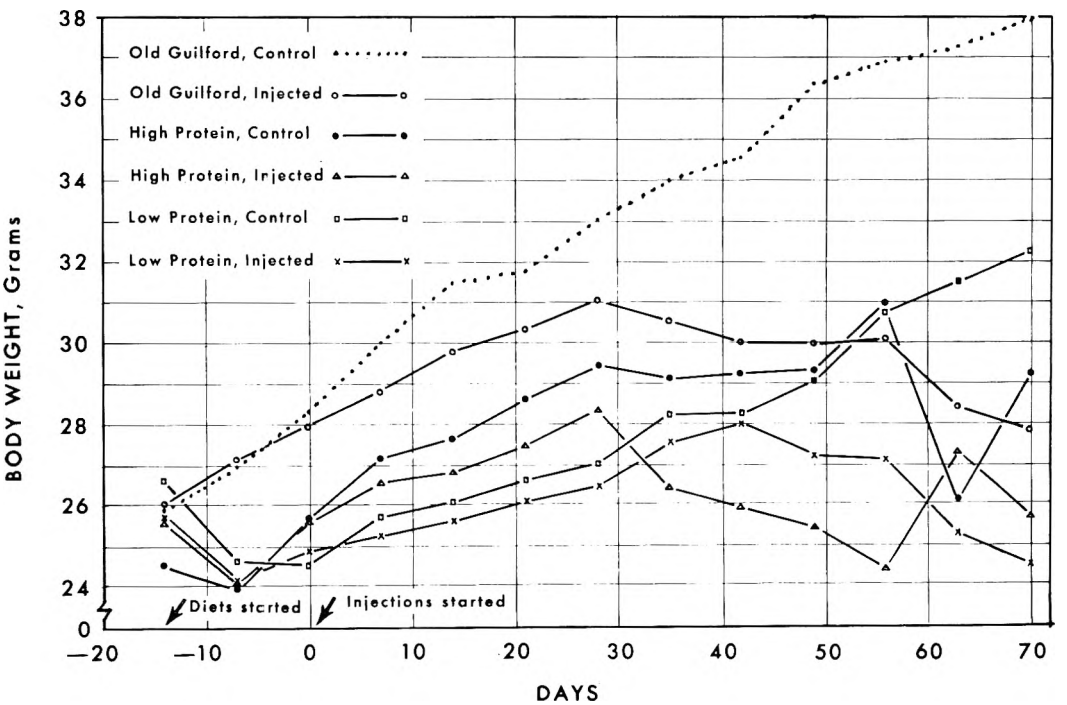

Fig. 1 Growth in body weight of injected and control mice fed various diets.

TABLE 1

Comparison of organ and body weights of control and amyloid animals fed various diets [1]

	Diet groups			P value
	Commercial pellet [2]	High casein	Low casein	
		Control animals		
	(10 animals)	(10 animals)	(10 animals)	
Body wt, g	37.97			0.01
		29.23	32.36	ns
Spleen wt, mg	113.5	111.0		ns
			89.5	0.01
Liver wt, mg	1846.5			0.01
		1543.5		0.01
			1188.0	0.01
		Injected animals		
	(14 animals)	(6 animals)	(18 animals)	
Body wt, g	27.87	25.7	24.54	ns
Spleen wt, mg	201.0 (77.2%) [3]	242.5 (118.5%)		ns
			139.4 (55.8%)	0.01
Liver wt, mg	1785.7 (96%)	1860.8 (120.5%)		ns
			1036.7 (87.5%)	0.01

[1] Those values for each parameter on the same horizontal line are not significantly different; those on different horizontal lines are significantly different from all others of same parameter.
[2] Old Guilford Mouse and Rat Breeder Diet.
[3] Percentage increase over control weight.

commercial pellets and lowest in those fed the low casein (table 1).

Experimental animals

Animals receiving subcutaneous injections of casein showed no macroscopic pathologic changes during the course of the experiment other than those expected as a result of the induction of amyloidotic disease. In general, the animals appeared less hardy and less well groomed than those not given caseinate injections. Typical signs of illness in the mouse, such as ruffled coat and lessened activity, were noted only in mice that died during the course of the experiment, and then only for a few days immediately preceding death. The injection sites showed no ulcerations nor evidence of infectious processes during the course of the experiment; however, at autopsy, some fibrosis was noted at these sites.

Significantly fewer animals fed the high casein diet survived until termination of the experiment than survived among those fed the commercial pellet and low casein diets (table 2). Although more animals survived of those fed the low casein diet than of those fed the commercial pellet diet, the difference was not statistically significant. Most of the deaths in all groups occurred during the last 2 weeks of the experimental period. All injected animals that survived until termination of the experiment showed macroscopic and microscopic evidence of amyloidosis in the liver, spleen and kidneys except for one animal fed the low casein diet; and that animal showed no evidence of amyloid disease.

TABLE 2

Survival of control and caseinate-injected animals fed various diets [1]

Animals	Diets			P value
	Commercial pellet [2]	High casein	Low casein	
Control	10/10 [3]	10/10	10/10	—
Casein-injected	14/20		18/20	ns
		6/20		< 0.02

[1] Number alive on same horizontal line not significantly different.
[2] Old Guilford Mouse and Rat Breeder Diet.
[3] Number surviving until termination over number at beginning of experiment.

Autopsies were performed on many of the animals that died. However, tissue samples were not taken from all these animals because in many cases postmortem changes were too far advanced. Grossly, the spleens of all these animals were quite enlarged and the kidneys exhibited some degree of lobulation. Microscopic study showed that the sampled spleens, livers and kidneys showed some degree of amyloidosis, the severity of the disease being, on a subjective basis, greatest in animals fed the high casein diet. No other signs of pathology were observed. The exact cause of death could not be determined, but may have been the result of renal failure brought on by amyloid deposits in the kidney. The histologic features of the amyloid deposits in these animals as well as those that survived until termination of the experiment were similar to those described by others (9–11).

Growth and weight data. The growth curves of the injected animals are shown in figure 1. The animals fed the purified diets showed an initial small weight loss during the first week; after this all animals showed growth similar to that of the control animals for 5 to 6 weeks. After this initial growth period, weight loss occurred in all injected animals and generally continued to termination of the experiment. The sudden gain of weight of the injected animals fed high casein (fig. 1) during the ninth week is correlated with the fact that a major number of deaths occurred in this group at this time, and only the less affected, higher weight animals contributed to the weight mean plotted here.

At termination of the experiment there was no significant difference in body weights among the injected dietary groups (table 1).

Among the injected animals the mean spleen weight of the animals fed low casein was significantly lower than the mean spleen weights of those fed commercial pellet and high casein diets; the latter 2 groups showed no significant difference in mean spleen weights (table 2). The mean spleen weights of the injected animals are substantially higher than those of the control animals in each dietary group, but the same interdiet relationship exists in both the control and the injected animals (table 1).

The mean liver weight of the injected animals fed low casein was significantly lower than that of those fed the commercial pellet and high protein; the latter 2 groups showed no significant difference in mean liver weights (table 1). Whereas the mean liver weight of animals fed high casein was significantly lower than that of those fed commercial pellets in the uninjected control groups, there was no difference between the two in the injected groups. Further, the injected animals fed

TABLE 3

Chalkley scores;[1] *quantitation of amyloid in spleens and livers from animals fed various diets*

Commercial pellet [2] (14 animals)	High casein (6 animals)	Low casein (18 animals)	
	"hits" [3]*/1000 total count*		
	Spleen		
243 (24.3%)	217.3 (21.7%)	199.2 (19.9%)	ns
	Liver		
	80.0 (16.0%)		
28.1 (5.6%)			
		11.2 (2.2%)	

[1] Counts for parameters on same horizontal line not significantly different. Counts on different horizontal lines significantly different from all others of same parameter ($P < 0.01$); percentage of total volume of the organ occupied by amyloid substance in parenthesis.
[2] Old Guilford Mouse and Rat Breeder Diet.
[3] "Hits" refer to the number of times a pointer in the ocular of the microscope impinges on amyloid material.

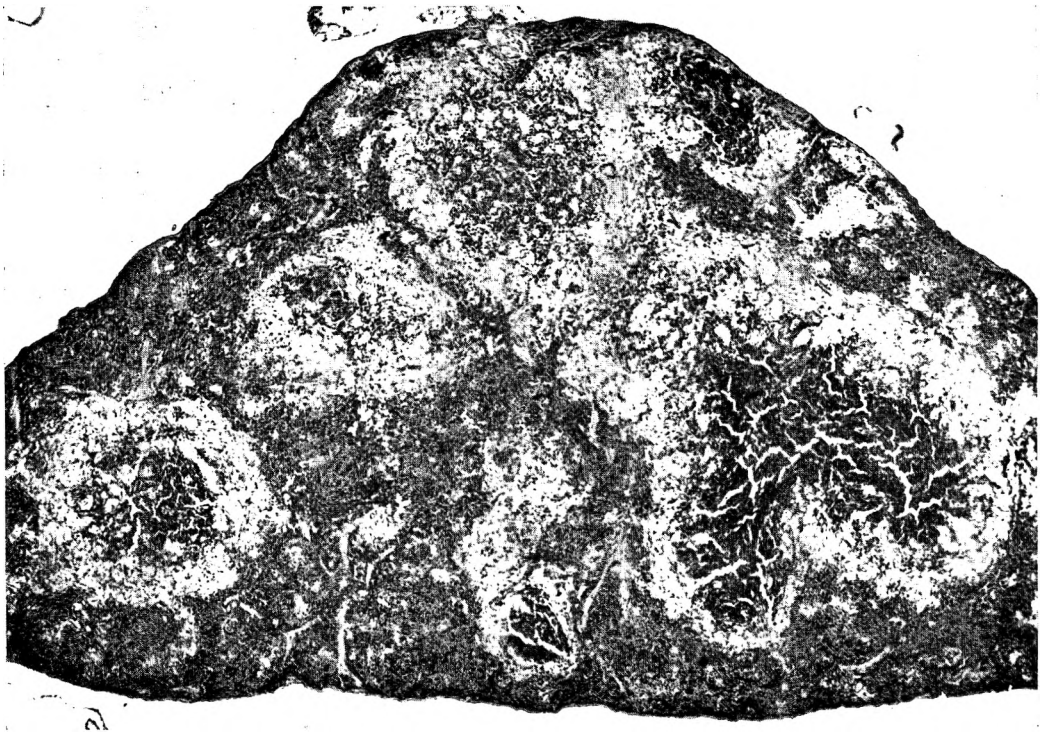

Fig. 2 Representative amyloid deposits in a spleen from an animal fed Old Guilford laboratory ration. H&E × 19.5.

high casein showed a higher mean liver weight than the uninjected control animals, and those fed commercial pellet and low casein diets showed a slightly lower mean liver weight (table 1).

Amyloid quantitation. The data derived by the Chalkley method for the quantitative morphologic analysis of tissues is presented in table 3. The proportion of amyloid in the spleens of the 3 dietary groups was not significantly different, ranging from 24.3% of the total spleen volume in the animals fed commercial pellets to 19.9% in those fed low casein. Figure 2 shows representative amyloid deposits in the spleen of an animal fed the commercial pellet diet.

There was a significant difference in the proportion of amyloid in the livers of the 3 dietary groups (table 3). The animals fed high casein showed the greatest proportion of amyloid and those fed the low casein, the least. Figure 3 shows representative amyloid deposits in the livers of animals fed the commercial pellet and high casein diets.

No effort was made to quantitate the amyloid volume of the kidney due to the difficulty in obtaining tissue sections which would give adequate sampling of this organ. Such data would be of value, however, and efforts are being made to overcome these difficulties. Amyloid deposits occurred in the distal two-thirds of the papilla, interstitially between the tubules, especially in the inner cortical region, and in the vascular portion of the glomeruli. In many cases, necrosis of the papilla and cortical lesions occurred, as previously described (12, 13). Figure 4 shows a kidney severely affected by amyloidotic disease.

General staining characteristics. Splenic, hepatic and renal amyloid deposits exhibited the same staining characteristics. Staining with Leib's crystal violet method (pH 1.5 to 1.9) rendered amyloid "metachromatic" in color; however, rinsing

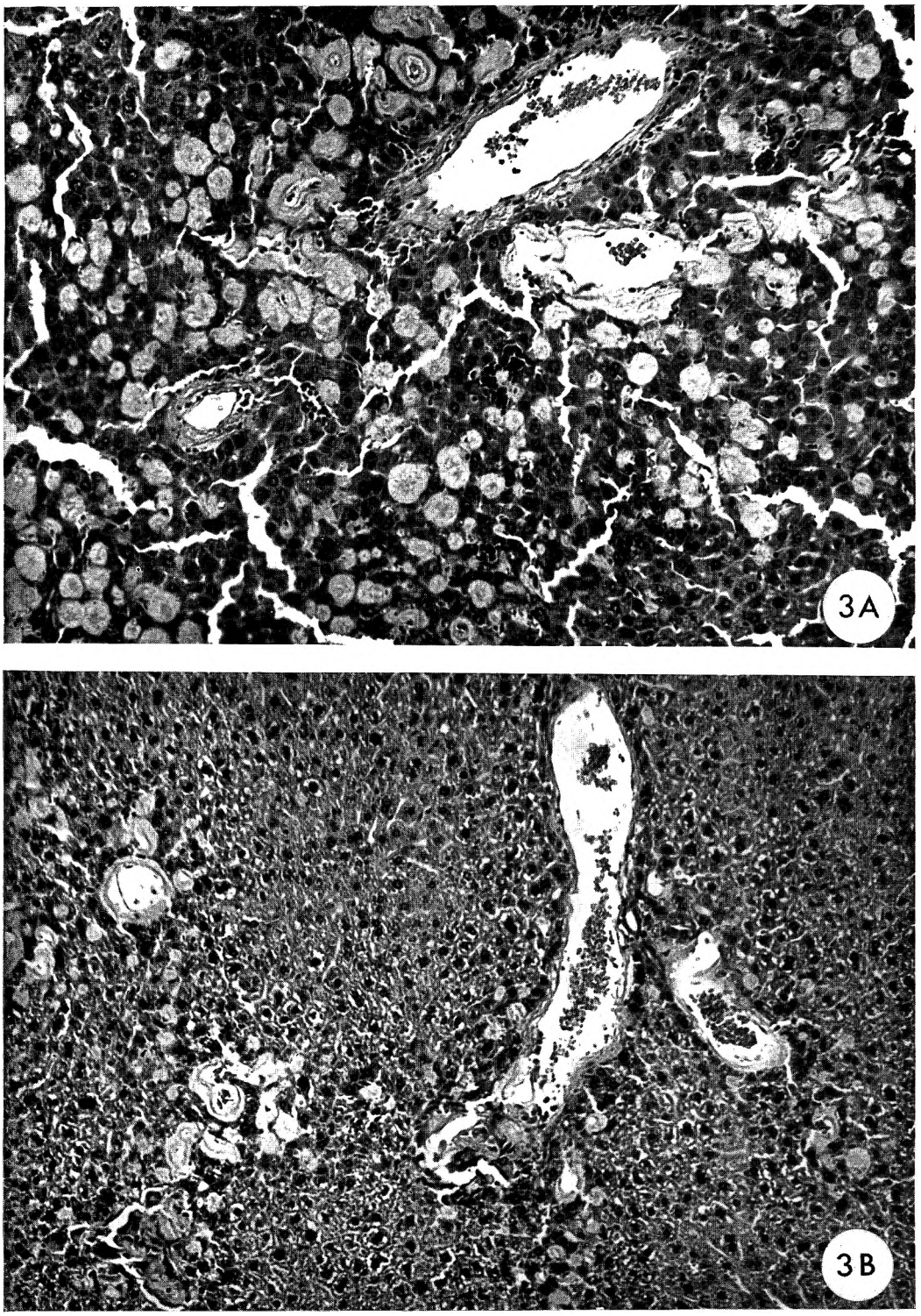

Fig. 3 Representative amyloid deposits in livers from animals fed (A) high casein diet, and (B) Old Guilford laboratory ration. Note the greater amount of amyloid in the liver of an animal fed the high casein diet. H&E × 60.

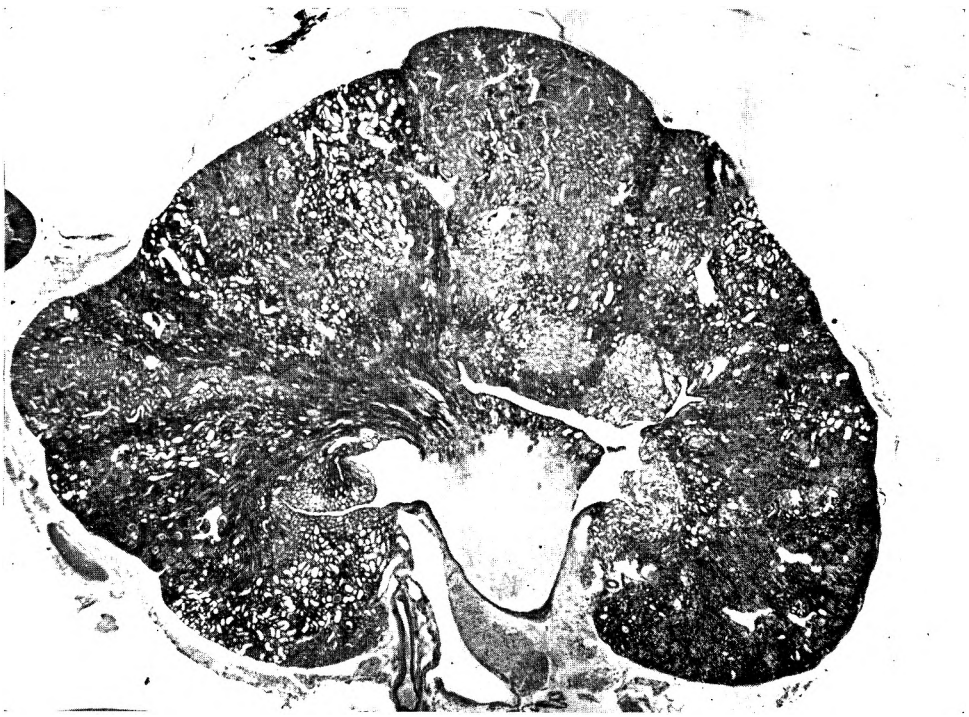

Fig. 4 Kidney severely affected by amyloidotic disease. Note the bland necrosis of the papilla and the wedge-shaped areas of tubular involvement in the cortex. H&E × 12.5.

the slides in water removed the metachromasia, leaving amyloid colorless. The same stain applied at pH 2.5 to 6.00 gave amyloid a "metachromatic" reaction which was water-fast. The induced amyloid material stained not at all with thioflavin-T; weakly with Congo red and was moderately birefringent after Congo red staining.

DISCUSSION

The results of this experiment indicate that variation of a single protein, casein, in the diet can affect the course of experimentally induced amyloid disease in mice under the conditions outlined. Neither the high casein nor the low casein diets prevented the development of amyloid disease. In general, however, a high casein diet tended to favor the development of amyloid disease as compared with a standard commercial ration of "normal" protein content. Although the results suggest that a low casein diet may inhibit somewhat the development of amyloid disease, the evidence is not conclusive.

Chalkley scores used to "quantitate" the proportion of amyloid in the organs of the animals clearly substantiate the above interpretations with respect to liver amyloid in the 3 diet groups (table 3). The scores indicate there is no difference in the proportion of splenic amyloid among the 3 groups. Examination of organ weight data (table 1), however, indicates that the same trend exists in the spleen as well as the liver. The spleens of the animals fed high casein increased more in weight as a result of the injections than those of animals fed the commercial food (table 1, 118.5% versus 77.2% increase). The spleens of the injected low casein animals increased the least in weight (55.8%). The liver weights of the high casein injected animals increased considerably over those of the control high casein animals, whereas those of the commercial food and low casein-fed animals actually weighed less than those of the respective control animals. The interpretations are strengthened because the least survival of animals

occurred in the injected high casein animals and the greatest survival in the low casein injected animals.

The manner in which the high and low casein diets exert their influence in the development of amyloid disease is not readily apparent. The low casein diet may lessen amyloid development because the diet itself is deficient in protein. The similarity of growth curves of both the normal and injected animals fed the low casein and commercial food diets contradict this interpretation and indicate that no overt nutritional deficiency was present in animals fed the low casein diet. The lack of lymphoid atrophy, the presence of fair amounts of body fat and the general good health of the control animals fed the low casein diet substantiate this interpretation.

Since the sole protein in the high and low protein diets was casein, the alteration in amyloid disease may represent a specific protein effect, that is, a qualitative rather than a quantitative effect on the part of the dietary protein. Pertinent to this point is that the diets used by Grayzel et al. (2) which favored amyloid production were, in general, notably high in milk products (up to 60% of the diet), whereas those in which amyloid induction was delayed or lessened were considerably lower in milk products (19%). In contrast, the diets of Ku and Simon (1) contained no milk products, yet the low protein diet decreased the incidence of amyloid disease as compared with a diet containing more normal protein content. Another argument against the quality of the protein being the major factor in the dietary effects on amyloid disease noted in this experiment is that in adult mammals proteins of dietary origin are rapidly hydrolyzed during digestion and few peptides or intact proteins are absorbed through the intestinal wall (14) into the portal circulation. Unfortunately, the experimental design is such that the role of protein quality cannot be eliminated as a possibility in causing the observed effects.

It might be argued that it is the variation of the quantity of carbohydrate rather than of protein in the diet that is responsible for the observed differences in amyloid production. However, this is rather doubtful for the following reasons. A diet very low in carbohydrate would be expected to bring about a rather large increase in amyloid substance; Jaffe (4) noted no amyloid production in mice fed a low carbohydrate diet consisting of dried beef heart powder mixed with water and a little yeast to make a dough. A diet very high in carbohydrate should greatly diminish the production of amyloid substance; Grayzel et al. (2), using a high carbohydrate stock diet containing 3 parts whole powdered milk and 2 parts ground white bread, produced amyloid earlier in mice than any other of their diets. Chemical analysis of isolated amyloid fibrils (15) indicated that amyloid contains little carbohydrate (4.6%), but a great deal of protein (15% nitrogen). Moreover, it is known that animals can synthesize sufficient carbohydrate for their needs from protein and fat provided these substances are not deficient.

Although the carbohydrates of the high and low casein diets are different in type (sucrose and starch), it is difficult to visualize that this difference would affect the outcome of the experiment. Starch and sucrose are eventually converted by the organism to glucose, the primary carbohydrate utilized by the body tissues. Thus, the interpretation that protein level is the factor affecting amyloid production in this experiment appears to be justifiable.

It is difficult to compare the dietary effects on experimental amyloid disease reported here with those in studies of other workers. Diets reported in the literature are composed of various combinations of natural and semipurified as well as exceedingly variable and different foodstuffs (1, 2, 4). Comparison is made even more complicated by the use of mice of different strains, ages and sex by these investigators. Although casein injections were used to induce amyloidosis in all these studies, the amount injected, the concentration of the casein solution, and the schedule of injections varied greatly. Finally, the means of determining the degree of amyloid disease varied among investigators; some used the percentage of mice showing amyloid disease as an end point, and others used the time of appearance of amyloid deposits.

The experiment reported here, as well as studies by other investigators, serve to point up the importance of nutrition in relation to experimental amyloid. Of even greater importance is the relation of nutrition to amyloid disease in humans. Although the incidence of so-called secondary amyloid disease has decreased with the advent of antibiotics, the primary or idiopathic forms are being more widely recognized. The relationships of nutrition to experimental and human amyloidosis remain to be fully delineated.

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Effect of Feeding Different Protein Sources on Plasma and Gut Amino Acids in the Growing Rat [1]

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ABSTRACT Experiments were conducted with growing rats to study the influence of feeding various protein and nitrogen-free diets on plasma amino acid levels and on the bulk amino acid composition of intestinal contents of rats. Feeding of a nitrogen-free, but calorically adequate diet to rats lowered total plasma essential amino acid levels below those noted for rats fed either 9% protein diets or fasted for 18 hours. Diets, whose protein source was solely rumen protozoal protein, when fed to rats resulted in significantly lower ($P < 0.01$) plasma histidine and valine levels than those noted in rats fed other protein diets or nitrogen-free diets. By 2 hours after feeding, endogenous nitrogen secretions into the gut had extensively diluted the exogenous nitrogen intakes; however, total intestinal amino acid levels were higher in the protein-fed rats than in those fed the nitrogen-free diet. The bulk amino acid compositions of either jejunal or ileal contents 2 hours after feeding were similar regardless of protein source or diet fed.

Plasma amino acid levels generally reflect, although not precisely, the amino acid composition of the ingested proteins (1–3). Dietary deficiencies of single essential amino acids may result in severely depressed plasma levels of that essential amino acid (4, 5), but Zimmerman and Scott (6) and Morrison et al. (7), have shown that a low plasma amino acid level did not always indicate a dietary amino acid deficiency, since plasma levels of individual essential amino acids were not elevated until dietary intakes exceeded requirements. Plasma amino acid levels do not, however, reflect only dietary amino acid intake but also the metabolic and nutritional status of the animal (8); and during fasting or starvation of mammals, protein stores are catabolized to help meet basal energy expenditures (9). In this unfavorable metabolic state, amino acids accumulated in blood plasma (6, 10), but refeeding with nitrogen-free diets, in turn, depressed the plasma amino acid elevations incurred during fasting (6, 10). This latter result has been interpreted to imply that either protein catabolism was depressed or amino acid reutilization was enhanced by the consumption of energy sources, or both (6).

The role of the gastrointestinal tract in mammalian protein metabolism is not well-understood. Dreisbach and Nasset (11) and Nasset and Ju (12) showed that the exogenous nitrogen intake was diluted several-fold with endogenous nitrogen secretions. It was also shown that both the total and the free amino acid composition of the intestinal contents remained rather uniform after single meals of proteins of varying amino acid composition were fed (13–15). Nasset (13, 14) theorized that the high influx of endogenous nitrogen into the gut and the relative constancy of the amino acid composition in gut contents after single meals of various proteins would imply that the gut possessed a homeostatic mechanism for the provision of a constant, most favorable amino acid mixture for intestinal absorption. Acceptance of this hypothesis necessitates, however, the experimental verification that the exogenous and endogenous nitrogen compounds are digested simultaneously since Snook and Meyer (16) and Snook (17) suggested that exogenous proteins were digested first and exerted a

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protective effect on the inactivation and digestion of the pancreatic proteases.

The primary objective of the present study was to gain information on the influence of various protein and nitrogen-free diets on plasma amino acid levels and the amino acid composition of intestinal contents of rats.

EXPERIMENTAL

Three protein sources, casein, rumen bacteria and rumen protozoa were used throughout this study. The rumen bacterial and protozoal proteins were collected and prepared as described previously.[3]

The bulk amino acid compositions of these two protein sources were similar to those reported by Purser and Buechler (18) for rumen bacteria and protozoa. Diets for rats were formulated containing 34% glucose monohydrate; 4% salt mix;[4] 5% corn oil; 1% vitamin mix;[5] and 56% cornstarch; the protein sources were substituted for the cornstarch to a final protein level of 9% in the diet. Since the rumen microbial protein sources were available only in limited quantities, 3 sets of weanling male rats were fed the various diets ad libitum in three separate 8-day feeding trials to facilitate statistical evaluation of the data. At the end of each of the three feeding trials food was removed from all rats and after approximately 2 hours, the rats were anesthetized with ether, blood was withdrawn by heart puncture and the small intestine was completely removed from each rat. Segments of jejum (15.3 cm measured from the duodenum) and of ileum (30.5 cm measured from the cecum) were subsequently stripped of their contents. The gut contents were then homogenized, subsamples were hydrolyzed in 6 N HCl for 18 hours at 121° and the amino acid content of the hydrolysates was determined with a Technicon Auto Analyzer. The percentage amino acid composition was determined for intestinal contents from trials 1 and 2; for trial 3 both total amino acid levels and percentage amino acid compositions were determined for the intestinal contents, but free gut amino acid levels were not determined in trials 1, 2 or 3. Another group of weanling male rats was fed a standard laboratory diet[6] for 8 days, fasted for 18 hours, and after the fasting period, a blood sample was withdrawn by heart puncture from each rat. Plasma amino acid levels were determined as described previously (19). Statistical evaluations of the results were made, using standard analysis of variance procedures.

RESULTS

The results from the study of the effect of the various dietary treatments on total plasma essential amino acid levels of rats are shown in table 1. These data show that feeding a nitrogen-free diet resulted in lower total plasma essential amino acid levels in rats than any of the other treatments, but these differences in the total plasma essential amino acid levels were not significantly different ($P < 0.05$). The percentage distributions of the plasma essential amino acids of rats fed various protein or nitrogen-free diets are given in table 2. The feeding of rumen protozoal protein to rats resulted in significantly

TABLE 1

Total essential amino acid levels in plasma of rats fed various diets [1]

Diet or treatment	*μg EAA/ml plasma*
9% casein	214.0 ± 32.07 [2,3]
9% protozoal protein	178.7 ± 17.03
9% bacterial protein	162.4 ± 20.70
Nitrogen-free	147.2 ± 18.40
Fasted 18 hours	183.6 ± 11.20

[1] Trials 1, 2 and 3.
[2] Mean ± SE.
[3] No significant difference between means at $P < 0.05$.

[3] Bergen, W. G., D. B. Purser and J. H. Cline 1967 Ration effects on protein quality parameters of rumen microbial fractions. J. Anim. Sci., 26: 1489 (abstract).

[4] The salt mixture contained: (in g/kg) ammonium alum, 0.09; calcium carbonate, 68.60; calcium citrate, 308.30; calcium phosphate (monobasic), 112.80; cupric sulfate, 0.08; ferric ammonium citrate, 15.28; magnesium carbonate, 35.20; magnesium sulfate, 38.30; manganese sulfate, 0.20; potassium chloride, 124.70; potassium iodide, 0.04; potassium phosphate (dibasic), 218.80; sodium chloride, 77.10; and sodium fluoride, 0.50 (obtained from General Biochemicals Incorporated, Chagrin Falls, Ohio).

[5] The vitamin mixture contained: (in mg/kg) retinyl palmitate (500,000 IU/g), 39.70; ergocalciferol (500,000 IU/g), 4.41; α-tocopheryl acetate (250 IU/g), 485.00; ascorbic acid (97%), 1017.52; *i*-inositol, 110.23; choline dihydrate citrate, 3715.12; menadione, 49.60; *p*-aminobenzoic acid, 110.23; niacin, 99.21; riboflavin, 22.05; pyridoxine·HCl, 22.05; thiamine·HCl, 22.05; Ca pantothenate, 66.14; biotin, 0.44; folic acid, 1.98; vitamin B_{12} (0.1% in mannitol), 29.77; and cornstarch diluent (Vitamin Fortification Mixture, obtained from General Biochemicals Incorporated).

[6] Ralston Purina Company, St. Louis.

TABLE 2

Essential amino acids in plasma of rats fed various diets [1]

	9% bacterial protein diet (5) [2]	9% protozoal protein diet (5)	9% casein diet (6)	Nitrogen-free diet (4)
Threonine	19.74 ± 1.05 [3]	15.34 ± 1.24	12.87 ± 1.81	26.32 ± 2.70
Valine	9.88 ± 0.64	5.54 ± 0.27 [4]	9.13 ± 0.49	7.50 ± 0.46 [4]
Methionine	2.30 ± 0.26	2.12 ± 0.08	2.85 ± 0.29	2.90 ± 0.19
Isoleucine	5.26 ± 0.55	5.12 ± 0.88	4.72 ± 0.14	3.78 ± 0.63
Leucine	7.46 ± 0.97	5.54 ± 0.41	7.73 ± 0.41	7.07 ± 0.56
Tyrosine	4.28 ± 0.43	5.96 ± 0.88	6.40 ± 0.80	3.30 ± 0.67
Phenylalanine	4.92 ± 0.56	4.62 ± 0.42	3.98 ± 0.41	4.58 ± 0.28
Lysine	41.78 ± 2.28	54.24 ± 2.04 [5]	43.03 ± 3.14	33.25 ± 2.69 [5]
Histidine	3.92 ± 0.68 [4]	1.48 ± 0.20 [4]	7.68 ± 0.65 [4]	12.83 ± 0.84 [4]

[1] Percentage distribution by weight (trials 1, 2 and 3).
[2] Numbers in parentheses indicate number of rats for each mean.
[3] Mean ± SE.
[4] Indicates significant differences between means at ($P < 0.01$).
[5] Indicates significant differences between means at ($P < 0.05$).

lower ($P < 0.01$) plasma histidine and valine levels as compared with the other dietary treatments; the feeding of nitrogen-free diets resulted in significantly higher ($P < 0.01$) plasma histidine levels than feeding of the other diets and resulted in significantly lower ($P < 0.05$) plasma lysine levels compared with feeding of casein or rumen bacterial protein.

The amino acid composition of intestinal contents is given in tables 3–6. Total amino acid levels in the upper jejunal and ileal contents from rats in trial 3 are given in table 3. During the feeding period immediately preceding the 2-hour deprivation periods in trial 3, the protein intakes were 590 mg, 540 mg and 545 mg, respectively, for the casein, bacterial protein and protozoal protein diet. Although these data represent only a few rats, the qualitative amino acid compositions of the gut contents were similar to the results from trials 1 and 2. Various dietary treatments resulted in decreasing sequences of total amino acid levels in the intestinal contents at various intestinal sites 2 hours after feed removal. For rats, in the upper jejunal contents the decreasing order of total amino acid levels with the various diets was in the sequence: casein, bacterial protein, protozoal protein, and nitrogen-free; but in the ileal contents, with these diets the decreasing order of total amino acid levels was changed to the sequence: bacterial protein, protozoal protein, casein and nitrogen-free. The contri-

TABLE 3

Total amino acids in the upper jejunum and ileum of rats fed different diets [1]

Diet	No. of rats	Upper jejunum	Ileum
		mg	*mg*
Nitrogen-free diet	4	21.49	49.10
9% casein diet	3	51.31	53.34
9% bacterial protein diet	3	41.37	72.67
9% protozoal protein diet	3	27.41	69.81

[1] Feeding trial 3.

TABLE 4

Amino acids in the upper jejunum and ileum from the exogenous protein sources [1,2]

Diet	No. of rats	Upper jejunum	Dilution ratio [3]	Ileum	Dilution ratio [3]
		mg		*mg*	
9% casein diet	3	29.82	1:1	4.24	1:10
9% bacterial protein diet	3	19.08	1:1	23.57	1:2
9% protozoal protein diet	3	5.92	1:4	20.71	1:2

[1] Feeding trial 3.
[2] Exogenous amino acids calculated according to the formula: total AA in gut segment (for given diet) minus total AA in gut segment (for nitrogen free diet).
[3] Dilution ratio: parts of exogenous amino acids to parts of endogenous amino acids.

TABLE 5

Amino acids in the HCl hydrolysates of jejunal and ileal contents of rats fed various diets [1]

Diet	Nitrogen-free	9% casein	9% bacterial protein	9% protozoal protein	Nitrogen-free	9% casein	9% bacterial protein	9% protozoal protein
Gut segment	Jejunum (6) [2]	Jejunum (6)	Jejunum (7)	Jejunum (7)	Ileum (6)	Ileum (6)	Ileum (7)	Ileum (7)
Aspartic acid	9.3 ± 0.44 [3]	9.9 ± 0.65	9.6 ± 0.68	9.4 ± 0.57	7.6 ± 1.67	8.5 ± 0.67	9.5 ± 0.40	9.6 ± 0.43
Threonine	5.8 ± 0.09	5.5 ± 0.25	6.4 ± 0.50	6.7 ± 0.78	6.1 ± 0.40	6.5 ± 0.41	5.7 ± 0.55	6.2 ± 0.39
Serine	5.9 ± 0.60	5.6 ± 0.29	6.2 ± 0.49	6.2 ± 0.35	5.8 ± 0.25	6.1 ± 0.56	5.5 ± 0.29	5.4 ± 0.29
Glutamic acid	11.9 ± 1.88	13.3 ± 0.63	12.5 ± 1.06	11.6 ± 1.30	11.5 ± 1.79	12.3 ± 1.67	12.2 ± 2.17	12.3 ± 1.09
Proline	4.3 ± 0.58	4.2 ± 0.62	4.0 ± 0.55	4.1 ± 0.56	5.3 ± 0.83	4.0 ± 0.29	4.7 ± 0.48	5.0 ± 0.92
Glycine	6.0 ± 1.09	5.3 ± 0.46	5.8 ± 0.41	5.3 ± 0.41	5.6 ± 0.56	5.00 ± 0.39	6.3 ± 0.41	5.5 ± 0.43
Alanine	6.3 ± 0.83	6.0 ± 0.52	6.3 ± 0.22	6.2 ± 0.29	7.4 ± 0.62	8.8 ± 0.42	7.1 ± 0.47	7.0 ± 0.39
Valine	5.6 ± 0.61	5.3 ± 0.65	5.1 ± 0.28	5.3 ± 0.31	6.5 ± 0.85	5.9 ± 0.57	6.0 ± 0.40	5.5 ± 0.32
Methionine	2.9 ± 0.03	2.9 ± 0.12	2.3 ± 0.06	2.7 ± 0.33	2.5 ± 0.31	2.8 ± 0.03	2.6 ± 0.16	2.3 ± 0.16
Isoleucine	5.1 ± 0.03	5.0 ± 0.22	5.1 ± 0.17	5.4 ± 0.16	5.2 ± 0.23	5.4 ± 0.23	5.5 ± 0.15	5.3 ± 0.18
Leucine	8.8 ± 0.26	8.8 ± 0.49	9.8 ± 0.44	8.8 ± 0.58	9.5 ± 0.34	9.1 ± 0.25	9.2 ± 0.40	8.7 ± 0.19
Tyrosine	4.7 ± 0.10	4.6 ± 0.18	4.6 ± 0.22	4.5 ± 0.21	4.0 ± 0.32	4.6 ± 0.18	4.4 ± 0.30	4.5 ± 0.32
Phenylalanine	5.5 ± 0.08	5.1 ± 0.22	5.1 ± 0.27	5.5 ± 0.24	5.1 ± 0.26	5.4 ± 0.08	5.6 ± 0.20	5.4 ± 0.32
Lysine	8.2 ± 0.44	8.6 ± 0.76	9.2 ± 0.81	8.6 ± 0.59	8.4 ± 0.18	8.4 ± 0.22	8.6 ± 0.43	9.2 ± 0.54
Histidine	3.2 ± 0.11	3.0 ± 0.13	2.8 ± 0.21	3.2 ± 0.13	3.3 ± 0.12	3.0 ± 0.05	3.0 ± 0.18	2.9 ± 0.22
Arginine	6.6 ± 0.56	7.1 ± 0.35	6.7 ± 0.38	6.7 ± 0.46	7.5 ± 0.40	7.1 ± 0.52	5.5 ± 0.63	6.8 ± 0.34

[1] Percentage distribution by weight.
[2] Numbers in parentheses indicate number of rats in each mean.
[3] Mean ± SE.

TABLE 6

Relative EAA concentration of HCl hydrolysates of protein-containing test meals and of jejunal contents from rats fed these rations

	Nitrogen-free diet		9% bacterial protein diet		9% protozoa protein diet		9% casein diet	
	Jejunal contents	Jejunal contents	Diet	Jejunal contents	Diet	Jejunal contents	Diet	Jejunal contents
				Threonine equal to unity				
Threonine	1.00 [1]	1.00 [2]	1.00 [1]	1.00 [1]	1.00 [1]	1.00 [1]	1.00 [1]	1.00 [1]
Valine	0.97	0.97	0.90	0.80	0.74	0.79 [3]	1.11	0.96
Methionine	0.50	0.16	0.50	0.36	0.44	0.40	0.61	0.53
Isoleucine	0.88	—	0.82	0.80	1.17	0.81	1.28	0.91
Leucine	1.52	0.93	1.11	1.52 [3]	1.49	1.31	2.28	1.60
Phenylalanine	0.94	0.56	0.85	0.80	1.00	0.82	1.28	0.93
Lysine	1.41	0.90	1.10	1.43 [3]	1.58	1.28	2.01	1.56
Histidine	0.56	0.32	0.33	0.44 [3]	0.42	0.48 [3]	0.60	0.55
Arginine	—	—	0.71	1.05 [3]	0.70	1.00 [3]	0.86	1.29 [3]
				Phenylalanine equal to unity				
Threonine	1.07 [1]	1.68 [2]	1.18 [1]	1.25 [1]	1.00 [1]	1.18 [1]	0.78 [1]	1.07 [1]
Valine	1.04	1.62	1.06	1.00	0.74	0.93 [3]	0.87	1.03
Methionine	0.53	0.27	0.59	0.45	0.44	0.47 [3]	0.48	0.57 [3]
Isoleucine	0.94	—	0.98	1.00	1.17	0.96	1.00	0.98
Leucine	1.62	1.56	1.31	1.90 [3]	1.49	1.54 [3]	1.78	1.73
Phenylalanine	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Lysine	1.51	1.51	1.30	1.80 [3]	1.58	1.50	1.57	1.68
Histidine	0.60	0.60	0.39	0.55 [3]	0.42	0.57 [3]	0.47	0.58
Arginine	—	—	0.84	1.31 [3]	0.70	1.18 [3]	0.67	1.38 [3]

[1] Present investigation.
[2] From Nasset (13).
[3] Higher relative concentration in jejunum than in the diet.

butions to the total gut amino acids from exogenous nitrogen intakes and from endogenous nitrogen secretions were estimated with the assumption that the total gut amino acid level of rats fed the nitrogen-free diet represented the endogenous nitrogen secretion level for the protein-fed rats. A similar assumption is made to correct for metabolic fecal nitrogen in the Thomas-Mitchell biological value determination procedure (20). Data on the response of nitrogen secretions of endogenous origin to protein ingestion (14) implied that with the use of this assumption total endogenous nitrogen secretions are likely to be underestimated in rats fed protein diets. On the basis of this assumption extensive dilution of the exogenous nitrogen intake by endogenous nitrogen secretions had occurred in the small intestine (table 4).

The percentage distribution of 16 amino acids in hydrolysates of the upper jejunal and ileal contents of rats fed the various protein or nitrogen-free diets are shown in table 5. Statistical evaluation indicated that neither the diets fed nor the intestinal loci significantly affected these amino acid distributions.

Nasset (13) compared the relative concentration of essential amino acids in jejunal contents at 1.5 hours post-feeding and the protein sources. These comparisons showed that a severe deficiency of an amino acid in a protein source could not be detected from the relative amino acid composition of the jejunal contents of dogs fed a single meal of this protein. Similar comparisons were made between the relative essential amino acid distribution of the jejunal contents from rats fed protein or nitrogen-free diets and of the various protein sources (table 6). The relative essential amino acid compositions of the jejunal contents from rats under the various dietary regimen (calculated either with threonine or phenylalanine as unity) were similar and comparable to the results of Nasset (13). The relative essential amino acid composition of the protein sources fed in this study were not similar. A comparison of the essential amino acid distribution of the jejunal contents and protein sources indicated that for some essential amino acids (see footnote 3, table 6) the relative concentration was higher in the jejunum than in the protein. It was of interest to observe that in most instances these same essential amino acids were implicated as either the limiting acid or included with the less available amino acids of these proteins (18).

DISCUSSION

Lowered total plasma essential amino acid levels in rats fed the nitrogen-free diet compared with total plasma essential amino acid levels in fasted rats confirmed the results of similar studies (10, 6) with chicks. These workers (6, 10) suggested that feeding nitrogen-free diets depressed protein catabolism or enhanced amino acid reutilization, thus accounting for the total plasma essential amino acid depressions. The total plasma essential amino acid levels 2 hours after feed removal in rats fed the three protein diets were not different from the total plasma essential amino acid levels in the fasted rats. Ganapathy and Nasset (21) reported that the ingestion of protein may cause increases or decreases in plasma amino acid levels with respect to fasting plasma amino acid levels. To explain these results Ganapathy and Nasset (21) reasoned that extensive neosynthesis of endogenous nitrogen secretions during the process of protein digestion may account for the unpredictable plasma amino acid response after feeding. McLaughlan (22), however, reported that certain essential amino acids increased markedly in plasma within 2 hours after protein ingestion.

The finding that the incorporation of protozoal protein in the diets depressed plasma histidine levels agreed well with a previous report that a dietary deficiency of an essential amino acid resulted in depressed plasma levels (4) since histidine was the limiting amino acid of this protein source.[7] The lowering of the plasma histidine level when protozoal protein was fed was also indirectly reported by Oltjen and Putnam (23); these workers found that when dietary treatments caused a depression of the rumen protozoa population,

[7] Bergen, W. G. 1967 Studies on the effect of dietary and physiological factors on the nutritive quality and utilization of rumen microbial proteins. Ph.D. Dissertation, The Ohio State University, Columbus, Ohio.

plasma histidine levels in steers were increased. Valine was not the limiting amino acid for protozoal protein, but the low valine plasma level might be explained with the observation in chickens that plasma levels of an amino acid will not increase until dietary intake exceeds the requirement (6).

Jones and Waterman (24) have indicated that protein disutilization in the small intestine may involve fecal elimination of a protein fraction containing disproportionate amounts of certain amino acids. The amino acid composition of contents of intestinal segments were therefore analyzed for disproportionate accumulations of amino acids implicated by the PAA-S method (25) as poorly available or limiting in the proteins fed.[8] The percentage distribution of the amino acids in both intestinal segments studied was similar regardless of the diet fed. Thus, disutilization of amino acids as indicated by a relative accumulation in gut contents was not observed and our initial goals in this study were not realized, although the determination of the amino acid composition of short segments of the ileum close to the cecum might prove to be more fruitful.

The amino acid concentration and composition in intestinal contents of rats showed that at 2 hours after feed removal the endogenous nitrogen secretions extensively diluted the ingested exogenous nitrogen, and that the amino acid composition of intestinal contents of rats was relatively constant when the rats were fed different proteins or nitrogen-free diets for 8 days. Differences in total amino acids from the exogenous proteins in the upper jejunum may be accounted for by the differences in total dietary protein intake by the rats during the preceding feeding period, whereas differences in total amino acids from the exogenous proteins in the ileum may be accounted for by differences in the digestibilities of the dietary proteins. The true digestibilities of casein, protozoal protein and bacterial protein were 97, 87 and 75,[9] respectively. The data (table 6) further imply the presence of a gut homeostatic mechanism by the endogenous nitrogen secretions to assure a favorable amino acid mixture for absorption. These results are an extension of the results by Nasset and co-workers (11–15) and appear to be in agreement with their contention that the gut possesses a homeostatic mechanism to assure a favorable amino acid mixture for absorption, but several factors that may influence the interpretation of these results must be considered. Studies by Nasset and co-workers (11, 12) as well as the present results, indicate that by 2 hours after feeding, the exogenous nitrogen intakes were extensively diluted by endogenous nitrogen secretions. Dawson et al. (26) reported that within 2 hours, approximately one-half of the labeled ^{14}C algal protein ingested by rats had disappeared from the intestinal lumen, whereas Snook and Meyer (16) and Snook (17) suggested that as long as dietary nitrogen sources were available for digestion they exerted a protective effect on the autodigestion of the proteolytic enzymes. These reports raise the question of whether simultaneous digestion of the exogenous nitrogen intakes and endogenous nitrogen secretions can occur at a sufficient level to provide the homeostatic mechanisms suggested by Nasset (13, 14). Experimental manipulations during the removal of the intestine and the washing procedure of the intestinal contents may also have influenced the present results since it had been shown that extensive mucosal shedding occurred in the gut of sheep during slaughter procedures (27). Finally, the present studies differed from the previous work (13, 14) in that rats were fed the various diets for 8 days and the bulk amino acid composition of intestinal contents was determined instead of the composition of free amino acids present in intestinal contents. From the above considerations the alternative suggestion may be offered that at the time of sampling of the gut contents, irrespective of dietary differences even for extended periods, endogenous nitrogen secretions account for the major part of the total gut amino acids and thus similarities in the amino acid composition of the gut contents may only be a direct reflection of these circumstances and not a demonstration of gut homeostasis.

[8] See footnote 7.
[9] See footnote 7.

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Response of Lipogenesis to Repletion in the Pyridoxine-deficient Rat [1,2]

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ABSTRACT Pyridoxine has been implicated in fat metabolism. To elucidate its role the response of lipogenetic activities in the liver and epididymal pad of deficient rats to pyridoxine administration was measured. Rats were fed a pyridoxine-deficient diet until they ceased to gain weight (40 days); they were then repleted by the subcutaneous injection of 300 μg pyridoxine·HCl/rat every day for 5 days. During repletion, diets varying in fat content (0 to 25%) were fed. The administration of the vitamin resulted in increases in the liver and epididymal glucose 6-phosphate dehydrogenase which were small in the pair-fed group and substantial in the ad libitum-fed controls. The enzyme activity was highest (five fold) in rats receiving the fat-free diet. The substitution of starch for sucrose resulted in lower levels of dehydrogenase acitvity. In animals fed ad libitum and repleted with the same basal diet, the liver lipids, the hepatic glucose 6-phosphate dehydrogenase activity and the extent of acetate-^{14}C incorporation into liver lipids were high on day 2 of repletion and returned to normal levels by day 11 with a concomitant increase in carcass fat, approaching the levels in controls maintained with a pyridoxine-sufficient diet throughout the experiment. The data demonstrate that the role of pyridoxine in lipogenesis may be mediated through the increase in food intake with the repletion of the vitamin.

The role of pyridoxine in fat metabolism has not been clearly elucidated. Withdrawal of the vitamin from the diet results in lower levels of body fat than those for the ad libitum- or pair-fed controls (1–5). The liver lipids have been reported to be lowered (6) or not to be affected in amount [4] or characteristics (7) by pyridoxine deficiency. Desikachar and McHenry (8) observed no effect of deficiency on the incorporation of glucose-^{14}C into liver fat. Wakil (9), however, presented evidence for the presence in liver mitochondria of an enzyme system that catalyzed the elongation of fatty acids, the activity of which was enhanced in vitro by the addition of pyridoxal phosphate to the system.

In the present studies, lipogenesis and other metabolic functions were determined on rats that were fed a pyridoxine-deficient diet until they ceased to gain weight, and were then repleted with the vitamin for a period of time. It appeared that this approach would show the metabolic responses in a more lucid manner than a comparison of rats maintained for a long period of time with deficient and complete diets. In view of recent reports that a change in the carbohydrate source (10), fasting and refeeding (11) or varying the fat content of the diets (12) alters lipogenesis and the activity of the dehydrogenases of the pentose phosphate pathway, the effects of feeding sucrose or starch and of varying the level of dietary fat during pyridoxine repletion were also investigated.

EXPERIMENTAL

In experiment 1, weanling male Wistar strain rats [5] (body weight 40–50 g) were fed a pyridoxine-deficient basal diet (diet 1 in table 1) for about 40 days, at which time they ceased to gain weight. At this stage their body weights were between 95 and 120 g. They were then divided into groups of 8 rats each, matched according to their body weights. Repletion was brought about by the subcutaneous injec-

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[4] Pardini, R. S., and H. E. Sauberlich 1967 The effect of pyridoxine deficiency on lipid metabolism. Federation Proc., 26: 412 (abstract).

[5] Obtained from Woodlyn Farm Ltd., Guelph, Ontario, Canada.

TABLE 1

Composition of experimental diets

	Diet 1	Diet 2	Diet 3	Diet 4
	g	*g*	*g*	*g*
Casein [1]	17	17	17	17
Sucrose	66.7	76.7	51.7	—
Cornstarch	—	—	—	66.7
Corn oil	10	—	25	10
Vitamin mixture [2]	2.5	2.5	2.5	2.5
Salts mixture [3]	3.8	3.8	3.8	3.8

[1] Vitamin-Free Casein, Nutritional Biochemicals Corporation, Cleveland, contained 88% protein by analysis.

[2] The vitamin mixture contained per 100 g; (in milligrams) thiamine·HCl, 20; riboflavin, 40; Ca pantothenate, 80; nicotinamide, 200; biotin, 1.2; folic acid, 4; vitamin B_{12}, 0.2; menadione, 0.8; vitamin D_2, 8; and (in grams) choline dihydrogen citrate, 20; alpha-cellulose, 77.5; and retinyl palmitate, 40,000 IU; and α-tocopheryl acetate, 440 IU.

[3] The salts mixture contained the following per 100 g; (in grams) $CaCO_3$, 19.08; $CaHPO_4 \cdot 2H_2O$, 37.63; Na_2HPO_4, 15.79; KCl, 19.21; $MgSO_4 \cdot 7H_2O$, 6.05; $MnSO_4 \cdot H_2O$, 0.4; ferric citrate, 1.55; $ZnCO_3$, 0.14; and KIO_3, 0.008.

tion of 300 μg pyridoxine·HCl/rat/day. The repleted rats received the experimental diets for 5 days before the termination of the experiment.

In experiment 2, control groups were included which were fed diet 1 with 10 mg pyridoxine·HCl/kg diet ad libitum throughout the experiment, that is, in both the depletion and repletion periods.

At the end of each experiment, the rats were decapitated, the livers and epididymal fat pads were quickly excised, transferred to cold solutions as required by the particular assay, and then washed free from the remaining blood and homogenized.

Carcass samples were prepared according to the method of Hartsook and Hershberger (13). The carcass nitrogen was determined by a micro-Kjeldahl technique (14) and the fat was estimated as the ethyl ether extract in a Soxhlet apparatus (15). Liver lipids were assessed according to Folch et al. (16) by extraction with chloroform-methanol. The protein content of tissue homogenates and supernatants was determined by the Lowry method (17).

Glucose 6-phosphate dehydrogenase activity was determined in liver and epididymal fat supernatants (40,000 × *g* for 30 minutes) as described by Lohr and Waller (18), using a system of 0.4 ml triethanolamine buffer (pH 7.5; 0.05 M), 50 μliters NADP (0.006 M in 1% sodium bicarbonate solution), 0.1 ml supernatant and 50 μliters glucose 6-phosphate (0.1 M). Glucose 6-phosphatase activity was measured in filtered 0.25 M sucrose-liver homogenate (19), using a system of 0.1 ml citrate buffer (0.2 M), 0.1 ml homogenate and 0.2 ml glucose 6-phosphate (0.08 M). The phosphorus released was measured by the method of Taussky and Schorr (20). L-Aspartate:2-oxoglutarate aminotransferase activity was assayed in the liver supernatants (40,000 × *g* for 100 minutes, in 0.25 M sucrose) by the method of Tonhazy et al. (21). In the isotope studies, sodium acetate-1-^{14}C (specific activity 44.4 mCi/mmole [6]) was diluted with stable acetate, dissolved in physiological saline and injected intraperitoneally one hour before killing at the rate of 2.16 × 10^6 dpm in 1.5 mg of sodium acetate/100 g body weight. Liver lipids were extracted as described previously (16), washed free from acetate, then taken up in toluene and counted in a Nuclear-Chicago MKI liquid scintillation counter using Liquifluor [7,8] as the scintillator. Counting efficiency was approximately 80% and quenching, as checked by the ratios method with an external standard, did not vary between samples.

RESULTS

Experiment 1. This experiment was carried out in 4 parts at one-week intervals. In the first 3 parts, the animals were depleted of pyridoxine with diet 1 (sucrose) then repleted for 5 days. During repletion, the animals were fed diet 1 in one case, or diet 2 (fat-free) or diet 3 (high fat) in the other 2 cases; all these diets contained sucrose as the carbohydrate. To test the effects of the carbohydrate source, animals studied in the fourth part of this experiment were fed the starch-based diet 4 during both depletion and repletion. To verify the uniformity of the experimental conditions, a group of animals fed diet 1 during depletion and repletion was included in each part of the experiment as a control. The responses of this group in the parameters measured were uniform.

[6] Obtained from Nuclear-Chicago Corporation, Chicago.

[7] See footnote 6.

[8] Final concentration, 4 g PPO and 50 g POPOP/liter in toluene.

The changes in body weight, the carcass fat content and liver and epididymal fat pad weights in response to pyridoxine repletion with variations of the source of dietary carbohydrate or the level of dietary fat are shown in table 2. The pair-fed repleted rats did not gain more weight and had no more body fat than the deficient rats regardless of the type of dietary carbohydrate. The pair-fed groups gained more weight than the deficient animals when a high fat diet was fed, whereas the ad libitum-fed repleted rats showed significant increases over the deficient animals in body fat and weight gain. The animals fed the high fat diet showed higher levels of carcass fat than any of the corresponding groups.

Data on enzyme activity and liver lipids are presented in table 3. The L-aspartate: 2-oxoglutarate aminotransferase activities show that under all dietary regimens the supernatant enzyme activity was significantly increased by the administration of pyridoxine.

The glucose 6-phosphatase activity of the liver homogenates failed to respond to pyridoxine repletion under all the conditions tested. However, glucose 6-phosphate dehydrogenase activity of the liver supernatants increased in response to pyridoxine repletion in the ad libitum-fed groups and was highest (over fivefold) when fat was excluded from the diet. The pair-fed animals that received the fat-free diet also showed a significant increase in the liver dehydrogenase activity. The response of the dehydrogenase of the epididymal fat was similar to that of the liver although of lower magnitude.

Experiment 2. Since the glucose 6-phosphate dehydrogenase activity was shown to respond to pyridoxine repletion, it was considered desirable to study the response at 2 and 11 days of repletion and to compare the levels of activity to those of animals maintained with a complete diet throughout the experiment. There was no alteration of the source of carbohydrate or the level of dietary fat in this experiment.

The data presented in table 4 show that the longer repletion period allowed the weight of epididymal fat to increase substantially in the ad libitum-fed groups. The increase in the weight of the fat pad over the 11-day repletion period was significant even in the pair-fed repleted groups.

The hepatic glucose 6-phosphate dehydrogenase showed an initial sharp response (within 2 days) to pyridoxine repletion, particularly in the ad libitum-fed group. As repletion was continued the enzyme activity decreased to a level close to that of the pair-fed group and yet still above that of the deficient or the sufficient controls. The dehydrogenase activity of the epididymal fat pad did not respond as sharply as the liver enzyme. The liver lipids also showed an initial and significant increase with pyridoxine repletion (by day 2), but this effect was not observed at the end of 11 days. Data on the incorporation of acetate-1-^{14}C into liver lipids, given as disintegrations/minute/milligram of liver lipid, showed a significant increase (5 times) in the early stage of repletion (2 days) followed by a decrease to normal levels by day 11.

The pattern of results in the sufficient controls was essentially the same in the groups killed at 2 and 11 days; hence the values obtained for the group killed along with the 11-day repleted rats are presented in table 4. The dehydrogenase activities of the liver and the fat pad were less in the sufficient controls than in any of the repleted groups. The liver lipids and acetate-^{14}C incorporation values were about the same in the sufficient controls and the 11-day repleted groups.

DISCUSSION

The withdrawal of pyridoxine from the diet and its administration resulted in the expected changes in body weight and in the liver supernatant aspartate aminotransferase.[9] In the present studies, the lowering of body and liver fat levels in pyridoxine-deficient rats was confirmed. This is in agreement with several earlier reports (2,3,6). It was further demonstrated that the body and liver fat levels responded to the repletion of the vitamin in deficient animals and to the increased food intake in the repleted ad libitum-fed animals. The magnitude of this response did not appear to be affected by the source

[9] Radhakrishnamurty, R., and Z. I. Sabry 1967 Mitochondrial and supernatant L-aspartate 2-oxoglutarate aminotransferases in vitamin B_6 deficiency. Federation Proc., 26: 414 (abstract).

TABLE 2

Effect of pyridoxine administration on body composition in pyridoxine deficient rats fed starch or sucrose with varying levels of fat [1]

	Deficient	Pair-fed	Ad libitum	Deficient	Pair-fed	Ad libitum
		Diet 1 (sucrose) [2]			Diet 4 (starch) [3]	
Avg wt, g/day	0.0 ± 0.3 [4]	0.9 ± 0.2	8.0 ± 0.6 [5]	0.1 ± 0.3	0.3 ± 0.4	7.3 ± 1.9 [5]
Diet consumed, g/day	6.4	6.4	12.3	6.3	6.3	12.5
Body fat, %	4.5 ± 0.7	4.4 ± 0.7	11.4 ± 1.3 [5]	6.0 ± 0.2	5.8 ± 0.5	10.9 ± 0.8 [5]
Epididymal pad wt, g/100 g body wt	4.11 ± 0.21	3.46 ± 0.2	5.58 ± 0.13 [5]	3.72 ± 0.12	3.20 ± 0.07 [5]	3.76 ± 0.24
Liver wt, g/100 g body wt	0.60 ± 0.08	0.58 ± 0.06	0.84 ± 0.10	0.81 ± 0.05	0.69 ± 0.03	1.04 ± 0.08
		Diet 2 (no fat) [2]			Diet 3 (high fat) [2]	
Avg wt, g/day	0.8 + 0.3	1.7 ± 0.4	8.1 ± 0.5 [5]	1.4 ± 0.5	3.8 ± 0.4 [6]	8.6 ± 0.5 [5]
Diet consumed, g/day	6.8	6.8	12.5	6.2	6.2	13.7
Body fat, %	5.0 ± 0.5	5.4 ± 0.8	12.8 ± 1.9 [5]	8.5 ± 0.87	9.8 ± 1.3	13.9 ± 1.1 [5]
Epididymal pad wt, g/100 g body wt	4.49 ± 0.11	3.87 ± 0.23	6.13 ± 0.28 [5]	3.95 ± 0.16	3.48 ± 0.11	4.65 ± 0.14 [5]
Liver wt, g/100 g body wt	0.62 ± 0.05	0.65 ± 0.05	0.86 ± 0.04 [5]	0.73 ± 0.07	0.88 ± 0.04	0.93 ± 0.11

[1] Eight rats/group.
[2] The 3 groups were depleted with diet 1 for about 40 days, then repleted with indicated diet for 5 days; data shown for repletion period.
[3] The 3 groups were fed diet 4 during about 40 days of depletion and the 5-day repletion period; data shown for repletion period.
[4] Mean ± SE.
[5] Values significantly different from those of the deficient group, $P < 0.01$.
[6] Values significantly different from those of the deficient group, $P < 0.02$.

See Erratum : 96, 287 1968

TABLE 3

Enzyme activities and liver lipid levels after 5 days repletion with pyridoxine [1]

	Deficient	Pair-fed	Ad libitum	Deficient	Pair-fed	Ad libitum
		Diet 1 (sucrose) [2]			Diet 4 (starch) [3]	
L-Aspartate aminotransferase, liver [4]	119 ± 10 [5]	314 ± 17 [6]	239 ± 13 [6]	115 ± 10	222 ± 6 [6]	208 ± 6 [6]
Glc-6-Pase, liver [7]	13.7 ± 0.9	13.2 ± 0.6	13.0 ± 0.4	14.2 ± 0.7	13.9 ± 0.6	14.9 ± 0.7
Glc-6-P dehydrogenase, liver [8]	193 ± 20	252 ± 28	713 ± 51 [6]	127 ± 24	136 ± 24	377 ± 13.0 [6]
Glc-6-P dehydrogenase, epididymal [8]	458 ± 46	642 ± 40 [6]	1068 ± 70 [6]	571 ± 63	674 ± 68	809 ± 14
Liver lipids, %	4.18 ± 0.63	4.64 ± 0.69	12.44 ± 1.59 [6]	3.77 ± 0.26	3.82 ± 0.16	9.86 ± 1.16 [6]
		Diet 2 (no fat) [2]			Diet 3 (high fat) [2]	
L-Aspartate aminotransferase, liver [4]	125 ± 10	336 ± 26 [6]	202 ± 10 [6]	111 ± 8	241 ± 15 [6]	208 ± 19 [6]
Glc-6-Pase, liver [7]	13.8 ± 0.9	13.3 ± 1.0	12.7 ± 0.9	10.9 ± 0.2	13.2 ± 0.5	12.4 ± 0.6
Glc-6-P dehydrogenase, liver [8]	493 ± 76	999 ± 81 [6]	2555 ± 214 [6]	91 ± 12	162 ± 22 [6]	258 ± 28 [3]
Glc-6-P dehydrogenase, epididymal [8]	526 ± 40	595 ± 79	725 ± 75	496 ± 37	410 ± 26	717 ± 97
Liver lipids, %	3.58 ± 0.12	4.08 ± 0.35	18.06 ± 1.20 [6]	4.92 ± 0.83	4.88 ± 0.23	8.51 ± 0.46 [6]

[1] Eight rats/group.
[2] The 3 groups were depleted with diet 1 for about 40 days, then repleted with indicated diet for 5 days; data shown for repletion period.
[3] The 3 groups were fed diet 4 during about 40 days of depletion and the 5-day repletion period; data shown for repletion period.
[4] Expressed as mg pyruvate formed/g liver/hour.
[5] Mean ± SE.
[6] Values significantly different from those of the deficient group, $P < 0.01$.
[7] Expressed as μg P released/mg protein/15 minutes.
[8] Expressed as Bucher units/100 mg tissue protein.

TABLE 4

Changes in liver, adipose tissue and acetate-1-^{14}C incorporation after 2 and 11 days of repletion [1]

	2 days [2]			11 days [2]			Sufficient control [3]
	Deficient	Pair-fed	Ad libitum	Deficient	Pair-fed	Ad libitum	
Avg gain, g/day	0.2 ± 0.8 [4]	3.5 ± 0.3 [5]	10.7 ± 0.7 [5]	0.1 ± 0.3	2.2 ± 0.1 [5]	7.8 ± 0.4 [5]	4.5 ± 0.34
Liver wt, g/100 g body wt	3.72 ± 0.14	3.81 ± 0.23	5.03 ± 0.27 [5]	4.37 ± 0.15	3.01 ± 0.54	4.04 ± 0.22	3.92 ± 0.12
Epididymal pad, g/100 g body wt	0.69 ± 0.02	0.72 ± 0.03	0.76 ± 0.08	0.57 ± 0.04 [5]	1.26 ± 0.05 [5]	1.26 ± 0.05 [5]	1.69 ± 0.16 [5]
Body fat, %	6.2 ± 0.5	6.0 ± 0.7	11.7 ± 1.0 [5]	6.7 ± 1.0	7.2 ± 1.0	15.0 ± 0.8 [5]	17.3 ± 2.1 [5]
Glc-6-*P* dehydrogenase, liver [6]	326 ± 58	540 ± 84	1181 ± 148 [5]	290 ± 62	536 ± 28 [7]	521 ± 53	336 ± 64
Glc-6-*P* dehydrogenase, epididymal [6]	459 ± 82	540 ± 57	587 ± 32	433 ± 36	479 ± 27	645 ± 34	373 ± 80
Liver lipids, %	3.02 ± 0.21	3.14 ± 0.34	6.61 ± 0.60 [5]	2.22 ± 0.24	2.13 ± 0.21	3.61 ± 0.67	4.32 ± 0.22 [5]
Acetate-^{14}C incorporation, dpm/mg liver lipid	102 ± 18	137 ± 30	545 ± 83 [5]	94 ± 25	95 ± 21	58 ± 27	65 ± 13

[1] Eight rats/group.
[2] All groups received diet 1 for about 40 days before the 2 or 11 days of repletion; data shown for repletion periods.
[3] The animals were fed diet 1 with 1 mg pyridoxine·HCl/100 g diet ad libitum throughout the experiment; data shown correspond to 11-day repletion.
[4] Mean ± SE.
[5] Values significantly different from those of the deficient group, $P < 0.01$.
[6] Expressed as Bucher units/100 mg tissue protein.
[7] Values significantly different from those of the deficient group, $P < 0.02$.

of dietary carbohydrate nor by the level of dietary fat during the repletion period. The pair-fed controls, unlike those fed ad libitum, showed no significant differences from the deficient animals in their body and liver fat levels and also in their body weight gains and liver and epididymal fat pad weights. In the ad libitum-fed repleted rats, the liver lipid levels were high (18% with the fat-free diet and 8–10% with the other diets) compared with those of the deficient animals. This is possibly due to increased lipogenesis as a result of increased intake and utilization of diet under the influence of pyridoxine. However, the increased activity of the liver glucose 6-phosphate dehydrogenase of the pair-fed controls indicates that increased food intake may not be the sole criterion responsible for favoring lipogenesis.

The ingestion of a high fat diet during the repletion period resulted in substantially higher levels of body fat, particularly in the deficient and pair-fed groups. There was no corresponding increase in the fat content of the liver nor in the activity of the hepatic glucose 6-phosphate dehydrogenase. This suggests that the increased fat depot in animals fed a high fat diet was mostly due to deposition rather than to *de novo* lipogenesis. These observations are in agreement with those of Hill et al. (12) who showed that a low fat diet favored lipogenesis whereas high dietary fat levels were inhibitory. The present data further demonstrate that even under conditions unfavorable to lipogenesis, the response of the hepatic glucose 6-phosphate dehydrogenase to pyridoxine repletion is significant in both the pair-fed and the ad libitum-fed animals.

The interrelationship of lipogenesis and pyridoxine nutriture is well-demonstrated as a response to the repletion of the vitamin in critically deficient animals. This response has been shown in the present studies over a repletion period of 11 days. Initially and within 2 days of the institution of the repletion the controls fed ad libitum exhibited substantially higher activity of hepatic glucose 6-phosphate dehydrogenase, increased incorporation of acetate-^{14}C in liver lipids and higher levels of body and liver fat. The hepatic glucose 6-phosphate dehydrogenase activity and the extent of the incorporation of acetate-^{14}C in the liver in the 2-day repleted animals were higher than those in comparable controls maintained with a pyridoxine-sufficient diet all their life. By day 11 of repletion, the hepatic glucose 6-phosphate dehydrogenase of the controls fed ad libitum had decreased to the level of the pair-fed control which was still higher than that of either the deficient group or the sufficient control. The incorporation of acetate-^{14}C in liver lipids decreased to a level comparable to those in the sufficient control, the deficient and the repleted pair-fed animals. Similarly, the level of liver lipids was reduced. Concomitantly, the level of body fat increased even further than in the 2-day repletion groups and became comparable to that of the sufficient control.

The increase in liver fat levels in the initial stages of repletion in the ad libitum-fed controls might be interpreted as a resultant of a spurt in lipogenesis to a level beyond the capacity of the lipid transport system which might be inadequate to meet the situation. This postulate is supported by the return to normal of liver lipid levels and the increase in carcass fat on prolonged repletion. It is not clear from the data whether this spurt in lipogenesis was in response to pyridoxine repletion per se or to the increase in food intake that accompanied repletion. Lipogenesis is known to be stimulated by fasting and refeeding (11), a condition that may be considered analogous to the sudden increase in food intake with repletion. In comparing the lipogenic response of the deficient rats and their pair-fed controls, it is important to recognize that the effect of pyridoxine repletion is not the only one measured and that the institution of a meal-eating pattern, inherent in pair-feeding, must have some influence on the response. Leveille (22) has reported that meal-eating results in increased lipogenesis when compared with a nibbling pattern. The sufficient control, although exhibiting a lipogenic activity similar in magnitude to that of the deficient animals, was able to store more body fat. This may be accounted for by the high food intake of the sufficient controls throughout the duration of the experiment.

It might be difficult to assign to pyridoxine a primary role in lipogenesis. However, its effect, even if mediated through an increase in food intake, is profound and well-demonstrated.

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Hepatic RNA Metabolism in Male and Female Obese Rats Fed Cholesterol [1,2]

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ABSTRACT DNA-dependent RNA polymerase activity was studied in liver nuclei from genetically lean or obese rats of the Zucker strain (males and females). Two recommended and modified assay procedures were adopted: a conventional Mg^{++}-dependent system (I); and an Mn^{++}-dependent-$(NH_4)_2SO_4$-stimulated system (II). Assays were carried out in liver nuclear fractions from rats fed either a control purified diet or a standardized cholesterol-containing diet. Liver nuclei from obese rats had higher levels of polymerase activity by either assay procedure; the activity measured in nuclear material derived from females (of either genotype) was consistently higher than those from males. However, cholesterol feeding resulted in a depression of nuclear RNA polymerase activity in obese and lean rats of both sexes only when method I was used, suggesting that this depression (which also reflects changes in microsomal protein synthesis) may be more closely related to ribosomal RNA synthesis. An examination of RNA synthesis in vivo generally supported these findings. Using ^{14}C-orotic acid, it was shown that the greatest percentage decrease was associated with a crude nucleolar fraction.

Amino acid incorporation into the total proteins of the liver in vivo or in vitro has been shown to be depressed significantly in those rats maintained with a diet containing cholesterol (1, 2). The dietary effect has been found to be more marked in a strain of genetically obese rats. Under normal dietary conditions, the obese rats spontaneously show a greater uptake of labeled amino acids into the proteins of at least 3 tissues, including liver, than do control lean rats of the same strain or commercially obtained rats (3). In all these studies, such differences were more closely related to the protein-synthesizing events of the microsomal fraction than to the cytoplasmic or cell sap fraction. A preliminary report of hepatic DNA-dependent RNA polymerase activity in liver nuclei from both lean and obese rats fed diets containing cholesterol showed that the activity of this enzyme reflected the changes in microsomal protein synthesis, i.e., cholesterol feeding resulted in a decrease in the activity of this enzyme (4). This report also indicated that the decreased capacity for protein synthesis in vitro in genetically obese rats was greater than expected on the basis of the observed decreases in polymerase activity, as well as of comparisons made in control lean rats. It was surmised that the conventional polymerase assay may reflect only part of the total polymerase activity since it had been recently postulated that two apparently different polymerase activities can be measured in rat liver systems (5, 6).

In the present report, an examination of nuclear and microsomal events was carried out in both lean and obese rats fed a standardized diet containing cholesterol. The RNA polymerase activities in liver nuclei were determined by modified and improved methods for measuring both an enzyme activity ascribed to ribosomal-like RNA synthesis as well as one purportedly for DNA-like RNA synthesis (5, 6). These data were compared with microsomal protein synthesis in vitro as well as RNA synthesis in vivo. Comparisons were made in both male and female rats.

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[2] Part of these data were included in a preliminary report at the annual meeting of the Federation of American Societies for Experimental Biology, Atlantic City, 1966.

MATERIALS AND METHODS

Young adult male and female rats (13 to 16 weeks old) were housed in a temperature- and humidity-controlled room, and maintained with commercial laboratory ration [3] before the introduction of a purified diet. The control and "cholesterol" purified diets used are described in table 1. Genetically obese and lean rats of the Zucker strain were used for these studies (7). Rats of the Charles River strain were included in the preliminary tests.

Each rat was decapitated after an overnight fast (16–18 hours) and the liver was immediately perfused *in situ* with 5 ml of ice-cold sterile 0.9% NaCl and then removed and placed in an ice-cold medium. Three to four grams were saved for nuclear RNA polymerase activity or for amino acid incorporation into microsomal protein in vitro. In studies carried out in vivo the tissue was initially prepared in the same way and kept at zero to 5° before analysis.

Nuclear RNA polymerase activity. After perfusion, the liver was placed in 0.25 M sucrose. Duplicate tissue samples weighing approximately 3 g each were finely minced and homogenized in 5 volumes of 0.25 M sucrose containing 5 mM $MgCl_2$ and 0.035 M Tris buffer, and adjusted to a pH of 7.8. The preparation was gently layered onto 10 ml of 0.34 M sucrose containing 5 mM $MgCl_2$ and centrifuged at 1000 × *g* in an International ultracentrifuge at zero to 2°. The subsequent crude nuclear pellet was washed with 0.25 M sucrose containing 5 mM $MgCl_2$; and a final 20-ml suspension of 2.2 M sucrose containing 5 mM $MgCl_2$ was centrifuged at 40,000 × *g* for 60 minutes. The purified nuclei were washed with 0.25 M sucrose containing 5 mM $MgCl_2$ and suspended in 10 ml of 0.05 M Tris buffer (pH 8.0). After the nuclei were allowed to lyse for 20 minutes, the resultant enzyme aggregate was isolated as a gel-like pellet following 10-minute centrifugation at 10,000 × *g*. The aggregate was lysed and suspended in 0.25 M sucrose based on the ratio of 3 to 5 ml of 0.25 M sucrose/6 g of original liver tissue. The activity of RNA polymerase was determined by the incorporation of 8-^{14}C-ATP [4] into RNA based on a modification of Weiss' method (8). The total volume of the final incubation of mixture was 0.5 ml. The incubation was carried out at 37° for 15 minutes and the 0.5 ml mixture contained 2.5 μmoles of $MgCl_2$, 3 μmoles of NaF, 50 μmoles of Tris phosphate buffer (pH 8.6), 10 μmoles cysteine (free base), 0.4 μmoles of the sodium salts of CTP, GTP, and UTP, and 0.02 μmoles of 8-^{14}C-ATP (0.5 μCi). Cold 0.2 N perchloric acid (PCA) was added to stop the reaction; and 0.5-ml duplicate samples of each enzyme aggregate were taken for nucleic acid and protein determinations. Samples were washed twice with 0.2 N PCA and finally with 5% TCA. The labeled RNA was completely extracted by two successive treatments with a 10% NaCl solution containing carrier RNA (0.5 μg sodium ribonucleate/ml) at 100° for 30 minutes. The RNA was precipitated from the combined supernatants with absolute ethanol overnight at zero to 2°. Finally the precipitate was dissolved in 1 ml of 0.1 N NH_4OH and placed on a stainless steel planchet for counting in a Nuclear Chicago thin-window gas-flow counter. The protein remaining after the RNA extraction was purified and determined by the method of Lowry et al. (9). The activity of the DNA-dependent RNA

TABLE 1

Composition of diets [1]

	Control	Cholesterol +
	%	%
Casein	20.0	20.0
Corn oil	20.0	20.0
Salt mixture [2]	4.0	4.0
Choline chloride	0.2	0.2
Inositol	0.1	0.1
Vitamin mixture [3]	0.1	0.1
Sucrose	55.6	53.6
Cholesterol	—	1.5
Cholic acid	—	0.5

[1] All dietary ingredients with the exception of corn oil (Corn Products Company, New York) and sucrose were purchased from Nutritional Biochemicals Corporation, Cleveland.

[2] Hegsted et al., J. Biol. Chem., *138*: 459, 1941.

[3] Vitamin mixture contained per kg of diet: (in milligrams) thiamine, 5.0; riboflavin, 5.0; niacin, 80.0; Ca pantothenate, 50.0; folic acid, 0.25; pyridoxine·HCl, 4.0; biotin, 0.20; menadione, 5.0; vitamin E acetate, 400.0; and vitamin A, 13,000 IU; vitamin D, 2,000 IU; mixture made up to 1 g with dextrose.

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

[4] ATP-8-^{14}C (specific activity, 16.8 to 17.1 mCi/mmole) was purchased from Schwarz BioResearch, Inc., Orangeburg, New York.

polymerase activity was expressed as counts per minute of ^{14}C-ATP incorporated per milligram of nucleoprotein or per milligram of DNA. These procedures are modified techniques described by others (10, 11).

RNA polymerase activity was also measured by the method of Widnell and Tata (5, 6). This "enzyme" activity is based on an Mn^{++}-dependent-$(NH_4)_2SO_4$-stimulated system. The incubation mixture (0.5 ml) contained 0.05 ml of a solution of $(NH_4)_2SO_4$ saturated at room temperature and adjusted to pH 7.8 with aqueous NH_3. 0.1 ml of nuclear suspension (containing 0.3 μg DNA), 2 μmoles of $MnCl_2$; 50 μmoles of Tris-HCl buffer, pH 7.8, 0.3 μmoles of GTP, CTP, and UTP and 0.015 μmoles of ATP. The system was preincubated for 15 minutes with the unlabeled ATP before 0.5 μCi of 8-^{14}C-ATP was added; an additional 45-minute incubation was then carried out.

The kinetics of incorporation of labeled ATP into the RNA expressed in terms of nuclear DNA was carried out in both obese and lean rats using both the above methods (fig. 1). Similarly, the pH optima for both assays was carried out in nuclear isolates from both genotypes (fig. 2). Other essential features of the assay, for example, cofactor requirements, were similarly studied in this laboratory and generally agree with published data. NAD pyrophosphorylase was also determined (12) in selected livers from 4 obese and lean rats. This precaution was taken to obviate any nonspecific effect following the use of labeled ATP. No differences were noted in NAD pyrophosphorylase activity between control and treated rats. In other studies with commercially obtained rats, no effect on the rate of the Mg^{++} system was noted between these two groups.[5]

Amino acid incorporation into microsomal protein in vitro. The microsomes were isolated after centrifugation at 105,000 × *g* for one hour. The final incubation medium contained 0.4 ml of the 105,000 × *g* supernatant (cell sap) and 0.4 ml of microsomal suspension, and 0.2 ml of supporting medium. This 1.0-ml suspension contained ATP 1 μM; GTP 0.25 μM; phosphoenol pyruvic acid (PEP)

[5] Yokono, O., and L. C. Fillios 1966 Liver RNA polymerase activity in obese rats fed cholesterol. Federation Proc., 25: 458.

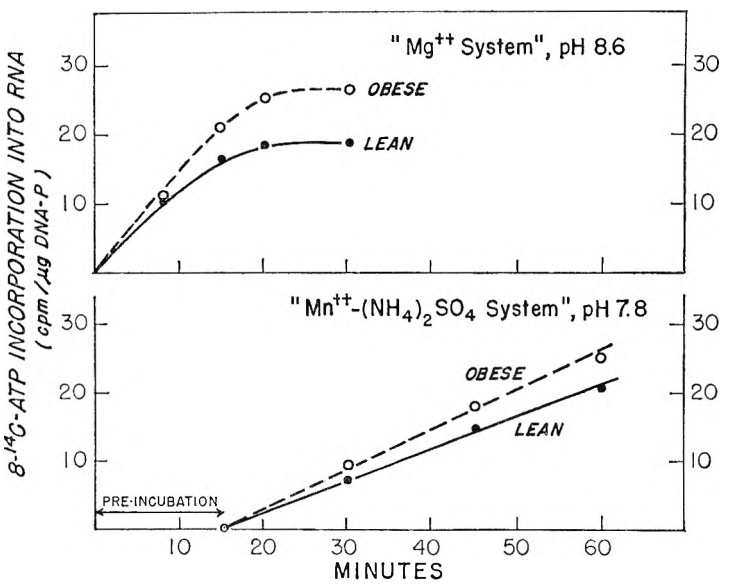

Fig. 1 DNA-dependent RNA polymerase activities; kinetics of incorporation of ^{14}C-ATP into nuclear RNA in vitro. For the Mn^{++}-$(NH_4)_2SO_4$ system, a 15-minute preincubation with unlabeled ATP was required before the introduction of ^{14}C-ATP. (See Methods for comparison of the two polymerase assays.)

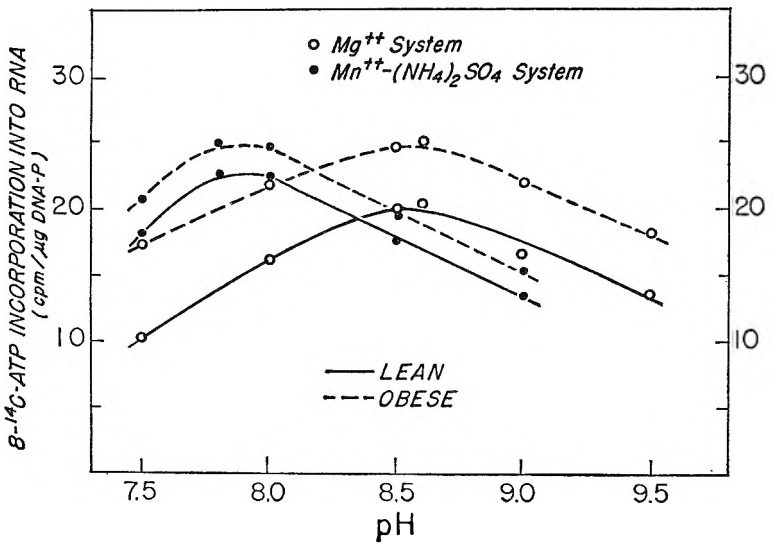

Fig. 2 DNA-dependent RNA polymerase activities. Establishment of pH optima of two assay methods for both lean and obese rats.

5 μM; pyruvate kinase, 50 μg; and 0.4 μCi of L-leucine-^{14}C.[6] The incubation was carried out for 20 minutes at 37°. The isolation and measurement of amino acid incorporation into microsomal protein was carried out as before (13). An aliquot was saved for the determination of microsomal protein by the method of Lowry et al. (9).

RNA "synthesis" in vivo. Each animal was injected intraperitoneally with orotic acid-6-^{14}C [7] (5 μCi/100 mg of body weight) and killed exactly 45 minutes later. This time-interval was selected on the basis of the experience of Munro et al. (14) who followed the incorporation at several intervals from 15 minutes to 3 hours. The kinetics of incorporation of ^{14}C from orotic acid into RNA have been followed and generally agree with the data of Munro et al. (14) who used labeled adenine. The isolation of several subfractions including nuclear material was also carried out by the methods used by Tata and Widnell (6). Both procedures gave similar values for the whole nuclear fraction. The cytoplasmic, mitochondrial, and microsomal fractions were studied by this latter procedure, whereas the procedures for isolating nuclear and nucleolar fractions were carried out by a modification of Munro's method (14).

RESULTS AND DISCUSSION

From data shown in figures 1 and 2, it was established that the two assay methods, developed originally by others, were suitable for comparing the effect of dietary cholesterol in either lean or obese rats. Irrespective of the assay system used, obese rats consistently had higher total polymerase activity than lean rats (tables 2 and 3). The implication is that the synthesis of one or more RNA is spontaneously higher in obese rats and accounts for the previously observed higher level of protein synthesis in this genotype.

If the DNA-dependent RNA polymerase activity is studied in rats fed a diet containing cholesterol and cholic acid for 21 days, there is a depression in polymerase activity only for the Mg^{++}-dependent system. Such differences were not noted for the Mn^{++}-dependent $(NH_4)_2SO_4$-stimulated system. This phenomenon was noted in both lean and obese rats of both sexes (tables 2 and 3). This observed depression of polymerase activity in rats fed choles-

[6] L-Leucine-1-^{14}C (specific activity, 17.4 mCi/mmole) was purchased from the New England Nuclear Corporation, Boston.

[7] Orotic acid-6-^{14}C (specific activity, 4.9 mCi/mmole) was purchased from New England Nuclear Corporation.

TABLE 2

RNA polymerase activities in rats fed cholesterol (males)

Genotype	Dietary treatment	Terminal body wt	1-^{14}C-leucine incorporation into protein in vitro	Nuclear protein / DNA-P	DNA-dependent RNA polymerase activities 8-^{14}C-ATP incorporation into nuclear RNA	
					Mg^{++} system	Mn^{++}-$(NH_4)_2SO_4$ system
		g	*cpm/mg microsomal protein*		*cpm/mg DNA-P*	
FaFa (lean)	Controls [1]	391	45.4 ± 1.8 [2]	11.5	13,450 ± 161	13,201 ± 170
	Cholesterol + [1]	380	** 36.1 ± 1.9	11.8	** 11,150 ± 117	13,405 ± 148
fafa (obese)	Controls	512	66.8 ± 2.8	12.8	22,366 ± 555	19,450 ± 401
	Cholesterol +	519	** 44.1 ± 2.0	13.2	** 13,889 ± 208	19,300 ± 333

[1] Controls represent animals fed a purified diet; cholesterol+, rats fed the same diet supplemented with cholesterol and cholic acid for 21 days.
[2] Mean ± SE of mean; duplicate samples from 6–8 rats/group. ** indicates values are significantly different from their respective controls ($P < 0.01$).

TABLE 3

RNA polymerase activities in rats fed cholesterol (females)

Genotype	Dietary treatment	Terminal body wt	1-^{14}C-leucine incorporation into protein in vitro	Nuclear protein / DNA-P	DNA-dependent RNA polymerase activities 8-^{14}C-ATP incorporation into nuclear RNA	
					Mg^{++} system	Mn^{++}-$(NH_4)_2SO_4$ system
		g	*cpm/mg microsomal protein*		*cpm/mg DNA-P*	
FaFa (lean)	Controls [1]	215	57.4 ± 2.7 [2]	12.1	18.252 ± 316	21,383 ± 276
	Cholesterol + [1]	221	** 39.7 ± 2.8	12.5	** 13,889 ± 184	20,040 ± 335
fafa (obese)	Controls	432	93.4 ± 4.5	13.0	25,911 ± 709	26,605 ± 552
	Cholesterol +	428	** 42.4 ± 3.8	13.5	** 16,050 ± 571	26,257 ± 480

[1] Controls represent animals fed a purified diet; cholesterol+, rats fed the same diet supplemented with cholesterol and cholic acid for 21 days.
[2] Mean ± SE of mean; duplicate samples from 6–8 rats/group. ** indicates values are significantly different from their respective controls ($P < 0.01$).

terol is supported by the data on total RNA synthesis in vivo (table 4).

Examination of the specific activity of the labeled total RNA in various subcellular fractions indicates that the nucleolar and nuclear fractions showed the greatest percentage decreases, respectively (following cholesterol feeding); the cytoplasmic and microsomal fractions (whose specific activity was, as expected, less) showed smaller but significant depressions, whereas the mitochondrial fraction showed no change. These observations on total RNA synthesis in vivo were useful inasmuch as they generally support the earlier observations on total protein synthesis and nuclear RNA polymerase activity in rats fed cholesterol, but do not provide direct information as related to any one species of RNA. It can be presumed that ribosomal RNA synthesis is nucleolar in origin (15) and the changes of this relatively high specific activity fraction may reflect largely ribosomal-like RNA synthesis.

Finally, female rats were consistently found to have higher DNA-dependent RNA polymerase activities than males (for both genotypes) and this reflects such a sex difference in terms of amino acid incorporation into the total proteins (3) (tables 2 and 3).

The present data support earlier observations on the protein-synthesizing apparatus of the liver cell in rats fed "hypercholesterolemic" diets and fasted overnight (1–3). It has been assumed that such changes may be directly related to the accumulation of hepatic lipids, particularly cholesterol, which resulted in a depres-

TABLE 4

6-^{14}C-orotic "incorporation" into RNA in vivo 30 minutes after killing (females)

Genotype	Dietary treatment, 21 days	Specific activities						
		Cytoplasmic	Mitochondrial	Microsomal	Whole nuclei [1]	"Nucleoplasmic" [1] "NaCl" extract	"Nucleoplasmic" [1] "PO_4" extract	Nucleolar residue [1]
		cpm/mg RNA–P						
FaFa (lean)	Control [3]	86 ± 5 [2]	20 ± 4	58 ± 3	1345 ± 30	788 ± 18	1388 ± 21	1680 ± 33
	Cholesterol + [3]	** 61 ± 3	20 ± 3	** 41 ± 5	** 901 ± 12	* 672 ± 20	** 1040 ± 25	** 988 ± 20
fafa (obese)	Control	101 ± 7	24 ± 3	93 ± 5	1890 ± 38	1505 ± 30	1967 ± 28	2785 ± 60
	Cholesterol +	* 85 ± 4	21 ± 2	** 50 ± 4	965 ± 27	** 1125 ± 15	** 1290 ± 27	** 1310 ± 35

[1] Nuclear fractions were derived from different groups of animals as compared with microsomal, mitochondrial, and cytoplasmic fractions.
[2] Mean ± SE of mean; duplicate liver samples were derived from 5–8 rats from each group. ** indicates values are significantly different from respective controls ($P < 0.01$); * indicates a suggestive difference ($P < 0.2$).
[3] Controls represent animals fed a purified diet; cholesterol+, rats fed the same diet supplemented with cholesterol and cholic acid for 21 days.

sion of protein synthesis, and that these changes are principally related to microsomal events; such changes are also accompanied by a decrease in the activity of DNA-dependent RNA polymerase (4). The effects of the diet are not due to anorexia since in all comparisons body weight responses were similar.

The present system for measuring polymerase activity is a modification of the original Mg^{++}-dependent assay proposed by Weiss (8). Comparing this enzyme activity with one based on an Mn^{++}-dependent $(NH_4)_2SO_4$-stimulated system, Widnell and Tata (5) postulated that the latter system directs the synthesis of a DNA-like RNA species, whereas the former yields ribosomal RNA (5, 16). This latter assay was used by these authors to study the mechanism of action of certain hormones, and they found that the Mn^{++}-$(NH_4)_2SO_4$ reaction was relatively unresponsive to their physiological manipulations, whereas the Mg^{++}-dependent reaction was responsive to certain hormone replacement therapies. This finding showed that the polymerase changes generally reflected the capacity of the liver system to incorporate amino acids into total protein (6, 17). In the present trials, the Mn^{++}-$(NH_4)_2SO_4$ system was unresponsive to our physiological manipulations since it gave similar results in control rats as well as in those fed the cholesterol-supplemented diet. It has been proposed that the use of $(NH_4)_2SO_4$ may result in the detachment of inhibitors so as to unmask more of the DNA template (18), or to result in the activation of an enzyme-DNA complex (19, 20). Aside from such possible considerations, there is no direct evidence to disregard the use of $(NH_4)_2SO_4$ since physiological relevance has been claimed for such a system by Widnell and Tata (5) as well as others (21).

The genetically obese rats spontaneously showed more polymerase activity by either assay method. This is not unexpected since this genotype has a greater capacity for amino acid incorporation into the total proteins in vivo or in vitro (3, 4). But the decreased capacity for amino acid incorporation into total proteins following cholesterol feeding was accompanied by a decrease in only the Mg^{++}-dependent ac-

tivity. Therefore, if one accepts the postulate that the Mg^{++}-dependent system is a reflection of the capacity of ribosomal RNA synthesis (5, 6) then our data can be interpreted to show that the changes following cholesterol feeding may at least be related to a direct or indirect control of ribosomal RNA synthesis or even ribosomal assembly and release. It is more reasonable, however, to assume that a depression in protein synthesis probably involves a depression also in the synthesis of all species of RNA to some degree. Whether only one specific RNA species is involved remains to be determined.

Other data from this laboratory are consistent with the previous observations.[8,9] It has been found, for example, that rats fed a cholesterol or cholesterol-cholic acid diet demonstrate a significant change in the "polysomal profile" of the liver; there is a relative decrease in larger polysomes and an increase in monosomes. This change in polysomal profile may be a direct or indirect consequence of the increased concentration of cholesterol at the endoplasmic reticulum. These events help to explain the decreased capacity for total "microsomal" protein synthesis observed in rats after cholesterol feeding. One may then argue in favor of some subsequent feedback control of RNA synthesis in the nucleus or nucleolus. How such a control operates can only be speculated. A postulated feedback control can conceivably be dependent on the accumulation of free ribosomes or their end products in the cytoplasm.

It is also of interest that female rats tend to have a higher capacity for liver microsomal protein synthesis. This sex difference has been a consistent observation for either lean or obese rats (1, 3). The present study supports this finding and further shows a higher level of RNA polymerase activity for females as compared with males. It is pertinent also that the female rat normally has a higher level of hepatic cholesterol synthesis as well as higher serum total cholesterol levels before (22) and after cholesterol feeding (23). At this time an explanation of this apparent sex difference should be deferred since the site of action of sex hormones remains a controversial issue. Nevertheless, the present report demonstrates another example of how dietary treatment can bring about significant alterations in the protein-synthesizing apparatus of the liver. This approach offers an opportunity to study the intracellular events that direct and control hypercholesterolemia.

The dietary levels of cholesterol and cholic acid were not toxic; all the male and female dietary groups showed comparable body weight gains and food intake. Therefore, the effects noted were due to a dietary-induced phenomenon and not due to any toxic effect. Furthermore, all rats were fasted overnight.

It is apparent that the accumulation of cholesterol and other lipids in rats fed "hypercholesterolemic" diets (2) can bring about significant alterations in the protein-synthesizing apparatus of the liver cell. The question still remains as to whether this represents a specific regulatory mechanism and the resultant "depression" in protein-synthesizing events reflects in part a redirection of events in favor of the proteins (and enzymes) to regulate the orderly removal or "storage" of excess lipid at the expense of other proteins.

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Interrelationship of Lighting Regimen and Virus Infection to Diurnal Rhythms in Liver Components Associated with Protein Metabolism in the Chick [1]

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ABSTRACT The interrelationships of lighting regimen and virus infection to diurnal rhythms of liver components were studied. Progressive within-day sampling of tissues of cockerel chicks conditioned to 12-hour light/dark (LD) and 24-hour (LL) lighting regimens and infected with Newcastle disease virus (NDV) showed that these exogenous and endogenous agents significantly affected diurnal rhythm patterns of liver weight, protein, DNA, RNA and free amino acids. Under the LL regimen the NDV increased liver weights, desynchronized DNA patterns and depressed RNA and free amino acid levels as compared with control values. Under the LD regimen diurnal patterns of these parameters were held in reasonable synchronization to controls. This breakdown in patterns is attributed to the additive effects of the stress of constant lighting plus the stress of the infection. Sampling periods spaced at 12-hour intervals are believed to be the minimal requirement to establish normal control rhythms and the presence of abnormal diurnal patterns resulting from unknown exogenous or endogenous agents which might confound treatment effects.

Progressive within-day sampling has revealed rhythms in the components of selected tissues obtained from noninfected control chicks and those infected with Newcastle disease virus (NDV). Rhythmic patterns of nucleic and free amino acids were significant and highly reproducible under a constant lighting regimen (1, 2). As light is considered a "dominant synchronizer" (3, 4), it became important to evaluate and compare biochemical changes in tissues of noninfected and infected chicks maintained under constant light with those on a normal 12-hour light-dark schedule. The biochemical parameters measured were especially chosen to fit current concepts of protein metabolism since this vital body function is affected by the presence of an infectious disease.

METHODS

The interrelationships of lighting regimen and Newcastle disease virus (NDV) infection were studied in 2 experiments involving a total of 500 White Leghorn cockerel chicks. Except for the lighting schedule, all conditions were the same for both trials. The birds were maintained in identical isolated air conditioned rooms and offered a standard diet and water ad libitum (5). All maintenance operations were carried out by a single caretaker between 0830 and 0930 hours each day. In experiment 1 the chicks were exposed to constant light (LL) and in experiment 2 the same type of illumination was used but on a 12-hour light-dark schedule (LD) with the light on from 0700 to 1900 hours. These schedules were initiated at day of hatch and continued throughout the trials. At 4 weeks of age, half the birds were injected intramuscularly with NDV (0.1 ml inoculum/bird) (5); the remainder served as noninfected controls. Starting at time of inoculation (0800 hours), 8 chicks from each treatment group were killed at 4 daily intervals: 0800, 1500, 2000 and 2400 over the 72-hour incubation period of the NDV (5). Livers were removed, weighed and frozen and later analyzed individually for tissue protein and nucleic and free amino acids (6, 7). Statistical analyses were carried out according to Snedecor (8) with values analyzed in terms of milligrams per gram of fresh tissue.

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RESULTS

Body weights for all treatment groups (fig. 1) showed a normal linear increase which fit reference growth curves. These results indicated (5) that feed and water intake of the NDV chicks were normal for the period of the disease cycle studied. Although the within-day weight fluctuations were not significant, at the final killing period 72 hours post inoculation, there was a significant spread ($P < 0.01$) between the control and NDV groups under both lighting schedules; by our definition, this significant weight loss signals the end of the NDV incubation period.

Liver weights in the LL controls (fig. 1) increased linearly, thus maintaining a constant liver-to-body ratio. In the NDV chicks the increase was also linear but the slope was significantly greater ($P < 0.01$) than that of the controls. Under the LD schedule the same increase in control liver weights was noted, but unlike the findings in experiment 1 there was a significant ($P < 0.01$) diurnal rhythm with distinct troughs at 0800 hours. Infected livers maintained the same rhythmic pattern in relation to the controls; however, 40 hours post-inoculation liver size began to increase significantly ($P < 0.01$) over that of the noninfected chicks.

Under the LL regimen DNA (fig. 1) in milligrams per gram of fresh tissue oscillated ($P < 0.03$) with peaks at 2000 to 2400 hours. It is recognized that the chick erythrocyte, being nucleated, may contribute to liver DNA levels; however, this would be equally distributed over time and treatments. In the NDV chicks DNA oscillations were significantly desynchronized in relation to the control pattern during the entire observation period. In the LD trial DNA also oscillated significantly in the controls ($P < 0.01$) but the peaks occurred at 0800 hours. For the first 12 hours post-inoculation, DNA in the NDV

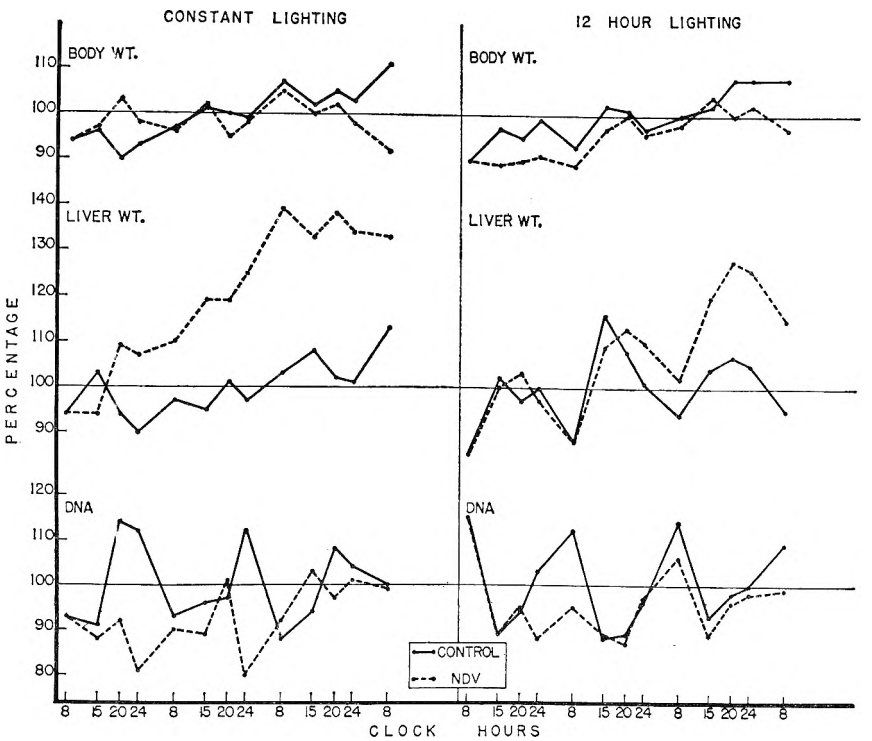

Fig. 1 Average body and liver weights and liver DNA of control and NDV-infected chicks on constant and 12-hour lighting regimens, in terms of percentage change from control average for a 72-hour period. DNA calculated on basis of milligrams per gram of fresh tissue. Each point represents 8 chicks.

group followed this same pattern but in the next 19 hours levels of DNA were lower and desynchronized in relation to the controls. Following this interval of desynchronization the oscillations then resynchronized with the controls. Total quantities of DNA (liver weight × mg DNA/g liver) showed similar oscillations but of somewhat less magnitude than those shown in figure 1.

RNA synthesis (fig. 2) was reasonably constant in the noninfected chicks in the LL experiment; however, NDV caused a linear depression ($P < 0.05$) from control RNA values. With the LD schedule, RNA in the noninfected and NDV livers oscillated in a similar pattern with peaks at approximately 0800 hours.

Under the LL schedule the free amino acids, similar to RNA, were significantly depressed ($P < 0.01$) by the NDV for most of the 72-hour incubation period. In the LD schedule the fluctuations were significant ($P < 0.05$), with troughs occurring in both the control and NDV groups during the evening hours. Changes in protein content were irregular and there were no pronounced effects from either lighting schedule.

The NDV effect on the individual free amino acids in the infected livers was obtained by plotting the values in terms of the controls at each sampling period (fig. 3). Under both the LD and LL regimens there were significant deviations ($P < 0.05$ — 0.01) from controls but with differing patterns.

DISCUSSION

Oscillations of tissue components were apparent when treatment groups were sampled at a sufficient number of within-day intervals and the data plotted in relation to sampling hours. Resulting patterns are normal phenomena of an intact system reflecting the ebb and flow of the various metabolic processes. The re-

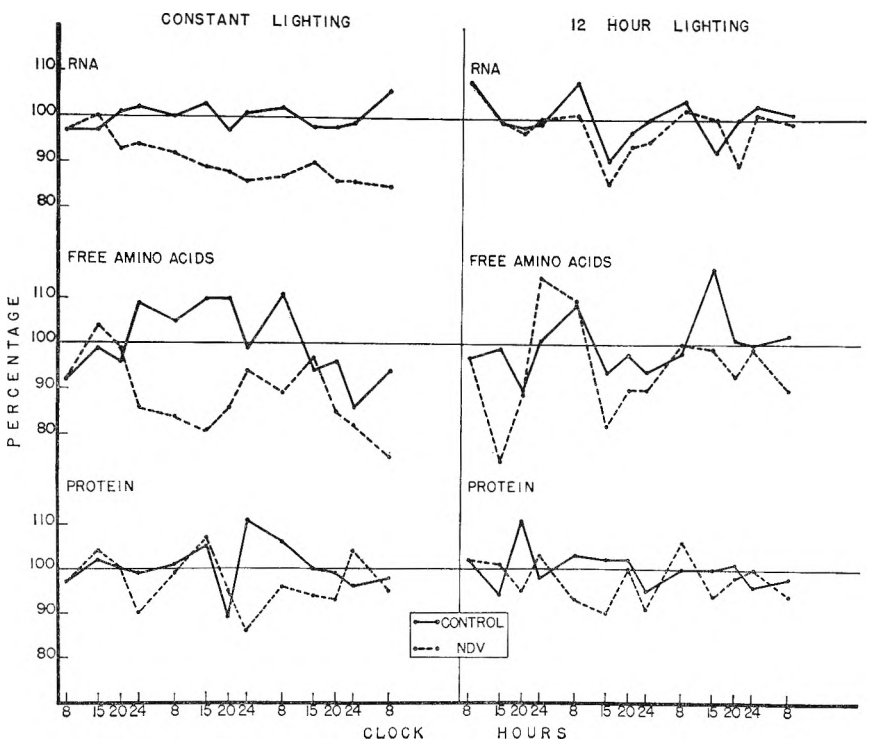

Fig. 2 RNA, tissue protein and free amino acid pool of control and NDV-infected chicks on constant and 12-hour lighting regimens, in terms of percentage change from control average for a 72-hour period. Calculations based on milligrams per gram of fresh tissue. Each point represents 8 chicks.

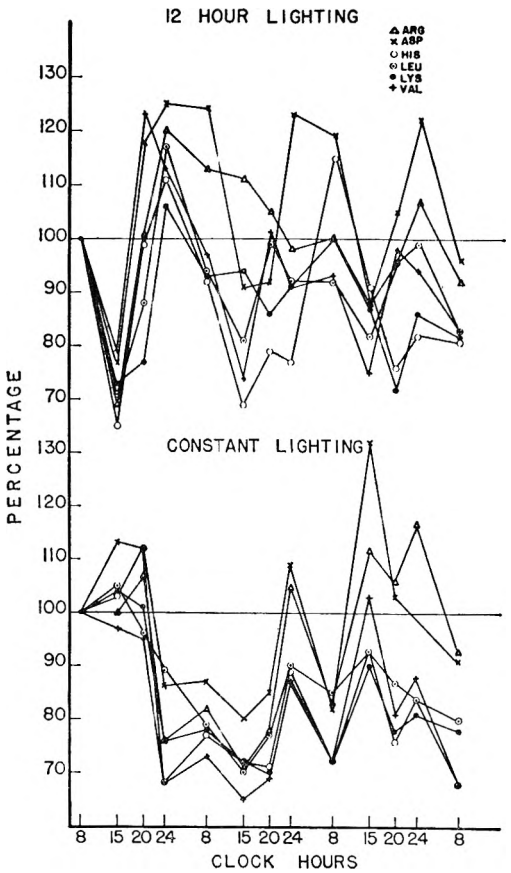

Fig. 3 Individual amino acids of chicks infected with NDV and subjected to constant and 12-hour lighting regimens, shown in terms of percentage change from control values at each sampling period. Calculations based on milligrams per gram of fresh tissue. Each point represents 8 chicks.

sults here show that fluctuations occur with considerable magnitude and may be changed by exogenous or endogenous agents.

The effect of lighting regimen on diurnal oscillations of the selected parameters must first be evaluated apart from disease interactions, which means a consideration of the changes in the patterns of the noninfected control groups. The LD regimen, considered normal, resulted in patterns of liver weight, DNA, RNA and free amino acids associated with definite clock hours. A comparison of the curves of the noninfected controls conditioned to LL with those of the LD controls shows obvious changes in the patterns. Of particular interest is the phase shift in DNA wherein peaks observed at 2000 to 2400 hours were shifted to 0800 hours. This reversal of DNA peaks also occurs in other species and tissue constituents when light schedules are inverted (9, 10). The LL regimen also appeared to dampen oscillations of liver weights and RNA and to desynchronize the free amino acids.

When the interactions between exogenous (light) and endogenous (disease) factors were examined, other changes were noted in the diurnal rhythm patterns. These observed interactions cannot be attributed to differences in the intensity of disease involvement as final mortality in both NDV groups was approximately 50%.

A comparison of the patterns of the various parameters in the NDV tissues under the two lighting regimens shows that the LD schedule was a powerful synchronizer during the 72-hour incubation period of the virus. This was evident from the fact that the LD schedule held liver weights, DNA, RNA and the total free amino acids in reasonable synchronization with the curves of the controls. However, under LL, infected livers showed a linear increase in weight, DNA values became desynchronized from controls, and RNA and total free amino acid levels were depressed. Body weights and liver protein values showed little evidence of entrainment to clock hours.

In addition to the disease × light interactions on the rhythms per se, phenomena related to the changing size of the liver merit comment. This is not solely an avian characteristic since Halberg (3) has shown that there are diurnal oscillations of liver weights in the mouse under an LD schedule; however, the troughs occur at night, which correlates with a difference in activity of the 2 species.

Another factor is the possible relationship of moisture content of the liver tissue to the rhythmic patterns. Determination of the dry matter in control and infected livers showed the averages were, respectively, 25.9 and 24.7% at 0800 hours and 26.5 and 25.4% at 2000 hours. Thus, from this and previously published data (2), changes in moisture content were

not responsible for the large within-day oscillations of values. Furthermore, all the curves of the various parameters did not show a parallelism. It also has been shown that diurnal changes in liver weights (11) and DNA rhythms (11, 12) in the rat are independent of moisture content.

Since the diurnal changes cannot be correlated with moisture content, they warrant further speculation, particularly in reference to the disease interactions. The increase in size of the infected livers during the latter stages of the NDV incubation period in the LD trial, and the constant linear increase observed under the LL regimen correlate with a rise in viral titer (13) and an increase in nitrogen retention (14); the latter also occurs in man during the initial stages of disease involvement (15).

Since the biochemical parameters determined here are intimately related to protein metabolism, it is logical to question the effect of diet intake on the oscillations. However, there is considerable evidence that many diurnal changes are independent of food consumption. For example, large daily oscillations of nucleic and free amino acids have been observed by Squibb (1) during the active involvement stage of NDV, a period when diet intake may be completely depressed. Halberg (16) has observed that rhythms persist in the mouse during complete starvation; and Beisel et al.[2] have found blood amino acid rhythms in man to be independent of diet loading.

The data *in toto* indicate that the effects of the infection on cellular constituents were more pronounced under the LL than the LD regimen. This is believed due to double jeopardy, for example, the stress of the constant lighting regimen plus the stress of the NDV.

The sampling of tissues once a day, even though at the same hour, is apparently inadequate since treatment effects may be confounded with normally occuring diurnal rhythms and the interaction of unknown exogenous synchronizers. To evaluate such phenomena it is recommended that tissue samples be taken at least at 12-hour intervals.

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Aspects of Sulfate Utilization by the Microorganisms of the Ovine Rumen [1,2]

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ABSTRACT Strained rumen fluid, obtained from sheep ingesting alfalfa hay of 14% protein content and trace-mineralized salt, was incubated with a small additive portion containing sulfate alone or with other sulfur sources. The incubation was at 39° and for 30, 90 and 180 minutes. With the added sulfate being labeled (with ^{35}S) and introduced in known concentration, its occurrence in protein was followed separately from that of undetermined total sulfate. About maximal utilization of added sulfate-S for protein synthesis occurred when the additive sulfate was present at a 500 μM level. Thus, this concentration of sulfate appeared to furnish the dominant part of the sulfate in the medium. To gain further knowledge of the role of sulfide as an intermediate of sulfate-S in the synthesis of ruminal protein, the occurrence of sulfide within the enclosed fluid was also observed. The rate of production of sulfide was increased materially by adding sulfate (500 μM level). This extra production of sulfide usually exceeded the measured conversion of the added sulfate-S to sulfide. The conversion was not affected by the presence of other sulfur (500 μM level) as casein, methionine, cystine or sulfide. However, the overall production of sulfide was increased mildly by casein or methionine and markedly by cystine and these increases were associated with somewhat commensurate decreases in the incorporation of the added sulfate-S into protein. This effect was not consistent for casein. Added sulfide lowered the utilization of sulfate-S even more than did the presence of methionine or cystine. Thus, the organisms which utilized sulfate upon reduction to sulfide were apparently adapted to utilizing sulfide from other sources. A fluid which represented a different ingesta showed a lesser utilization of added sulfate (500 μM level) for protein synthesis. This was attributed to a higher content of original sulfate in the second fluid.

Dietary sulfate has been shown to facilitate growth in ruminants when used as the only sulfur source (1). Block et al. (2) showed that the ruminal organisms can use sulfate in the synthesis of cystine and methionine of microbial protein. Lewis (3) observed that sulfide was formed from sulfate in the rumen. The sulfate appeared to be quantitatively reduced to sulfide. Anderson (4) found that the mixed microorganisms consume sulfide during incubation. It remained for Henderickx (5) to show that sulfate is reduced to sulfite and on to sulfide and then the sulfide-S is incorporated into the protein. Ruminal microorganisms are also known to form sulfide from protein (4) and from free cysteine (6). According to Hungate (7), ". . . it is highly probable that hydrogen sulfide can be used as a source of sulfur by most ruminal organisms." Since the factors that may affect the utilization of sulfate-S for protein synthesis in the rumen are not well-understood, a study with rumen fluid from sheep was conducted.

EXPERIMENTAL

The rumen fluid was obtained from fistulated wethers receiving a diet of alfalfa hay and trace-mineralized salt.[5] The salt was fed separately from the hay and ad libitum. The allotment of hay per animal per day was 1.8 kg, one-half being fed at 9:00 AM and the remainder at 4:30 PM. The hay contained 14% of crude protein except that in one instance it contained 21% of crude protein. The trace-mineralized salt contained 0.46% manganese as

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[5] Formula no. 1, Hardy Salt Company, St. Louis.

manganese sulfate, 0.01% iodine as iodized fish meal, 0.01% cobalt as cobalt carbonate, 0.17% iron as ferrous sulfate and iron oxide, 0.05% copper as copper oxide, 0.1% sulfur as sulfate and 97% sodium chloride. At about 1:00 PM on the day of an experiment, 200 to 400 ml of rumen contents were aspirated from the dorsal or ventral portion of the rumen. The contents were strained through cheesecloth and then kept at about 39° during the interval of 15 to 40 minutes before incubation. One or two animals served as the source of the fluid in a particular trial, but several animals were sampled during the course of the 2-year study. The animals were housed in unheated quarters. They had continuous access to water. The pH of the freshly collected fluid varied from 6.7 to 7.4 in different instances. If incubated for 180 minutes, the pH dropped 0.1 to 0.6 unit. The sulfate content of the fluid was not determined.

In an experiment, aliquots of 9.5 ml of the fluid and 0.5 ml of additive solution or of distilled water were introduced into incubation chambers after each had been flushed with carbon dioxide. The glass chambers, which had Tygon fittings, were vented to an assembly unit containing carbon dioxide under slight positive pressure or else they were sealed. Sealed chambers were used whenever an experiment involved sulfide determinations. After mixing the contents, the chambers were placed in a 39° water bath and held there without agitation for 30, 90 and 180 minutes. At each time interval, one chamber from each treatment was used for analysis. The chambers were constructed to permit introduction of trichloroacetic acid (TCA) solution and flushing of the acidified medium with gas at the conclusion of an incubation period.

Two additive treatments were involved. In one treatment, $K_2{}^{35}SO_4$ plus carrier were added to the fluid so as to supply the equivalent of 50, 250, 500 and 1000 μmoles of added sulfate per liter of medium in addition to the original (undetermined) sulfate of the fluid. The ^{35}S activity was kept constant, and the carrier was varied. In the other treatment, 500 μmoles of ^{35}S-labeled potassium sulfate was added per liter of the fluid along with other sulfur sources (unlabeled). Each other sulfur source was added individually with sulfate and at a 500 μM level with respect to the sulfur atom. The other sulfur sources were vitamin-free casein,[6] DL-methionine, L-cystine and sodium sulfide (monohydrate). The cystine was prepared for use in the medium by solubilizing it with a minimal amount of dilute sodium hydroxide. The casein was introduced into the medium in powdered form. With either treatment, the sulfate-^{35}S activity of the label was equivalent to about 7 mCi/liter of medium.

At the termination of the incubation periods, 1.1 ml of 100% TCA solution (w/v) were added to the contents of each chamber. The volatile matter was flushed out with nitrogen into a trap containing 5 ml of 0.5% cadmium acetate to collect sulfide. The trapped sulfide was measured by iodometric titration (8). On dispersing a portion of the titrated solution in scintillation fluid containing Hyamine hydroxide,[7] the ^{35}S activity of the trapped sulfide was measured with a Tri-carb scintillation spectrometer. An internal standard (as $K_2{}^{35}SO_4$), which contributed an activity corresponding to 100% conversion of sulfate-^{35}S to sulfide-^{35}S, was counted. Background counts were made on the blank reagents that were used for titration. From the counts of the internal standard, the sample counts were expressed as a percentage of the added sulfate-^{35}S activity. These percentages were multiplied by the micromolar level of added sulfate used in the medium. The product was the micromolar level of added sulfate converted to sulfide during incubation.

Block et al. (2) have observed that the TCA-insoluble matter of rumen fluid that is incubated with sulfate-^{35}S contains the organic or fixed ^{35}S. Emery et al. (9) have reported that a major part of the fixed ^{35}S occurs as cystine and methionine after acid hydrolysis. From these observations, the measurement of radioactivity (from sulfate-^{35}S) in TCA-insoluble matter was used as the means of studying the microbial utilization of sulfate-S for protein synthesis. Thus, a portion which was equivalent

[6] Nutritional Biochemicals Corporation, Cleveland.
[7] Packard Instrument Company, Inc., Downers Grove, Illinois.

to 1.8 ml of medium was removed from each incubation chamber at about 12 hours after the TCA addition. The chamber was well-shaken before the sampling. The portion was placed in a 13 mm × 100 mm glass centrifuge tube and centrifuged at 4000 × g for 15 minutes. The supernatant part was discarded and the precipitate was retained and then washed 4 times with 2-ml portions of 10% TCA. This washing procedure removed essentially all of the soluble ^{35}S from the precipitate. The washed precipitate was dispersed in 2 ml of 3 N hydrochloric acid and hydrolyzed at 99° to 100° under reflux for 18 to 24 hours. This hydrolysis was as effective as one with 6 N hydrochloric acid with respect to ^{35}S release. The hydrolysate was diluted to a 10-ml volume, centrifuged to remove particulate matter and then used for ^{35}S measurement.

Either Geiger-Müller or scintillation counting methods were used to measure the ^{35}S activity of the protein hydrolysates. If Geiger-Müller methods were used, duplicate 0.2-ml portions were placed on stainless steel planchets. These were allowed to dry at room temperature for a few hours and then counted. If scintillation methods were used, a 0.2-ml portion of a hydrolysate was handled and counted according to the procedure already described for determining the ^{35}S activity of the trapped sulfide. An internal standard was used to establish the count which equaled 100% conversion of sulfate-^{35}S to protein-^{35}S. A background count was made. The count of a hydrolysate was expressed as the percentage of the added $K_2{}^{35}SO_4$ activity. The product of this percentage multiplied by the micromolar level of added sulfate gave the level of incorporation of added sulfate-S into microbial protein.

RESULTS AND DISCUSSION

On incubating rumen fluid from a sheep that received alfalfa hay of 14% protein content, added sulfate-^{35}S was incorporated into protein to a considerable degree (table 1, fluid 1). With the use of different levels of carrier (sulfate) along with the additive sulfate-^{35}S, i.e., 50, 250, 500 and 1000 μM levels, the values for added sulfate-S which were incorporated into protein tended to increase as the level of added sulfate in the medium was increased. However, the rate of increase in incorporation was less than proportional to the increase in added sulfate. When the level of added sulfate was as high as 500 μM, only a slight additional incorporation of the added sulfate-S resulted from an increase to a 1000 μM level. Low levels of added sulfate, i.e., 50 and 250 μM, appeared inadequate in the fluid since their utilization diminished noticeably in the course of the incubation.

Somewhat similar results were obtained in 2 other trials with rumen fluid 1 (data not presented). In these instances, the incorporation of the added sulfate-S into protein was indistinguishable after 30 minutes of incubation if the level of added sulfate was 250, 500, 1000 or even 1500 μM and after 90 or 180 minutes of incubation if the level of additive was 500, 1000 or even 1500 μM. Geiger-Müller methods were used for measuring this ^{35}S activity in protein. The finding that added sulfate-S contributed the same toward protein synthesis whether supplied at levels that were 500, 1000 and 1500 μM indicated that the added sulfate supplied the dominant part of the sulfate in the medium under each condition. Thus, the observed incorporation of added sulfate-S should have been an approximate measure of the total sulfate-S incorporation when as much as a 500 μM level of added sulfate was used.

The results obtained with a rumen fluid of different background (fluid 2) are also shown in table 1. This fluid was from a sheep that received alfalfa hay of 21% protein content. The effect of variance of level of added sulfate (alone) on the utilization of the added sulfate-S for the synthesis of sulfide and of protein was observed. The added sulfate-S was incorporated into protein in a lesser degree than had been observed with fluid 1. With levels of added sulfate of 500 μmoles per liter and below and an incubation for 90 or 180 minutes, the micromolar incorporation of the additive sulfur into protein and its appearance as sulfide were each rather proportional to the level of the added sulfate. The sulfide accounted for much more of the additive sulfur than the protein, however. If the added sulfate was introduced at a 1000 μM level, the additional

TABLE 1

Use of ^{35}S-labeled sulfate in rumen fluid to study the utilization of added sulfate-S when used at different levels

Incubation time	Extent of added sulfate [1]	Fluid 1 [2]		Fluid 2 [2]				
		Occurrence of added sulfate-S in protein [3]		Occurrence of added sulfate-S in protein [3]		Occurrence of added sulfate-S in sulfide [3]		Extent of total sulfide
min	*μM level*	*%*	*μM level*	*%*	*μM level*	*%*	*μM level*	*μM level*
0 [4]	—							91
	50	1.38	0.69	0.00	0.00	0.24	0.12	54
	250	0.20	0.50	0.18	0.44	0.05	0.13	90
	500	0.05	0.25	0.02	0.09	0.07	0.35	79
	1000	0.18	1.80	0.01	0.06	0.06	0.60	84
30	—							160
	50	15.06	7.53	0.19	0.09	1.23	0.62	153
	250	5.03	12.58	0.17	0.41	1.35	3.38	162
	500	2.96	14.80	0.06	0.32	1.18	5.90	159
	1000	1.75	17.50	0.04	0.38	0.95	9.50	170
90	—							282
	50	21.54	10.77	0.30	0.15	4.06	2.03	274
	250	11.42	28.55	0.28	0.70	3.45	8.63	256
	500	6.64	33.20	0.28	1.41	3.61	18.05	276
	1000	3.56	35.60	0.20	1.95	2.96	29.60	289
180	—							417
	50	22.38	11.19	0.69	0.34	4.94	2.47	392
	250	15.92	39.80	0.55	1.37	6.19	15.48	394
	500	10.37	51.85	0.70	3.52	6.35	31.75	398
	1000	5.75	57.50	0.47	4.70	4.26	42.60	390

[1] The added sulfate, as the potassium salt, was in addition to original (undetermined) sulfate of the fluid. The ^{35}S activity of the added sulfate was held constant while the carrier was varied; the additive portion diluted the fluid about 5%.

[2] Rumen fluid 1 and rumen fluid 2 refer to that from animals which received alfalfa hay of 14 and 21% protein content, respectively.

[3] The first column refers to the percentage of the added sulfate-^{35}S activity that appeared in protein or in sulfide. The second column refers to the added sulfate-S that appeared in protein or in sulfide as determined by multiplying the percentage of the first column by the micromolar level of added sulfate used in the medium.

[4] This incubational time was less than 1 minute.

utilization appeared to be less than proportional to the increase in sulfate, however. The original content of sulfate in this fluid should have been large to account for the finding that the relation between level and utilization of added sulfate-S was rather linear until the level of added sulfate was as much as 500 μmoles per liter. The observation that adding sulfate to the fluid did not augment the production of sulfide was also evidence that the original content of sulfate in the fluid was large. Thus, the use of labeled sulfate-S at a 500 or 1000 μM level did not offer a direct means of quantitating the conversion of sulfate-S to sulfide and to protein with fluid 2. Scintillation methods were used for measuring ^{35}S incorporation into protein in this instance.

On returning to the rumen fluid which was obtained from animals receiving alfalfa hay of 14% protein content (fluid 1), the effect of other sulfur sources (500 μM level) on the utilization of added ^{35}S-labeled sulfate (500 μM level) was studied. The results of 3 trials are presented in table 2. The measurements on sulfide are of particular importance. Without added sulfate, the production of sulfide within the fluid was generally small and frequently short-lived. With added sulfate, the production was noticeably increased and then maintained. The extra production was frequently more than the observed contribution from the added sulfate-S. The further addition of sulfur as casein, methionine or cystine caused additional quantities of sulfide to be produced but the presence of any of these along with added sulfate in the medium did not affect the conversion of the sulfate-S to sulfide. This was indicated because the percentage of the sulfate-^{35}S activity which occurred in sulfide was the same after in-

TABLE 2

Use of ^{35}S-labeled sulfate in rumen fluid to study the utilization of added sulfate-S when other sulfur sources were introduced

Incubation time	Sulfur sources added [1]	Fluid 1A [2]			Fluid 1B [2]			Fluid 1C [2]		
		Occurrence of added sulfate-S		Total sulfide	Occurrence of added sulfate-S		Total sulfide	Occurrence of added sulfate-S		Total sulfide
		In protein [3]	In sulfide [3]		In protein [3]	In sulfide [3]		In protein [3]	In sulfide [3]	
min		*μM level*			*μM level*			*μM level*		
0	—			2			27			117
30	—			4			48			279
	Sulfate	1.3	1.6	14	1.4	36	84	6	—	—
	Sulfate + casein	0.8	1.2	9	3.0	43	79	6	61	344
	Sulfate + methionine	0.2	0.8	12	1.5	32	92	6	47	357
	Sulfate + cystine	0.2	1.7	28	1.6	41	279	5	52	750
	Sulfate + $Na_2S \cdot 9H_2O$	0.2	3.2	332	0.9	38	471	4	45	1033
90	—			7			50			419
	Sulfate	4.5	5.8	22	6.3	114	166	13	128	633
	Sulfate + casein	3.6	4.3	29	6.7	117	187	13	134	649
	Sulfate + methiomine	2.5	4.4	32	4.3	112	219	11	112	685
	Sulfate + cystine	1.8	6.9	166	3.4	118	518	8	124	1300
	Sulfate + $Na_2S \cdot 9H_2O$	0.6	8.4	368	2.8	110	544	6	108	1377
180	—			15			53			469
	Sulfate	12.4	12.4	54	10.0	175	262	16	205	898
	Sulfate + casein	6.1	11.2	64	12.9	187	344	20	208	1051
	Sulfate + methionine	7.8	10.5	92	7.4	154	340	15	195	1060
	Sulfate + cystine	1.9	12.8	364	7.1	207	613	12	204	1597
	Sulfate + $Na_2S \cdot 9H_2O$	2.0	9.9	385	4.7	150	602	7	—	1393

[1] Each sulfur component of the additive portion was introduced at a 500 μM level with respect to the sulfur atoms; the sulfate component was labeled (with ^{35}S); the additive portion diluted the fluid about 5%.

[2] The A, B and C designations refer to studies made with rumen fluid 1 which was collected in November, February and August, respectively (see footnote 2 of table 1 for dietary reference on fluid 1).

[3] See footnote 3 of table 1 for the explanation of the calculations.

cubation whether or not another additive was present along with sulfate. This was true even if sulfide itself was the other additive. The extra production of sulfide which was caused by added cystine was considerably greater than that caused by added sulfate, whereas that caused by added casein or methionine was less. Since the sulfide in a medium containing added cystine or added sulfide was about the same after incubation, it appeared that cystine-S was quantitatively converted to sulfide.

In cognition that sulfide is an intermediate in the utilization of sulfate-S for protein synthesis in the rumen (5), the depressive effect which added sulfide or sulfide-forming substances had on the incorporation of added sulfate-S into protein was not unexpected (table 2). When the production of extra sulfide was small as when methionine was added in the medium, the incorporation of the sulfate-S was depressed in moderate or small degree. With a greater contribution of sulfide to the medium as when cystine was added, the incorporation of the sulfate-S was often depressed considerably more. Neither methionine nor cystine was as effective in depressing the incorporation as was added sulfide, however. This suggested that the sulfide produced from methionine or cystine could explain the inhibitory effect of the methionine or cystine. That casein caused a small increment of sulfide to be produced and yet its presence was associated with both increases and decreases in incorporation of added sulfate-S was not readily explained. Either Geiger-Müller or scintillation methods were used to measure ^{35}S incorporation into protein in these observations. The findings lead to the interpretation that sulfide from different sources was available to those microorganisms which could utilize sulfide obtained from sulfate. Sulfate-utilizing microorganisms have been reported to be in a minority in rumen fluid (9). With the present findings suggesting that these organisms utilize sulfide from a variety of sources, it is more difficult to conceive that their representation is minor in the rumen.

Cysteine has been reported to depress the utilization of sulfate-S for protein synthesis with an isolated ruminal microorganism, i.e., *Lachnospira multiparus* (10). This organism shows little growth if sulfur is supplied as sulfate only, but much growth if cysteine is also present. The organism appears to utilize cysteine directly since sulfide is not formed (11). Obviously, sulfate-S and cysteine-S are not interchangeable with this organism and hence the depressive effect of the cysteine on sulfate-S utilization cannot be one of mere competition. With rumen fluid 1 of the present study, sulfate-S was converted to sulfide, and cystine did not interfere. Cystine-S was also converted to sulfide and hence its interference with the utilization of sulfate-S could be explained on the basis that the sulfide formed from either source was interchangeable or competitive for use in microbial synthesis of protein.

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Amino Acid Synthesis from Glucose-U-^{14}C in *Argyrotaenia velutinana* (Lepidoptera: Tortricidae) Larvae

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ABSTRACT In previous studies using the dietary amino acid deletion technique, it was found that the insect *Argyrotaenia velutinana* (Walker) required the same 10 amino acids indispensable for the rat. To further verify the dietary amino acid requirements of this insect, amino acid synthesis from glucose-U-^{14}C was investigated in the larval stage. For this, fifth-instar larvae were reared aseptically on a synthetic medium containing glucose-U-^{14}C. The relatively high specific activity measurements for aspartic acid, glutamic acid, glycine, serine, alanine and cysteine indicate that the insect is capable of synthesizing these amino acids from glucose. Lysine, histidine, arginine, valine, methionine, isoleucine, leucine, phenylalanine and threonine showed low specific activity measurements; therefore, these amino acids are classified as nutritionally indispensable. These results agree with those obtained by the amino acid deletion technique. Although tyrosine showed no radioactivity it may be considered a dispensable amino acid because it was shown to be dispensable by the deletion technique, and its synthesis from phenylalanine has been demonstrated in other insects. Proline was shown to be dispensable by the deletion technique and the low specific activity measurement obtained for proline in this study was accounted for by dilution of the ^{14}C with unlabeled components of the diet during the indirect synthetic route from glucose to proline.

Previous investigations have shown that the insect, *Argyrotaenia velutinana* (Walker), a plant-feeding lepidopteran, can be perpetuated aseptically on a chemically defined medium. By deleting one amino acid at a time from the medium, it was established that *A. velutinana* required the same 10 amino acids indispensable for the rat. Because no larvae developed beyond first instar on a medium lacking an indispensable amino acid, it was concluded that there was little nutrient reserve or synthesis of these amino acids in newly hatched larvae. Although the insect could be perpetuated on an amino acid mixture containing only the 10 indispensable amino acids at an adequate dietary nitrogen level, optimal growth was achieved only when the indispensable amino acids were supplemented with a mixture of dispensable amino acids (1, 2).

An independent method using substrates that contain ^{14}C has been used to determine the nutritional requirements of several insects (3). This method has been of special value in nutritional studies in which the deletion method has been hindered by the difficulty in preparing chemically defined diets which are acceptable to insects. However, in the present work the approach was used to verify the amino acid requirements of *A. velutinana* as indicated by the amino acid deletion method.

EXPERIMENTAL

Rearing methods. The methods for mass-rearing the insect on a purified diet were the same as those reported in a previous paper (4).

The composition of the casein medium used for aseptic rearing of the insect is shown in table 1. Unless designated otherwise, the dietary constituents were obtained commercially.[2] Aseptic rearing techniques and methods for preparing the casein medium were generally the same as reported previously (5). A 200-g batch of the medium was prepared and heat sterilized at 120° for 15 minutes. Following

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[2] Nutritional Biochemicals Corporation, Cleveland.

TABLE 1

Composition of the casein medium

	g/100 g diet
Casein	3.000
Dextrose	2.500
Safflower oil	0.300
Cholesterol	0.200
Salt mixture W [1]	1.200
Vitamin mixture [2]	0.132
Tween 80 [3]	0.300
Agar	3.000
2 M KOH added to give pH 6.2	
Water to 100 g	

[1] Osborne, T. B., and L. B. Mendel 1932 A modification of the Osborne-Mendel salt mixture containing only inorganic constituents. Science, 75: 339.

[2] Composition of vitamin mixture: (mg/100 g diet) folic acid, 2; riboflavin, 2; Ca pantothenate, 4; pyridoxine·HCl, 6; thiamine·HCl, 1.2; nicotinic acid, 12; biotin, 0.025; vitamin B_{12}, 0.004; choline chloride, 100; and α-tocopherol, 15.

[3] Polyoxyethylene sorbitan monooleate, Atlas Powder Company, Wilmington, Delaware.

sterilization the medium was dispersed in portions of 4 to 5 g into sterilized 20 mm × 90 mm culture vials stoppered with sterilized cotton. Newly hatched aseptic larvae, sterilized by methods described previously (5), were added to the culture vials, one larva to each vial. The insects were reared at 21 ± 0.5° with an 18-hour illumination period each day until larval development reached fifth instar. Ten fifth-instar larvae were then transferred aseptically to culture vials containing freshly prepared casein medium made radioactive by the addition of 100 μCi of glucose-U-^{14}C. For this, the glucose-U-^{14}C (7.2 mg) was dissolved in 1 ml of distilled water and sterilized by passage through membrane filters (Millipore, 0.45 mμ). The 1 ml of solution was added to a 2-ml aliquot of the heat-sterilized casein medium just before the agar hardened. The medium was prepared so that the addition of the 1 ml of glucose-U-^{14}C solution to the 2-ml medium aliquot gave approximately a 10% solid composition. The constituents of the casein medium containing the glucose-U-^{14}C were the same as listed in table 1, except that dextrose was purposely eliminated from the diet to force the insects to utilize as much glucose-U-^{14}C as possible. The 3 ml of medium was dispersed so that 10 sterilized culture vials each contained approximately 0.3 ml medium. The larvae were allowed to feed ad libitum for 48 hours on the radioactive casein medium.

Preparation for scintillation counting. The amino acids were determined by ion-exchange chromatography as described by Spackman et al. (6). The 10 fifth-instar larvae (approximately 200 mg fresh weight) were placed in heavy-walled Pyrex test tubes, and 10.0 ml of 6.0 N HCl was added. The tube was then filled with N_2 and sealed in an oxygen flame. After heating for 24 hours at 121°, the cooled tubes were opened, and the contents were filtered. One-half of the total filtrate was dried in vacuo to remove the HCl, and then redissolved in 15 ml of buffer (pH 2.2) to give a final concentration of about 20 μmoles N/ml. Aliquots of 2.0-ml volume were then used for analysis on a Model 120 Beckman/Spinco amino acid analyzer. The eluted amino acid fractions were collected manually from the amino acid analyzer after emerging from the colorimeter. By determining the time-interval from when the amino acid peak was first recorded on the chart of the amino acid analyzer to when the ninhydrin color first emerged from the colorimeter, it was possible to collect the complete fraction of individual amino acids. This procedure permitted the quantitative analysis of 17 amino acids and the pooling of each fraction for radioactive counting.

The ^{14}C analysis of the amino acids was carried out in a Tri-Carb liquid scintillation spectrometer by use of 0.2 ml of the collected fractions dispersed in 15 ml of liquid scintillator composed of 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis-[2-(5-biphenyloxazolyl)] benzene in a solvent consisting of 20% ethanol in toluene. Blanks and internal standards consisting of ^{14}C-benzoic acid of known absolute disintegration rate were used to determine counting efficiency. Knowing the micromolar concentration, the total volume and the total counts per minute in each fraction, the specific activity was calculated. Three complete analyses were performed on the sample.

RESULTS AND DISCUSSION

The results of the analyses are shown in table 2. The specific activity measurements showed that aspartic acid, glutamic acid, glycine, serine, alanine and cysteine were highly labeled. However, lysine, his-

TABLE 2

Amino acid content and ^{14}C labeling of Argyrotaenia velutinana fed diet containing glucose-U-^{14}C

Amino acid	Content	Total count	Specific activity	Dietary requirement	
				^{14}C studies	Deletion studies (1)
	μmoles [1]	*cpm*	*cpm/μmole*		
Lysine	0.748	396	528 ± 33 [2]	+	+
Histidine	0.338	318	932 ± 457	+	+
Arginine	0.497	313	631 ± 97	+	+
Valine	0.666	76	113 ± 87	+	+
Methionine	0.193	239	1,243 ± 493	+	+
Isoleucine	0.447	139	313 ± 232	+	+
Leucine	0.736	111	152 ± 48	+	+
Phenylalanine	0.389	216	550 ± 354	+	+
Threonine	0.445	826	1,859 ± 119 [3]	+	+
Tryptophan					+
Aspartic acid	0.955	5,255	5,501 ± 224	−	−
Glutamic acid	1.304	9,680	7,423 ± 476	−	−
Glycine	0.955	10,920	11,427 ± 275	−	−
Alanine	0.769	14,944	19,366 ± 4172	−	−
Cysteine	0.059	1,175	20,083 ± 3517	−	−
Serine	0.427	10,662	24,923 ± 2563	−	−
Tyrosine	0.557	242	430 ± 333	−	−
Proline	0.748	246	328 ± 77	−	−

[1] Micromoles in approximately 15 mg of larvae.
[2] Mean of 3 analyses ± SD.
[3] See text for explanation of the high specific activity.

tidine, arginine, valine, methionine, isoleucine, leucine, phenylalanine, tyrosine, proline and threonine showed relatively low specific activities.

These results agree with those obtained by the amino acid deletion method, in that relatively little radioactive carbon from the ingested glucose-U-^{14}C was incorporated into the carbon chain of the indispensable amino acids, lysine, histidine, arginine, valine, methionine, isoleucine, leucine, phenylalanine and threonine. Relatively large amounts of radioactive carbon were incorporated into the dispensable amino acids, aspartic acid, glutamic acid, glycine, serine, alanine and cysteine (table 2).

Because tryptophan was destroyed in acid hydrolysis, no conclusion concerning this amino acid was possible. However, tryptophan was shown to be an indispensable nutrient for *A. velutinana* by the single deletion method (1).

The lack of radioactivity in tyrosine showed that this amino acid was not synthesized from the carbon chain of glucose nor its breakdown products. Phenylalanine is known to be the principal precursor and probably the sole precursor of tyrosine in the rat (7, 8). Among insects the synthesis of tyrosine from phenylalanine was shown in the silkworm larva (9). Kasting and McGinnis (10) injected phenylalanine-U-^{14}C into the pale western cutworm and recovered radioactive tyrosine. The results of the present study, coupled with results of feeding tests showing tyrosine to be a dispensable dietary nutrient (1), give evidence that tyrosine is synthesized from phenylalanine by *A. velutinana*.

The specific activity obtained with the indispensable amino acid, threonine, indicates appreciable synthesis of this amino acid. Kasting and McGinnis (10) interpreted similar data to indicate threonine synthesis from glucose-U-^{14}C in the pale western cutworm. More recently, Rodriguez and Hampton (11) reported synthesis of threonine by the phytophagous mite, *Tetranychus urticae*, in radioactivity studies similar to these. In all these studies the possibility existed that the ^{14}C in the threonine fraction was due not to synthesis of threonine from glucose, but to incomplete separation of the threonine fraction from the aspartic acid and serine fractions during collection. To clarify this

point, the remaining one-half of the total sample filtrate in 5 ml of 6 N HCl was dried in vacuo, redissolved in 2 ml of buffer (pH 2.2), and then the total sample was used for analysis on the amino acid analyzer. Aliquots of 0.65 ml were successively collected as the aspartic acid, threonine and serine fractions emerged from the analyzer. From each 0.65-ml aliquot, 0.20 ml was used for ^{14}C analysis as described above. The remaining portion was diluted with 2 ml of distilled water and the optical densities were read at 570 mμ.

Figure 1 shows the ninhydrin color yield and ^{14}C data for the aspartic acid, threonine and serine fractions. There was no ^{14}C from the threonine and the threonine fraction was contaminated with ^{14}C from the tail of the aspartic acid fraction and from the beginning of the serine fraction. This contamination resulted in a misleading value for the specific activity of threonine as originally calculated. The absence of ^{14}C from threonine shows that little, if any, threonine is synthesized from glucose. The specific activity values for other indispensable amino acids are also likely too high because these fractions may not have been absolutely free from admixture with adjacent amino acids having high specific activity. From this observation, acceptance of the peak emerging from an amino acid analyzer as unquestionably pure is not warranted, and the relatively high specific activity for threonine obtained by other investigators (10, 11) was probably due to ^{14}C contamination of the threonine fraction and not to threonine synthesis from glucose.

Methionine became significantly labeled in these studies, although clearly indispensable in the single deletion experiments (table 2). In this instance the ^{14}C undoubtedly moved into methionine through the one-carbon precursor of the labile methyl group (8).

Single deletion experiments indicated proline was dispensable. In the present experiment, however, the low radioactivity

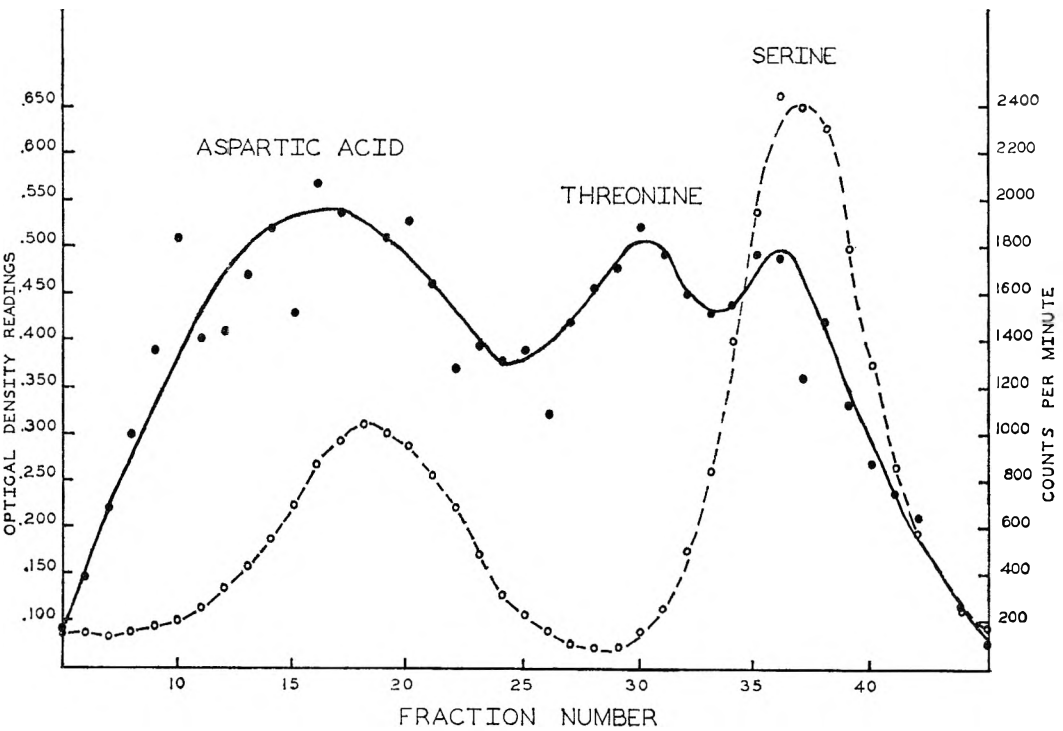

Fig. 1 Optical density curves read at 570 mμ (solid line) and count per minute curves (broken line) for 0.65-ml aliquots of the ninhydrin-reacted fractions of aspartic acid, threonine and serine.

in the isolated proline suggests at first that it is indispensable. The synthetic route to the carbon chain of proline from glucose is quite indirect, and multiple intermediates exist at which dilution of the isotope would be expected from unlabeled components of the diet. For amino acids such as serine, alanine and cysteine, deriving from pyruvate, the specific activities are in the range of 19,000 to 24,000 cpm/μmole. Dilution with unlabeled carbon between this stage in metabolism and glutamate reduced the specific activity to about one-third of that level, and in the few steps on to aspartate the specific activity decreased to about one-fourth the value at the 3-carbon level. Proline derived from glutamate would be expected to have undergone even more dilution and this effect is apparent in the data (table 2).

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Protein Quality of Various Algal Biomasses Produced by a Water Reclamation Pilot Plant [1]

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ABSTRACT A study was made to evaluate the protein quality of 7 algal biomasses produced by a water-reclamation experimental plant. The protein efficiency ratios obtained ranged from 0.68 to 1.98. The biomass with the highest protein quality compared favorably with soybean meal. The nitrogen balance experiments suggest that the variation was due largely to digestibility differences, and to a lesser extent to differences in nitrogen retention. The factors responsible for these differences were not determined in these experiments, but the importance of trying to control these factors is apparent if a consistently good quality product is to be obtained. The protein quality of *Chlorella* grown on a chemically defined medium was determined. The effects of storage and moist heat on the protein quality of algae were also evaluated.

Interest in the protein quality of an algal biomass continues to grow because of an increasing shortage of protein sources and because of the development of water reclamation projects which have the potential for producing algal biomass as a by-product (1,2). The economic feasibility of algal-water reclamation projects depends on the value of the algal biomass as feed.

Past nutritional studies indicate that the protein quality of algal biomasses varies considerably. The difference is not attributable to any one factor, since growth conditions and drying procedures have varied in each experiment (3–8). Cook (3) reported a protein efficiency ratio (PER) of 1.69 and a "true digestibility" of 65% for a waste-grown mixture of 10:1 *Scenedesmus* and *Chlorella*. Lubitz (7) demonstrated that *Chlorella* grown on a chemically defined medium resulted in a PER of 1.66 and "true digestibility" of 86%. Leveille et al. (6) reported that the PER of 4 species of algae produced under different conditions varied from 0.34 (*Spongiococcum*) to 1.38 (*Scenedesmus-Chlorella* mixture).

Amino acid analyses (9–12) and feeding experiments (6,7) indicated that the limiting amino acid is methionine. However, a fairly high protein quality (PER 2.63) resulted when algal protein served as the main protein source, supplemented with protein sources such as oatmeal, cracked wheat and milk (13). Lee et al. (14) demonstrated that *Chlorella* grown on a chemically defined medium can replace one-third of the protein of egg and up to two-thirds of the protein of fish without impairment of nitrogen retention in human adults. The protein quality of rice was improved by partial replacement with algal protein. Hintz and Heitmann (5) demonstrated that, for the growth of swine, waste-grown algae in a barley ration have the same protein quality as meat and bone meal. Several studies have evaluated algae as a feed for chickens (6,15). The algae tested were inferior to soybean meal even after amino acid supplementation (6).

The primary objective of the present experiment was to determine the protein quality of the different algal biomasses harvested from a water reclamation pilot plant.

EXPERIMENTAL

Protein quality was evaluated by determining PER (grams weight gained per gram crude protein consumed) and by a modification of the nitrogen balance method described by Mitchell (16) and Mitchell and Carman (17). Male rats of the Sprague-Dawley strain,[2] were used as test animals. For the PER determinations, 3-week weanling rats were housed separately and fed a 3% egg protein diet for

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[1] The funds for this research were provided by North American Rockwell Corporation.
[2] Purchased from Berkeley Pacific Laboratories, Inc., Berkeley, California.

a 6-day adjustment period before the 28-day experimental period. After the adjustment period, the rats were weighed and arranged in groups as similar as possible with respect to weight distribution and weight average. During the experimental period, animal weights and food intakes were recorded twice a week. Food and water were fed ad libitum. At the end of the 28-day period the animals were killed and autopsies were performed to determine the general condition of the internal organs. For the nitrogen balance study, rats were housed in metabolism cages [3] and an adjustment period of 5 to 6 days was allowed before beginning the 4-day collection period. A 3% egg protein diet was used to determine endogenous and metabolic nitrogen. Fecal nitrogen was analyzed by a nitrogen analyzer,[4] and urinary nitrogen was analyzed by the semimicro-Kjeldahl method.

Water-soluble vitamins were dissolved in 5% ethanol, and the fat-soluble vitamins in corn oil. Vitamins were fed 3 times a week at the following levels: (µg/rat/day) thiamine·HCl, 48; riboflavin, 72; niacinamide, 540; Ca pantothenate, 360; pyridoxine·HCl, 84; folic acid, 12; biotin, 12; vitamin B_{12}, 0.36; menadione, 12; α-tocopherol, 180; and 20.3 IU calciferol, and 321 IU vitamin A acetate.

The algal biomasses used in these experiments were grown in industrial photosynthesis reactors,[5] flocculated by Purifloc (Dow C-31),[6] separated by a dissolved air flotation unit, and dried on sand beds. Table 1 lists the known variables in the production of the various biomasses tested: species composition (determined by counting 150 cells), drying time, drying temperature, and storage time before the first test began. The weather determined the length of drying time. The dried algae consisted of airy fragments from 2.5 to 5 cm thick, and formed a bright green pow-

[3] Acme Metal Products, Inc., Chicago.
[4] Coleman Instruments, Inc., Maywood, Illinois.
[5] North American Rockwell Corporation Pilot Facility located at the L. A. County Sanitation Districts Water Pollution Control Facility, Lancaster, California. SID 67-401, The Use of Biomass Systems in Water Renovation, Space Division, North American Rockwell Corporation, Downey, California.
[6] Dow Chemical Company, San Francisco.

TABLE 1

Species composition, drying time, drying temperature, moisture, crude protein and ash content, and storage time of various algal biomasses

Harvest	Species composition	Drying time	Drying temperature	H_2O	Crude protein (N × 6.25)	Ash	Storage time before first test
		days	°	%	%	%	*weeks*
No. 1	30% *Closterium* 30% *Chlorella* 25% *Scenedesmus dimorphus* 10% *Scenedesmus quadricauda*	10–30	unrecorded	5	27	43	52
No. 2	75% *Scenedesmus dimorphus* 18% *Chlorella*	4–7	10–25	7	40	30	6
No. 3 [1]	95% *Scenedesmus quadricauda*	2–4	12–35	7	36	35	6
	95% *Chlorella*	2–4	12–35	7	35	33	6
No. 4	95% *Scenedesmus intermedius*	4–6	8–35	7	50	17	2
No. 5	75% *Chlorella* 20% *Scenedesmus* 4% *Ankesterodesmus*	4	4–22	7	40	26	1
No. 6	50% *Chlorella* 30% *Scenedesmus*	2–3	40–80	7	29	40	12
Laboratory-grown *Chlorella*		1	−20	7	46	6	120

[1] Harvest no. 3 resulted in two different biomasses because of maintaining 2 reactors at different depths.

der when ground in a Wiley mill,[7] using a 1-mm sieve. The ground algae were analyzed for crude protein (N × 6.25) by a nitrogen analyzer.[8] Moisture and ash content were determined on all samples; fat content was determined on harvest no. 2 by the Soxhlet method. Ash varied from 17 to 43%, and crude protein varied from 27 to 50%. The moisture content was about 7%. Fat content of harvest no. 2 was 5%. The approximate caloric content of each biomass harvest was calculated by using the composition figures obtained from the above analyses, and by considering the availability figures obtained by earlier workers (3, 7). The algal biomass was considered to be 3% chlorophyll, 3% fiber, and the remainder carbohydrate. The calculated caloric values ranged from 1.70 to 2.28 kcal/g. An attempt was made to equalize the caloric content of the test and control diets (at approximately 3.6 kcal/g) by increasing the corn oil content and decreasing the cornstarch content accordingly.

The level of available protein in the test diets was approximately 10%, though the PER's were calculated on a crude protein basis of 15 to 16%. This approximate level of available protein was calculated by considering the crude protein of a waste-grown algal biomass to be 65% available protein. The experimental diets consisted of the following: (in percent) algae, 30 to 50; USP salt mixture XIV, 2.5; corn oil, 5 to 14; powdered sugar, 15; cornstarch, 25 to 50; choline chloride, 0.2. The casein control diets consisted of: (in percent) high nitrogen casein,[9] 11.7; USP salt mixture XIV, 5; cellulose, 2; corn oil, 2; powdered sugar, 15; cornstarch, 55.1; choline chloride, 0.2; clean dried sand, 6. The casein control diet contained 10.6% crude protein (N × 6.38).

Harvests of algae were gathered (by harvest number indicated) on the following dates: no. 1, January 22, 1965; no. 2, June 7, 1965; no. 3, August 18, 1965; no. 4, September 29, 1965; no. 5, March 8, 1966; and no. 6, April 19, 1966. Harvest no. 3 (August 18) yielded 2 species, *Chlorella* and *Scenedesmus*, because the reactors were maintained at different depths.

The effect of algae as the main source of protein in a typical chicken feed was evaluated by using a test diet with 60% of the dietary protein from harvests no. 2, 4 or 5, 20% of the protein[10] from fish meal, and 20% of the protein from ground yellow corn. The algae were compared with soybean meal by using a test diet with 60% of the protein[11] from soybean meal, 20% from fish meal and 20% from corn. The effect of moist heat was evaluated by reconstituting the dried algae with water and heating to 80° for 30 minutes. The effect of storage was evaluated by storing the dried algae (7% moisture) at room temperature for periods of 4 to 14 months.

A high-temperature strain of *Chlorella* was grown on a chemically defined medium in a microterrella.[12] The unit consisted of 6 Plexiglas[13] tanks (91 cm × 129 cm × 1.2 cm) lighted by fluorescent lights. Temperature was controlled and the input of air enriched with carbon dioxide was controlled by flow meters. The *Chlorella* was harvested by centrifugation, frozen within 1 hour, and kept in a freezer for periods of up to 1 month before freeze-drying. Some of the *Chlorella* was heated to 80° before freezing; the heated *Chlorella* and some of the unheated *Chlorella* was stored under nitrogen gas for approximately 8 months before testing. The remainder was unheated and stored in air for 4 months before testing.

RESULTS

The results in tables 2, 3 and 4 show large differences in protein quality of the 7 waste-grown algal biomasses. The casein-corrected PER values ranged from 0.68 to 1.98; true protein digestibility (TPD) ranged from 51 to 75 and biological values (BV) ranged from 60 to 76. The differ-

[7] Thomas Wiley Mill, Model ED-5. Arthur H. Thomas Company, Philadelphia.

[8] See footnote 4.

[9] ANRC Test Diet High Nitrogen Casein, Animal Nutrition Research Council, Sheffield Chemical Company, Norwich, New York.

[10] Peruvian fish meal (67% protein), Nebraska whole yellow corn (8% protein) and soybean meal (50% protein) were purchased from Rykebosch poultry Farms, Lancaster, California.

[11] See footnote 10.

[12] Information & Life Sciences Systems Division, North American Rockwell Corporation, El Segundo, California.

[13] Rohm-Haas, Philadelphia.

TABLE 2

Protein efficiency ratios (PER) of casein, various algal biomasses, and algae or soybean combined with fish meal and corn

Test diet	No. of rats	Avg wt gain	Avg crude protein intake	PER	Casein-corrected PER
		g	*g*		
Casein control	20	84.6	32.2	2.63 ± 0.06 [1]	2.50
Harvest no. 2	12	77.1	45.2	1.71 ± 0.03	1.62
60% protein from harvest no. 2 + 20% fish meal + 20% corn	6	98.0	42.9	2.28 ± 0.02	2.17
Harvest no. 3, *Chlorella*	15	27.5	38.1	0.72 ± 0.04	0.68
Harvest no. 3, *Scenedesmus*	15	37.5	41.0	0.92 ± 0.04	0.87
Harvest no. 4	12	53.8	46.5	1.15 ± 0.04	1.09
60% protein from harvest no. 4 + 20% fish meal + 20% corn	10	80.1	41.1	1.95 ± 0.05	1.85
Harvest no. 5, sun-sand bed dried 4 days	6	139.2	66.9	2.08 ± 0.02	1.98
Harvest no. 5, air-dried 4 days out of sun	6	138.7	68.6	2.02 ± 0.01	1.92
60% protein from harvest no. 5 + 20% fish meal + 20% corn	6	102.7	37.5	2.74 ± 0.09	2.60
60% protein from soybean + 20% fish meal + 20% corn	10	134.6	42.0	3.20 ± 0.07	3.04

[1] Mean ± SE of mean.

ence in protein quality between harvest no. 5 and harvest no. 2 appears to be due to digestibility rather than retention.

Large increases in protein efficiency ratios were observed when algal protein was supplemented by corn and fish meal. Harvest no. 5 resulted in a PER of 2.60 when combined with corn and fish, but this PER was still below the PER of 3.04 obtained when soybean meal was supplemented by corn and fish.

The protein efficiency ratios obtained for the *Chlorella* grown on a chemical medium were 1.54 and 1.41, but decreased to 0.78 when the *Chlorella* was heated before drying. Moist heat appeared to improve protein quality of the waste-grown algae when applied after drying. The protein quality of the algae appeared to decrease slightly when stored in air as 7% moisture. The result of the test using harvest no. 5 indicates that drying in the sun has no effect on protein quality.

DISCUSSION

Since both the highest and the lowest PER were obtained from a biomass which was predominantly *Chlorella*, the species composition does not appear to be the cause for the protein quality differences in these experiments. The 2 biomasses which were dried at temperatures up to 25° had higher protein quality than the biomasses dried at temperatures up to 35°. Despite long drying times, none of the biomasses tested had an odor or showed

TABLE 3

Protein efficiency ratios (PER) of casein, algal biomass stored or heat treated, and Chlorella *grown on a chemically defined medium*

Test diet	No. of rats	Avg wt gain	Avg crude protein intake	PER	Casein-corrected PER
		g	*g*		
Casein control	11	81.0	29.7	2.73 ± 0.05 [1]	2.50
Harvest no. 2, stored 14 months	8	42.2	29.1	1.45 ± 0.07	1.33
Harvest no. 3, *Scenedesmus* stored 12 months	8	25.2	29.5	0.85 ± 0.09	0.78
Harvest no. 5, stored 4 months, 16% crude protein in diet	6	86.8	50.2	1.73 ± 0.03	1.58
Harvest no. 5, stored 8 months, 16% crude protein in diet	6	107.0	59.5	1.80 ± 0.02	1.65
Harvest no. 5, stored 4 months, 10% crude protein in diet	6	37.8	23.8	1.58 ± 0.10	1.45
Harvest no. 5, stored 4 months, 10% crude protein in diet after moist heat treatment	6	39.3	23.5	1.67 ± 0.08	1.53
Harvest no. 6	6	29.8	20.4	1.46 ± 0.15	1.34
Harvest no. 6, after moist heat treatment	6	33.2	20.6	1.61 ± 0.07	1.47
Harvest no. 6, stored 6 months	6	25.2	23.3	1.08 ± 0.09	0.99
Chlorella stored under N	6	70.3	41.6	1.68 ± 0.07	1.54
Chlorella air-stored	6	67.8	44.0	1.54 ± 0.06	1.41
Chlorella, heated before drying, stored under N	6	20.8	24.2	0.85 ± 0.10	0.78

[1] Mean ± SE of mean.

brown discoloration. The high amount of air incorporated in the fragments may have prevented anaerobic breakdown. The total aerobic bacterial count in harvest no. 5 (analyzed before drying) was 4×10^9 cells/g dry algae. The count was performed by plating. After 1 week at 9% moisture, the aerobic bacterial count was 1×10^4 cell/g dry algae. The bacteria were mainly coliforms and *Proteus*. Variation in PER may be a result of non-photosynthetic organisms present and should be considered a major variable in future experiments.

The variables in the production of an algal biomass have not received much attention from other investigators. Species composition is always reported, but in many cases conditions of growth and drying are not. Golueke and Oswald (2) reported that sand-bed dried, sewage-grown algae were less digestible than drum- or

TABLE 4

Nitrogen balance data, biological value (BV), true protein digestibility (TPD) and net protein utilization (NPU) of various algal biomasses compared with casein

Test diet	No. of rats	Avg wt	Avg N intake	Avg urinary N	Avg fecal N	Avg endogenous N	Avg metabolic N	BV	TPD	NPU
		g	*g*	*g*	*g*	*g*	*g*			
Casein [1]	7	250	1.08	0.382	0.090	0.144	0.087	77 ± 2.2 [2]	97 ± 2.2	75
Harvest no. 5	8	220	1.57	0.381	0.480	0.102	0.092	76 ± 4.6	75 ± 1.7	57
Harvest no. 2	7	247	2.01	0.477	0.830	0.134	0.126	73 ± 1.0	65 ± 1.4	48
Harvest no. 1, stored over 1 yr	7	240	1.81	0.482	0.733	0.139	0.135	71 ± 2.3	67 ± 1.7	47
Harvest no. 3, *Scenedesmus*	6	160	1.33	0.342	0.728	0.074	0.079	60 ± 2.5	51 ± 1.6	31
Harvest no. 5, stored 4 months	8	236	1.43	0.414	0.554	0.129	0.108	71 ± 1.9	69 ± 1.2	50
Casein [1]	5	228	1.06	0.327	0.110	0.126	0.092	81 ± 1.4	98 ± 0.8	79

[1] Determined approximately 6 months apart.

[2] Mean ± SE of mean.

spray-dried sewage-grown algae. The criteria for digestibility were not given.

However, the sand-bed dried algae in the present experiment, harvest no. 5, resulted in a PER higher than that for any other algal biomass reported in the literature. Harvest no. 5 resulted in a PER of 1.95, a TPD of 75, and a BV of 76. These figures show this biomass to be only slightly lower in protein quality than heated soybean meal, which has a PER of 2.15 (18), a TPD of 85, and a BV of 75 (19). The waste-grown mixture of *Scenedesmus-Chlorella* (PER 1.69) used in Cook's study (3) was spread on aluminum sheets and placed over a heat source for about 60 hours. The *Chlorella* grown on a chemically defined medium (PER 1.66) in Lubitz's study was freeze-dried in 7 hours. The PER values reported in Leveille's study (6) varied from 1.38 for a *Scenedesmus-Chlorella* mixture to 0.34 for Spongiococcum, but conditions of growth and drying were not given.

The effect of moist heat when applied before drying suggests that the algae, if unheated, may be improved by the action of microorganisms or enzymes. Similar results were obtained in one experiment using waste-grown algae, but not enough algal biomass was processed to obtain a 4-week PER. Heated biomass to which a small portion of fresh biomass was added before drying had a much higher protein quality than biomass which was only heated. Since the results could not be repeated with another harvest of biomass, these data need further substantiation. These results are not in agreement with the work of Vanderveen et al. (20) which indicated that sterile algae were more digestible than contaminated algae (*Chlorella pyrenoidosa*). The beneficial effect of moist heat when applied after drying appears to correlate with the work of Cook (3).

The increased PER of the algal biomasses when combined with corn and fish meal is also in agreement with the work of Cook (13) on algae combined with grain and milk; a high PER can be obtained by using algae as the main source of protein. The nitrogen balance experiments indicate that failure of the algae tested to result in a PER equal to the

soybean meal, corn, and fish meal mixture is due to the slightly lower availability of algal nitrogen. Lee et al. (14) felt that, though undesirable, the characteristic indigestibility of algal protein may not be a significant problem when used in a supplement.

These experiments indicate that an algal biomass can have a protein quality high enough to be valuable as an animal feed. Several factors need to be investigated if the product is to be consistently of good protein quality. Other attributes which add to the value of algae as a feed are the vitamins and minerals present (3), and, for chickens, the xanthophyll pigments are highly desirable (15). If a consistently good quality product can be obtained, swine and poultry farmers may be able to use a replacement for vegetable protein sources that must be shipped long distances.

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Lipid Composition of Heart, Kidney and Lung in Guinea Pigs Made Anemic by Dietary Cholesterol [1]

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ABSTRACT The lipid composition of heart, kidney and lung of cholesterol-fed anemic guinea pigs was investigated and compared with normal control guinea pigs. The heart showed a decrease of triglyceride, an increase in unesterified cholesterol, and a decrease of polyunsaturated fatty acids in the cholesterol-fed guinea pig. A relative increase in size of kidney was observed. The level of triglyceride decreased, and the unesterified cholesterol content increased. The enlarged lungs showed a marked increase of esterified cholesterol, a decrease of triglycerides, and increased amounts of polyunsaturated fatty acids in the cholesterol ester and phospholipid fractions. All 3 organs showed a relatively high linoleate level in normal guinea pigs as compared with other laboratory rodents. These results were compared with data in the literature on rats and rabbits, species of rodents that show vaying degrees of susceptibility to a cholesterol-induced anemia. They are interpreted to indicate that these changes in lipid composition of heart, lung and kidney may be related in part to the increased functional burden to the organs due to the anemia and in part to a species-specific handling of lipids in the guinea pig.

The guinea pig reacts to dietary cholesterol by changes in the lipid composition and morphology of many tissues, accompanied by the development of a hemolytic anemia (1–3). We have previously reported on the histopathology of the tissues (4) and on the lipid composition of plasma, red blood cells, liver, spleen, adrenals and bone marrow (5–7). We are now reporting on the lipid composition of heart, kidney and lung of the cholesterol-fed, anemic guinea pig. The response of kidney lipids to dietary cholesterol is of interest because of a glomerulosclerosis observed in the anemic guinea pig (8). The lipid composition of lung and heart tissue might be expected to change in response to the increased functional burden imposed on these organs by a severe hemolytic anemia.

The results of this study are discussed in relation to the lipid composition of the same organs in rabbits and rats, species that show different degrees of susceptibility to a cholesterol-induced anemia.

MATERIALS AND METHODS

Groups of young, male guinea pigs were fed a semipurified diet containing 30% casein, 10% cottonseed oil and amounts of vitamins and minerals adequate for normal growth, with or without addition of 1% cholesterol (7).

After the cholesterol-fed group had become anemic, with a blood count of less than 3 million/mm^3 which required 8 to 10 weeks, at least 5 to 7 animals of each group were killed. Tissue lipids of each animal were extracted with 15 volumes of chloroform-methanol (1:1) (containing 0.1 ml of 1% hydroquinone) and separated, by silicic acid-column chromatography, into fractions containing cholesteryl ester, triglycerides, unesterified cholesterol (containing also free fatty acids and partial glycerides) and phospholipids (9).

Total lipid (10), cholesterol (11) and phosphorus (12) were determined in the unfractionated extract and in the appropriate fractions. The fatty acid composition was determined by gas-liquid chromatography (GLC), using a Wilkens Aerograph, model 204, with flame-ionization detector. The phospholipids were separated into subclasses by thin-layer

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chromatography (TLC), using the solvent system described by Skipski et al. (13). After visualization by dichlorofluorescein (0.2% in methanol), the individual components were eluted twice with the developing solvent followed by 3 portions of methanol, and transmethylated by controlled heating with 1% H_2SO_4 in methanol. The fatty acid composition of each phospholipid class was determined by GLC after extraction of the methyl ester with petroleum ether. The phosphorus distribution was determined in the remaining aqueous layer.

RESULTS

Weight gains of the animals, size of organs and hematological data are summarized in tables 1 and 2. As observed previously (5), the control animals grew better than those fed cholesterol. In relation to body weight, kidney and lungs were significantly enlarged, whereas the heart remained unchanged.

The composition of the organs according to major lipid classes is shown in table 3. Hearts and kidneys of anemic animals showed a decrease in the amount and proportion of total lipid resulting from a decrease in the triglyceride fraction. In the kidney the decrease in total lipid did not reach statistical significance. The proportion of cholesterol increased in both organs, primarily as a result of increases in the unesterified cholesterol, although there was also a tendency for an increase in the cholesterol ester fraction. Because of the decrease in total lipids of heart, the proportion of lipid represented by esterified and by unesterified cholesterol was doubled, and the ratio of unesterified cholesterol to phospholipid increased. A noteworthy increase occurred in the cholesterol ester content of lung lipids, with only minor increases of unesterified cholesterol and a decrease of triglyceride similar to that in heart and kidney.

The fatty acid composition of the major lipid classes of the 3 organs is shown in table 4. In the heart, the concentration of linoleate decreased in the cholesterol ester and the triglyceride fractions. Lung lipids, however, responded to dietary cholesterol by increases of the amounts of cholesterol ester and phospholipid polyunsaturated fatty acids (PUFA). Cholesterol feeding did not result in significant changes in

TABLE 1

Gross effects of feeding 1% cholesterol to guinea pigs [1]

Animals	Time fed diet	Wt at autopsy	Total wt gain	Erythrocyte count	Packed cell volume
	days	*g*	*g*	*x 10^6/mm³*	%
Control	76	741	500	normal [2]	normal [3]
C-fed [4]	77	491	207	2.25	24

[1] Six animals/group.
[2] Normal value, 5–6 million/mm³.
[3] Normal value, 50%.
[4] Control diet plus 1% cholesterol.

TABLE 2

Organ weights in control and cholesterol-fed anemic guinea pigs [1]

Animals	Heart		Kidney		Lung	
	Wet wt	Body wt [2]	Wet wt	Body wt	Wet wt	Body wt
	g	%	*g*	%	*g*	%
Control	1.94 [3]	0.26 ± 0.02	4.58	0.61 ± 0.018 [3]	3.97	0.54 ± 0.045 [3]
C-fed [4]	1.36	0.28 ± 0.02	3.99	0.82 ± 0.063	4.02	0.83 ± 0.076
+C/−C [5]	0.70	1.08	0.87	1.34	1.01	1.54

[1] Values are means or means ± SE for groups of 6 animals each, fed the control or cholesterol-containing diet for 11 weeks.
[2] Based on body weight immediately before autopsy; animals had been fasted 14 to 18 hours.
[3] Difference between groups is statistically significant at $P < 0.01$.
[4] Control diet plus 1% cholesterol.
[5] Ratio of means for control and C-fed group.

TABLE 3

Lipid composition of heart, kidney and lung in control and cholesterol-fed anemic guinea pigs [1]

Animals	Total lipid		Phospho-lipid	Esterified cholesterol	Unesterified cholesterol	Triglycerides
	mg/organ	*g/100 g*	*g/100 g*	*mg/100 g*	*mg/100 g*	*g/100 g*
			Heart			
Control	70.7 ± 6.0 [2]	3.64 ± 0.10 [3]	2.35 ± 0.02	20 ± 3	96 ± 2.5 [2]	1.34 ± 0.15 [2]
C-fed [4]	39.6 ± 5.0	2.92 ± 0.20	2.31 ± 0.05	36 ± 6	142 ± 8.0	0.48 ± 0.10
			Kidney			
Control	293 ± 55.0	6.20 ± 0.72	3.10 ± 0.12	56 ± 10	299 ± 6.0 [2]	2.77 ± 0.31 [2]
C-fed	188 ± 8.0	4.72 ± 0.10	3.18 ± 0.12	92 ± 18	342 ± 9.0	1.10 ± 0.03
			Lung			
Control	142 ± 11.0	3.78 ± 0.09	2.58 ± 0.13	18 ± 3 [2]	375 ± 13 [2]	0.83 ± 0.12 [2]
C-fed	172 ± 18.0	4.02 ± 0.18	2.77 ± 0.13	389 ± 84	474 ± 12	0.37 ± 0.07

[1] Values are means or means ± SE for groups of 6 animals each, fed the control or cholesterol-containing diet for 11 weeks.
[2] Difference between groups is statistically significant at $P < 0.01$.
[3] Difference between groups is statistically significant at $P < 0.02$.
[4] Control diet plus 1% cholesterol.

the fatty acid composition of the kidney lipid classes.

When the phospholipids of the heart and kidney were separated into individual phospholipid classes, it became evident that the only effect of dietary cholesterol was a decrease, although not statistically significant in the proportion of heart phosphatidylethanolamine (table 5).

Table 6 presents the fatty acid composition of the individual phospholipid classes of heart and kidney. Cholesterol feeding resulted in moderate increases in stearic acid of the major phospholipid classes of heart which was compensated for by a decrease in one or both of the PUFA's. These changes reached statistical significance only in the lysolecithin fraction.

In the kidneys of anemic animals the proportion of arachidonate of sphingomyelin increased at the expense of the other unsaturated fatty acids. In the fractions containing phosphatidylserine (PS) and phosphatidylinositol (PI) linoleate increased at the expense of stearate.

DISCUSSION

Different species of rodents respond differently to dietary cholesterol. In the following discussion we are comparing the lipid composition of heart, lung and kidney of the guinea pig to that of the rabbit and the rat. All 3 species show increased red cell production in bone marrow when fed cholesterol (7, 14). Rabbits have been reported to become anemic to varying degrees depending on the cholesterol and fat content of the diet (14–17). Rats, however, develop a frank anemia only when fed a severely atherogenic diet for 18 to 20 weeks (18, 19). The purpose of this comparison is an attempt to decide whether the changes in lipid composition of three important organs observed in the cholesterol-fed, anemic guinea pigs are related to inherent differences of lipid metabolism in this species compared with species more resistant to this injury, or whether they are consequences of the functional burden due to the anemia.

We are not aware of any reports on the lipid composition of tissues from cholesterol-fed rabbits that also comment on the hematology of the animals. Reports on the cholesterol-induced anemia in rabbits, on the other hand, do not describe their tissue lipid composition. For the purpose of this discussion we are assuming that the cholesterol-fed rabbits were mildly anemic.

The organ weights of guinea pigs reported in this study closely resemble those reported in the literature (20–22). Rabbits have been reported to give a response to dietary cholesterol similar to that of guinea pigs, with unchanged relative size of the heart (23) and increases in relative lung weights (24).

A decrease of triglyceride content of heart and kidney in cholesterol-fed rabbits

TABLE 4

Fatty acid composition of lipid classes of heart, kidney and lung in control and cholesterol-fed anemic guinea pigs [1]

	Fatty acid [2]						Total fatty [3] acid
	16:0	16:1	18:0	18:1	18:2	20:4	
	% of total methyl ester measured						*mg/100 g fresh wt*
Heart							
Cholesterol ester							
Control	18.4±0.7	5.8±0 7	6.9±0.5	17.8±0.7	44.0±2.1 [5]	2.6±0.3	28
C-fed [4]	21.2±0.4	7.8±0.8	7.3±0.6	18.4±0.8	34.6±0.9	2.8±0.4	36
Triglycerides							
Control	28.0±1.9	2.4±0.1	6.7±0.2 [5]	25.1±1.5	34.8±1.4 [5]	0	1275 [5]
C-fed	29.5±3.7	4.2±0.8	10.6±1.2	25.0±1.4	22.8±2.5	0	458
Phospholipid							
Control	14.0±1.1	0.7±0.1	21.2±1.4	8.3±0.7	33.0±0.95	19.2±2.9	1286
C-fed	15.1±0.8	0.8±0.1	23.7±1.8	8.6±0.3	31.6±0.93	16.2±1.8	1127
Kidney							
Cholesterol ester							
Control	25.6±2.0	1.8±0.8	7.3±1.0	21.4±1.7	32.9±2.5	3.0±1.0	66
C-fed	20.3±1.8	2.4±0.4	8.5±1.0	18.3±0.8	33.4±1.6	5.2±0.5	61
Triglycerides							
Control	25.6±0.7	3.1±0.2	7.4±0.6	25.7±0.9	35.8±1.7	0	2692 [6]
C-fed	27.6±0.8	3.1±0.2	7.5±0.5	25.6±0.7	32.8±1.1	0	905
Phospholipids							
Control	14.0±0.4	0.1±0.1	21.9±0.3	9.4±0.2	26.7±0.4	23.5±0.8 [6]	1837
C-fed	13.8±0.5	0.2±0.1	23.1±0.4	9.1±0.5	28.2±0.3	20.7±0.5	1616
Lung							
Cholesterol ester							
Control	28.9±1.6 [5]	13.7±0.7 [5]	5.6±0.4	15.1±1.0 [5]	16.8±2.6 [5]	0.7±0.4 [5]	8 [5,7]
C-fed	21.3±1.6	1.3±0.4	5.2±0.4	21.9±0.5	32.1±1.7	6.6±1.0	310
Triglycerides							
Control	28.6±1.4	2.6±0.6	6.9±0.8	26.2±1.6	29.3±1.6	0	790 [5]
C-fed	29.6±1.6	3.6±0.8	8.2±0.8	25.7±1.1	28.9±3.0	2.0±1.2	350
Phospholipids							
Control	36.3±3.2	2.3±0.6	12.1±1.2	16.7±1.1	21.5±1.1	4.5±1.6 [6]	1370
C-fed	30.1±2.2	1.2±0.5	12.9±1.3	15.0±0.8	22.4±1.6	10.6±1.3	1560

[1] Values are means or means ± SE for groups of 6 animals each, fed the control or cholesterol-containing diet for 11 weeks.
[2] Major fatty acids only are listed.
[3] Calculated from sum of peak areas as compared with that of 17:0 added before methylation of sample.
[4] Control diet plus 1% cholesterol.
[5] Difference between groups is statistically significant at $P < 0.01$.
[6] Difference between groups is statistically significant at $P < 0.05$.
[7] Three samples had been lost.

has been reported (24), but of a lesser magnitude than our findings in the guinea pig. In rats, however, the triglyceride content of these tissues increased in response to dietary cholesterol (25).

An increase in the cholesterol content of tissues from cholesterol-fed rats and rabbits has been reported frequently (24–28). In contrast with our observation in guinea pigs, however, most of this increase was in the esterified fraction, not only in lung tissue but also in heart and kidney.

Our findings indicate that normal guinea pig tissues contain a large proportion of linoleate both as cholesterol ester and as triglycerides, whereas the reports in the literature for rats and rabbits indicate values of less than 20% linoleate in the

TABLE 5

Phospholipid classes of heart and kidney in control and cholesterol-fed anemic guinea pigs [1]

Animals	Origin	Lysolecithin [2]	Sphingomyelin	Lecithin	Phosphatidylinositol + phosphatidylserine	Phosphatidyl-ethanolamine	Solvent front	Phosphorus recovery
				% total phosphorus				%
				Heart				
Control	1.6 ± 0.2	0.8 ± 0.1	4.4 ± 0.4	40.8 ± 1.0	6.4 ± 0.9	26.2 ± 1.4	19.9 ± 1.1	73
C-fed [3]	3.6 ± 0.1	3.0 ± 1.6	6.5 ± 1.2	40.2 ± 0.6	8.5 ± 0.5	16.7 ± 3.9	21.5 ± 1.4	76
				Kidney				
Control	1.4 ± 0.1	0.8 ± 0.1	7.0 ± 0.7	45.3 ± 3.1	10.2 ± 1.1	27.0 ± 2.8	8.3 ± 0.5	73
C-fed [3]	0.8 ± 0.1	1.0 ± 0.1	7.1 ± 0.2	41.9 ± 1.8	12.5 ± 1.3	29.4 ± 1.2	7.3 ± 0.8	77

[1] Values are means ± SE of 3 animals each fed the control or cholesterol-containing diet for 11 weeks.
[2] Phospholipid classes were identified by comparison of their position on TLC plates with those of appropriate standards run on the same plate.
[3] Control diet plus 1% cholesterol.

neutral lipids of heart and kidney and 10% linoleate in those of lungs (29–32). No report on the response of the fatty acid composition of neutral lipids of heart, kidney or lungs of rats or rabbits to dietary cholesterol has come to our attention. A decrease in the PUFA content of total lipids in cholesterol-fed rat hearts has been reported (25). This may correspond to our observation that the linoleate content of cholesterol ester and triglycerides decreased in the hearts of cholesterol-fed guinea pigs.

The phospholipids of guinea pig heart and kidney appear to have a lower proportion of sphingomyelin and of the fraction containing PI and PS than those of rats (34) and of a series of other mammals (35). These authors reported 12 to 20% sphingomyelin and 10 to 25% PI and PS. The total recovery of phosphorus from the TLC plates by our methods was, however, only 75%, and it is possible that this loss was not equally distributed among the individual phospholipid subclasses. The methods used by the other authors have been reported to yield phosphorus recoveries of 81 to 89% and 93 to 96%, respectively. The fatty acid composition of the unfractionated phospholipids from guinea pig hearts (over 50% PUFA and a relatively high proportion of stearic acid) was not too different from that reported for rats (29, 30). The fatty acid composition of the heart phosphatidylcholine and phosphatidylserine was also similar to that reported for the rat (36). Similar to neutral lipids, the phospholipids of guinea pig kidney and lung were richer in PUFA's than those reported for other mammalian species (30, 31, 33).

This investigation has shown that the lipid composition of heart, kidney and lung of normal guinea pigs differs from that in comparable organs of other laboratory rodents by reason of a considerably higher content of linoleate in the three major lipid classes and possibly certain differences in the proportions of phospholipid subclasses. This indicates there may be, in fact, species-specific differences in lipid metabolism between the guinea pig and some other rodents.

The heart responded to dietary cholesterol by a large decrease in triglyceride

TABLE 6

Fatty acid composition of phospholipid classes of heart and kidney in control and cholesterol-fed anemic guinea pigs [1]

Phospholipid class	Fatty acids [2]					
	16:0	16:1	18:0	18:1	18:2	20:4
	% of total methyl ester measured					
			Heart			
Origin	24.8	9.4	21.7±6.4	11.6±2.9	11.1±4.4	1.4±1.0
Lysolecithin	18.5	4.8	24.2±4.4 [3]	10.2±1.2	20.3±2.6	10.7±1.4
Sphingomyelin	26.4	4.7	40.3±4.8	5.5±0.5	4.0±0.5	3.7±1.1
Lecithin	24.0	0.4	18.1±2.9	13.8±1.0	30.0±2.6	9.7±1.7
PI+PS [4]	5.9	0.7	43.9±6.4	7.1±0.7	11.7±2.9	26.8±3.2
Phosphatidyl-ethanolamine	6.7	0.9	39.2±2.4	4.9±0.6	10.1±1.2	33.3±5.2
Solvent front	5.9	1.1	22.4±2.8	6.1±0.5	39.2±5.7	20.7±4.6
			Kidney			
Origin	16.5	3.1	6.8±2.0	19.5±5.0	17.8±4.3	3.6±2.1
Lysolecithin	24.1	5.4±1.1	11.7±1.7	12.2±2.3	16.4±5.0	1.9±1.4
Sphingomyelin	32.5	3.1	10.2±0.6	12.6±3.5	16.1±4.7	4.4±2.9 [5]
Lecithin	25.8	0.6	19.5±0.9	13.9±1.1	32.1±0.7	4.7±0.6
PI+PS [4]	8.7	0.4	40.7±3.3 [6]	11.1±1.9	16.5±1.4 [6]	16.9±1.5
Phosphatidyl-ethanolamine	8.7	0.4	21.6±2.4	12.7±4.8	18.9±1.9	33.8±6.5
Solvent front	14.3	2.1	10.7±1.2	15.0±3.1	38.9±1.9	11.4±1.8

[1] Values are means or means ± SE of 6 animals of which 3 had been fed the control diet and 3 the cholesterol-containing diet for 11 weeks. The data of the 2 groups were pooled because there were no statistical differences between them.
[2] Major fatty acids only are listed
[3] Control group 15.8 ± 3.7, C-fed group 32.6 ± 3.3; difference statistically significant at $P < 0.05$.
[4] Phosphatidylinositol + phosphatidylserine.
[5] Control: none detected; C-fed: 8.8 ± 2.5.
[6] 18:0 control: 47.0 ± 2.1, C-fed: 34.4 ± 3.3; 18:2 control: 13.5 ± 2.8, C-fed: 19.6 ± 0.3; differences between groups statistically significant at $P < 0.05$.

content, a moderate increase in unesterified cholesterol and a decrease in the PUFA in the cholesterol ester, triglycerides and phospholipids. The decrease in triglyceride content is similar to that reported for the hearts of cholesterol-fed rabbits. Such a decrease is absent, however, in cholesterol-fed rats that normally do not show this anemia, and may therefore be related to the increased functional burden caused by the hemolytic anemia. The decrease in the proportions of PUFA of heart lipids has been observed in cholesterol-fed rats and may therefore be unrelated to the anemia.

The lungs of the cholesterol-fed guinea pig responded with an increase in relative size, a decrease in triglyceride content, a marked accumulation of esterified cholesterol and increased amounts of PUFA in cholesterol ester and phospholipids. The decrease in lung triglyceride may be related, as in heart, to the functional burden imposed by the anemia. Aylward and Stott (24) did not report a similar decrease in their cholesterol-fed rabbits. The degree of anemia, if any, in these animals is unknown however. We have previously reported a marked increase of esterified cholesterol and of cholesterol ester-linoleate in spleens of cholesterol-fed guinea pigs (5). Both lung and spleen are highly vascular tissues, and both tissues have been shown previously to be important for the removal of cholesterol from the circulation of rats and guinea pigs (4, 37).

The kidneys responded to dietary cholesterol with an increase in relative size, a decrease in triglyceride content and a moderate accumulation of unesterified cholesterol. The relationship of these changes to the glomerulosclerosis and the accumulation of fat globules of the glomeruli, which we have observed in the cholesterol-fed anemic guinea pig (8), remains obscure.

The data presented appear to indicate that the changes in lipid composition of heart, lung and kidney of cholesterol-fed, anemic guinea pigs are in part related to

the increased functional burden to the organs and in part to a species-specific handling of lipids in the guinea pig.

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Effect of Ascorbic Acid Deprivation in Guinea Pigs on Skeletal Metabolism [1]

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ABSTRACT The skeletal response to ascorbic acid deficiency in guinea pigs was studied. A reduction in skeletal deposition of both radioactive calcium and phosphorus was consistently observed following 14 days of vitamin C deprivation; and the lability of deposited bone salts was increased in the deficient animals. Subsequent calculations showed a highly significant inverse relationship between these parameters. Development of each was dependent on the time of deficiency. The close relationship between deposition and bone salt release leads to the suggestion that the type and amount of matrix formed was altered by the ascorbic acid deficiency. It is suggested that ascorbate deprivation, similar to that of some other deficiencies, may impede the transition of amorphic bone salts to the crystalline form.

The requirement of ascorbic acid for bone tissue formation has been well-established (1). It appears to influence collagen formation (2) and is also involved in skeletal alkaline phosphatase activity (1). More recent efforts have been directed toward the elucidation of the mode of action involved. Evidence had been obtained which indicated that ascorbic acid was involved in the hydroxylation of proline during the formation of collagen (3). In this light, it has been suggested that ascorbate acts as a hydrogen donor in the hydroxylation reaction (4).

The possibility that vitamin C may influence skeletal calcification, independent of the matrix effect, has also been considered (5). Bourne (6) observed that bone tissue regeneration was dependent on ascorbic acid, and others (7) have noted that skeletal ^{32}P deposition was significantly decreased in scorbutic animals. In the latter case bone was the only tissue of several tested so influenced. Thus, while it is clear that vitamin C is required for bone formation, the means by which it influences this processes remains to be shown.

The amount of bone tissue present at any one time is obviously dependent on a relative balance between bone formation and destructive activities. Another factor which also seems important to bone tissue maintenance is the stability of those crystals which have been formed. In the current study, bone salt deposition and stability were measured in an effort to determine skeletal response to ascorbic acid deficiency.

METHODS

Male guinea pigs weighing 300 to 400 g were used for the first 2 studies. Before the experiments the animals were fed a commercial guinea pig ration [2] and were given a scorbutogenic diet [3] or the same diet supplemented with crystalline ascorbic acid (400 mg/kg) during the experiment. Both feed and water were supplied free-choice during the preliminary and experimental periods. A 15-day feeding period was followed in each experiment.

For the first study, 12 deficient and 12 control animals were divided equally and given either $^{45}CaCl_2$ (10 μCi/100 g body weight) or $H_3{}^{32}PO_4$ (5 μCi/100 g) after 14 days of dietary treatment. The isotopic materials were suspended in physiological saline solution and administered intraperitoneally. After 24 hours the animals were killed; the tibiae were immediately re-

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[2] Purchased from Ralston Purina Company, St. Louis.

[3] This diet had the following percentage composition: ground oats, 40; ground wheat bran, 15; alfalfa meal, 8; skim milk powder (treated to destroy vitamin C), 20; vitamin-free casein, 10; vegetable oil (corn), 5; NaCl, 0.5; $CaCO_3$, 1; and $MgSO_4$, 0.5 (obtained from Nutritional Biochemicals Corporation, Cleveland).

moved, cleaned, and placed in iced physiological saline solution. When cooled, the proximal cancellous region was separated from the compact, blotted with paper towels to remove marrow and apparent moisture, weighed, and placed in 3.0 ml of cool incubation medium (8) which had been adjusted to a pH 7.4 before introduction of the sample. All tissue was incubated for 1 hour in the Dubnoff metabolic shaker at 37°, using air as the gas phase in an attempt to assess the bone salt stability. Following the 1-hour incubation the radioactivity of the medium and the bone tissue was determined and the values were used to calculate the percentage of isotope released during this period. Chemical determinations of similar bone tissue not incubated include citric acid (9) and alkaline phosphatase (10).

A similar procedure was used in the second study with the following exceptions. Radioactive phosphorus (10 μCi/100 g body weight) was given either 12 hours or 6 days before killing (the animals were killed after 15 days of dietary treatment). This was done in an attempt to study the influence of the vitamin C deficiency on stability of bone salts which had been deposited for some time (6-day group) as well as those recently deposited (12 hours). In addition, studies were conducted on inactivated bone tissue. Inactivation was effected by submerging samples in boiling water for 5 minutes before incubation. Food consumption and body weight changes were carefully recorded for all animals used in these experiments.

Next, we wished to determine the amount of time required to cause changes in bone salt deposition and lability when an ascorbate-deficient diet was fed. Thirty young, male guinea pigs were equally divided and given commercial rabbit ration [4] or this feed supplemented with ascorbic acid as in the first 2 studies. This diet was chosen for the present work because the animals consumed it more readily. Previous studies had illustrated that it had scorbutogenic properties comparable to the diet used in the first-described investigations, and it supported an excellent growth rate in guinea pigs when supplemented with ascorbic acid (400 mg/kg). At 4, 9, and 14 days following initiation of dietary treatment, 5 animals from each group were given $H_3{}^{32}PO_4$ (15 μCi/100 g body weight). After 24 hours the animals were killed and observations similar to those of the first 2 studies were made.

Concentration of the skeletal isotopes was determined in the following manner. Bone tissue was ashed overnight at 600°. The resulting ash was taken up with 1 N HCl and a small portion of the liquid sample plated on a stainless steel planchet at infinite thickness. Distribution of the plated sample was enhanced with ethanol. Radioactivity of the medium was measured to determine the degree of skeletal release of isotope during incubation. Media samples were treated in a manner similar to that for the bone ash samples. Both bone ash and media were counted with a gas-flow detector (Nuclear-Chicago) with appropriate calculations (physical decay, etc.) used.

RESULTS

The reduction of bone alkaline phosphate activity in animals given the scorbutogenic diet (table 1) was expected. A number of workers have noted that this change is associated with scurvy, as reviewed by Bourne (1). However, this as-

TABLE 1

Metabolic changes effected by vitamin C deficiency in the guinea pig [1]

Assessment	Control	Scorbutic
Skeletal alkaline [2] phosphatase activity	23.1 ± 0.25 [3]	8.7 ± 0.15 [4]
Bone citric acid concn, μg/100 mg	609 ± 31	605 ± 34
Food intake [5]	100	115
Body wt change, % [6]	+3	−6

[1] Each value represents a mean of 24 animals used in the first 2 experiments.
[2] Refers to the micromoles of *p*-nitrophenyl phosphate split/hour by extract from 100 mg of bone.
[3] Mean ± SE of mean.
[4] Difference between means of scorbutic and control animals is significant ($P < 0.01$).
[5] Intake/unit of body weight; scorbutic value relative to control.
[6] Change in body weight compared to weight when the experiment was started.

[4] Purina Rabbit Chow, Ralston Purina Company. This diet contained the following ingredients of unknown quantities: alfalfa meal, ground yellow corn, dehydrated alfalfa meal, wheat middlings, soybean oil meal, cane molasses, vitamin and mineral supplements; no ascorbic acid was added.

sessment was considered an important addition to this experiment because it provided supporting evidence of the scorbutic condition even though the deficient diet was fed only 15 days. Bone citric acid concentration did not appear to be influenced by the ascorbic acid deprivation which suggested that no influence on bone resorptive function had occurred.

It was evident that quantitative skeletal deposition of both isotopes was inhibited in the deficient animals (tables 2, 3, and 4) which substantiates the results of Friberg and Ringertz (7). Evidence has also been presented suggesting that the rate of skeletal calcium deposition is impeded by this deficiency (11). Skeletal deposition of the isotopes appeared to be inversely related to the length of time of the deficiency (table 4). A calculation of the correlation coefficient between time of deficiency and skeletal deposition (12) (table 4) showed a highly significant value ($r = -0.900$).

Lability, which was estimated by measuring the release of the isotope from the bone to the medium during incubation, was consistently and significantly greater for bone tissue from deficient animals (tables 2, 3 and 4). This was true for both isotopes (table 2), for isotopes deposited for some time or only recently (table 3), in both live and deactivated tissue (table 3), and was influenced by the amount of time the animals were fed the scorbutogenic diet (table 4). In the latter case (table 4)

TABLE 2

Influence of ascorbic acid deficiency on skeletal mineral deposition and release in the guinea pig (12 animals/group)

Assessment	Control	Scorbutic
Skeletal ^{45}Ca [1] activity [2]	21.7 ± 1.4 [3]	11.8 ± 0.9 [4]
Skeletal ^{45}Ca released [5]	0.25 ± 0.03	0.57 ± 0.10 [4]
Skeletal ^{32}P [6] activity	15.5 ± 0.7	9.4 ± 0.6 [4]
Skeletal ^{32}P released	0.62 ± 0.02	1.53 ± 0.16 [4]

[1] Administered intraperitoneally as $^{45}CaCl_2$ (10 μCi/100 g body wt).
[2] Counts/minute × 10^3 per mg of bone ash.
[3] Mean ± SE of mean.
[4] Differences between means of scorbutic and control animals is significant ($P < 0.01$).
[5] Percentage of total skeletal isotope content released during incubation.
[6] Administered intraperitoneally as $H_3{}^{32}PO_4$ (5 μCi/100 g body wt).

TABLE 3

Additional evidence concerning skeletal deposition and release of radioactive minerals (6 animals/group)

Assessment	Control	Scorbutic
Isotope administered 6 days before killing		
Skeletal ^{32}P concn [1]	59.0 ± 10.2 [2]	24.8 ± 2.7 [3]
Skeletal ^{32}P released [4]	0.17 ± 0.03	0.42 ± 0.06 [3]
Skeletal ^{32}P released [5]	0.05 ± 0.01	0.13 ± 0.04 [6]
Isotope administered 12 hours before killing		
Skeletal ^{32}P concn [1]	61.5 ± 10.8	18.1 ± 2.0 [3]
Skeletal ^{32}P released [4]	0.55 ± 0.04	1.85 ± 0.23 [3]
Skeletal ^{32}P released [5]	0.21 ± 0.04	0.54 ± 0.14 [6]

[1] Counts/minute × 10^3/mg of ash. Isotope administered intraperitoneally as $H_3{}^{32}PO_4$ (10 μCi/100 g body wt).
[2] Mean ± SE of mean.
[3] Difference between means of scorbutic and control animals is significant ($P < 0.01$).
[4] Percentage of isotope released from bone to medium by live bone tissue/hour.
[5] Percentage of isotope released from bone to medium by inactivated bone tissue/hour.
[6] $P < 0.05$.

TABLE 4

Development of skeletal deficiency symptoms in the guinea pig

Days of dietary treatment	Group	Food intake [1]	Wt gain (cumulative)	Skeletal ^{32}P [2]	^{32}P released [3]
			g		
5	Control	100.0	22 ± 3 [4]	86 ± 29	0.50 ± 0.08
	Scorbutogenic	123.4	25 ± 3	91 ± 23	0.50 ± 0.05
10	Control	100.0	43 ± 9	90 ± 8	0.62 ± 0.03
	Scorbutogenic	100.2	39 ± 12	61 ± 10 [5]	0.74 ± 0.10
15	Control	100.0	59 ± 5	86 ± 5	0.64 ± 0.03
	Scorbutogenic	98.4	48 ± 17	42 ± 7 [5]	1.01 ± 0.14 [5]

[1] Scorbutogenic values relative to control. Each group (5, 10, and 15 days) was composed of 5 animals for each dietary treatment.
[2] Bone ^{32}P concentration in counts/minute × 10^3/mg ash. Isotope was administered intraperitoneally as $H_3{}^{32}PO_4$ (15 μCi/100 g body wt).
[3] Percentage of the total skeletal activity released from the bone to the medium during a 1-hour incubation period.
[4] Mean ± SE of mean.
[5] Difference between means of scorbutic and control animals is significant ($P < 0.01$).

the degree of lability was increased as deposition decreased, which suggested that the 2 factors were inversely related. A calculation of the correlation coefficient (12) between these factors showed a significant association ($r = -0.785$).

Food intake (tables 1 and 4) was measured in case inanition was an influential factor. The results suggested that the deficient group consumed considerably more feed during the early stages of the deprivation period (table 4) and was comparable to that of controls during the latter phase of the experiment. Despite the greater intake of the scorbutogenic diet, the animals failed to gain as much weight as controls (tables 1 and 2); however, the weight gain differences were not statistically significant.

DISCUSSION

The expected bone lesions in scurvy are generally considered to be a consequence of an impaired function of the osteoblast. This implies that the calcification processes remain unchanged in this deficiency state; or, if changed, such alterations result from a malformed or insufficient amount of matrix.

It was clear that skeletal deposition of the injected isotopes was reduced in the ascorbic acid-deficient animals (tables 2, 3, and 4). It does not seem logical to attribute this result to an increased resorption rate because no change in bone citric acid occurred (table 1). Neuman et al. (13) demonstrated directly that parathyroid hormone enhanced the production of citric acid by bone; and Mecca et al. (14) found that the addition of this hormone extract to organ cultures stimulated the output of citrate by bone tissue. Further, the effect was evident and as extensive in animals injected 12 hours before killing as it was in those given the isotope 6 days before (table 3). Finally, these results agree with earlier observations (15) that the osteoclast population did not increase in scurvy although evidence of bone rarefaction was evident.

It appears most probable that the reduced isotope deposition (tables 2, 3, and 4) in the vitamin C-deficient animals was partially due to an impedance of osteoblastic activity. Direct results have been obtained showing that ascorbic acid is required for collagen formation (3) and many others have provided indirect evidence (1). Additionally, some of the observations and calculated relationships which were made in this work suggest other implications. The decreased stability of the bone salts formed in the deficient animals (tables 2, 3, and 4) appears to be of consequence. Since isotope deposition and lability were inversely related and time-dependent in development, it is possible that not only quantity but also matrix quality was influenced by the deficiency. Although no direct assessments were made regarding the nature of the bone salts deposited, certain inferences can be made based on the results of others. Harper and Posner (16) have shown quantitatively that bone tissue contains amorphous calcium phosphate as a second major mineral phase in addition to crystalline apatite. Termine and Posner (17) found that rickets was associated with a decrease in the crystalline apatite fraction and a subsequent increase in the amorphic phase. Since deficiencies of vitamin D, calcium, and phosphorus had a similar impact (17), it follows that the response was general.

Generally it is belived that amorphic solids are more reactive chemically than crystalline solids; thus, it appears that amorphism would be associated with increased lability. The in vitro results of this study were consistently suggestive of increased lability in vitamin C-deficient bone tissue (tables 2, 3, and 4). Perhaps this particular deficiency (ascorbic acid) is similar to vitamin D, calcium, or phosphate deprivation (17) in that the metastasis of bone salts from the amorphic to crystalline phases is impeded by its absence.

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Changes in Liver Xanthine Dehydrogenase and Uric Acid Excretion in Chicks during Adaptation to a High Protein Diet [1,2]

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ABSTRACT Studies were conducted to examine the relationship between changes in liver xanthine dehydrogenase with those in uric acid excretion during a 10-day adaptation period in chicks fed diets containing 25 or 75% isolated soybean protein. Food consumption, weight gain and liver nitrogen were also determined. Marked reductions in food consumption and weight gain were noted the first 24 hours in chicks fed the high protein diet as compared with chicks fed a control diet containing 25% isolated soybean protein. Consumption of the high protein diet and weight gain increased markedly after the initial 24 hours and remained at approximately 80% of the levels observed for control chicks throughout the remainder of the experiment. Liver weight, expressed on a body weight basis, was significantly increased in chicks fed the high protein diet for the first 2 days, after which time the differences between the 2 treatment groups largely disappeared. Elevated liver nitrogen levels were observed throughout the experiment for birds fed the high protein diet. Liver xanthine dehydrogenase activity increased twofold in chicks fed the high protein diet for 1 day and reached a maximum fourfold increase by day 4. A direct relationship between the elevated activity of hepatic xanthine dehydrogenase measured in vitro and the uric acid excretion was observed in these studies.

The results of a previous series of experiments conducted in this laboratory showed a marked elevation in liver xanthine dehydrogenase levels in 3-week-old chicks fed a diet containing 75% isolated soybean protein for 10 days as compared with control chicks that had been fed a diet containing 25% isolated soybean protein (1). In vitro measurements of specific enzyme activities have stimulated interest with respect to their physiological significance in vivo.

The present study investigated the changes in xanthine dehydrogenase activity with time when chicks were changed from a diet containing a normal level of protein to a diet containing a high level of protein. The relationship between liver xanthine dehydrogenase levels and uric acid formation and the influence of dietary protein level on weight gain, feed consumption, liver weight, and liver nitrogen during the 10-day adaptation period are also presented.

EXPERIMENTAL

Animals. One-day-old White Rock cockerels were fed a diet containing 25% isolated soybean protein (1) for a pre-experimental period of 10 days. At this time, 4 pens of 5 chicks per pen were changed to a diet containing 75% isolated soybean protein (1) and 4 pens of 5 chicks per pen were continued with the 25% isolated soybean protein diet (control diet). The average weights of the 2 groups of chicks were 139.5 and 140.1 g, respectively. The 25% diet contained 25% isolated soybean protein, 0.5 DL-methionine, 5% corn oil, 3% cellulose, vitamins, minerals and glucose monohydrate to make 100%. The 75% isolated soybean protein diet contained 3 times the levels of isolated soybean protein and DL-methionine used in the 25% diet added at the expense of glucose monohydrate. Feed and water were offered ad libitum. Feed consumption on a per pen basis and individual chick weights were recorded daily.

Additional groups of 6 chicks each that were fed the 2 diets described above were

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killed at 1, 2, 4, 7, and 10 days of the experiment for the liver xanthine dehydrogenase assays.

Uric acid collection and assay. Excreta were collected in stainless steel pans containing 0.5% lithium carbonate at 1, 2, 4, 7, and 10 days of the experimental period. Excreta collections were made for each of the 4 pens per treatment, so that each sample represented the total excreta from 5 birds for a 24-hour period. The excreta were quantitatively transferred to volumetric flasks, made to volume, and then homogenized in a large stainless steel blender. Aliquots of excreta taken immediately after homogenization were stored in polyethylene bottles at $-10°$ until uric acid analyses were conducted.

Duplicate aliquots of uniformly dispersed excreta were transferred to volumetric flasks, the pipettes washed several times, and the samples diluted to the appropriate volume with sodium hydroxide-boric acid buffer (pH 9.1). The flasks containing the diluted excreta were covered with watch glasses and placed in boiling water for 15 minutes (2). After cooling, an aliquot of the diluted excreta was removed for uric acid determination using the uricase method as described in a technical bulletin.[3]

Xanthine dehydrogenase assay. Xanthine dehydrogenase activity was measured spectrophotometrically at 340 mμ as the rate of formation of NADH. Assay procedures were identical to those reported previously (1).

Nitrogen was determined by the Kjeldahl method on duplicate samples of liver and excreta.

Data obtained in these studies were analyzed statistically by analysis of variance. Treatment means were tested for significant differences by the sequential method of Newman and Keuls (3).

RESULTS

Comparisons of the effects of the level of dietary protein on daily feed consumption, nitrogen consumption, and weight gain in chicks during the 10-day experimental period are presented in table 1. After one day, feed consumption was reduced to half that of control levels in birds fed the high protein diet. By the second day, consumption of the high protein diet was increased considerably and continued to increase throughout the remainder of the experiment. Although feed consumption was significantly lower ($P < 0.01$) at each day for birds fed the high protein diet compared with control chicks fed a diet containing 25% isolated soybean protein, nitrogen consumption was markedly higher for chicks fed the high protein diet. The

[3] Technical Bulletin no. 680, 1965, Sigma Chemical Company, St. Louis.

TABLE 1

Effect of dietary protein level on daily feed consumption, nitrogen consumption, and weight gain in chicks during adaptation to a high protein diet

Day of exp. [1]	Feed consumed/ chick/day		Nitrogen consumed/ chick/day		Wt gain/ chick/day	
	Isolated soybean protein, %		Isolated soybean protein, %		Isolated soybean protein, %	
	25	75	25	75	25	75
	g	g	g	g	g	g
1	25.6 **	13.2	0.86 *	1.33	16.7	9.7
2	28.4 **	19.0	0.95 **	1.91	17.5	14.4
3	29.6 **	22.6	0.99 **	2.28	17.4	16.0
4	31.7 **	24.4	1.06 **	2.47	19.2	14.8
5	33.0 **	25.9	1.10 **	2.61	20.3	15.2
6	35.9 **	26.2	1.19 **	2.65	21.6	14.7
7	36.8 **	30.4	1.22 **	3.08	21.1	15.8
8	38.9 **	32.1	1.30 **	3.24	23.5	17.5
9	42.2 **	31.7	1.41 **	3.20	24.8	16.0
10	42.8 **	33.5	1.43 **	3.38	22.4	20.6

[1] All chicks were fed the 25% isolated soybean protein diet for a pre-experimental period of 10 days at which time one-half of the chicks were transferred to a diet containing 75% isolated soybean protein and one-half were continued with the 25% diet.

*, ** Significantly different from birds fed the 75% isolated soybean protein diet ($P < 0.05$ and $P < 0.01$), respectively.

total protein content (N × 6.25) of the 25 and 75% isolated soybean protein diets was 21.0 and 63.9%, respectively.

The ability of the chick to rapidly adapt to a diet containing high levels of protein is also indicated by the daily weight gain data. Weight gain was markedly reduced in chicks fed the high protein diet for 24 hours as compared with gains of control chicks, 9.7 vs. 16.7 g, respectively. The weight gain of chicks fed the high protein diet returned toward normal during the second 24 hours, the average gain being only slightly lower than that of the control chicks, 14.4 vs. 17.5, respectively. Although weight gain of the control chicks was higher each day, the differences between the 2 treatments were not statistically significant ($P > 0.05$). The average weights of the 20-day-old chicks at the end of the experimental period were 344.3 and 294.4 g for chicks fed the 25 and 75% isolated soybean protein diets, respectively. These values indicate the accumulative changes in weight over the 10-day experimental period and when they were analyzed separately the differences are statistically significant ($P < 0.01$).

Significantly higher ($P < 0.01$) amounts of total nitrogen and uric acid were excreted by birds fed the high protein diet as compared with control chicks at each day excreta were collected (table 2). Both total nitrogen and uric acid excretion by chicks within each treatment increased as the experiment progressed. Expressed as a percentage of the nitrogen intake, nitrogen excretion averaged 42.4 and 78.2% for the experimental period for the chicks fed the control and high protein diets, respectively. Expressed as a percentage of nitrogen excretion, uric acid nitrogen averaged 55.4 and 63.1% of the nitrogen in the excreta throughout the experimental period for chicks fed the control and high protein diets, respectively. The value of 55.4% of the excreta nitrogen as uric acid for the control group is in close agreement with the observations of Creek and Vasaitis (4) in studies with chicks fed a similar level of protein. Uric acid nitrogen excretion, expressed as a percentage of the nitrogen consumed, was approximately 2 times greater for birds fed the high protein diet. Although somewhat variable, the percentage of ingested nitrogen excreted as uric acid increased with age in chicks fed either level of protein. Data from tables 1 and 2 show that chicks fed the high protein diet retained less nitrogen than the control chicks but that weight gain per unit of nitrogen retained was comparable between the 2 groups.

Additional groups of chicks fed either the 25 or 75% isolated soybean protein diets were killed on days 1, 2, 4, 7 and 10 of the experiment for liver weight, liver nitrogen, and liver xanthine dehydrogenase determinations (tables 3 and 4). Compared with control values, fresh liver weights were larger at days 1 and 2 for birds fed the high protein diet but the differences were not statistically significant ($P > 0.05$). When expressed as liver weight per 100 g body weight, however, the initial liver hypertrophy was shown to

TABLE 2

Effect of dietary protein level on nitrogen and uric acid excretion in chicks during adaptation to a high protein diet

Day of exp. [1]	Total N excreted/chick/day		Uric acid excreted/chick/day		N excreted as uric acid	
	Isolated soybean protein, %		Isolated soybean protein, %		Isolated soybean protein, %	
	25	75	25	75	25	75
	g	*g*	*g*	*g*	*% N consumed*	
1	0.37 **	1.01	0.54 **	1.76	20.5 **	44.2
2	0.41 **	1.40	0.67 **	2.67	24.3 **	46.8
4	0.44 **	1.98	0.73 **	4.13	22.8 **	55.8
7	0.54 **	2.52	0.92 **	4.60	24.9 **	50.0
10	0.57 **	2.70	1.06 **	5.12	24.8 **	50.5

** Significantly different from birds fed the 75% isolated soybean protein diet ($P < 0.01$).

[1] All chicks were fed the 25% isolated soybean protein diet for a pre-experimental period of 10 days at which time one-half of the chicks were transferred to a diet containing 75% isolated soybean protein and one-half were continued with the 25% diet.

TABLE 3

Effect of dietary protein level on liver weight and liver nitrogen in chicks during adaptation to a high protein diet [1]

Day of exp. [2]	Liver wt		Relative liver wt		Nitrogen/liver	
	Isolated soybean protein, %		Isolated soybean protein, %		Isolated soybean protein, %	
	25	75	25	75	25	75
	g	*g*	*g/100 g body wt*		*mg*	*mg*
1	7.0 [a]	8.0 [a]	4.4 [a]	5.3 [b]	147.5 [a]	196.7 [d]
2	7.0 [a]	8.5 [a]	4.3 [a]	5.2 [b]	146.6 [a]	227.0 [e]
4	8.7 [ab]	8.7 [a]	4.1 [a]	4.4 [a]	207.3 [b]	259.5 [f]
7	10.5 [b]	11.5 [bc]	3.9 [a]	4.2 [a]	214.1 [b]	306.2 [g]
10	12.9 [c]	12.3 [bc]	3.8 [a]	4.1 [a]	298.7 [c]	330.8 [h]

[1] Values represent means of 6 observations each. Means pertaining to any one variable which have the same lettered superscript in a vertical column or in a horizontal row are not statistically different ($P > 0.05$).
[2] All chicks were fed the 25% isolated soybean protein diet for a pre-experimental period of 10 days at which time one-half of the chicks were transferred to a diet containing 75% isolated soybean protein and one-half were continued with the 25% diet.

TABLE 4

Effect of dietary protein level on liver xanthine dehydrogenase activity in chicks during adaptation to a high protein diet [1]

Day of exp.[3]	Xanthine dehydrogenase activity [2]							
	Isolated soybean protein, %		Isolated soybean protein, %		Isolated soybean protein, %		Isolated soybean protein, %	
	25	75	25	75	25	75	25	75
	units/g liver		*units/liver*		*units/100 g body wt*		*units/100 mg liver N*	
1	0.52 [a]	1.16 [b]	3.69 [a]	9.36 [c]	2.31 [a]	6.20 [b]	2.47 [a]	4.75 [b]
2	0.52 [a]	1.54 [c]	3.62 [a]	13.33 [d]	2.23 [a]	8.24 [b]	2.47 [a]	5.83 [bc]
4	0.49 [a]	1.87 [c]	4.32 [a]	16.13 [d]	2.00 [a]	8.10 [b]	2.04 [a]	6.21 [bc]
7	0.44 [a]	1.86 [c]	4.57 [a]	21.41 [e]	1.71 [a]	7.98 [b]	2.15 [a]	7.02 [c]
10	0.47 [a]	1.77 [c]	6.04 [b]	21.72 [e]	1.77 [a]	7.27 [b]	2.05 [a]	6.62 [bc]

[1] Values represent means of 6 observations each. Means pertaining to any one variable which have the same lettered superscript in a vertical column or in a horizontal row are not statistically different ($P > 0.05$).
[2] Units represent enzyme activity catalyzing the reduction of 1 μmole NAD/min at 25°.
[3] All chicks were fed the 25% isolated soybean protein diet for a pre-experimental period of 10 days at which time one-half of the chicks were transferred to a diet containing 75% isolated soybean protein and one-half were continued with the 25% diet.

be significant ($P < 0.05$). Liver weights, expressed on a body weight basis, for birds fed the high protein diet were also significantly larger ($P < 0.05$) at days 1 and 2 than observed in birds on the same treatment on subsequent days of the experiment. The small differences in relative liver weight between the 2 treatments on days 4, 7, and 10 were not statistically significant ($P > 0.05$). Total liver nitrogen levels were significantly higher ($P < 0.05$) for birds fed the high protein diet throughout the experiment, although fresh liver weights were similar to those of control chicks on days 4, 7, and 10.

Since liver size, liver nitrogen, body weight, and liver xanthine dehydrogenase levels were changing during the experiment, a number of methods were used for expressing enzyme activity (table 4). Regardless of the basis chosen for expressing enzyme activity, birds fed the high protein diet exhibited significantly higher ($P < 0.05$) xanthine dehydrogenase levels than control chicks at each day reported. Compared with control levels, xanthine dehydrogenase activity per gram of liver was approximately 2 times greater in chicks fed the high protein diet for one day. Xanthine dehydrogenase activity per gram of liver did not change significantly with age for control chicks, which is in contrast with that observed for chicks fed the high protein diet in which maximal activity was not reached until the fourth day. The approximate fourfold elevation in xanthine dehydrogenase levels per gram of liver, observed at day 4 in birds fed the high protein diet, persisted throughout the remainder of the experiment.

Expressing enzyme activity as units per liver shows that xanthine dehydrogenase activity increased in both groups of chicks as the experiment progressed. The increases are essentially reflections of the increased liver size in older birds. When expressed as units per 100 g body weight, enzyme activity remained relatively unchanged within protein levels. Xanthine dehydrogenase activity per 100 mg liver nitrogen remained unchanged with age for control chicks but was shown to increase gradually with age for the first 7 days birds were fed the high protein diet. These data suggest that there was an initial synthesis of xanthine dehydrogenase at rates greater than the rate which could be accounted for by the general increase in liver protein alone in birds fed the high protein diet.

The relationship between total liver xanthine dehydrogenase as measured in vitro and uric acid production in vivo is illustrated in figure 1. A point at either protein level corresponds to enzyme activity and uric acid excretion at a given day of the experiment. These data clearly indicate a physiological role for the elevated levels of liver xanthine dehydrogenase observed during these studies with chicks subjected to wide differences in protein intake.

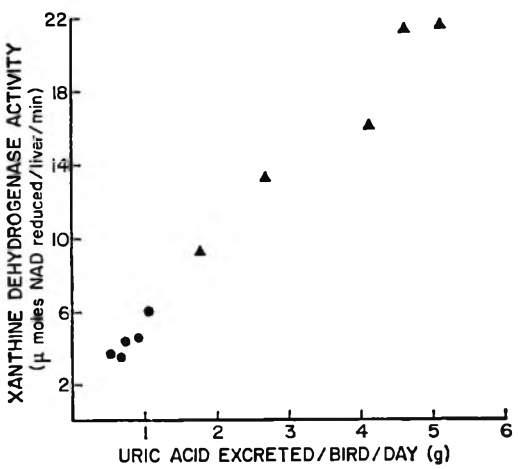

Fig. 1 Total liver xanthine dehydrogenase activity and total uric acid excretion of chicks fed diets containing 25 (●) or 75% (▲) isolated soybean protein. A point at either protein level corresponds to enzyme activity and uric acid excretion at a given day of the experiment.

DISCUSSION

If the homeostatic integrity of the animal organism is to be maintained in cases in which wide variations of a major dietary constituent occur, certain regulatory mechanisms must be present. The research presented in this report was designed to elucidate a physiologic basis for the elevated levels of liver xanthine dehydrogenase reported earlier when chicks were fed a diet containing 75% isolated soybean protein (1). Since uric acid is the primary nitrogenous excretory product formed in the avian species, the biosynthetic pathway for its formation may be considered analogous physiologically to the formation of urea in ureotelic species. In this context, xanthine dehydrogenase would assume a similar functional role in birds as arginase does in mammals.

In the present study, chicks fed the high protein diet for one day exhibited a sharp reduction in feed consumption and weight gain compared with control chicks fed a diet containing 25% isolated soybean protein. Subsequently, consumption of the high protein diet increased and rate of gain was not greatly different from that observed in control chicks. In reviewing similar studies with rats, Harper (5) noted that the return of adequate feed consumption and weight gain patterns could be associated with elevated levels of enzymes required for the utilization of large amounts of dietary amino acids.

The liver hypertrophy and elevated levels of liver nitrogen observed when chicks were fed a high protein diet have also been reported for the rat under similar dietary conditions (6, 7). In the present study, the hypertrophic liver condition observed during the first 2 days chicks were fed the high protein diet was not observed on subsequent days of the experiment. From the results of the xanthine dehydrogenase assays, it is suggested that the initial liver hypertrophy and elevated levels of liver nitrogen represents, in part, a rapid synthesis of many enzymes or enzyme systems involved in the catabolism and utilization of the high levels of dietary protein for energy purposes as well as protein synthesis. Since xanthine dehydrogenase is associated with the formation of the major excretory product of amino acid catabolism

in birds, it is likely that elevations of enzymes involved more directly in the catabolism of amino acids per se had occurred at times earlier than those observed for xanthine dehydrogenase. Preliminary investigations conducted in this laboratory concerned with the in vitro oxidation of several isotopically labeled amino acids have demonstrated a marked elevation of amino acid catabolism in liver homogenates from birds fed the high protein diet.[4] More detailed reviews of the changes in specific enzyme levels in animals as influenced by alterations in protein intake have been presented by Harper (5) and Knox et al. (8).

The increased activity of xanthine dehydrogenase observed in chicks fed excessive levels of dietary protein was reflected by a concomitant elevation of uric acid excretion. Maximal enzyme activity per gram of liver or per 100 g body weight was observed 4 days after initiation of the high protein diet and xanthine dehydrogenase levels remained at these levels throughout the remainder of the experiment. This effect appears to be analogous to the elevated activities of the urea cycle enzymes and increased urea formation reported in rats under similar conditions of high protein intake (9–11).

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Effect of Diethylstilbestrol on the Blood Plasma Amino Acid Patterns of Beef Steers Fed Finishing Diets

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ABSTRACT Three trials were conducted to determine the effect of diethylstilbestrol (DES) on the blood plasma free amino acid patterns of steers fed finishing diets ad libitum. Results from the trials indicate that when steers were fed diets based upon corn, DES treatment caused a reduction in the concentration of the essential amino acids, except for methionine and histidine which increased. Lysine concentration was especially decreased. Blood plasma concentrations of urea and serine were also depressed due to DES, whereas the concentration of hydroxyproline was markedly increased. When steers were fed a diet based upon wheat in one trial the effect of DES was somewhat different, suggesting that the composition of the diet may have influenced the response.

The majority of beef cattle being finished for slaughter in the United States receive diethylstilbestrol (DES) either as a diet ingredient or as an ear implant (1). This synthetically produced estrogenic compound is very effective in stimulating gains and improving the feed efficiency of growing and finishing ruminants. Research results (1–3) indicate that weight gain and feed efficiency are improved approximately 15 and 12%, respectively, through the proper use of DES. Heavier carcasses containing a higher percentage of protein and moisture and a lower percentage of fat are obtained (4–6) from the use of this compound. Increased retention of nitrogen (7–9) calcium and phosphorus (8, 9) has been observed in metabolism trials.

Although a great deal of research has been conducted with DES in respect to its effect on weight gain, feed efficiency and carcass characteristics, little is known of its mode of action in the ruminant body. In 1958, Riggs (2) wrote concerning its use, ". . . no compound has ever had as much an impact on the beef cattle industry with as little information concerning its mode of action and effects in the animal body." This is still largely true today.

Since DES significantly increases the weight gain and nitrogen retention of ruminants, it is possible that the blood plasma amino acid patterns may be altered by its use. The present experiment was conducted to study the influence of DES on blood plasma amino acid patterns of steers fed finishing diets ad libitum.

EXPERIMENTAL PROCEDURE

Trial 1. Twenty yearling steers were removed from summer pasture and placed in a drylot. Adjustment of the steers from grazing to the finishing diets was accomplished in 21 days by the gradual reduction of roughage in a standardization diet. At the end of this period the steers averaged 355 kg, were assigned at random to 2 treatments and were group-fed ad libitum the 2 all-concentrate diets shown in table 1. Five steers in each group were selected at random and implanted subcutaneously at the base of the ear, with 24 mg of DES [2] at the start of the trial. The DES was administered in pellet form (two 12-mg pellets) which allows for the slow absorption over a period of time.

Jugular blood samples were taken from each steer at 8 AM after the trial had been in progress for 6 weeks. The blood samples were prepared for amino acid analysis (10) and the amounts of the individual amino acids and other ninhydrin-positive compounds were determined by ion exchange chromatography using an automatic amino acid analyzer (11).

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TABLE 1

Composition and crude protein analysis of the experimental diets

	Finishing diets		
	All-concentrate		Conventional
	Trials 1 and 3	Trial 1	Trials 2 and 3
	%	%	%
Cracked corn	90.6	—	63.0
Cracked wheat [1]	—	90.9	—
Chopped timothy hay [2]	—	—	12.5
Chopped alfalfa hay [2]	—	—	12.5
Linseed meal	—	—	5.0
Cottonseed meal	—	—	5.0
Molasses	6.0	6.0	—
Urea	1.4	1.1	—
Trace mineral salt [3]	1.0	1.0	1.0
Dicalcium phosphate	0.5	0.5	1.0
Calcium carbonate	0.5	0.5	—
Vitamins A and D [4]	—	—	—
Analysis, % as fed:			
Crude protein	11.9	12.9	13.0

[1] Soft red winter wheat.
[2] Ground through 38-mm screen.
[3] Trace mineral analysis: (in percent) Mn, 0.200; Fe, 0.160; Cu, 0.033; S, 0.011; Co, 0.010; Zn, 0.010; I, 0.007.
[4] Trials 1 and 3, 8 g; trial 1, 11 g; contained 20,000 USP units of vitamin A palmitate and 2,500 USP units of vitamin D-activated plant sterol/g.

Trial 2. Four yearling steers were removed from summer pasture, placed in a drylot and adjusted to a conventional finishing diet (table 1) according to the procedure described for trial 1. The steers were fed ad libitum. At the end of 4 and 6 weeks jugular blood samples were taken from each steer at 8 AM. Each steer was implanted with 36 mg of DES at the end of 6 weeks. The higher level was used in an attempt to obtain more definite results than with the 24-mg level. The steers averaged 360 kg at the time of implantation. Additional blood samples were taken again at 2 weeks and 4 weeks after implantation for analysis. The samples collected from each steer were analyzed individually. In this trial each steer served as its own control, and by bleeding at 2-week intervals it was possible to remove the time effect in the statistical analysis. The preparation and analyses of the blood samples were the same as described in trial 1.

Trial 3. Ten steers averaging 290 kg were allotted at random to 2 groups of 5 steers each. One group was fed the all-concentrate diet and the other the conventional finishing diet (table 1) on an ad libitum basis. Experimental design and procedure for taking blood samples were as described for trial 2. Twenty-four milligrams of DES were implanted in the ear of each steer because of the lower weight of the steers compared with those in trial 2. In this trial norleucine was used as an internal standard to determine the amino acid losses during sample preparation, and the amino acid determinations were adjusted accordingly. The data obtained from the 3 trials were statistically analyzed using the least squares analysis.

RESULTS

The influence of DES on the average daily gains of the steers in the 3 trials is shown in table 2. The growth response from DES was marked in all comparisons except in trial 2 in which a smaller response was noted.

Trial 1. The effect of DES on the blood plasma amino acid patterns is shown in table 3. Since it has been suggested that 9 amino acids are essential for the ruminant per se (12, 13) these particular amino acids were grouped together in the table for comparative purposes. The amino acids observed in greater concentrations when the steers were fed the corn diet as compared with the wheat diet were threonine ($P < 0.05$), leucine ($P < 0.05$) as-

partic acid ($P < 0.01$), serine ($P < 0.01$) and tyrosine ($P < 0.05$). Lysine ($P < 0.05$) and urea ($P < 0.01$) were detected in lesser concentrations. Interactions between type of grain and DES were found for threonine ($P < 0.05$), lysine ($P < 0.05$), tryptophan ($P < 0.05$), serine ($P < 0.01$), glycine ($P < 0.05$) and citrulline ($P < 0.05$).

TABLE 2

Effect of diethylstilbestrol (DES) on average daily gain [1] of steers fed the experimental diets

Diet and trial	Avg daily gain	
	Control	DES
	kg	*kg*
90% wheat, trial 1	1.7	1.9
90% corn, trial 1	1.5	1.9
63% corn, trial 2	0.8	0.9
90% corn, trial 3	1.1	1.4
63% corn, trial 3	1.1	1.9
Avg	1.2	1.6

[1] Average daily gains of steers for the first 42 days of trial 1 and average daily gains for a 28-day period before implantation (control) and a 28-day period after implantation (DES) for trials 2 and 3.

Trial 2. The results are shown in table 4. Glutamic acid was found in greater concentration ($P < 0.05$) after the implantation of DES. The reverse trend was true for threonine ($P < 0.05$), valine ($P < 0.01$), lysine ($P < 0.01$), histidine ($P < 0.05$), tryptophan ($P < 0.01$), urea ($P < 0.01$), and arginine ($P < 0.01$).

Trial 3. The results are also shown in table 4. Methionine ($P < 0.01$), tryptophan ($P < 0.01$), hydroxyproline ($P < 0.05$), asparagine plus glutamine ($P < 0.01$), citrulline ($P < 0.05$) and ammonia ($P < 0.01$) concentrations all increased after DES implantation. Urea ($P < 0.01$), serine ($P < 0.05$), and glycine ($P < 0.01$) concentrations decreased after implantation. An interaction between diet and DES was found for glycine ($P < 0.05$). Aspartic acid ($P <$

TABLE 3

Influence of diethylstilbestrol (DES) on the blood plasma amino acids of steers fed the all-concentrate diets (trial 1)

	90% corn diet		90% wheat diet		SE [1]
	Control	DES	Control	DES	
	μmoles/100 ml blood plasma				
Essential amino acids					
Threonine	6.8 [2]	5.2	4.5	5.2	0.5
Valine + cystine [3]	12.2	11.2	13.0	15.3	1.7
Methionine	2.9	2.5	2.1	2.3	0.3
Isoleucine	8.9	6.8	7.8	8.8	1.2
Leucine	17.6	13.3	10.1	9.6	2.0
Phenylalanine	5.5	4.4	4.3	4.0	0.5
Lysine	10.0	8.6	10.0	13.6	1.0
Histidine	6.8	7.7	5.2	7.0	0.9
Tryptophan	2.3	1.6	1.4	2.5	0.3
Total	73.0	61.3	58.4	68.3	7.0
Other ninhydrin-positive compounds					
Taurine	3.3	3.9	2.6	3.2	0.4
Urea	318.2	310.5	478.0	467.2	40.1
Hydroxyproline	1.6	2.0	1.4	1.3	0.3
Aspartic acid	1.4	1.3	0.7	0.8	0.2
Serine	12.3	8.8	6.2	9.4	0.7
Asparagine + glutamine	23.6	22.4	22.5	23.5	2.0
Proline	9.4	7.0	5.8	6.7	1.0
Glutamic acid	10.3	9.3	7.7	9.4	1.0
Citrulline	7.1	5.9	5.0	7.0	0.7
Glycine	25.5	25.1	20.5	29.0	1.5
Alanine	20.5	20.7	18.0	18.7	1.5
Tyrosine	5.5	4.7	3.3	4.2	0.6
Ornithine	5.2	6.0	5.4	5.6	1.4
Ammonia	17.3	16.0	15.8	14.3	1.2
Arginine	9.2	7.8	8.5	9.4	0.7

[1] Standard error.
[2] Each value is the mean of 5 steers.
[3] These acids eluted together on chromatogram.

TABLE 4

Influence of diethylstilbestrol (DES) on the blood plasma amino acids of steers fed all-concentrate and conventional finishing diets (trials 2 and 3)

	Blood plasma amino acids							
	63% corn diet					90% corn diet		
	Trial 2			Trial 3				
	Control	DES	SE [1]	Control	DES	Control	DES	SE [1]
	μmoles/100 ml blood plasma							
Essential amino acids								
Threonine	6.2 [2]	5.3	0.4	9.0 [3]	8.8	6.2 [3]	6.8	0.4
Valine	20.4	15.1	0.6	27.0	26.4	19.4	18.4	1.0
Methionine	2.1	2.4	0.1	2.8	3.2	2.3	3.1	0.2
Isoleucine	8.4	7.3	0.5	13.6	13.1	8.1	7.9	0.5
Leucine	13.8	12.6	0.7	18.7	19.4	16.8	14.8	0.9
Phenylalanine	4.1	4.4	0.2	7.1	6.8	5.3	5.0	0.2
Lysine	9.4	5.8	0.4	14.6	13.4	7.7	7.5	0.6
Histidine	5.6	5.0	0.3	6.3	6.9	5.8	6.4	0.3
Tryptophan	1.1	0.7	0.1	2.1	2.7	1.9	2.3	0.2
Total	71.1	58.6	2.2	101.2	100.7	73.5	72.2	3.2
Other ninhydrin-positive compounds								
Taurine	2.6	2.6	0.2	3.1	3.7	3.9	3.9	0.3
Urea	380.6	232.6	45.4	347.7	276.5	335.6	331.3	20.8
Hydroxyproline	1.6	2.9	0.3	4.3	5.1	4.0	5.1	0.4
Aspartic acid	0.4	0.5	0.1	0.6	0.6	0.8	0.8	0.1
Serine	7.3	6.6	0.6	8.0	7.8	7.9	6.7	0.3
Asparagine + glutamine	21.8	21.8	1.5	27.3	31.5	25.3	31.1	1.1
Proline	6.8	6.6	0.4	9.8	9.8	8.3	8.4	0.5
Glutamic acid	5.8	8.5	0.8	5.7	6.1	8.6	9.2	0.3
Citrulline	6.9	7.0	0.7	6.1	6.7	5.6	6.4	0.3
Glycine	20.0	22.3	1.8	25.4	23.3	34.8	25.8	1.2
Alanine	19.6	18.7	0.8	25.4	23.7	19.6	18.9	1.0
Cystine	1.8	1.6	0.3	1.8	1.9	1.6	1.8	0.1
Tyrosine	4.2	4.7	0.3	6.5	7.0	5.1	5.8	0.3
Ornithine	7.1	5.8	0.7	7.7	7.3	6.3	6.3	0.4
Ammonia	18.4	18.4	1.0	22.4	24.0	23.4	25.4	0.6
Arginine	7.5	5.9	0.3	10.4	9.9	7.5	8.1	0.4

[1] Standard error.
[2] Each value is the mean of 4 steers.
[3] Each value is the mean of 5 steers.

0.01), glutamic acid ($P < 0.01$), and glycine ($P < 0.01$) were observed in greatest concentrations in steers fed the 90% corn diet. Threonine ($P < 0.01$), valine ($P < 0.01$), isoleucine ($P < 0.01$), leucine, ($P < 0.05$), phenylalanine ($P < 0.01$), lysine ($P < 0.01$), alanine ($P < 0.05$), tyrosine ($P < 0.01$), and arginine ($P < 0.05$) were present in greater quantities in the blood plasma of the steers fed the 63% corn diet than in steers fed the 90% corn diet.

Table 5 summarizes the results obtained when steers were fed the 2 corn-based diets in the 3 trials. The results from the steers fed the wheat-based all-concentrate diet were not included in the table because of the several significant interactions between type of grain and DES response. There were several significant differences in amino acid concentrations between the 63% and 90% corn diets but DES influenced both similarly. Data in the table indicate that DES implantation resulted in a depression of all of the essential amino acids except for methionine and histidine which increased. Lysine was especially depressed. Urea and serine concentrations were depressed, whereas the plasma concentration of hydroxyproline was markedly increased.

DISCUSSION

The mode of action of DES in the ruminant is not well-understood. However, a stimulation of growth hormone secretion, a stimulated adrenal androgen secretion or an increased production of thyroid hormone have been suggested to act singly

TABLE 5

Summary of the influence of diethylstilbestrol (DES) on blood plasma amino acids when data from all steers fed the corn based diets are averaged

	Blood plasma amino acids		
	Control	DES	DES/control
	μmoles/100 ml blood plasma		*%*
Essential amino acids			
Threonine	7.1	6.5	92
Valine	19.8	17.8	90
Methionine	2.5	2.8	112
Isoleucine	9.8	8.8	90
Leucine	16.7	15.0	90
Phenylalanine	5.5	5.2	95
Lysine	10.4	8.8	85
Histidine	6.1	6.5	107
Tryptophan	1.9	1.8	95
Total	79.8	73.2	92
Other ninhydrin-positive compounds			
Taurine	3.2	3.5	109
Urea	345.5	287.7	83
Hydroxyproline	2.9	3.8	131
Aspartic acid	0.8	0.8	100
Serine	8.9	7.5	84
Asparagine + glutamine	24.5	26.7	109
Proline	8.6	8.0	93
Glutamic acid	7.6	8.3	109
Citrulline	6.4	6.5	102
Glycine	26.4	24.1	91
Alanine	21.3	20.5	96
Cystine	1.7	1.8	106
Tyrosine	5.3	5.6	106
Ornithine	6.6	6.4	97
Ammonia	20.4	21.0	103
Arginine	8.7	7.9	91

or in combination to elicit the response. The probable role of the anterior pituitary gland is indicated in all these proposals (6).

Diethylstilbestrol does not appreciably improve the digestibility of the diet components but an improvement occurs in the efficiency of the utilization of absorbed nitrogen, but not energy after absorption (6, 14, 15) and, therefore, it appears that DES stimulates directly or indirectly the utilization of energy for protein synthesis rather than fat synthesis (6).

The results in table 2 indicate a definite response to DES as shown by the greater growth rates of the steers following implantation. This response was similar for all groups except in trial 2 in which a smaller response was noted. It was not apparent why this occurred, especially since 36 mg of DES was used. In all trials, and especially trials 1 and 3, the response to DES was clearly noted within 2 weeks after implantation. In general, ruminant response to DES has been noted to be most pronounced shortly after administration (9, 16, 17). Pellets containing 12 to 15 mg of DES are absorbed at the rate of about 2 mg/week for 3 to 4 weeks and more slowly as the surface area decreases (16). In a characteristic trial (17) the response was evident within 2 to 3 weeks after implantation and a 40% improvement was noted after the steers were on test 42 days. In typical finishing studies with cattle being fed ad libitum, weight gain is usually the greatest shortly after the trial begins and the combination of these 2 factors undoubtedly contributed to the high average daily gains and to the DES response being greater than normal (1–3) in the present trials.

The response of DES on the free amino acid patterns of the steers fed wheat was different from that of steers fed corn, which suggested that diet composition was

important in the response. Slyter et al.[3] studied the rumen microbial population of the steers used in the trial and reported an interaction ($P < 0.01$) between type of grain and DES on total bacterial counts. The counts were highest in steers fed corn plus DES and lowest in steers fed wheat plus DES. Ciliated protozoa were found in steers fed wheat but not in steers fed corn. Although it has been reported that the amino acid composition of 22 individual strains of rumen bacteria were essentially similar (18), an enzymatic digest of several of the individual strains indicated that the proportion of essential amino acids being released as free amino acids varied between 2.5 and 52.6% (19). The distribution of the essential amino acids within this fraction varied markedly also. Thus, in the present study, the substitution of corn for wheat resulted in a somewhat different microbial population which in turn was differently influenced by DES. The response was probably further complicated by the quality and quantity of microbial population made available to the host animal and which in turn was ultimately reflected in a differing amino acid pattern in the blood of the steers fed wheat.

Although the amino acid results obtained in the 3 trials in which steers were fed the 2 corn-based diets are not in complete agreement, it appears that the data show a definite trend (table 5). When the steers were fed the corn-based diets, DES implantation resulted in a depression in the concentration of 7 of the essential amino acids in the blood plasma. Lysine appeared to be the amino acid most affected in this respect followed by the branched chain amino acids. Plasma concentrations of methionine and histidine, however, increased due to DES treatment.

Ogilvie et al.[4] reported that feeding 30 mg DES daily to steers greatly improved daily gains, but analysis of blood samples taken from the steers before and after a 20-hour fast at zero, 30 and 60 days indicated that DES did not significantly alter the individual concentrations of the amino acids or the nonprotein-nitrogen level in the blood plasma. A lowering (nonsignificant) of the amino nitrogen in the blood plasma of sheep fed DES or diallylhexestrol was found with sheep (20).

It has been reported that a general lowering of the total free amino acid content in the blood plasma along with lowered urea levels is associated with rapid-growing calves (21) and the hypothesis was put forth that these calves have greater ability to draw nitrogenous substances from the plasma for building body tissues.

Preston et al.[5] reported that implanting steers with 36 mg DES lowered blood urea levels and stimulated the average daily gains of steers. The DES effect on both criteria was the greatest 28 days after implantation. Both ammonia and urea in the blood of sheep (22) were significantly reduced due to DES and it was suggested that DES acts through a direct action on the tissues to promote better utilization of NPN. In the present studies, urea concentration was depressed due to DES but ammonia was not influenced.

The elevation of hydroxyproline and the depression of serine seems noteworthy in the present studies. It has been shown that collagen in connective tissue contains a relatively high precentage of hydroxyproline (6–7%) and that DES treatment increases the connective tissue in the skeletal muscle of lambs in a direct proportion to the amount of DES used (23). Furthermore, the rates of turnover of collagen are much greater in young animals than in adults (24). In the present study, the elevated plasma levels of hydroxyproline in the DES-treated steers may be related to increased synthesis and metabolism of collagen. In a previous study (10) in which urea was compared with isolated soy protein in isonitrogenous purified diets, and fed to steers, the blood plasma levels of serine and glycine were detected in significantly greater quantities when the steers were fed the urea diet. Nitrogen retention was significantly greater when steers were fed the isolated soy diet and

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although not significant ($P < 0.05$), blood plasma urea was 20% less when steers were fed the isolated soy diet. Elevated plasma concentrations of glycine (25, 26)[6] and serine (27) have been associated with reduced nitrogen retention in other purified diet studies with ruminants. It is possible that DES not only lowers the plasma concentration of urea but also serine and glycine and this may contribute toward improved nitrogen retention. Relatively high levels of serine and glycine may be indicative of amino acid imbalance and lowered nitrogen utilization.

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Effect of Hypophysectomy on the Metabolism of Essential Fatty Acids in Rat Testes and Liver [1]

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ABSTRACT The effect of hypophysectomy on lipid classes and interconversion of linoleic acid in livers and testes of essential fatty acid (EFA)-deficient rats is described. Male rats of the Sprague Dawley strain made EFA-deficient by feeding them a fat-free diet from weaning to 26 weeks of age were used. Hypophysectomy caused a marked elevation of the percentage of cholesteryl esters, glyceryl ether diesters and triglycerides at the expense mainly of phosphatidylcholine and phosphatidylethanolamine in the testes. There was a small effect of hypophysectomy on the composition of the liver lipids with only a slight increase in the percentage of polar lipids and corresponding slight decrease in the percentage of the neutral lipid fraction 3 weeks after the operation. The characteristic pattern of fatty acid interconversions, on feeding linoleate, occurred in both livers and testes of hypophysectomized (hypox) animals. However, the conversion of linoleic acid to higher polyunsaturated fatty acids was not as efficient in the testes of hypox as normal animals. Despite the marked change in fatty acid composition produced from feeding linoleate there appeared to be little effect on the pattern of changes in lipid class composition caused by hypophysectomy. The results suggest that the hypophyseal hormones exercise control over the transformation of fatty acids among the lipid classes.

Among the effects of a deficiency of essential fatty acids (EFA) in the rat is atrophy of the testes with impairment of spermatogenesis (1–3). Similar effects occur as a result of hypophysectomy (4). Ahluwalia et al. (5) suggested that degeneration of the gonads observed in the EFA-deficient rabbit may be due to primary impairment of anterior hypophyseal function. Spermatogenesis is also impaired by a vitamin A deficiency (6) which also produces quantitative changes in the lipid classes of rat testes (7). These changes may be reversed in part by injection of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), suggesting hormonal control of the metabolism of the testicular lipids. Gambal and Ackerman (8) observed an increase in percentage of lipids, particularly of the nonphospholipid fraction, as well as changes in the relative amounts of the individual phospholipids in the testicular lipids of the hypophysectomized rat. A recent study in this laboratory (9) showed that changes in the composition of the testicular lipids of rats as a result of hypophysectomy were due mainly to an increase in the percentage of cholesteryl esters and glyceryl ether diesters at the expense of phospholipids. Changes were also observed in fatty acid composition of the testicular lipids in this study, but the effect of hypophysectomy on the metabolism of the fatty acids was not delineated. Since the response of EFA-deficient rats to feeding a supplement of linoleate in the diet is well-characterized, a study was made on the effect of hypophysectomy on this response in an effort to determine whether there was an effect of the hypophyseal hormones on the metabolism of EFA. The results of this study are reported here.

EXPERIMENTAL

Male weanling rats of the Sprague Dawley strain were kept in individual cages and given ad libitum a basic fat-free diet consisting of (by weight) 30% vitamin-test casein, 60% sucrose, 4% cellulose,[2] 4% salt mixture,[3] and 2% vitamin mix.[4]

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[2] Non-nutritive cellulose, Alphacel, Nutritional Biochemicals Corporation, Cleveland, Ohio 44128.

[3] Wesson modified, Osborne-Mendel salt mix (19), from General Biochemicals, Chagrin Falls, Ohio 44022.

[4] The percentage composition of the vitamin mix was: vitamin A acetate (crystals), 0.025; vitamin D_2 conc (400,000 USP units/g), 0.0175; α-tocopherol, 1.70; *i*-inositol, 0.96; choline chloride, 5.0; menadione, 0.0085; *p*-aminobenzoic acid, 0.325; niacin, 0.43; riboflavin, 0.13; pyridoxine·HCl, 0.035; thiamine·HCl, 0.13; Ca pantothenate, 0.43; biotin, 0.001; folic acid, 0.045; vitamin B_{12}, 0.0002; and casein diluent, 90.77.

After the diet had been fed for 26 weeks, a number of the rats were hypophysectomized.[5] The rats surviving the operation were maintained with the basic diet supplemented with skim milk and were given drinking water containing 5% glucose for 6 days following hypophysectomy. The hypophysectomized rats were then divided into 2 groups, one maintained with the basic diet supplemented with skim milk and glucose water and the other transferred to the same diet further supplemented with methyl linoleate[6] (10 g methyl linoleate to 100 g of basic fat-free diet). Of the nonhypophysectomized animals, one-half were sham-operated and divided into two dietary groups; one of these groups was given the supplemented, methyl linoleate containing diet and the other was maintained on the basic diet. The other half of the rats were similarly split into two dietary groups to serve as normal controls. The rats were fed the experimental diets for 3 weeks, with the exception of the hypophysectomized rats maintained with the basic fat-free diet. Only 2 animals in this group remained in good condition and were killed 16 days after the operation. At the end of the experimental periods the animals were exsanguinated under ether anesthesia. Tissues were excised, weighed in the natural state, quick-frozen on dry ice and stored at $-20°$ until they were extracted. Extraction of lipids from the tissue was carried out with 20 volumes chloroform-methanol (2:1), a second extraction with 10 volumes of the same mixture and finally with 10 volumes of chloroform-methanol (1:2). Ganglioside-like material and non-lipid contaminants were removed by Sephadex[7] column chromatography by the method of Wuthier (10) scaled-up for large samples (9).

Lipid class analyses. Neutral lipids composition was determined by thin-layer chromatography (TLC) using the densitometry-charring technique described by Blank et al. (11). The polar lipids remained at the point of origin on the plate in the solvent systems used in this method (petroleum ether:ethyl ether:acetic acid) and are estimated by difference. Glyceryl ether diesters separated between the triglycerides and cholesteryl esters in this solvent system and were identified as described by Thompson (12) and Thompson and Lee (13) on a small sample isolated by preparative TLC. Neither spraying of the plates with dinitrophenylhydrazine reagent nor 2-dimensional separation-reaction-separation chromatography according to Schmid and Mangold (14) gave any evidence of aldehydes or aldehydogenic lipids among the neutral lipids.

The composition of the polar lipids was also determined by TLC via the charring and densitometry technique (11). The analysis of these compounds was carried out in 2 stages. In the first stage diphosphatidylglycerol, phosphatidylethanolamine, and sulfatides were separated on plates using chloroform:methanol:acetic acid:water (80:20:8:4) (15). The mixtures that remained unresolved in this system were then separated by a two-dimensional system similar to that described by Rouser et al. (16), consisting of chloroform:methanol:water (65:25:4) in the first dimension and *n*-propanol:methanol:water (60:20:20) in the second dimension. Application of the procedure to standard mixtures of polar lipids gave highly reproducible results (a standard error of the mean of ± 0.4 to $\pm 1.1\%$).

Fatty acid composition of the lipid classes was determined by gas-liquid chromatography (GLC) of methyl esters prepared by interesterification with methanol as described by Privett et al. (17). GLC was carried out with an F&M gas chromatograph equipped with a hydrogen flame detector and a 2.44 m $\times$ 6.4 mm column packed with 10% EGSS-X on Chromosorb W[8] at 190°. Nitrogen was used as the carrier gas and quantification was made directly from the proportionalities of peak areas determined by triangulation. Identification of the peaks was made on the basis of the retention time of known compounds, chain length analysis (GLC of sample after hydrogenation) and by degree of unsaturation (GLC of compounds separated by argentation-TLC). The ether moiety of glyceryl ether diesters was also

[5] Hormone Assay Laboratories, Chicago 60652.
[6] 99% Purchased from the Lipids Preparation Laboratory of The Hormel Institute.
[7] Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey 08854.
[8] Applied Science Labs., State College, Pennsylvania 16801.

determined by GLC by analysis of the isopropylidene derivatives (18) of the corresponding glycerol ethers prepared according to procedures described by Thompson (12) and Thompson and Lee (13). Identification of components was made by comparison with the retention times of the derivatives prepared in a similar manner from the C_{16} and C_{18}-alkoxy glycerol ethers.

RESULTS

At the time of hypophysectomy all animals exhibited advanced symptoms of an essential fatty acid deficiency and their growth had leveled off at approximately 260 g. Data on the animals in the various groups at the time they were killed are summarized in table 1.

No data on the sham-operated animals are reported because they were not significantly different from those of the normal control groups. The data in table 1 indicate the general condition of the animals in the various groups. Growth was impaired by hypophysectomy as evidenced by comparison of the weights of the normal fat-free group with the linoleate supplemented groups. The hypox animals fed the fat-free diet could not maintain their weights, which resulted in a high mortality among the animals of this group. Although only 2 animals were available for analysis in this group the results were expressed as the mean with its standard error because they show the closeness of the agreement between the values obtained on these animals.

Hypophysectomy had an effect on both the size and amount of lipid in the liver and testes. Both organs decreased in size, particularly the testes which were one-quarter the size of those of the normal EFA-deficient animals. The amount of lipid in these organs was also decreased as a result of hypophysectomy, but in the case of the testes the percentage of lipid was actually greater.

Results of the lipid class analyses in table 2 show the pronounced effect of hypophysectomy on lipid class composition of the testes, and follow the general pattern reported previously (9) for commercially raised adult rats that were fed a diet adequate in EFA. The major changes in the testicular lipids consisted of an increase in the percentage of neutral lipids, particularly cholesteryl esters and glyceryl ether diesters, at the expense of mainly phosphatidylcholine and phosphatidylethanolamine. Changes in the composition of the lipids of the liver as a result of hypophysectomy were less than the change in the testes, and in the opposite direction in that the percentage of neutral lipid was decreased. The decrease in neutral lipid came mainly as a result of decreases in the free fatty acids and triglycerides. Phosphatidylcholine and phosphatidylethanolamine were increased slightly in the liver lipids of the hypox groups but the increases in the polar lipids of the liver did not appear to involve any specific components. Table 2 also shows that the same pattern of changes in lipid composition as a result of hypophysectomy occurred in both the linoleate and fat-free groups.

Despite the marked effects of hypophysectomy, characteristic interconversions of fatty acids occurred in both liver and testes of the hypox animals fed linoleate (table 3). The conversion of linoleic acid to higher polyunsaturated fatty acids in the

TABLE 1

Analytical data on animals

	Fat-free group		Linoleate group	
	Hypox (2) [1]	Normal (3)	Hypox (7)	Normal (5)
Wt of animals, g	188 ± 3 [2]	269 ± 39	234 ± 7	314 ± 24
Liver, g	6.3 ± 0.7	8.7 ± 0.4	6.9 ± 0.4	9.3 ± 0.3
Lipid, mg	296 ± 18	486 ± 40	249 ± 45	434 ± 75
Liver lipid (wt %)	4.7 ± 0.2	5.6 ± 0.4	4.1 ± 0.3	4.6 ± 0.6
Testes wt, g	0.43 ± 0.03	1.19 ± 0.53	0.37 ± 0.02	1.78 ± 0.23
Lipid wt, mg	19.5 ± 0.1	33 ± 15	15.8 ± 0.9	39.4 ± 5.6
Testes lipid (wt %)	4.5 ± 0.3	2.8 ± 0.2	4.3 ± 0.3	2.2 ± 0.2

[1] Numbers in parentheses indicate number of animals/group.
[2] Mean ± SE.

TABLE 2

Lipid class analysis (wt %)

	Liver [1]				Testes [2]			
	Fat-free group		Linoleate group		Fat-free group		Linoleate group	
	Hypox	Normal	Hypox	Normal	Hypox	Normal	Hypox	Normal
Cholesteryl esters (CE)	6.9	4.0	1.4	1.9	20.2 ± 0.9	4.5 ± 1.3	17.2 ± 1.0	5.0 ± 0.6
Glyceryl-ether diesters (GEDE)	tr	tr	tr	tr	9.1 ± 1.1	4.3 ± 1.3	6.8 ± 1.1	2.3 ± 0.3
Triglycerides (TG)	7.2	12.1	6.4	11.3	20.0 ± 3.0	9.0 ± 1.7	12.4 ± 1.0	10.2 ± 0.5
Free acids (FFA)	7.7	18.9	13.5	19.4	7.6 ± 4.4	7.7 ± 1.4	5.4 ± 0.7	3.8 ± 0.3
Cholesterol (C)	8.4	6.5	7.2	6.8	13.8 ± 0.1	9.8 ± 0.7	10.4 ± 0.9	10.0 ± 0.8
Total neutral lipid (NL)	30.2	41.5	28.5	39.4	70.5 ± 5.5	35.0 ± 2.8	52.0 ± 3.1	31.6 ± 0.2
Cardiolipin (CL)	7.6	6.6	4.9	—	5.8 ± 3.0	8.0 ± 0.6	7.1 ± 0.9	2.0 ± 0.9
Phosphatidic acid (PA)	1.6	—	2.6	—	—	—	—	—
Phosphatidyl ethanolamine (PE)	20.9	17.1	24.1	22.4	4.1 ± 0.6	19.7 ± 1.4	9.1 ± 0.7	20.2 ± 0.8
Sulfatide (SU)	—	—	2.3	1.9	1.0 ± 0.1	3.8 ± 0.8	2.6 ± 0.5	5.0 ± 0.1
Phosphatidyl choline (PC)	23.4	20.2	22.0	21.8	7.2 ± 3.2	16.7 ± 2.2	11.1 ± 0.6	19.8 ± 0.2
Phosphatidyl inositol (PI)	9.8	7.3	8.2	6.9	1.4 ± 0.9	3.3 ± 1.8	3.2 ± 0.7	1.1 ± 0.5
Sphingomyelin (SPH)	4.3	3.5	5.4	4.7	4.9 ± 2.1	4.5 ± 1.4	9.6 ± 0.7	9.2 ± 0.9
Phosphatidyl serine (PS)	0.8	1.0	1.0	1.1	2.3 ± 1.0	5.7 ± 0.4	2.6 ± 0.5	3.2 ± 0.6
Lysophosphatides (LP)	1.4	2.9	1.0	1.7	2.0 ± 0.9	4.1 ± 0.5	5.3 ± 0.4	6.6 ± 0.6
Total polar lipid (PL) (100% – NL%)	69.8	58.5	71.5	60.6	29.3 ± 5.4	65.3 ± 2.4	48.3 ± 3.0	68.2 ± 0.4

[1] Analyses performed on pooled samples of each experimental group.

[2] Analyses performed on individual animals, values expressed as mean ± SE.

TABLE 3

Fatty acid composition of liver and testicular lipids (wt %) [1]

	Testes				Liver			
	Fat-free group		Linoleate group		Fat-free group		Linoleate group	
	Hypox	Normal	Hypox	Normal	Hypox	Normal	Hypox	Normal
16:0	19.4 ± 0.9 [2]	26.3 ± 3.5	20.6 ± 0.7	28.2 ± 1.1	18.3 ± 1.7	16.4 ± 0.2	18.4 ± 0.3	15.4 ± 0.4
16:1	2.8 ± 0.6	2.7 ± 0.2	1.8 ± 0.4	1.4 ± 0.2	12.5 ± 1.4	11.7 ± 0.2	3.1 ± 0.5	3.1 ± 0.4
18:0	6.4 ± 0.3	7.0 ± 0.7	6.5 ± 0.3	6.9 ± 0.3	12.8 ± 2.3	13.0 ± 0.6	18.5 ± 0.4	15.7 ± 0.8
18:1	18.9 ± 2.9	17.8 ± 1.1	12.5 ± 0.5	11.7 ± 0.3	33.7 ± 3.7	32.7 ± 1.5	9.9 ± 0.6	12.4 ± 0.9
18:2	0.9 ± 0.1	0.6 ± 0.1	3.7 ± 0.4	5.2 ± 0.3	3.1 ± 0.3	3.1 ± 0.3	22.5 ± 0.7	21.4 ± 2.0
18:3	1.0 ± 0.2	0.8 ± 0.2	1.2 ± 0.3	tr	0.5 ± 0.2	1.5 ± 0.4	tr	tr
20:3ω9	5.2 ± 1.0	8.4 ± 1.2	2.8 ± 0.3	0.7 ± 0.3	10.1 ± 1.4	12.6 ± 0.5	0.7 ± 0.1	0.7 ± 0.1
20:3ω6	—	—	2.3 ± 0.3	1.6 ± 0.2	—	—	2.3 ± 0.2	1.1 ± 0.1
20:4	6.2 ± 1.1	5.7 ± 0.9	9.3 ± 0.4	14.8 ± 0.5	4.6 ± 0.4	3.2 ± 0.4	17.7 ± 0.5	21.7 ± 0.4
22:4ω9	3.3 ± 0.9	2.3 ± 0.3	2.8 ± 0.4	1.0 ± 0.4				
22:4ω6	2.0 ± 0.1	0.6 ± 0.1	3.3 ± 0.2	2.3 ± 0.3				
22:5	15.4 ± 3.6	10.3 ± 2.5	12.2 ± 1.3	15.8 ± 1.0				
24:4	1.1 ± 0.3	0.8 ± 0.2	3.0 ± 0.4	1.3 ± 0.1				
24:5	2.6 ± 0.2	1.7 ± 0.2	4.0 ± 0.3	2.7 ± 0.1				

[1] Unidentified minor component fatty acids not included.
[2] Mean ± se.

testes was not as efficient in the hypox as in the normal animals. To obtain more detailed information, the fatty acid composition of the major lipid classes of the testicular lipids was examined (table 4). These analyses also showed that the conversion of linoleic acid to arachidonic acid was less efficient in the testes of hypox than of the normal EFA-deficient animals. Hypophysectomy appeared to have some effect on the interconversions of 22- and 24-carbon chain polyunsaturated fatty acids but the changes were not well-defined.

Data on the glyceryl ether diester show a number of interesting points (table 4). Almost 60% of the fatty acids had 22- and 24-carbon chains and the close similarity between the hypox and normal animals receiving the linoleate supplement indicated further that hypophysectomy exerted little effect on fatty acid composition despite marked effects on the lipid class composition. Another feature of interest concerning these compounds, and one that sets them aside from those isolated from most other sources, is the absence of aldehydogenic species among them. There were quantitative differences between the glyceryl ether moiety of the compounds isolated from hypox and normal animals, but no marked differences were observed, and considerably more study is required to define the course of the metabolism of these compounds.

DISCUSSION

Hypophysectomy had little effect in liver on the interconversion of fatty acids or metabolism of the lipid classes although there were some quantitative changes in the latter. The normal pattern of interconversions of fatty acids also appeared to take place in the testes of hypox animals, but the conversion of linoleic acid to higher polyunsaturated fatty acids was not as efficient in these animals as in normal EFA-deficient animals fed linoleate. Nevertheless, it may be concluded that hypophysectomy did not disrupt the interconversion of fatty acids in the liver or testes.

Changes in the lipid class composition in the testes of hypox animals may occur as a result of degeneration of cell structure changing the concentration of en-

TABLE 4

Fatty acid composition of testicular lipid classes

Lipid [2] class	Rat groups [2]	Acid types [1]	18:1	18:2	20:3ω9	20:4	22:4ω9	22:4ω6	22:5	24:4	24:5
CE	FF	Hx	13.2 ± 0.4 [3]	0.5 ± 0.3	8.2 ± 1.0	2.3 ± 0.1	5.2 ± 1.8	1.5 ± 0.7	20.4 ± 3.2	2.7 ± 2.7	12.4 ± 0.8
		N	17.3 ± 2.4	1.6 ± 0.2	5.6 ± 0.9	1.9 ± 0.4	3.2 ± 1.1	—	8.6 ± 3.0	4.3 ± 0.6	9.6 ± 0.4
	LIN	Hx	12.9 ± 0.5	2.7 ± 1.2	6.0 ± 0.7	4.3 ± 0.6	4.0 ± 0.5	1.8 ± 0.1	16.7 ± 1.9	5.4 ± 0.6	10.1 ± 0.6
		N	10.1 ± 1.2	5.6 ± 0.3	0.6 ± 0.1	8.5 ± 1.0	1.9 ± 0.5	8.6 ± 2.1	21.6 ± 3.0	0.5 ± 0.2	7.1 ± 2.3
GEDE [4]	FF	Hx	10.3 ± 1.0	0.4 ± 0.1	3.7 ± 0.8	2.5 ± 0.6	6.0 ± 3.2	2.9 ± 0.4	13.6 ± 1.7	2.4 ± 1.7	2.5 ± 0.3
	LIN	Hx [5]	3.8	0.8	0.2	1.6	6.5	6.8	17.1	8.4	11.2
		N [5]	4.9	2.0	0.6	1.6	5.2	6.6	18.3	8.5	10.3
TG	FF	Hx	18.7 ± 8.2	0.8 ± 0.1	2.7 ± 0.1	1.7 ± 0.3	3.5 ± 0.9	1.6 ± 0.8	13.8 ± 5.0	1.6 ± 1.1	0.3 ± 0.3
		N	19.8 ± 1.3	1.0 ± 0.2	4.5 ± 1.2	1.7 ± 0.3	5.1 ± 1.9	0.6 ± 0.3	11.2 ± 4.3	0.4 ± 0.2	1.0 ± 0.6
	LIN	Hx	13.9 ± 1.9	2.8 ± 2.0	2.5 ± 0.5	2.2 ± 0.1	3.5 ± 0.7	3.5 ± 0.2	11.8 ± 1.7	2.4 ± 0.6	0.8 ± 0.4
		N	11.7 ± 0.8	7.0 ± 0.7	0.8 ± 0.3	3.4 ± 0.4	1.6 ± 0.6	4.0 ± 0.5	14.9 ± 1.5	1.5 ± 0.3	2.5 ± 0.6
PL	FF	Hx	18.8 ± 1.0	0.6 ± 0.3	5.5 ± 0.9	11.0 ± 2.9	1.1 ± 0.3	1.8 ± 0.1	11.4 ± 1.7	0.2 ± 0.2	—
		N	19.2 ± 1.0	0.5 ± 0.1	9.4 ± 1.7	7.1 ± 0.5	2.8 ± 1.8	—	10.1 ± 1.6	1.7 ± 0.2	0.4 ± 0.1
	LIN	Hx	13.0 ± 0.9	5.1 ± 0.5	2.8 ± 0.7	15.9 ± 1.4	0.5 ± 0.2	2.9 ± 0.3	8.1 ± 0.9	3.4 ± 0.3	—
		N	12.2 ± 0.3	4.3 ± 0.4	0.5 ± 0.2	17.0 ± 0.9	0.5 ± 0.3	1.8 ± 0.1	14.8 ± 1.0	1.4 ± 0.4	0.7 ± 0.3

[1] Not included are major nonessential fatty acids and unidentified minor component fatty acids.

[2] Abbreviations: *Lipid classes*: CE, cholesteryl esters; GEDE, glyceryl ether diesters; TG, triglycerides; and PL, polar lipids. *Rat groups*: FF, fat-free group; LIN, linoleate group; Hx, hypophysectomized; and N, non-operated ("normal").

[3] Mean ± SE.

[4] 22:3 is a major acid in the glyceryl ester diesters: FF Hx, 12.2±5.6%; LIN Hx, 11.3%; and LIN N, 9.7%.

[5] Analyses performed on pooled samples.

zymes involved in the metabolism of these compounds. Another possibility is the absence of hormonal control in the metabolism of lipid classes. Since large changes in fatty acid composition from feeding linoleate had little effect on the pattern of changes in the lipid classes caused by hypophysectomy, it appears that the main process effected by the lack of hypophyseal hormones was the transformations of fatty acids among the lipid classes. Moreover, since phospholipids in particular are an integral part of membrane structure hormone control in the testes may be exercised through an effect on membrance function via metabolism of these compounds.

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Effect of Dietary Carbohydrates on Intestinal Disaccharidases in Germfree and Conventional Rats [1]

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ABSTRACT The effects of various dietary carbohydrates on intestinal maltase, invertase, trehalase, lactase and cellobiase were studied in intestinal homogenates of germfree and conventional rats fed chemically defined, water-soluble diets containing either glucose, maltose, sucrose or lactose. In all dietary treatments, germfree rats showed higher disaccharidase levels than conventional rats. Lactose in the diet in comparison with glucose increased lactase and cellobiase activities at 30 days of age in both germfree and conventional rats. In 60-day-old conventional rats, lactose in the diet had a slight but not significant effect on lactase activity. Maltose in the diet increased maltase and trehalase levels compared with other dietary treatments in both germfree and conventional rats. A sucrose-containing diet increased invertase activity significantly in conventional rats and slightly but not significantly in germfree rats. Disaccharidase levels in germfree rats raised from birth with a glucose diet were comparable to those weaned from mother's milk and fed a glucose diet subsequently. These results indicate that changes in disaccharidase levels caused by feeding different disaccharides occur independently of the intestinal microflora.

Many attempts, both inconclusive and controversial, have been made to determine whether the addition of a particular disaccharide to the diet can enhance the formation of the corresponding disaccharidase in the small intestine (1–7). Diets containing large amounts of sucrose produced a significant increase in invertase activity in adult rats as compared with a carbohydrate-free, high casein diet (2). But oral administration of sucrose or isomaltose to 12-day-old rats for 2 days did not stimulate the appearance of invertase activity (1). Other experiments failed to show an effect of lactose administration on lactase activity in adult rats (5, 6). Koldovsky and Chytil (7) attributed this last result to the fact that the animals had previously consumed the normal laboratory diet, which had led to an irreversible decline in lactase activity.

In most of the above studies adult rats raised with commercial diets were either transferred to experimental diets or were injected intraperitoneally with a disaccharide solution. In addition, these diets were not well-defined and contained many unknown variables which might possibly affect the adaptive response of disaccharidases. Our previous study showed that germfree rats have higher disaccharidase levels in the intestinal mucosa than conventional rats (8). Since the microflora can change disaccharidase levels, it is conceivable that dietary changes could alter enzyme levels by changing the composition or properties of the microflora rather than by a direct effect on enzyme production. Thus, better design of experiments, a completely defined diet, and an experimental animal free from microbial influences appeared necessary to study the adaptive response of disaccharidases. Germfree animals offer an excellent tool in these studies since the substrate (disaccharides) present in the diet are completely at the disposal of the host. Similar experiments in conventional animals would indicate to what extent the intestinal microflora could influence the effect of the dietary changes. In addition, the chemically defined, low molecular weight, water-soluble diets developed in our lab-

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oratory are ideally suited to this purpose because all nutrient components are qualitatively and quantitatively defined and easily absorbable (9).

The present investigation was designed to study the effect of glucose, maltose, sucrose or lactose as the source of carbohydrate in water-soluble diets on maltase, invertase, trehalase, lactase and cellobiase activities of the small intestine of germfree and conventional rats.

MATERIALS AND METHODS

The germfree and conventional rats of Wistar origin (Lobund strain, mixed sex) used in this study were genetically closely related. Unless otherwise specified, all rats were housed and maintained as described in our previous investigation (8). To prevent the infant rats from consuming the solid diet offered to the mothers the following procedures were followed. Germfree rats at 10 to 12 days of age were transferred along with their mothers to stainless steel cages in plastic germfree isolators. At 14 days of age, the young were put into a cage where only the experimental diet was available. Out of each 2 hours, the mother spent one hour with its young and one hour in a different cage where it consumed its regular diet. This procedure enabled us to wean the rats directly from mother's milk to the experimental diets without access to solid diets. The same procedure was followed for conventional rats except that these animals were transferred to stainless steel cages in open colony rooms. The animals were killed for analysis at 30 and 60 days of age. In one series of experiments, to study the effect of a complete lack of disaccharide intake on disaccharidase activities, germfree rats were separated from the mother at birth and maintained by the hand-rearing methods of Pleasants et al. (10) with a liquid diet containing glucose as the source of carbohydrate. These animals were killed at 90 days of age.

The basal (chemically defined, water-soluble) diet L-479E9 used in this study was that of Pleasants [2] as described by Reddy et al. (9) consisting of amino acids, fat, vitamins, and minerals (table 1). The basal diet always contained about 2% fructose as calcium salt of fructose 1,6-diphosphate because this was the only way to keep calcium and phosphorus in solution. The carbohydrate under investigation (glucose, maltose, sucrose or lactose) was added to the basal diet. The diet was sterilized by filtration through a Millipore filter of 0.22-μ pore size. The first diet offered to 15-day-old germfree and conventional rats contained 12% solids with the sugar under investigation constituting 47% of the solids. The percentage of solids in the water-soluble formula and the percentage of sugar in the solids were increased gradually until the rats were 35 days old, when they were fed a formula containing

TABLE 1

Composition of chemically defined, water-soluble diet (L-479 E9)

	g/100 g solids
L-Lysine·HCl	1.25
L-Histidine·HCl·H_2O	0.55
L-Tryptophan	0.40
L-Phenylalanine	0.90
L-Isoleucine	0.50
L-Leucine	0.80
L-Threonine	0.50
L-Methionine	0.85
L-Valine	0.70
L-Arginine	0.75
L-Asparagine	1.20
L-Proline	3.00
Monosodium L-glutamate	6.00
Glycine	0.50
L-Serine	1.55
L-Alanine	0.75
Tyrosine-ethylester·HCl	2.00
Carbohydrate (glucose, maltose, sucrose or lactose)	70.50
Choline chloride	0.25
B-vitamin mixture-111 E2 [1]	0.066
Ladek-62 (fat-soluble vitamins) [2]	—
NaCl	0.377
CH_3COOK	1.06
Monocalcium fructose 1,6-diphosphate	5.00
Mg fructose l, 6-diphosphate	0.50
Trace mineral mixture [3]	0.074

[1] B-vitamin mixture-111 E2 contained: (in milligrams) thiamine·HCl, 0.50; riboflavin, 0.75; pyridoxine·HCl, 0.63; niacin, 3.75; inositol, 25; Ca pantothenate, 5.0; *p*-aminobenzoic acid, 30; biotin, 0.10; folic acid, 0.15; and cyanocobalamin, 0.03.

[2] Ladek-62 was administered orally (200 mg/day/animal). Daily supplement contained: (in milligrams) *dl*-α-tocopherol, 2.0; *dl*-α-tocopheryl acetate, 4.0; vitamin A palmitate, 0.33; vitamin K_1, 0.55; vitamin D_3, 0.00035; and ethyl linoleate to make 200 mg.

[3] Trace mineral mixture contained: (in milligrams) ferrous gluconate, 35; KI, 3.0; $Mn(C_2H_3O_2)_2 \cdot 4H_2O$, 26; $ZnSO_4 \cdot H_2O$, 5.5; $Cu(C_2H_3O_2)_2 \cdot H_2O$, 2.5; $Co(C_2H_3O_2)_2 \cdot 4H_2O$, 0.9; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.6; Na_2SeO_3, 0.011; and $Cr(C_2H_3O_2)_2 \cdot H_2O$, 0.48.

[2] Pleasants, J. R. 1966 Development of chemically defined, water-soluble diets nutritionally adequate for germ-free rats and mice. Ph.D. thesis, University of Notre Dame, Notre Dame, Indiana.

44% solids with 70.5% of solids present as a specific sugar.[3] The mothers of infant rats were maintained with a steam-sterilized commercial laboratory ration [4] with water ad libitum.

At the end of the experimental period, germfree and conventional rats were starved for 6 hours with free access to water and subsequently killed by stunning, followed by decapitation. The entire small intestine was excised immediately and chilled in ice-cold 0.9% NaCl solution. After the adhering fat and mesentery had been removed, the small intestine was slit open longitudinally, cleaned carefully with ice-cold saline to remove contents, blotted gently and weighed. All enzyme measurements were made on the supernatant fraction from the small intestine, homogenized and centrifuged as described in our previous report (8). Preparative procedures were carried out in a cold room at 4°. Maltase, invertase, trehalase, lactase and cellobiase activities were determined by the procedures previously described (8, 11), using 0.056 M maltose, sucrose, trehalose, lactose and cellobiose in 0.1 M maleate buffer (pH 6.5 in case of maltose and pH 6.0 in case of the other disaccharides) as substrates, respectively.

One enzyme unit corresponds to the activity hydrolyzing 1 μmole of corresponding disaccharide per 60 minutes at 37°. Protein was determined by the procedure of Lowry et al. (12) on all supernatant fractions. The results are expressed as units of enzyme activity per milligram of protein. Data were analyzed statistically to compare each treatment with every other treatment using Duncan's new multiple range test (13) as modified by Kramer (14) for unequal number of replications.

RESULTS

The protein content of the homogenate of the small intestine was similar in germfree rats fed various carbohydrates. Conventional rats fed various carbohydrates also showed comparable protein levels. However, there was 10 to 15% more protein/g of tissue in germfree rats than in conventional rats. In all dietary treatments, germfree animals showed higher disaccharidase activities than the conventional rats. This confirmed earlier findings (8).

Effect of dietary carbohydrates on intestinal disaccharidase activities in 60-day-old germfree and conventional rats. Tables 2 to 4 show the maltase, invertase, trehalase, lactase, and cellobiase activities in the small intestine of germfree and conventional rats fed diets containing maltose, sucrose or lactose as the sole source of carbohydrate, and these activities are compared with those in animals maintained with a glucose-containing formula. Germfree rats fed a maltose-containing diet showed a significant increase in maltase activity (table 2). Similarly there was

[3] See footnote 2.
[4] Purina Laboratory Chow, 5010 C, Ralston Purina Company, St. Louis.

TABLE 2

Effect of dietary carbohydrates on intestinal maltase and trehalase activities in 60-day-old germfree and conventional rats

Carbohydrate fed	Maltase activity [1]		Trehalase activity [1]	
	Germfree	Conventional	Germfree	Conventional
Glucose, G	25.0±3.3 [2] (5) [3]	10.9±1.0 (5)	4.76±0.33 (5)	2.04±0.13 (5)
Maltose, M	37.7±3.4 (6)	16.2±2.0 (7)	6.64±0.46 (6)	3.98±0.21 (7)
Sucrose, S	27.9±1.6 (9)	11.5±1.2 (8)	4.98±0.39 (9)	2.12±0.11 (8)
Lactose, L	—	9.1±0.8 (4)	—	2.00±0.14 (4)
	Statistical significance, P value			
G vs. M, S, L	< 0.05, ns, [4] —	< 0.05, ns, ns	< 0.05, ns, —	< 0.05, ns, ns
M vs. S, L	< 0.01, —	< 0.05, < 0.01	< 0.05, —	< 0.05, < 0.05
S vs. L	—	ns	—	ns

[1] Enzyme activities are expressed as micromoles of respective disaccharide hydrolyzed/hour/milligram of protein.
[2] Averages ± SE of mean.
[3] Number of rats.
[4] Not significant, $P > 0.05$.

TABLE 3

Effect of dietary carbohydrates on intestinal invertase activity in 60-day-old germfree and conventional rats

Carbohydrate fed	Invertase activity [1]	
	Germfree	Conventional
Glucose, G	3.58 ± 0.50 [2] (5) [3]	1.52 ± 0.14 (5)
Maltose, M	3.65 ± 0.34 (6)	1.84 ± 0.12 (7)
Sucrose, S	4.01 ± 0.42 (9)	2.03 ± 0.17 (8)
Lactose, L	—	1.28 ± 0.08 (4)
Statistical significance, P value		
G vs. M, S, L	ns,[4] ns, —	ns, < 0.05, ns
M vs. S, L	ns, —	ns, < 0.05
S vs. L	—	<0.01

[1] Invertase activity is expressed as micromoles of sucrose hydrolyzed/hour/milligram of protein.
[2] Averages ± SE of mean.
[3] Number of rats.
[4] Not significant, $P > 0.05$.

TABLE 4

Effect of dietary carbohydrates on intestinal lactase and cellobiase activities in 60-day-old germfree and conventional rats [1]

Carbohydrate fed	Lactase activity [2]		Cellobiase activity [2]	
	Germfree	Conventional	Germfree	Conventional
Glucose	1.40 ± 0.08 [3] (5) [4]	0.54 ± 0.05 (5)	0.39 ± 0.02 (5)	0.15 ± 0.02 (5)
Maltose	1.52 ± 0.15 (6)	0.53 ± 0.05 (7)	0.37 ± 0.05 (6)	0.13 ± 0.02 (7)
Sucrose	1.53 ± 0.07 (9)	0.49 ± 0.04 (8)	0.42 ± 0.05 (9)	0.14 ± 0.03 (8)
Lactose	—	0.63 ± 0.06 (4)	—	0.17 ± 0.02 (4)

[1] No significant difference ($P > 0.05$) among the dietary treatments in either germfree or conventional rats.
[2] Enzyme activities are expressed as micromoles of respective disaccharide hydrolyzed/hour/milligram of protein.
[3] Averages ± SE of mean.
[4] Number of rats.

an increase in maltase activity in conventional rats receiving maltose over those animals fed other diets. No significant difference in maltase activity was found between germfree rats fed glucose and sucrose and among conventional rats fed glucose, sucrose and lactose. The data in table 1 also show that, in both germfree and conventional rats, maltose in the diet appeared to increase trehalase activity.

The results presented in table 3 show that there was a slight but not significant increase in invertase activity in germfree rats fed sucrose compared with animals fed either glucose or maltose. In conventional rats, however, feeding of sucrose caused a significant increase of invertase activity when compared with animals fed either glucose or lactose. Maltose-fed conventional rats appeared to demonstrate a slight but not quite significant increase of invertase levels over animals fed glucose. There was no significant difference in activity between maltose- and sucrose-fed conventional animals.

The data summarized in table 4 show there was a slight but not significant increase of lactase activity in conventional rats fed lactose compared with other treatments. No significant change occurred in cellobiase activities among dietary treatments in either germfree or conventional rats.

Disaccharidase activities in the small intestine of 30-day-old germfree and conventional rats fed lactose or glucose diet. Because we were unable to raise the germfree rats to 60 days of age with the lactose-diet (see discussion below), and also to study whether the decrease in lactase activity in rats at the time of weaning (8) could be slowed down by feeding lactose, we determined enzyme activities in the small intestine of 30-day-old germfree and conventional rats fed lactose or glucose diets. The data in table 5 show that in

TABLE 5

Effect of dietary lactose or glucose on intestinal disaccharidase activities in 30-day-old germfree and conventional rats [1]

Disaccharidase	Enzyme activity [2]			
	Germfree		Conventional	
	Lactose diet	Glucose diet	Lactose diet	Glucose diet
Maltase	16.9 ± 1.11 [3]	18.0 ± 0.26	7.53 ± 0.53	8.32 ± 0.42
Invertase	1.98 ± 0.05	2.10 ± 0.08	0.67 ± 0.04 [4]	0.86 ± 0.04
Trehalase	2.32 ± 0.12	2.50 ± 0.09	0.92 ± 0.05	1.02 ± 0.03
Lactase	1.52 ± 0.12 [4]	1.10 ± 0.03	0.75 ± 0.03 [5]	0.55 ± 0.02
Cellobiase	0.39 ± 0.02 [5]	0.26 ± 0.01	0.18 ± 0.006 [5]	0.14 ± 0.005

[1] Four rats/group.
[2] Enzyme activities are expressed as micromoles of respective disaccharide hydrolyzed/hour/milligram of protein.
[3] Averages ± SE of mean and *t* test used to compare groups.
[4] Differs from glucose diet significantly, $P < 0.025$.
[5] Differs from glucose diet significantly, $P < 0.005$.

comparison with glucose, lactose in the diet increased lactase and cellobiase activities in both germfree and conventional rats and possibly decreased invertase activity in conventional rats. Replacement of glucose by lactose in the diet had no effect on maltase and trehalase activities in either germfree or conventional rats.

Disaccharidase activities in the small intestine of 90-day-old germfree rats raised from birth with the glucose diet. Disaccharidase activities of germfree rats raised from birth with the glucose diet or weaned from mother's milk and thereafter fed the glucose diet are shown in table 6. Maltase, invertase, trehalase, lactase and cellobiase activities in the animal raised with the glucose diet from birth were comparable to those weaned from mother's milk and fed the glucose diet subsequently.

DISCUSSION

The inability of lactose-fed germfree rats to survive for 60 days apparently resulted from poor utilization of lactose at high dietary levels. Since lactase activity of the intestinal epithelial cells was comparatively small, the conventional animals appear to depend to a considerable extent on bacterial lactase, especially in the cecum, for the utilization of the large amount of lactose in the diet (15).

The increase in maltase activity in the small intestine of 60-day-old germfree and conventional rats fed the maltose diet (table 2) indicated a direct dietary influence. The increase in invertase activity (table 3) observed in conventional rats fed sucrose confirms the observations of Blair et al. (2). A similar but much smaller (nonsignificant) effect observed in germfree rats suggests that the dietary adaptation of invertase activity in the small intestine of rats may be influenced by the microflora. However, analysis of variance showed no significant interaction between the status (germfree and conventional) and the diet (glucose and sucrose). This indicates that the status and diet act independently of each other and that microflora may not play a significant role in the dietary adaptation of invertase activity.

Since trehalase appears to have absolute specificity for its substrate unlike the other intestinal disaccharidases (16), the increase of this enzyme activity observed in maltose-fed germfree and conventional rats (table 2) presumably is not a case of substrate induction. Blair et al. (2) have

TABLE 6

Effect of feeding glucose diet from birth and from weaning on intestinal disaccharidase activities in 90-day-old germfree rats

Disaccharidase	Enzyme activity [1]	
	Glucose from birth [2] (3 rats/group)	Glucose from weaning [2] (5 rats/group)
Maltase	26.4 ± 2.1 [3]	29.0 ± 2.8
Invertase	2.0 ± 0.18	2.4 ± 0.14
Trehalase	4.2 ± 0.29	4.6 ± 0.24
Lactase	0.90 ± 0.01	1.1 ± 0.01
Cellobiase	0.21 ± 0.01	0.19 ± 0.01

[1] Enzyme activities are expressed as micromoles of respective disaccharide hydrolyzed/hour/milligram protein.
[2] No significant ($P > 0.05$) difference between the groups using *t* test.
[3] Averages ± SE of mean.

reported that feeding either galactose, fructose, maltose or melizitose increased the invertase activity. Our data (table 3) show also a slight increase of invertase activity in conventional rats fed the maltose diet. It has been suggested that the stimulatory effect of these sugars might be due to stabilization of the enzyme by retardation of the rate of breakdown rather than by activation or synthesis of the enzyme (2). The increase observed in trehalase activity in animals fed the maltose-containing diet might be the result of a similar mechanism.

The observation that feeding lactose increased lactase levels only slightly but not significantly in 60-day-old conventional rats (table 4), whereas it increased the levels significantly in 30-day-old germfree and conventional rats (table 5), suggests the complexity of regulation of this enzyme in mammals. In 30-day-old rats, an increase in lactase levels following the feeding of the lactose-containing diet from before weaning may result from a retardation of the normal rapid decline in activity of this enzyme which occurs just before weaning. This mechanism was first suggested by Koldovsky and Chytil (7). In 60-day-old rats, the lowest levels of enzyme activity had already been reached (8), and the feeding of lactose even from before weaning was not sufficient to affect lactose levels significantly.

Since this investigation established that diet has a certain effect on intestinal disaccharidase activities, it is pertinent to elaborate on the mechanism involved. Since hormones influence the production of certain disaccharidases, as suggested by the stimulatory effect of hydrocortisone on invertase activity in infant rats (17, 18) and by the effect of adrenalectomy on the rate of decline of lactase in weaning rats (7), we should not rule out the possibility that the dietary effect may be mediated by changes in hormonal activity. However, data presented in many investigations (19–25) indicate that following corticosteroid administration increases in enzyme activity in the liver are generally accompanied by an increase in protein. In the present experiments, increases in enzyme activity in the small intestine were never associated with changes in the protein content. This makes it less likely that the dietary effect was mediated by hormonal influences.

Another indirect mechanism by which disaccharide feeding could affect disaccharidase levels would be through the effect of the dietary carbohydrate on the intestinal microflora. Even though the presence of a microflora considerably decreases the absolute disaccharidase levels in the small intestine, the effects of a change in disaccharide on disaccharidase levels were always in the same direction in both germfree and conventional rats, and of comparable magnitude. Thus, although the microflora clearly affects disaccharidase levels, whether by affecting substrate availability, enzyme inactivation, or the metabolism and maturation of mucosal cells (8), the changes in enzyme levels caused by feeding different disaccharides occur independently of the microflora.

Two additional direct effects of dietary disaccharides on disaccharidase levels must therefore be considered. Feeding a specific disaccharide could either increase the rate of production of the corresponding enzyme, or decrease its rate of breakdown. Schimke et al. (23) showed that substrate (tryptophan) administration resulted in an increase in substrate specific enzyme (tryptophan pyrrolase) activity which was mediated through prevention of normal rate of enzyme breakdown, whereas synthesis continued at a normal rate. Blair et al. (2) suggested a similar mechanism for the adaptive response of invertase to the feeding of various sugars. The present data do not exclude the hypothesis of enzyme induction, but they appear to favor the hypothesis of enzyme stabilization suggested by these investigators (2, 23) because increases in disaccharidase activities in the small intestine were never associated with changes in protein content. This stabilization could be relatively nonspecific and thereby account for our finding that the feeding of maltose increased trehalase and invertase levels. That this effect was also observed in germfree animals, thus excluding the effects of microflora on enzyme stability, strengthens the argument for substrate stabilization of the enzyme involved, which retarded their rate of

breakdown, as an hypothesis deserving increased consideration and investigation.

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Tissue Selenium Levels during the Development of Dietary Liver Necrosis in Rats Fed Torula Yeast Diets [1]

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ABSTRACT Blood, liver and kidney selenium levels were studied in male rats fed a Torula yeast diet with variations in selenium, vitamin E, and protein content. Kidney selenium levels were the highest of all tissues studied; liver levels were intermediate; and blood levels were lowest. A profound drop in the selenium levels of these tissues was observed in animals fed low selenium diets. Variations in dietary protein and vitamin E content did not affect selenium levels. Vitamin E administration prevented liver necrosis in animals receiving no supplementary selenium but had essentially no effect on their selenium levels. Blood and liver selenium levels were maintained at an almost constant level in animals receiving selenium regardless of whether it was supplied as 0.50 ppm or 0.25 ppm of the diet. Kidney selenium levels were maintained at the pre-experimental level when a diet containing 0.25 ppm Se was fed but rose sharply when 0.50 ppm was fed.

Torula yeast diets deficient in selenium and vitamin E predictably produce liver necrosis and death when fed to weanling rats. Selenium, vitamin E, or both, added to the diet in appropriate amounts prevent liver necrosis. The action of selenium and of vitamin E in preventing this condition is unknown although several hypotheses have been proposed (1, 2).

Considering the number of publications dealing with selenium deficiency liver necrosis, the lack of published data on tissue selenium levels during its development is remarkable. Accordingly, in this study observations were made on the effects of dietary selenium, vitamin E, and protein levels on blood, liver and kidney selenium levels and on plasma vitamin E levels in the rat.

EXPERIMENTAL

The basic diet used in this work was the Torula yeast diet used by Schwarz and Foltz (3) in their early studies. In the form used here (table 1) the diet contained 15% protein. The diet for the low protein group (see below) was modified by reducing the amount of Torula yeast to 18% and making up the deficit with sucrose. This provided a diet containing only 9% protein. Selenium was added to the diet as sodium selenite at 2 levels, 0.50 ppm and 0.25 ppm. Hereafter these levels are referred to as Se and 1/2Se, respectively. The selenite was mixed with sucrose before being added to the diet. Vitamin E was added to some diets as 200 mg of *dl*-α-tocopherol/kg of diet.

Male weanling albino rats of the Sprague-Dawley strain were fed commercial laboratory ration [2] for one day upon receipt, and were then divided into 7 dietary groups. A control group of 5 rats was killed to obtain baseline analytical data at the time the other groups were fed their special diets. Table 2 shows the various diets fed. The animals were housed in individual cages and were weighed twice a week. Food and distilled water were provided ad libitum. Three rats from each group (2 from the 1/2Se-vitamin E group) were killed at the end of the first and second weeks and the remainder in each group was killed at the end of the fourth week. The animals were anesthetized with ether and exsanguinated by drawing heart blood into a heparinized syringe. A microhematocrit was carried out and about 1 ml of whole blood was frozen for Se analysis.

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

TABLE 1

Torula yeast diet

	%
Torula yeast	30
Sucrose	49
HMW salt mixture [1]	5
Vitamin mixture [2]	1
Stripped lard (with or without vitamin E added)	5
Sucrose (with or without Se added)	10

[1] Hubbell et al. J. Nutr., *14*: 273, 1937.
[2] Amounts of vitamins per 100 g of diet: (in milligrams) thiamine, 0.04; riboflavin, 0.25; pyridoxine·HCl, 0.2; Ca D-pantothenate, 2; niacin, 10; menadione, 0.1; folic acid, 0.2; biotin, 0.1; vitamin D_2, 0.8; vitamin B_{12}, 0.01; choline chloride, 100; and vitamin A (aqueous), 1,400 USP units.

TABLE 2

Modification of basal Torula yeast diet

Group	No. rats	Dietary supplement	
		Selenium	Vitamin E
		ppm	*mg/100 g diet*
No Se-no vitamin E	12	—	—
No Se- vitamin E	10	—	20
Se-no vitamin E	10	0.5	—
Se- vitamin E	10	0.5	20
Low protein [1]	10	0.5	20
1/2Se- vitamin E	6	0.25	20

[1] This group was fed a diet containing only 9% Torula yeast protein.

The remaining blood (in some cases there was none) was centrifuged to obtain plasma for vitamin E levels which were measured by the micromethod of Quaife et al. (4). Liver and kidneys were removed and frozen for Se analysis. Liver samples were taken from the animals killed after 4 weeks, for histological study. Sections were stained with hematoxylin and eosin.

The determination of selenium was carried out by a modification of the method of Watkinson (5). This method uses digestion of the wet tissue sample in nitric and perchloric acids followed by complexing of the selenium with diaminonaphthalene and extraction of the selenium-diaminonaphthalene with diaminonaphthalene complex into cyclohexane with subsequent measurement of its fluorescence in an Aminco-Bowman spectrophotofluorometer. A recovery study run on blood and liver from rats injected with $^{75}SeO_3$ 2 weeks before death yielded recoveries for the entire procedure (digestion and extraction) of 88% for liver, 88.6% for blood, and 83.4% for the standard solution.

RESULTS AND DISCUSSION

Study 1. This preliminary study was designed to measure blood, liver, and carcass selenium levels in 2 groups of 20 rats that consumed either the Torula yeast basal diet containing no selenium supplementation or a similar diet with 0.5 ppm Se added. Both males and females were included in this study. The results are instructive even though for unknown reasons the animals in this experiment gained little weight, and there were no deaths in the Se and vitamin E-deficient group. In addition, no gross or microscopic abnormalities were noted in any livers.

The liver, blood and carcass selenium levels are shown in figure 1. Liver selenium concentrations decreased markedly by the end of the first week and then more slowly in the group fed the diet without the selenium supplement. A level of 15% of the initial value was noted at the end of 4 weeks. The liver selenium levels in the the rats fed the diet containing added selenium increased somewhat, then declined, but remained well above values for the low selenium group.

The blood selenium concentration of rats fed the diet containing selenium increased slightly during the experiment and that of rats fed the low selenium diet decreased to approximately half of the original value. During the first 3 weeks the carcass concentrations decreased almost linearly with time in the latter group. The slight increase during the last week of the experiment is unexplained. In animals consuming the diet containing added selenium, carcass selenium concentrations were maintained throughout the study. No sex differences in tissue selenium levels were noted. No further details are reported on this preliminary study because no liver necrosis was produced.

Study 2. This study was described in detail in the experimental section. Growth curves are shown in figure 2. Ten animals from the same shipment, fed laboratory ration, and that were 20 g heavier than the other rats at the start of the experiment, grew rapidly during the study, reaching a weight of 231 g in 4 weeks. Conversely, rats fed the Torula yeast diet grew at a considerably reduced

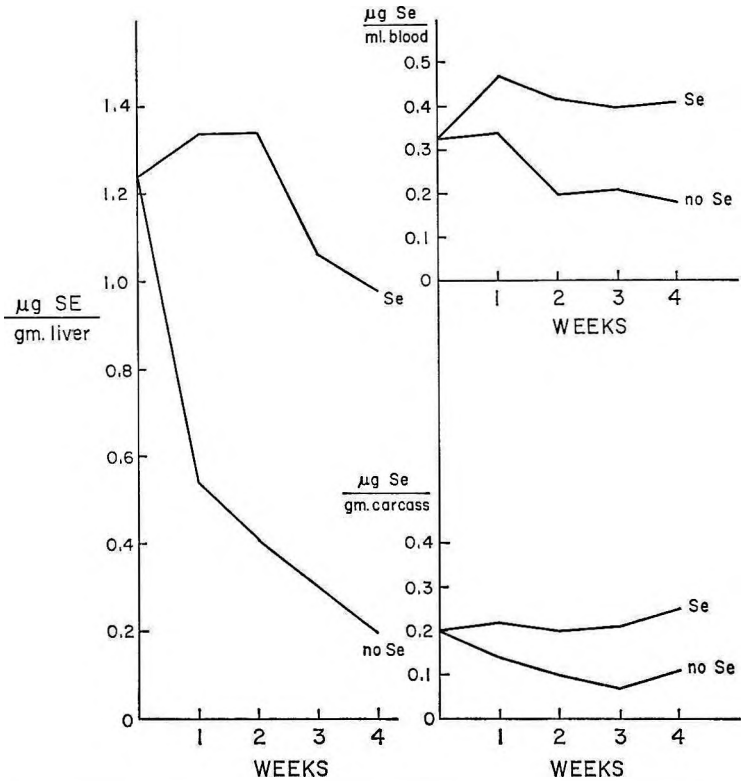

Fig. 1 Liver, blood, and carcass selenium levels from study 1. Each point represents the mean value of 4 rats.

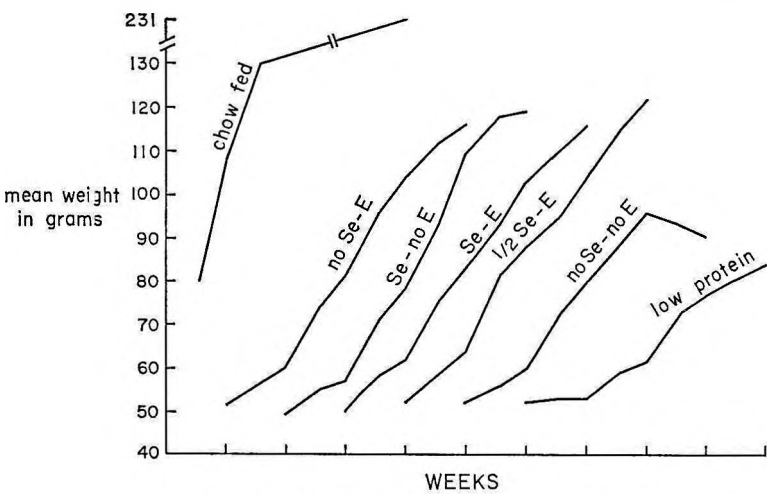

Fig. 2 Growth curves of all groups in study 2.

rate, and, at the end of 4 weeks, had body weights only half those of the rats fed laboratory ration. The group receiving no Se or vitamin E began to lose weight in the fourth week, whereas all other groups continued to gain. As expected, the low protein group gained less weight. Mean group food consumption data are shown in table

3. The rats fed the low protein and the no Se-no vitamin E diets ate somewhat less but otherwise the intake differences were not remarkable. No animals were anemic as judged by the hematocrits.

Of the 6 rats surviving more than 2 weeks in the no Se-no vitamin E group, five developed gross liver changes within 4 weeks. One animal died on the sixteenth day of the experiment. Gross and microscopic examination of the liver showed acute hemorrhage and necrosis. Of the 5 animals killed at 4 weeks, 4 had grossly visible pale areas of the liver and a few 1-mm hemorrhages. One liver with pale areas looked fatty on gross examination, and staining of a section with Sudan IV established the presence of a large amount of fat. The other rat liver appeared normal on gross inspection, and no definite areas of necrosis and hemorrhage were identified microscopically. Thus, five of the 6 animals fed the no Se-no vitamin E diet showed signs of liver necrosis at the end of the 4-week feeding period, but no other animals had gross liver lesions at that time. The 6 animals fed the necrogenic diet (no Se-no vitamin E), killed during the first 14 days of the study, did not have gross liver lesions. Microscopic lesions were not present in 2 livers from each of the other groups killed at 4 weeks.

The variability of the time needed to produce necrosis and the reaction of each animal to an episode of necrosis is of interest. The probable time of onset of necrosis varied from 16 to 28 days. One rat succumbed to the first attack, and 4 others showed evidence of several attacks but were still living. Similar findings have all been described before (6).

In figure 3 the mean blood selenium levels of all groups consuming selenium-supplemented diets are compared with those of all groups consuming unsupplemented diets. In selenium-supplemented animals the blood selenium levels remained at about 0.3 μg/ml; the level of those not receiving selenium decreased to 0.05 μg/ml. There was a significant difference between these 2 categories after 1 week. Figure 4 shows the mean blood selenium concentrations by group. There was no significant difference between the blood selenium concentrations of groups no Se-no vitamin E and no Se- vitamin E. Likewise no difference was found between groups Se-no vitamin E and Se- vitamin E. This indicates that vitamin E does not affect blood selenium levels under these

TABLE 3

Mean food consumption of the various groups

Week no.	No Se-no vitamin E	No Se-vitamin E	Se-no vitamin E	Se-vitamin E	Low protein	½Se-vitamin E
1	51 [1]	53	54	60	50	62
2	111	118	118	122	99	125
3	213	257	285	273	213	279

[1] Values are in grams and are averages for animals surviving to time indicated.

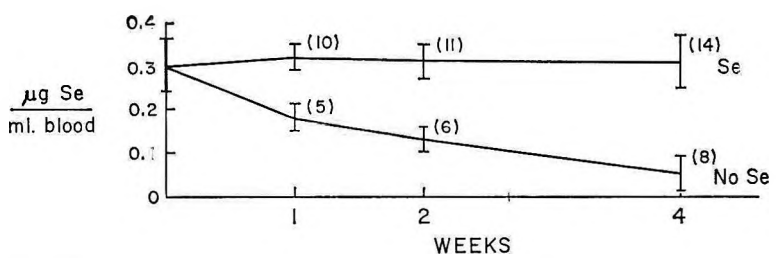

Fig. 3 Blood selenium levels of animals in study 2 divided into animals receiving selenium supplementation (0.25 ppm or 0.50 ppm) in the diet and animals receiving none. Vertical lines (I) indicate ± 1 SD. Numbers in parentheses indicate the number of animals averaged to determine the point.

conditions. The diets of group Se- vitamin E and the low protein group differed only in their protein content. There was no significant difference in their total plasma solids (plasma proteins) as measured by an American Optical TS Meter nor was there a significant difference in the blood selenium levels. There was no significant difference between the blood selenium levels of groups Se- vitamin E and 1/2Se-vitamin E, suggesting that (at least within these limits) blood selenium level may be homeostatically maintained.

The approximate initial liver selenium levels of 0.75 μg/g wet weight were maintained in animals fed the diets supplemented with selenium, but levels in animals fed low selenium diets decreased to 0.07 μg/g (fig. 5). Figure 6, which shows liver selenium concentrations by group, permits the same conclusions here as those for blood levels. In addition, group no Se-vitamin E, that had no liver necrosis, had a lower liver selenium level than the no Se-no vitamin E group that had liver necrosis, which indicates that vitamin E administration does not prevent liver necrosis by preventing a decrease in liver selenium level. Why these liver levels are somewhat less than those recorded in the preliminary study is not known.

Unlike the selenium concentrations in liver and blood, those in the kidney increased as the experiment progressed in the groups fed diets containing supplementary selenium. In addition, the concentration also appeared to be a function of the amount of selenium in the diet (fig.

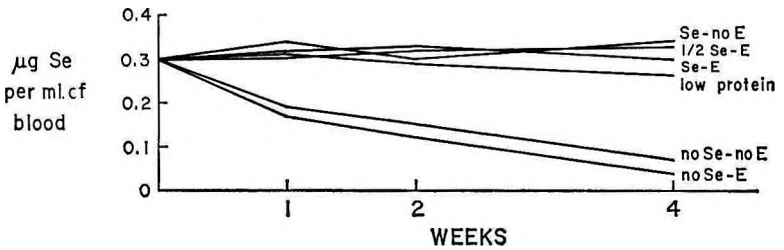

Fig. 4 Blood selenium levels by groups in study 2. Each point represents the mean level of 2 to 4 rats.

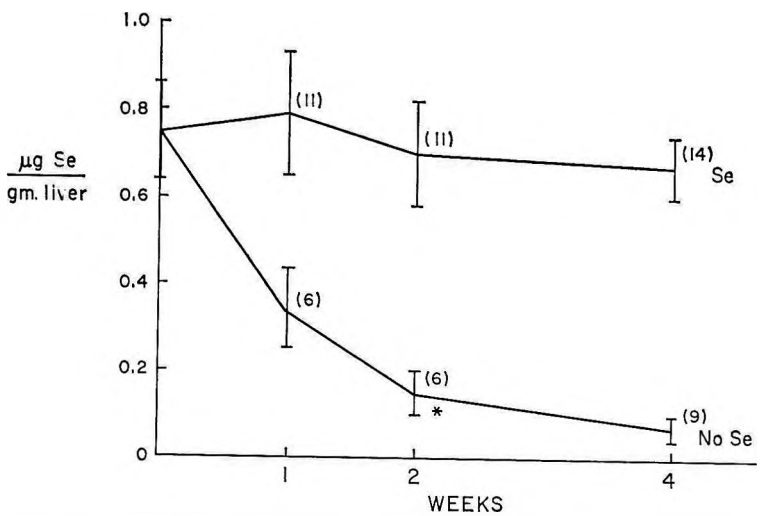

Fig. 5 Liver selenium levels of animals in study 2. All groups receiving Se vs. all groups not receiving Se Vertical lines (I) indicate ± 1 SD. Numbers in parentheses indicate the number of animals averaged to determine the point. The asterisk marks the liver selenium level of the no Se-no vitamin E rat that died at 16 days.

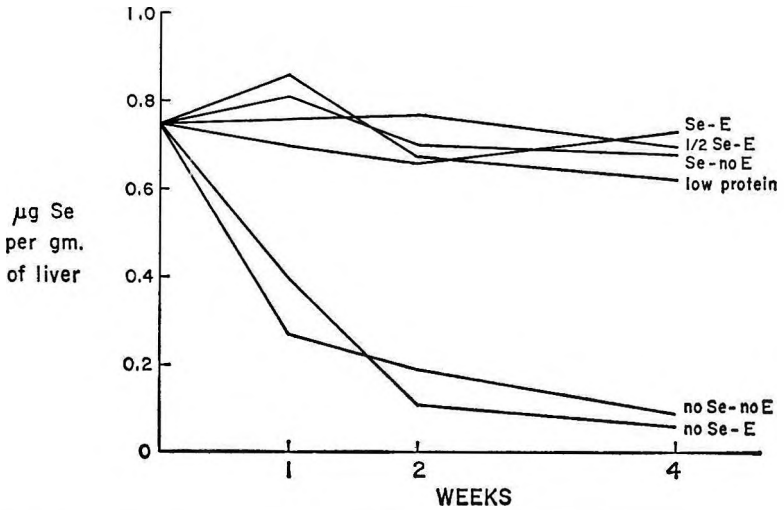

Fig. 6 Liver selenium levels by groups in study 2. Each point represents the mean of 3 to 4 rats in all groups except the 1/2Se- vitamin E group in which 2 were averaged.

7). Initially kidney levels of 0.96 μg/g of wet tissue fell to 0.27 μg/g in rats fed the basal diet without added selenium. Figure 8, which shows kidney selenium levels by group, indicates the variability of the values. In these studies, vitamin E and the level of dietary protein did not affect kidney selenium levels. The level of selenium in the diet, however, appeared to make a difference. Groups Se- vitamin E and 1/2Se- vitamin E, that consumed diets differing only in selenium content, had significantly different kidney selenium levels.

Figures 3, 5, and 7 show blood, liver, and kidney selenium concentrations plotted on the same scale. The kidney levels were highest. These graphs show the marked similarity of the liver and blood selenium concentrations after 2 weeks in rats receiving no selenium supplementation. On this regimen, blood, liver and kidney selenium levels decreased to 17%, 9% and 28%, respectively, of the original levels of the control group. The liver and kidney selenium concentrations in the no Se-no vitamin E rat that died on day 16 of the experiment are designated in figures 5 and 7. Although the liver selenium level of this animal was only slightly below the group average, its kidney level was considerably below the group average and about the same as that for the group on termination of the experiment 12 days later.

The mechanism whereby blood and liver selenium levels remain the same whether the rats consume diets containing 0.50 ppm or 0.25 ppm Se is unknown. Such regulation does not occur in the kidney. It is likely that the form of selenium in the liver may depend on the form fed. Cummins and Martin (7) have recently reported that selenite binds to sulfur compounds in rabbit liver but is not incorporated into cystine or methionine. Excess selenite may be converted to dimethyl selenide in the liver and excreted through the lungs (8), thus maintaining liver stores at a constant level. This might not be the case if ^{75}selenomethionine were fed because dimethylselenide is not generated from this compound, at least during the first hour after administration (9). Possibly the blood level could also be regulated by the liver, although a recent publication describes the incorporation of selenium given as ^{75}Se-selenomethionine into the α-globulin of the plasma protein in hepatectomized rats (9). No such study with selenite has been reported. The kidney is the principal organ of selenium excretion when selenium is given as selenite (8) or as selenomethionine (9). Accordingly, the kidneys of rats fed diets containing the higher level of selenium would be exposed to more se-

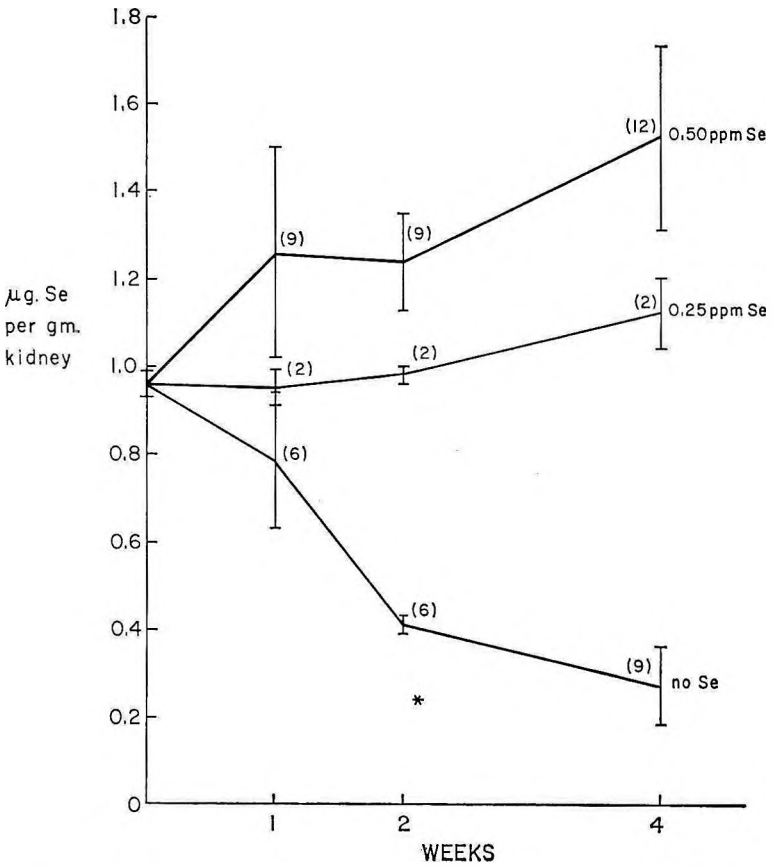

Fig. 7 Kidney selenium levels of animals in study 2. All groups receiving 0.5 ppm Se vs. groups receiving 0.25 ppm Se and no ppm Se. Vertical lines (I) indicate ± 1 SD. Numbers in parentheses indicate the number of animals averaged to determine the point. The asterisk marks the kidney selenium level of the no Se-no vitamin E rat that died at 16 days.

lenium for excretion and this might be reflected by higher kidney selenium levels.

The low protein group had selenium levels similar to those of its Se- vitamin E control; in figures 4 and 6, however, which show blood and liver selenium levels by groups, the low protein group showed the lowest levels at 4 weeks. Conversely, the kidney selenium levels in this group were the highest (fig. 8). If, in fact, kidney levels are related to excretion of selenium by that organ, low dietary protein might result in a failure to retain dietary selenium in the liver and blood. Over a period of several months this could lead to tissue selenium levels lower than anticipated from the selenium content of the diet. Considering the relatively small differences observed, apparently long-term studies are required to investigate this possibility.

Although there was considerable variability in the plasma vitamin E levels, they fall clearly into 2 groups (fig. 9). Groups no Se- vitamin E, Se- vitamin E, low protein, and 1/2Se- vitamin E all consumed diets containing vitamin E and all values fell within normal limits. Groups no Se-no vitamin E and Se-no vitamin E consumed diets lacking vitamin E and had very low plasma vitamin E levels. A finding of interest is that every animal tested in the Se-no vitamin E group had a zero plasma vitamin E level, whereas those in the no Se-no vitamin E group had small but measurable amounts at all times. The plasma levels were determined in a random fash-

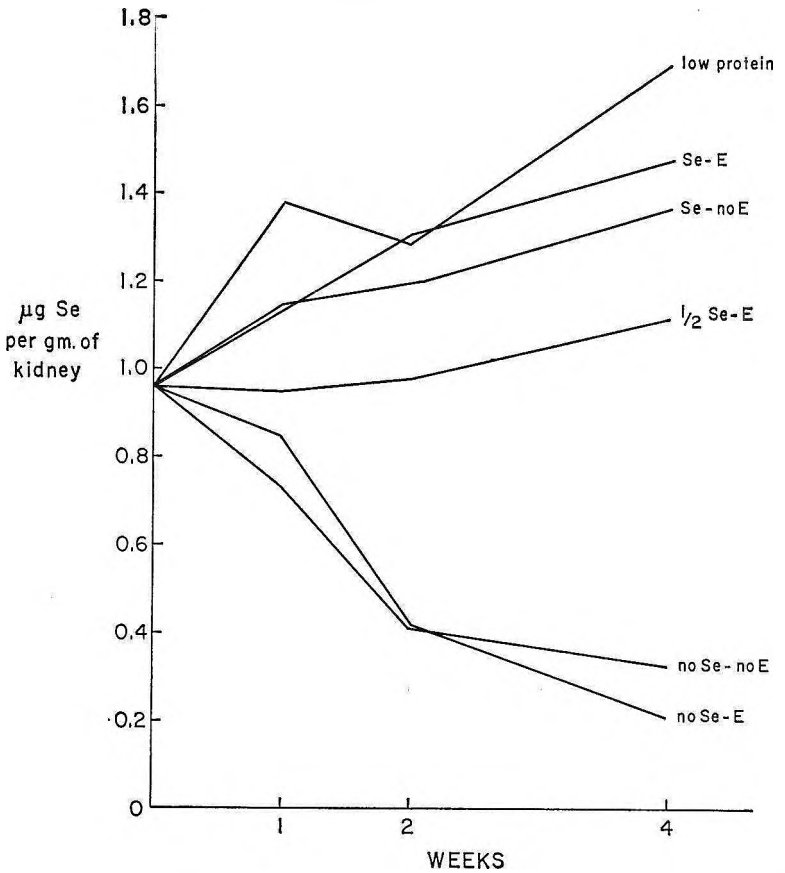

Fig. 8 Kidney selenium levels by groups in study 2. Each point represents the mean of 3 to 4 rats in all groups except the 1/2Se- vitamin E group in which 2 were averaged.

ion and hence the findings can not be explained by day-to-day methodological variation. It thus appears that dietary selenium may enhance the disappearance of vitamin E from the plasma when no vitamin E is given in the diet. Whether liver vitamin E levels were affected by selenium was not studied, but their determination would be of interest in view of the proposal of Scott (10) that a selenium compound may be a carrier for *d*-α-tocopherol.

Blood selenium levels only half as high as those of controls have been reported in infants with kwashiorkor (11), raising questions as to the cause and meaning of this finding. This study provides partial answers to each question if these data can be extrapolated to the human being. With respect to the possible cause of low blood levels of selenium in kwashiorkor, dietary intake of selenium was the only variable tested which lowered blood selenium levels. A low protein diet with adequate selenium did not markedly alter tissue selenium levels. The presence or absence of vitamin E in the diet had no pronounced effect on blood selenium levels. Accordingly, it appears likely that infants with kwashiorkor have low blood selenium levels primarily because their dietary intake of the element is low.

With respect to the meaning of the low blood selenium levels observed in kwashiorkor, these rat experiments may be of significance also. It was observed that low blood selenium levels are associated with low concentrations of selenium in liver, kidney and carcass. The reduction of selenium level is most pronounced in the liver. It is therefore likely that children with low

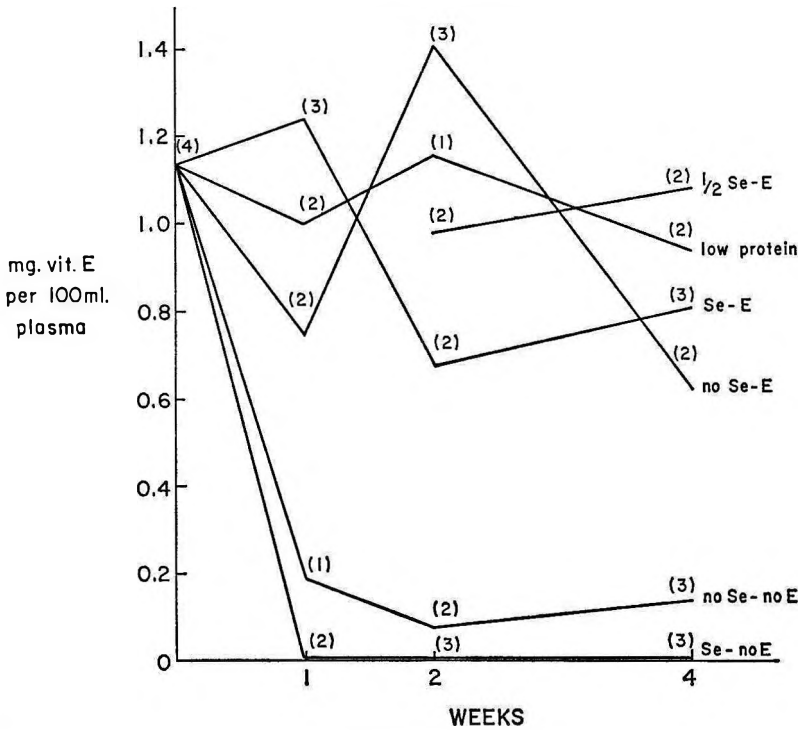

Fig. 9 Plasma vitamin E levels by groups in study 2. Numbers in parentheses indicate the number of animals averaged to determine the point.

blood selenium levels also have low total body selenium stores and more specifically low liver selenium levels. Whether low selenium levels contribute to the pathology in kwashiorkor is not known.

ACKNOWLEDGMENTS

We are grateful to Peter Heseltine for the vitamin E analyses and to Fran Adams for animal care.

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Effect of Supplementary Copper on Blood and Liver Copper-containing Fractions in Rats [1,2]

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ABSTRACT Fifty rats were fed rations containing copper, ranging from subnormal levels to toxic amounts, in order to study the influence of dietary copper on the copper-containing fractions of the blood and liver. Copper in the blood was determined as erythrocyte copper and plasma copper. Most of the plasma copper was in the protein ceruloplasmin, and the remainder in a loosely bound form reacting directly with Na diethyldithiocarbamate (DR Cu). Erythrocyte and DR Cu appear to be directly related to the Cu intake with values ranging from 0.95 to 2.04 μg/ml and 0.19 to 1.52 μg/ml, respectively. Ceruloplasmin values were depressed in the low rations (6.6 IU) and remained constant (35 IU) with Cu intakes greater than 10 ppm. In liver of normal rats (ca. 18 ppm Cu) the distribution is: debris, 12.8%; mitochondria, 13.5%; microsomes, 17.9%; and soluble fraction, 54.8%. Liver Cu levels were depressed in the low (1 ppm intake) group (10 ppm), normal in the range of 10 to 100 ppm intake (20 ppm), and elevated with 200 ppm intake (69 ppm). The distribution in the liver fractions was affected by copper content, with the soluble fraction being the most sensitive to changes in liver Cu, and the debris relatively insensitive to the Cu content of the liver.

Blood and liver can be fractionated into several copper-containing components. In humans, the serum copper level is 144 μg/100 ml compared with 89 μg/ml in the packed red blood cells (1). Adams and Haag (2) found this distribution to be equal in cattle. Copper is present in the serum in 2 forms. A blue copper protein, ceruloplasmin, accounts for most of the copper (1). The copper in ceruloplasmin is tightly bound and will not react with chelating agents without first destroying the molecule. The remainder of the copper in serum is loosely bound and will react directly with diethyldithiocarbamate and is termed "direct-reacting" copper (1, 3).

Brinkman (4) fractionated rat liver by differential centrifugation. The copper was distributed as follows: debris, 15%; mitochondria, 20%; microsomes, 10%; and soluble fraction, over 50%.

Dietary copper strongly influences the levels of copper in the blood and liver of most species fed either toxic (5–7) or copper-deficient diets (8–10). The present study was an investigation of the effects of dietary copper on the distribution of copper in blood and liver cellular fractions in rats, an area of limited research.

MATERIALS AND METHODS

Fifty 21-day-old rats (O.S.U. Brown) with equal sex distribution were selected from litters with five or more pups of the same sex. Five littermates from each of 10 litters were distributed at random between five experimental groups. The 5 groups of rats were fed a basal ration (table 1) supplemented with different levels of copper as $CuSO_4 \cdot 5H_2O$ as follows (g/kg): group 1, no supplement; group 2, 0.0392; group 3, 0.196; group 4, 0.392; and group 5, 0.784. Since the basal ration contained about 1 ppm copper, the copper content of the different rations was approximately 1, 10, 50, 100, and 200 ppm, respectively.

After 8 weeks, during which growth rates and feed intake were recorded, the rats were anesthetized with CO_2 and most of the blood was removed by heart puncture and citrated. Livers were obtained,

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[2] This report constitutes a portion of a thesis submitted by David B. Milne in partial fulfillment of the requirements for the Ph.D. degree.

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TABLE 1

Composition of copper-deficient diet no. 304–0

	g/kg diet
Casein	200
Glucose monohydrate [1]	650
Vegetable oil [2]	100
Mineral mixture [3]	40
Vitamin mixture [4]	10
α-Tocopherol	0.2
	μg/kg diet
Vitamin D (calciferol)	200

[1] Cerelose, Corn Products Company, Argo, Illinois.
[2] Wesson, refined cottonseed oil, Wesson Sales Company, Fullerton, California.
[3] Mineral mixture contained: (in grams) NaCl, 292.5; KH_2PO_4, 816.6; $MgSO_4$, 120.3; $CaCO_3$, 800.8; $FeSO_4 \cdot 7H_2O$, 56.6; KI, 1.66; $MnSO_4 \cdot H_2O$, 8.45; $ZnCl_2$, 0.5452; and $CoCl_2 \cdot 6H_2O$, 0.0476.
[4] Vitamin mixture contained: (in milligrams) thiamine·HCl, 40; riboflavin, 25; pyridoxine·HCl, 20; Ca D-pantothenate, 200; menadione, 10; folic acid, 20; biotin, 10; and (in grams) choline chloride, 10; niacin, 1; vitamin B_{12}(1%), 1; and lactose to make 100 g.

blotted, weighed, and frozen for subsequent analyses.

All copper analyses were performed by a method of Eden and Green (11) after digestion in perchloric, nitric, and sulfuric acids.

Blood samples were centrifuged shortly after removal and the erythrocyte volume was noted. The erythrocytes, plasma, and a portion of liver were analyzed for copper. Direct-reacting copper in the plasma was determined by the method of Gubler et al. (3). Ceruloplasmin was assayed as *p*-phenylenediamine oxidase by a method of Houchin (12) as modified by Rice (13).

Three livers were selected at random from each of the 5 groups, without regard to sex, homogenized in 9 parts of a 0.25 M sucrose solution, and then centrifuged at 700 × *g* for 10 minutes. The particles were resuspended in the sucrose solution and recentrifuged at 700 × *g* for another 10 minutes. This fraction was termed debris. The combined supernatants were then centrifuged at 5,000 × *g* for 10 minutes, the particles resuspended and centrifuged at 5,000 × *g* another 10 minutes to obtain the mitochondria. The combined supernatants from this step were centrifuged at 75,000 × *g* for 30 minutes to obtain the microsomes. The supernatant from this step was termed the soluble fraction. Each fraction was analyzed for its copper content.

RESULTS AND DISCUSSION

Tables 2 and 3 show the distribution of copper in blood and liver fractions of 12-week-old O.S.U. Brown rats maintained with a commercial laboratory ration.[4,5] In "normal" adult rats the plasma copper is slightly greater than in the erythrocytes. Most of the plasma copper is present in the tightly bound form in ceruloplasmin.

As noted in table 3, over half of the liver copper was in the soluble fraction and the remainder was distributed through the particulate fraction.

The difference in growth (table 4) could be attributed to decreased feed consumption as the copper level increased. Copper levels in the erythrocytes reflected the copper intake (table 5). Low copper rats had a significantly lower erythrocyte copper level. Rats receiving 100 and 200 ppm copper had a significantly higher level of copper in the erythrocytes compared with other treatments. Hemolytic jaundice, one of the symptoms of copper toxicity (14), was observed in group 5.

Plasma copper levels also increased with increasing amounts of supplementary copper. The direct-reacting copper appeared to directly reflect the copper intakes better than any other fraction. This indicates that copper is transported from the intestine to the tissues in a more

TABLE 2

Average copper levels in blood fractions for adult stock rats fed normal rations

Whole blood Cu, μg/ml	1.31
Erythrocyte vol, ml/100 ml	48.0
Erythrocyte Cu, μg/ml	1.22
Plasma Cu, μg/ml	1.40
Direct-reacting Cu, μg/ml	0.19
Ceruloplasmin, IU	39.4

TABLE 3

Average relative distribution of copper in rat liver (with normal rations)

Distribution of whole liver Cu (18.3 ppm dry wt)	
	%
Debris	12.8
Mitochondria	13.5
Microsomes	17.9
Soluble	55.8

[4] Purina Laboratory Chow, Ralston Purina Company, St. Louis.
[5] 15.6 ppm Cu.

TABLE 4

Growth and feed intakes as influenced by copper supplement

Group	Sex	Cu supplement	8-week wt gain	Avg daily food intake
		ppm	*g*	*g*
1	M	0	192	12.7
	F		153	12.2
2	M	10	189	11.5
	F		143	10.7
3	M	50	134	9.1
	F		124	9.3
4	M	100	85	5.0
	F		74	6.6
5	M	200	61	5.9
	F		62	6.1
		LSD 5%	20.1	0.4

loosely bound form. Ceruloplasmin activities, however, were significantly depressed in the group receiving the unsupplemented ration. This is in agreement with Starcher and Hill (15), who found reduced ceruloplasmin levels in copper-deficient sheep. No significant differences from normal values were found in the ceruloplasmin activities of the 4 groups receiving copper supplements. Copper is apparently necessary for inducing activity and excessive amounts of copper (i.e., 200 ppm) will not inhibit ceruloplasmin as *p*-phenylenediamine oxidase activity in vivo. No sex differences were noted in blood and liver copper levels.

The influence of dietary copper intake on whole liver copper levels is shown in figure 1. Rats receiving diets low in cop-

TABLE 5

Average copper content of blood fractions as influenced by copper

Group	1	2	3	4	5	LSD	P value
Supplementary Cu, ppm	—	10	50	100	200		
Erythrocyte vol, ml/100 ml	49.6	46.4	47.5	41.8	39.6	5.44	0.01
Erythrocyte Cu, μg/ml	0.95	1.63	1.45	1.89	2.04	0.37	0.05
Ceruloplasmin activity, IU	6.6	40.5	34.6	31.1	35.2	7.7	0.05
Direct-reacting Cu, μg/ml	0.19	0.35	0.54	1.17	1.52	0.62	0.05
Plasma Cu, μg/ml [1]	0.62	1.13	1.16	2.34	—		

[1] Pooled samples.

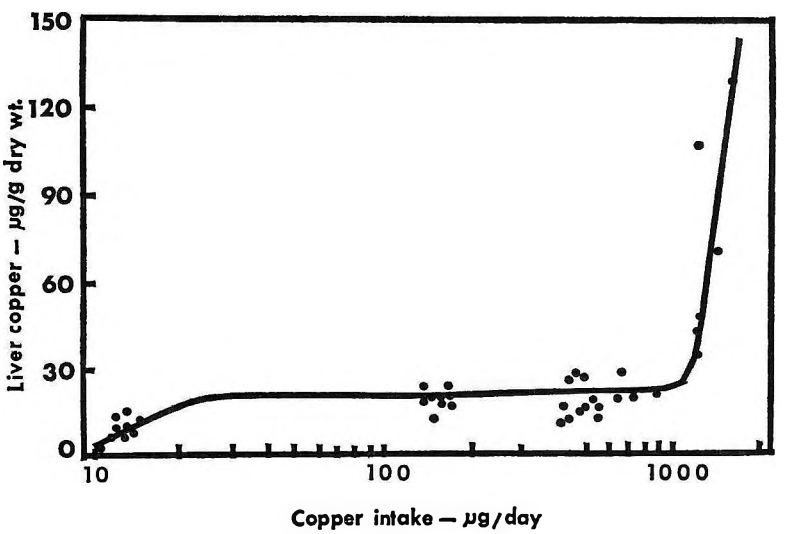

Fig. 1 Influence of copper intake on liver copper.

TABLE 6

Percentage distribution of copper in liver fractions

Group	Cu supplement	Whole liver Cu (dry)	Debris	Mitochondria	Microsomes	Soluble
	ppm	*ppm*	%	%	%	%
1	—	8.7	20.3	12.4	13.9	53.4
2	10	19.9	14.3	12.7	17.1	55.9
3	50	18.2	15.2	11.9	20.5	52.4
4	100	19.7	14.1	15.1	18.1	52.7
5	200	68.5	14.3	11.0	23.8	50.8

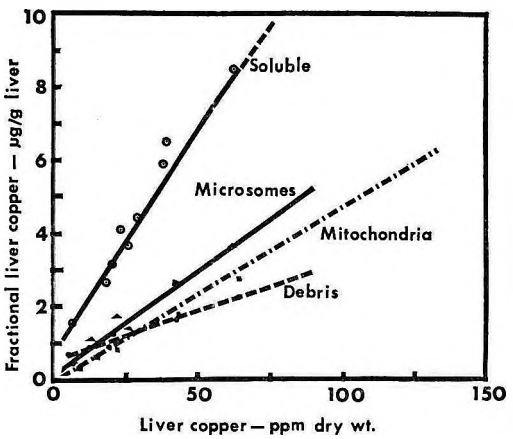

Fig 2 Relationship between liver copper and copper in fractions.

per had significantly reduced liver copper stores. With supplementation, liver copper levels remained normal until an intake of about 1,000 µg of copper per day was reached. This corresponds to approximately 200 ppm copper in the ration (see table 4). When this threshold was reached, liver copper increased rapidly, which suggested an overloading of the excretory capacity of the rat This threshold differs from species to species. Fifty parts per million of dietary copper are sufficient to elevate the liver stores in cotton rats,[6] while this threshold is not reached in swine until some point between 70 and 130 ppm dietary copper.

Cattle and sheep present an entirely different picture. In both cases the blood values reflect copper intakes only at subnormal levels (14, 16). When normal blood levels were reached, the liver copper content ranged from 30 to 1,000 ppm in cattle with no significant change in blood copper values (14) and from about 50 to 4,000 ppm in sheep with little change in blood levels (16). Beck (17) suggested that differences in these species were due mainly to differences in the excretory mechanism. However, it would be difficult to use this hypothesis to explain why the blood copper levels in ruminants remain relatively constant while liver stores change so markedly. Apparently, there must also be an increased ability to bind copper in the liver so as not to elevate blood levels.

Table 6 summarizes the average distribution of copper between liver fractions in the 5 groups of rats. The relative amount of copper in the soluble fraction and mitochondria remained essentially constant. The only apparent differences were a decrease in the relative amount in the debris and an increase in the relative amount of copper in the microsomes from the depleted group to the supplemented groups.

Generally, there was a linear increase in the amount of copper in each fraction with the total amount of copper in the liver (fig. 2). The soluble fraction was by far the most sensitive to changes in liver copper, whereas the copper content of the debris fraction changed relatively little. Copper levels changed about the same in both the mitochondria and microsomes with liver copper concentration. Thus, it was apparent that most of the excess copper was stored in the soluble fraction and that there was an equilibrium between the copper in the soluble fraction and the copper in the mitochondria and microsomes.

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Effect of an Amino Acid Imbalance on the Metabolism of the Most-limiting Amino Acid in the Rat [1]

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ABSTRACT Rats trained to consume their entire daily ration in 1.5 hours were used to study the effect of an amino acid imbalance on the uptake of the limiting amino acid into the protein and protein-free portions of a number of tissues. They were fed 7.5 g of one of the following 3 diets containing L-histidine-U-^{14}C: a 6% casein basal diet (B); and imbalanced diet (I) consisting of B plus an indispensable amino acid mixture devoid of histidine; or a corrected diet (C) consisting of diet I plus histidine. Eight and 12 hours after the animals were fed, samples of brain, liver, stomach, small intestine, large intestine plus cecum, muscle and portal and heart blood were obtained. After adjustment is made for dilution of histidine-U-^{14}C by the additional histidine in the corrected diet, the amounts of radioactivity in acid extracts of tissues and plasma from the corrected group (C) were highest followed in order by those from the basal (B) and the imbalanced groups (I). The specific activity of liver protein from the corrected group was also highest followed in order by that from the imbalanced and the basal groups. The results (basal vs. imbalance) suggest that amino acids added to this diet to create the imbalance induced the liver to make more protein and thus increased the efficiency of utilization of histidine by this organ. The relative deficiency of histidine in the imbalanced diet (imbalance vs. corrected) limits the ability of the animal fed this diet to synthesize liver protein. Rats fed the imbalanced diet for 1.5 hours/day did not exhibit the typical growth depression observed in animals fed this type of diet ad libitum.

The general topic of amino acid imbalance has been reviewed regularly and hence a general review of the subject here is not warranted (1–5). Recently studies using ^{14}C-labeled amino acids have added to our understanding of the metabolism of the limiting amino acid by rats fed a diet in which there is an amino acid imbalance (6). These studies have shown that animals fed a single meal of such a diet containing threonine-U-^{14}C expire less, rather than more, of the limiting amino acid as CO_2 than controls and that they incorporate as much if not more of the limiting amino acid into carcass and tissue proteins.

The purpose of the present investigation was to extend our knowledge of the metabolism of histidine by rats fed a diet in which an imbalance was created by adding a mixture of amino acids devoid of histidine to a low protein diet. Several tissues not previously studied were examined to determine whether an amino acid imbalance influenced the competition among organs for the limiting amino acid and whether amino acid uptake into protein was enhanced in the entire intestinal tract as well as in the liver. Measurements were made at 2 time-intervals to determine how the distribution of radioactivity in tissue fractions would change with time after ingestion of the meal. Also, a group of rats receiving an imbalanced diet containing supplemental histidine (corrected) was included to permit comparisons between groups fed the 2 types of deficient diets (basal and imbalanced) and between these 2 groups and one fed a much more adequate diet (corrected).

MATERIALS AND METHODS

The 36 male rats of the Sprague-Dawley strain (120–130 g) used in this experiment were housed in individual screen-bottom cages in a room maintained at 25°.

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They were trained to eat for only 1.5 hours daily. Two weeks before the experiment all rats were switched from a 15% casein diet to one containing 6% of casein. One week before the trial the diet was changed to one containing 3% of casein and 3% of amino acids. The composition of this diet was: (in percent) casein, 3; indispensable amino acid mixture, 1.5 (7); histidine·HCl·H_2O, 0.1; 3:1 mixture of glutamic acid and glycine, 1.4; L-methionine, 0.3; vitamin mixture, 0.5 (8); salt mixture, 5.0 (8); choline-chloride, 0.2; sucrose, 28.5; dextrin, 54.5; and corn oil,[2] 5.0. The diet was prepared as an agar gel (8). This diet was used to allow the rats to adjust to the taste of free amino acids in the hope that they would readily consume the diets containing amino acids on the day of the experiment.

On the morning of the isotope experiment, 6 rats were fed 5 g of the basal diet, six the corrected and six the imbalanced diet.[3] Each 5-g portion contained 13 μCi of L-histidine-U-^{14}C. As soon as they had eaten the 5 g portion containing the labeled amino acid they received an additional 10 g of the same agar gel diet without labeled histidine. Analysis of the histidine-^{14}C by ion exchange chromatography indicated that 97% of the radioactivity chromatographed in a single peak.

Eight and 12 hours after the animals were fed, 18 of the rats (6/group) were killed and samples of brain, liver, stomach, small intestine, large intestine plus cecum, muscle, and portal and heart blood were obtained. The tissue samples were immediately weighed and then frozen until analysis was performed. The tissue samples were homogenized in distilled water. One portion of the homogenate was used to determine total nitrogen by the Kjeldahl method and another portion was precipitated with trichloroacetic acid (TCA) or sulfosalicylic acid (SSA) and then centrifuged. The radioactivity of aliquots of the TCA and SSA supernatants from tissues and plasma were measured using the scintillation fluid described by Bruno and Christian (9). The TCA-precipitable material from the tissue samples was then resuspended in its original volume with 10% TCA and incubated at 80° for 1 hour to extract the nucleic acids from the protein. The sample was then filtered on a small piece of fine hard filter paper (Whatman no. 50), wrapped in a large filter paper, and extracted in a Goldfisch extractor for 6 hours with anhydrous diethyl ether, then for another 6 hours with chloroform. The protein samples were dried in a vacuum oven at 60° and stored until they were analyzed for nitrogen and radioactivity. Analysis of the residue from the diethyl ether and chloroform extracts showed that only 1% of the nitrogen was lost by the extraction procedure used. Munro (10) had previously indicated that from 20 to 50% of the nitrogen in the TCA precipitate could be removed by conventional procedures. Ten-milligram portions of the dried protein powder were weighed into a scintillation vial and then solubilized in 2 ml of N sodium hydroxide as described by Yoshida (6), neutralized and 15 ml of scintillation fluid (9) added, and counted without and with internal standard (toluene-^{14}C) to determine the radioactivity of the protein. Similar amounts of protein were used for determination of nitrogen content by the Kjeldahl method.

The proportion of the total radioactivity remaining in the intestinal tract was estimated by washing the contents of the complete tract into a beaker. The sample was acidified and stored in a freezer, and later homogenized and neutralized before the radioactivity of a 2-ml sample was determined, using the same technique described for the tissue TCA extracts.

The radioactivity of the "carcass" was estimated by dissolving it in a solution made up of 400 ml of 40% KOH and 400 ml of 95% ethanol. The dissolved material was diluted to one liter with distilled water and a small aliquot neutralized and counted using the same techniques used for the TCA supernatants.

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[2] Mazola, Corn Products Company, New York.

[3] The basal diet contained: (in percent) casein 6; L-methionine 0.3; vitamin mix (7) 0.5; salt mix (7) 5.0; choline chloride 0.2; sucrose 28.5; dextrin 54.5; and corn oil 5.0. In the imbalanced diet 6% of the carbohydrate was replaced with an amino acid mixture lacking histidine (6). The corrected diet contained the amino acid mixture plus 0.1% histidine HCl·H_2O. The diets were prepared as gels by suspending them in an equal weight of 3% agar solution in 10 ml beakers. (Note: disposable syringes with the ends cut off are much more convenient.)

Since the literature indicated that rats could grow normally with the imbalanced diet if they ate enough food (2, 7, 11), the rats not used for the metabolism study were assigned to the basal, imbalanced or corrected diet and maintained with these diets for 3 weeks with food available only 1.5 hours out of each twenty-four. The objective of this part of the study was to learn whether animals trained to consume their food in a 1.5-hour period exhibit the depressed food intake and growth and undergo the adaptation to the imbalanced diet observed in animals fed the diet ad libitum.

RESULTS

The growth rates of rats fed the imbalanced or basal diets were considerably less than those of rats fed the corrected diet when the diet was offered 1.5 hours a day (fig. 1). The daily dry matter consumed by the 3 groups of rats is shown in figure 2. Rats fed the imbalanced diet voluntarily restricted their food intake only after the second day. This observation differs from observations made on rats offered these diets ad libitum; the latter restrict their intake in as short a period as 4 hours (2, 5, 12). If it were not for the fact that these rats decreased their voluntary food intake (fig. 2), one would readily conclude that rats trained to eat for only 1.5 hours a day are unaffected by an amino acid imbalance which will cause a reduction in the growth of rats with free access to the diet (1–3, 5).

The carcass composition of rats consuming the imbalanced diet must be different from that of rats fed the basal diet because the former gained the same amount of weight while eating less food (figs. 1 and 2). Therefore, carcass composition was determined at the end of the experiment by drying the whole rat to a constant weight. The carcass composition was calculated from the water loss, assuming that the lean body contains 74.5% water (13). The weight difference between the live body weight and the lean body mass was assumed to be fat. The values for the total body fat content of the basal, corrected and imbalanced groups were 6.9, 5.4 and 4.5%, respectively, and although the fat content of those fed the imbalanced diet was low, the values were not different from each other at the 5% level of probability. These total carcass fat percentages appear to be quite low; possibly the factors derived for the water content of the lean body mass from rats fed ad libitum cannot be used for rats offered food only 1.5 hours per day.

A summary of the body and organ weights of rats used in the isotope study is presented in table 1. The values for the rats killed at 8 and 12 hours did not differ significantly.

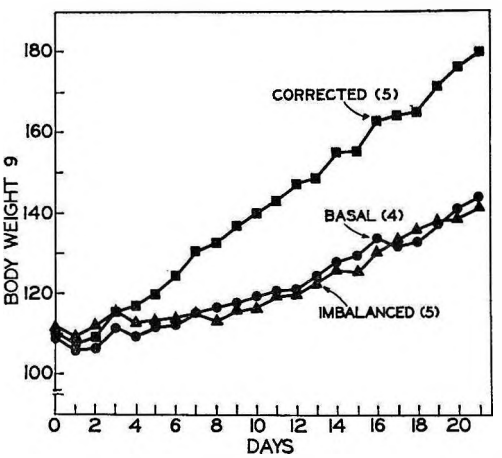

Fig. 1 Growth rates of rats fed the basal, corrected or imbalanced agar-gel diets for only 1.5 hours each day. Figures in parentheses indicate the numbr of animals/group.

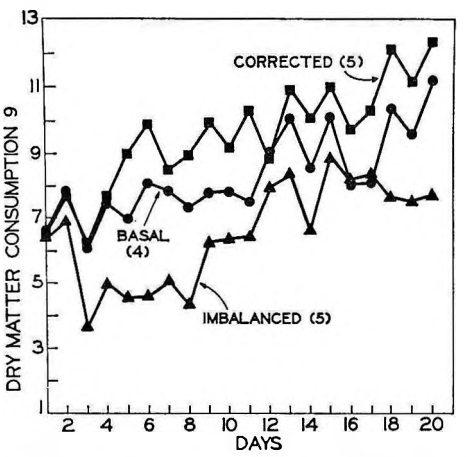

Fig. 2 Dry matter consumption of rats fed the basal, corrected and imbalanced agar-gel diets for only 1.5 hours each day. Figures in parentheses indicate the number of animals/group.

TABLE 1

Rat and organ weights [1]

Group	Body	Liver	Stomach	Small intestine	Large intestine + cecum	Brain
	g	*g*	*g*	*g*	*g*	*g*
			8 hours			
Basal	125.1 ± 2.8 [2]	6.60 ± 0.32	1.03 ± 0.04	4.46 ± 0.20	1.43 ± 0 07	1.31 ± 0.04
Corrected	127.3 ± 3.2	6.48 ± 0.23	1.14 ± 0.09	4.48 ± 0.24	1.49 ± 0.06	1.36 ± 0.03
Imbalanced	125.5 ± 2.7	6.41 ± 0.29	1.08 ± 0.03	4.44 ± 0.13	1.55 ± 0.09	1.32 ± 0.04
			12 hours			
Basal	118.6 ± 2.4	6.59 ± 0.30	1.08 ± 0.04	4.00 ± 0.06	1.29 ± 0.04	1.35 ± 0.02
Corrected	111.0 ± 8.0	6.77 ± 0.61	1.08 ± 0.05	4.15 ± 0.17	1.43 ± 0.06	1.38 ± 0.03
Imbalanced	121.3 ± 2.3	6.01 ± 0.20	1.05 ± 0.02	3.91 ± 0.19	1.36 ± 0.04	1.34 ± 0.03

[1] Six animals/group.
[2] Mean ± SE.

Values for total nitrogen content of the liver, stomach, small intestine and large intestine plus cecum at the 8- and 12-hour sampling times are shown in table 2. The values for the gastrointestinal tracts of the 3 groups did not differ at either of the killing times. The values for total liver nitrogen content at 8 and 12 hours for the corrected and imbalanced groups tended to be higher than for the basal group, although the differences do not exceed the 5% level of probability.

The amounts of radioactivity from the 13 μCi of L-histidine-U-^{14}C in the diet recovered in the intestinal tract washings are shown in table 3. The washings from rats fed the imbalanced diet contained about 5% more ($P < 0.05$) of the dose at the 8-hour sampling time. There were no significant differences at 12 hours. The 8-hour values for the various tissues were not corrected for this difference because the majority of the values obtained at 8 and 12 hours were not different from each other. This suggests that the major portion of the metabolism of the label had occurred before 8 hours and that such a correction would not be valid.

One point should be emphasized with respect to the presentation of the results of the isotope study. The values for the corrected group in the tables and figures were not adjusted for the dilution of the radioactive histidine by unlabeled histidine added to correct the imbalance. Precise corrections can be made only when the specific activities of the precursor pools over the time-period studied are known with certainty. This cannot be done in experiments of this type but a rough correction can be made on the assumption that specific activities of the pools are proportional to specific activities of the diets. The values for the corrected group, which received additional histidine, should therefore be multiplied by 1.5 in making direct comparisons with basal and imbalanced groups. Although statistical comparisons have been made on the basis of the actual radioactivity measurements, the effects of corrections of this type are

TABLE 2

Total nitrogen in liver and three segments of the intestinal tract

Group	Liver	Stomach	Small intestine	Large intestine + cecum
	mg	*mg*	*mg*	*mg*
		8 hours		
Basal	129.5	25.1	90.0	29.1
Corrected	143.8	25.6	89.3	29.1
Imbalanced	140.9	27.0	90.9	30.8
		12 hours		
Basal	124.1	25.6	81.9	21.4
Corrected	146.0	25.3	90.7	26.7
Imbalanced	130.1	27.1	73.9	26.7

TABLE 3

Recovery of isotope in intestinal contents [1]

Time after feeding	Basal	Corrected	Imbalanced
hr	*μCi*	*μCi*	*μCi*
8	1.50 [a]	1.86 [ab]	2.18 [b]
12	0.99	1.48	1.62

[1] Means with same letter not significantly different at $P < 0.05$.

shown on figures 2, 3 and 4 by the unshaded areas above the shaded bars for the corrected group.

Values for radioactivity in the acid-soluble portion of the cell at the 8- and 12-hour sampling times are shown in figure 3. Limited chromatographic analysis of these samples showed that histidine and carnosine were the only amino compounds labeled with ^{14}C. In general, with the exception of the values for the small intestine and stomach, amounts of radioactivity in the tissue extracts from the basal group tend to be greater than those for the imbalanced group. The correction for dilution increased the values for the corrected group to or above those for the basal group. For each group the amounts of radioactivity in blood taken from the heart and the portal vein were similar, which suggests that little material was being absorbed from the intestinal tract at the time of sampling.

The relatively high levels of soluble radioactivity found in the entire intestinal tissues suggest that the intestinal tract competes well with the remainder of the body for amino acids circulating in the blood. By using the organ weights shown in table 1, it can be calculated that the gastrointestinal tract contains as much soluble radioactivity as the liver, if not

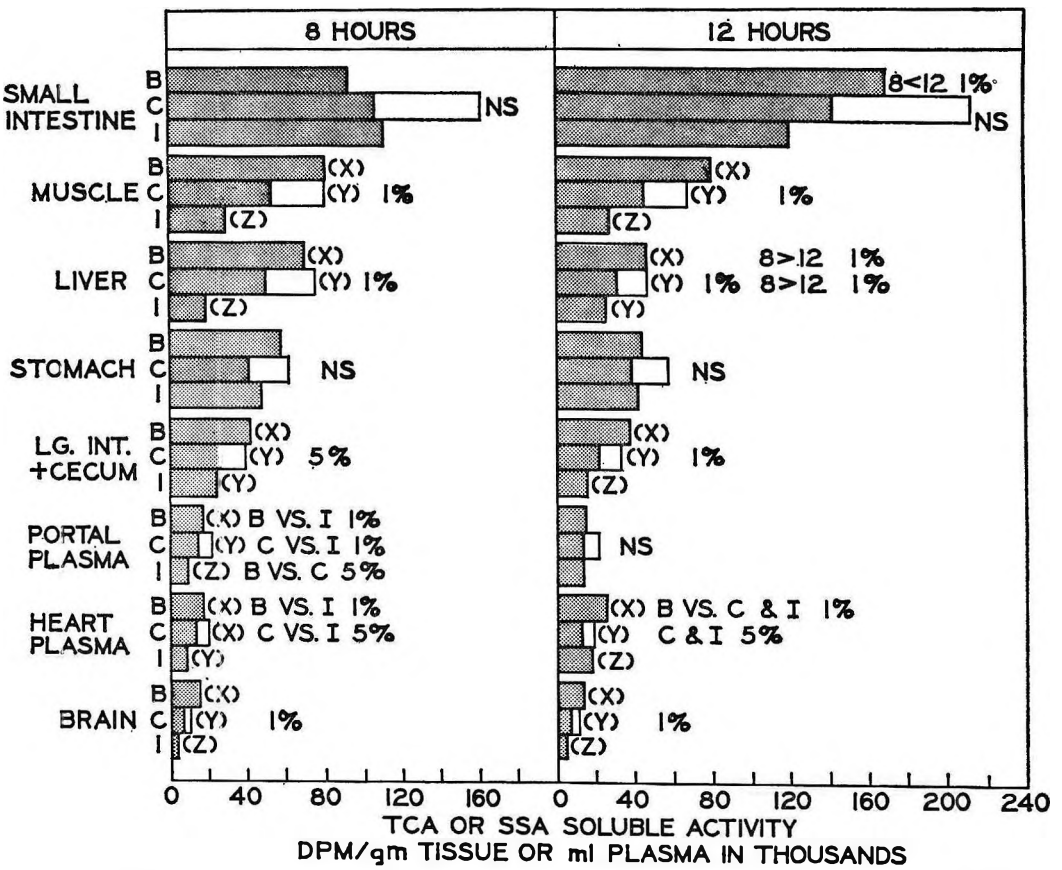

Fig. 3 Radioactivity soluble in TCA or SSA extract of 1 g tissue or 1 ml of plasma 8 or 12 hours after feeding L-histidine ^{14}C to rats receiving basal (B), corrected (C) or imbalanced (I) diets. The unshaded sections of the bars for the corrected group represent the values adjusted for dilution of the radioactivity by the histidine supplement. The non-adjusted values were used for the statistical analyses. Means with the same letter (x, y, z) are not different from each other. Those with different letters are different from each other at the level of probability indicated. Differences between time-periods are indicated by $8 < 12$ 1%.

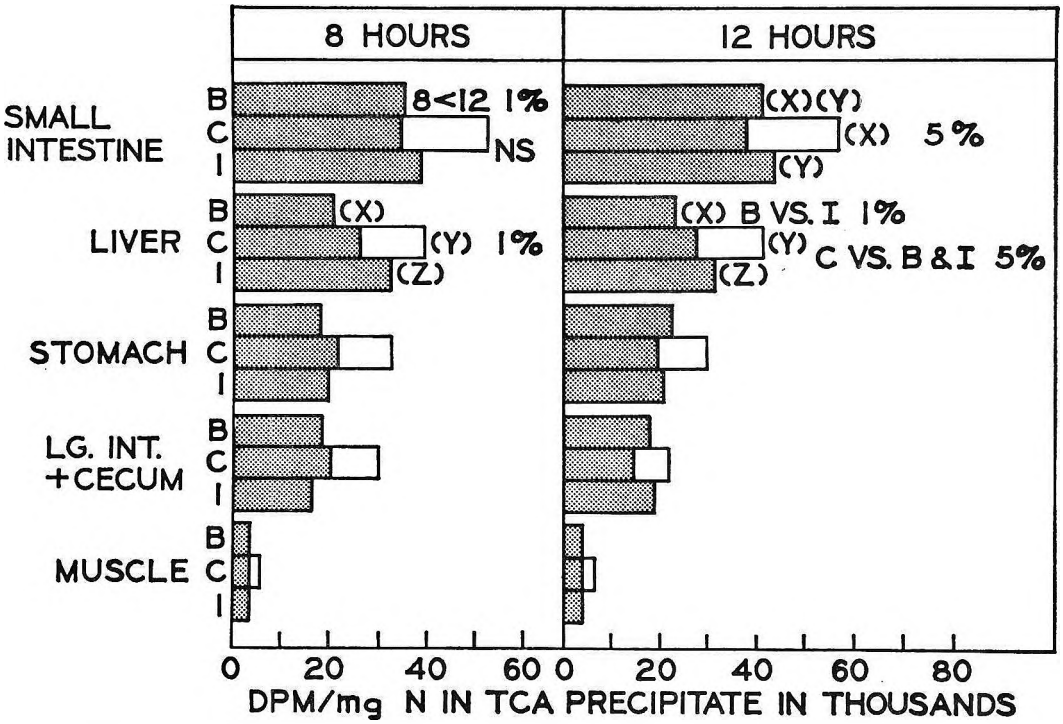

Fig. 4 Radioactivity of semipurified protein from the TCA precipitate 8 and 12 hours after feeding L-histidine ^{14}C to rats eating basal (B), corrected (C), or imbalanced (I) diets. The unshaded sections of the bars for the corrected group represent the values adjusted for dilution of the radioactivity by the histidine supplement. The non-adjusted values were used for the statistical analyses. Differences between means and time-periods are indicated as per figure 3.

more. Although the amounts of radioactivity per gram in muscle are not the highest, the major portion of the soluble radioactivity is in muscle because it makes up such a large portion of the total weight of the animal.

Although no great differences were observed between the 8- and 12-hour tissue samples, some tissues changed significantly. The TCA-soluble radioactivity of the small intestine increased significantly only in rats fed the basal diet although the other 2 groups increased slightly over the 4-hour time-period. The TCA-soluble radioactivity in the livers of the basal and corrected groups decreased significantly from the 8-hour to the 12-hour sampling times.

The amounts of radioactivity found in the protein fraction of each tissue are shown in figure 4. Only in the liver were there significant differences among all of the groups. The amount of radioactivity in the small intestine of the basal group increased from 8 to 12 hours, but as none of the other differences were significant, it appears that the major portion of the metabolism of the labeled amino acid had been completed before 8 hours. Correction for dilution of radioactivity (unshaded bars) gave values for the corrected group for all tissues that were significantly higher than those for the other 2 groups.

Again it should be pointed out that the total amount of radioactivity in the protein fraction of the intestinal tract is greater than that in the liver when one takes into account the weights of the various segments of the intestinal tract recorded in table 1. The entire intestinal tract competes effectively with the remainder of the body for the limiting amino acid. This notion is supported by the work of Hansson (14), who showed, using autoradiograms, that rats injected with ^{35}S-DL-methionine and killed after only 5 minutes have considerable radioactivity in the entire in-

testinal tract, and by Hartman and King (15), who found results similar to ours using ^{15}N-labeled histidine.

The values for total radioactivity per gram of tissue (fig. 5) were calculated by determining the milligrams of nitrogen per gram of tissue and multiplying the disintegrations per minute per milligram of nitrogen of the protein fraction of the tissue by this number and adding to this the amount of radioactivity extracted from 1 g of tissue by TCA. These calculations probably overestimate the radioactivity per gram of tissue because the nitrogen of the nucleic acid fraction is assigned the same ^{14}C content relative to nitrogen as the purified protein of the cell. The values given for carcass are direct measurements.

Even though the differences in the soluble and protein fractions of the liver of basal, corrected and imbalanced groups were opposite in direction (figs. 3 vs. 4), the amount of radioactivity retained in the protein fraction of the liver was so great that the calculated total radioactivity per gram of liver is greatest in the imbalanced group followed by the corrected and basal groups. However, after correction for dilution (unshaded bars), the values for the corrected group for liver and for other tissues were significantly higher than those for the other 2 groups. The total radioactivity of the small intestine of the basal group increased significantly from 8 to 12 hours. It is not unexpected that the total radioactivity per gram of tissue in the 3 portions of the

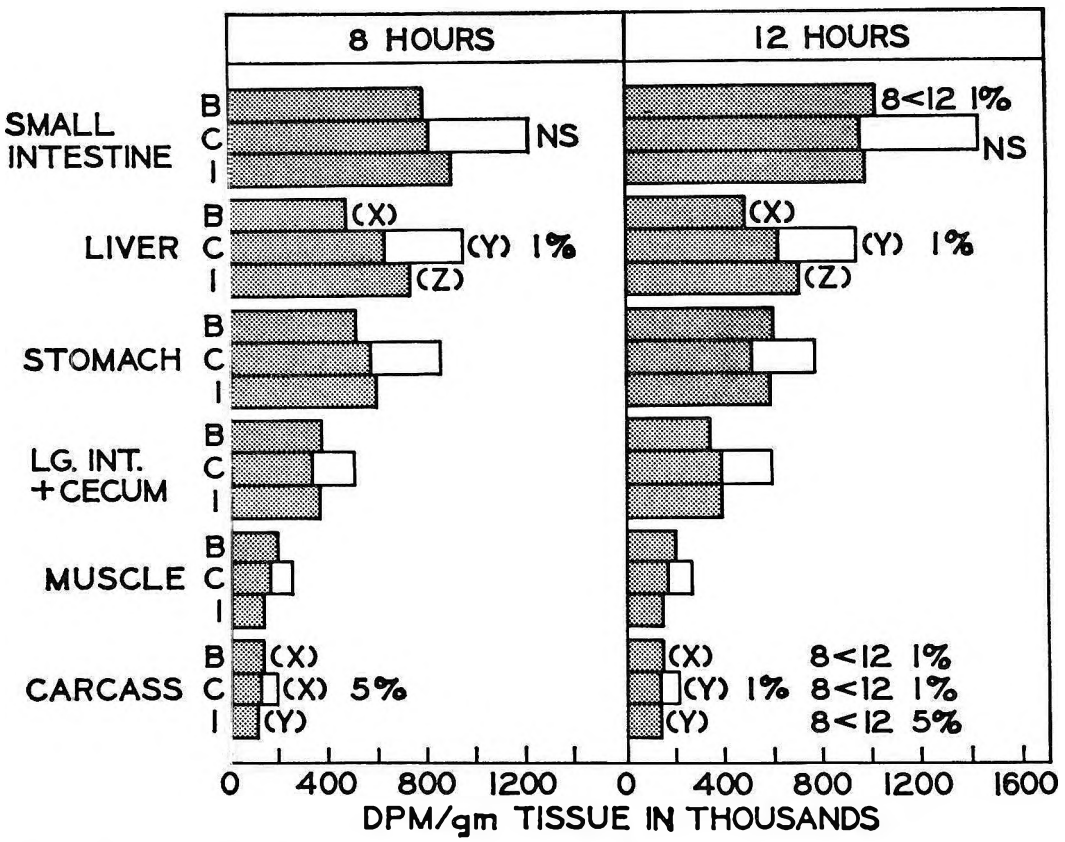

Fig. 5 Calculated total activity/g of tissue 8 and 12 hours after feeding L-histidine ^{14}C to rats consuming basal (B), corrected (C), or imbalanced (I) diets. The unshaded sections of the bars for the corrected group represent the values adjusted for dilution of the radioactivity by the histidine supplement. The non-adjusted values were used for the statistical analysis. Differences between means and time-periods are indicated as per figure 3.

intestinal tract is so high since the amounts of radioactivity in the soluble and protein fractions of these samples were greater than or nearly equal to those in the liver. The values for radioactivity in "carcass" for the basal group were above those for the imbalanced group and, when the correction is made for dilution, the values for the corrected group are significantly greater than those for the other two. All the values were higher at 12 hours than at 8 hours.

The values in table 4 were obtained by multiplying the amount of radioactivity per unit weight (fig. 5) of each fraction by the actual weight of the organ or fraction and expressing each value relative to that of the liver. It is apparent that the proportions of radioactivity in the liver and the entire intestinal tract or individual segments did not change materially between groups or with time. The imbalanced and corrected groups appear to retain a greater portion of the radioactivity in the visceral organs at both time-periods than the basal group.

DISCUSSION

The effect of the amino acid imbalance on efficiency of utilization of histidine can be assessed directly in this experiment because the group fed the basal and that fed the imbalanced diet consumed the same total amount of radioactive and nonradioactive histidine. The effect of a relative deficiency of histidine on protein synthesis can be assessed by comparing the results for the group fed the imbalanced diet with those for the group fed the corrected diet after adjustment is made for the lower specific activity of histidine in the corrected diet. This adjustment is shown by the unshaded parts of the bars for the corrected group in figures 3, 4 and 5.

The observations on the effect of an imbalance agree with those of Yoshida et al. (6) in that the protein fractions of liver from rats fed the imbalanced diet contained more radioactivity than those of rats fed the basal diet; and in that the TCA extracts of liver from rats fed the imbalanced diet contained less radioactivity than those from rats fed the basal diet. Our observations that the specific activities of muscle proteins from the basal and imbalanced groups were not different whereas the amount of radioactivity in the SSA extract of muscle from the basal group was much higher than that for the imbalanced group, are also similar to those reported by Yoshida et al. (6). Our observations that the specific activities of intestinal proteins from basal and imbalanced groups are not significantly different also agree with the results of the previous study (6); however, differences which resemble those noted in the liver may occur early (i.e., 1 to 3 hours) in the small intestine and disappear by the 8-hour sampling time.

TABLE 4

Ratio of total radioactivity in the intestinal tract and carcass relative to that in the liver

	8 hours			12 hours		
	Basal	Corrected	Imbalanced	Basal	Corrected	Imbalanced
Stomach	0.17	0.16	0.13	0.20	0.13	0.14
Large intestine + cecum	0.19	0.16	0.14	0.13	0.10	0.11
Small intestine	1.09	0.89	0.84	1.28	0.94	0.89
Liver	1.00	1.00 (1.28) [2]	1.00 (1.50)	1.00 [1]	1.00 (1.33)	1.00 (1.33)
Carcass	4.30	3.30	2.53	5.62	3.75	3.69
Intestinal contents	1.06	1.03	1.02	0.70	0.77	0.85

[1] Values for 8 and 12 hours identical.
[2] Numbers in parentheses show the activity of the liver of the corrected and imbalanced groups relative to that of the basal group.

The adjusted values for specific radioactivity of proteins and tissues of rats fed the corrected diet (figs. 4 and 5) are without exception higher than those for the other 2 groups. This would be predicted, as the corrected group, in effect, receives nearly twice as much of a balanced protein and amino acid mixture as the basal and imbalanced groups and always outgrows them. This comparison and the one above indicate clearly the difference between an amino acid imbalance and an amino acid deficiency.[4] Effects of an amino acid deficiency are attributable to an inadequate supply of one amino acid and comparisons are made between 2 groups fed diets containing different quantities of the growth-limiting amino acid. Effects of an amino acid imbalance are attributable to a surplus of amino acids other than the one that is growth-limiting and comparisons are made between 2 groups fed diets containing the same amount of the growth-limiting amino acid but different amounts of several other amino acids.

An increase in amino acid incorporation into liver protein, resembling that reported above in rats fed imbalanced diets, has been observed by Sidransky and associates (16-18) and by Nimni and Bavetta (19) in rats fed diets devoid of one essential amino acid. However, comparing a group fed a diet devoid of one indispensable amino acid (16-18) with a group fed an adequate diet resembles more closely our comparison of the imbalanced with the corrected group than of the imbalanced with the basal group.

The conclusion drawn from comparing the imbalanced and "adjusted" corrected groups in this study are just the opposite of those reported by Sidransky (16–18), but are in full accord with those reported by Hartman and King (15) as expressed by their calculation of net assimilation of histidine. The rats used in this study and that of Hartman and King were fed at the time of the incorporation experiment, whereas those used by Sidransky had been force-fed an amino acid-devoid diet for 3 or 7 days and then fasted for 18 hours before the isotope study. Our study and that of Hartman and King involved the use of an isotope in the diet, whereas Sidransky used an intraperitoneal injection. The single injection of an isotope and its concomitant use in metabolism is complete within a very short time as shown by Borsook et al. (20) who found that labeled amino acids injected directly into the blood stream may be completely removed from the blood within 10 minutes. They also observed little ^{14}C in CO_2 after the first hour of collection, suggesting that the metabolism of the isotope was complete by that time. Hence, results of experiments in which the isotope is administered by injection probably reflect events occurring over a relatively short time-interval, whereas those in which the isotope is fed would reflect events occurring over a much longer time-interval. Whether these differences in experimental technique are sufficient to explain the differences between the results of this study and that of Sidransky remains to be clarified. Variations in enzyme, hormone and substrate patterns as a result of the different pretreatments may also contribute to the divergent results.

In contrast with the observations on enhanced liver protein synthesis by rats fed various amino acid-devoid diets discussed above, Munro and associates (23, 24) and Sidransky and associates (25) have reported that incorporation of radioactive amino acids by microsomal preparations of liver from rats fed a tryptophan-devoid diet is depressed. This decrease in incorporation was associated with a shift in the polysome profile such that there were relatively fewer polysomes and more monosomes and disomes. Yet Wunner[5] has shown that diets devoid of arginine, phenylalanine or methionine-cystine do not materially affect the liver polysome profile and Sidransky and associates (17)[6] have shown increased activity of liver ribosomes from animals fed methionine- and isoleucine-devoid diets.

The increase in protein synthesis observed in the livers of rats fed the imbal-

[4] This may be of some interest in relation to the recent exchange of letters on this subject between Dr. Hans Fisher and Dr. Kendall King (J. Nutr., *93*: 564, 1968).

[5] Wunner, W. H. 1967 Amino acid deficiency and rat-liver polysome stability. Biochem. J., *103*: 71p (abstract).

[6] Sidransky, H., and M. Bongiorno 1967 The response of mouse liver polyribosomes to amino acid mixtures — complete or devoid of single essential amino acids. Federation Proc., *26*: 409 (abstract).

anced diet (fig. 4) suggests that their liver polysome patterns are not altered in the same manner as in animals fed tryptophan-devoid diets (23–25).

The observations on uptake of ^{14}C into muscle proteins do not resemble those of Sidransky and Verney (18, 26) in animals fed a threonine-devoid diet since they found decreased amounts of ^{14}C in muscle proteins. However, in part this decrease may be accounted for by the 15 to 30% reduction in muscle weight of their animals (16, 18) because their results are reported as total radioactivity in the gastrocnemius muscle (18, 26). They also observed a decrease in the activity of muscle ribosomes. This would not be expected in our rats fed an imbalanced diet which showed no depression in incorporation of radioactivity into muscle protein.

Increased free amino acid concentrations in liver may contribute to increased incorporation of the limiting amino acid into liver proteins in animals fed the imbalanced diet. Leung [7] found that livers of rats fed threonine-imbalanced diets contained 1.4, 3.2 and 1.4 times as much free amino acids as livers of animals fed the basal diet 3, 5 and 8 hours, respectively, after they had consumed their diets. Similar results have recently been reported by Sanahuja and Rio (27). The effect of cellular amino acid concentrations on protein synthesis was shown by Riggs and Walker (28) and Eagle et al. (29) because protein synthesis of tumor cells and cultured human cells was greatly elevated when the cellular concentration of amino acids was increased. Hanking and Roberts have reported (30, 31) that high levels of phenylalanine, threonine or valine — 3 times normal plasma levels — increased the incorporation of leucine into the protein of rat liver slices, and intubation of 4 mg of tryptophan to fasted mice significantly increased leucine incorporation into liver proteins (25). Liver ribosome protein-synthesizing activity is enhanced some 40 to 50% when liver slices are incubated in high concentrations of phenylalanine and threonine (22). More direct evidence is required before definite conclusions may be drawn as to whether high concentrations of free amino acids in the livers of rats fed imbalanced diets induce the synthesis of more protein and thus effectively remove enough of the limiting amino acid from the circulation to distort the plasma amino acid pattern and, in one way or another, cause a reduction in voluntary food consumption.

Some of the results obtained in this study suggest that the experimental animals were not synthesizing liver and intestinal proteins at a constant rate. The observation that only one of the protein samples taken at 12 hours contained significantly more radioactivity than those taken at 8 hours even though 10 to 15% of the isotope consumed remained in the intestinal tract at 8 hours, appears to indicate that the rate of incorporation of amino acids into liver and intestinal proteins decreased with time after ingestion of the meal. This suggests that the rate of protein synthesis in visceral organs is greater during the early period of nutrient absorption than during the later period. This thesis is, in general, supported by the recent report of Hartman and King (15).

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Urinary Aminoimidazolecarboxamide in the Rat as Influenced by Dietary Vitamin B_{12}, Methionine and Thyroid Powder [1,2,3]

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ABSTRACT The objectives of this investigation were to 1) determine whether aminoimidazolecarboxamide (AIC), like formiminoglutamic acid, was elevated in the urine of rats fed a vitamin B_{12}-methionine-deficient diet; 2) assess the value of thyroid powder supplementation in enhancing the development of vitamin B_{12} deficiency; and 3) study the effect of supplementary methionine on AIC excretion. Vitamin B_{12}-supplemented controls averaged about 20 μg of urinary AIC/day. Rats fed the vitamin B_{12}-deficient diet for 6 weeks or more excreted at least twice as much. Thyroid powder did not alter urinary AIC excretion, nor did it enlarge the difference in weight gain between vitamin B_{12}-supplemented and deficient animals. Injection of 10 μg of vitamin B_{12} or supplementation of the diet with 2% of DL-methionine lowered AIC excretion of vitamin B_{12}-deficient rats to the level of vitamin B_{12}-supplemented controls, but had no effect on the AIC excretion of the control animals. The results of this investigation are consistent with the theory that vitamin B_{12} influences folic acid metabolism at the level of methionine biosynthesis.

In humans, megaloblastic anemia may occur with deficiencies of either folic acid or vitamin B_{12}. When this anemia is caused by a vitamin B_{12} deficiency alone (such as pernicious anemia), temporary remission can be induced by the administration of large doses of folic acid, which suggests that the 2 vitamins interact metabolically (1). Furthermore, formiminoglutamic acid (FIGLU), a metabolite dependent upon tetrahydrofolic acid (THF) for its degradation to glutamic acid in histidine catabolism (2), is elevated in the urine of either vitamin B_{12}- or folic acid-deficient rats (3) and chicks (4) fed low methionine diets. FIGLU excretion is lowered in such animals by feeding a methionine supplement. The same appears to occur in humans (5).

These clinical and nutritional observations, in conjunction with the knowledge that both vitamin B_{12} and folic acid cofactors participate in methionine biosynthesis in higher animals, have led to the hypothesis (6–8) that tissue levels of various THF cofactors are regulated at the site of methionine biosynthesis. According to this theory, in the event of dietary deficiencies of both vitamin B_{12} and methionine, there exists a functional folic acid deficiency (THF "trapped" as 5-methyl-THF) which can be overcome by either methionine or vitamin B_{12}. If this is true, then a methionine-vitamin B_{12} deficiency should affect all folic acid cofactor-dependent reactions as it does FIGLU metabolism.

Aminoimidazolecarboxamide (AIC) is the hydrolysis product of AIC-ribotide, an intermediate of purine biosynthesis which is dependent upon 10-formyl-THF for eventual conversion to inosinic acid (9). Clinical data (10–12) indicate that pernicious anemia is accompanied by elevated urinary AIC. Dietaries of the patients studied are not available, and hence evaluation of the influence of methionine intake on AIC excretion has not been possible. The present study was therefore

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[3] Portions of these data were presented at the VIIth International Congress of Nutrition, Hamburg, August, 1966. (Stokstad, E. L. R., S. M. Oace and C. Kutzbach 1967 Proceedings of the VIIth International Congress of Nutrition, vol. 5. F. Vieweg and Son, Braunschweig, p. 583.)
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undertaken to investigate the influence of dietary methionine on the AIC excretion of vitamin B_{12}-deficient rats. Since a variety of thyroid-active substances have been used to aggravate vitamin B_{12} deficiency in rats (13,14), the effects of thyroid powder on growth and AIC excretion of vitamin B_{12}-deficient and supplemented rats were also studied. Our results are in accordance with, and lend additional support to, the theory that vitamin B_{12} and methionine regulate folic acid metabolism at the level of methionine biosynthesis.

MATERIALS AND METHODS

Male, 21-day-old weanling rats of the Sprague-Dawley strain [5] were housed individually in screen-bottomed metabolism cages in a temperature- and light-controlled room. Animals were assigned to dietary groups by pair-weighing, so that the initial group-average weights were always about 50 g. Both food and water were allowed ad libitum. Fresh food was given every 2 to 3 days; water bottles were changed twice weekly.

The diets (table 1) were made in batches of 20 kg and stored in a refrigerator. Thyroid powder and DL-methionine were added at the expense of glucose and soy protein, respectively. Two separate vitamin mixes (with and without vitamin B_{12}) were prepared for incorporation into the respective diets. At certain points, 10 μg of vitamin B_{12} was administered by intraperitoneal injection in one milliliter of physiological saline.

TABLE 1

Composition of basal low methionine diet [1]

	g/kg diet
Soy assay protein [2]	200
Glucose monohydrate [3]	704
Corn oil [4]	40
Salts [5]	35
Fat-soluble vitamin mix [6]	10
Water-soluble vitamin mix [7]	10
Choline chloride [8]	1

[1] Supplements: 3 g thyroid powder replaced 3 g glucose; 5 or 20 g methionine replaced 5 or 20 g protein.
[2] General Biochemicals, Inc., Chagrin Falls, Ohio; provided 1.8 g methionine/kg diet.
[3] Cerelose, Corn Products Company, Argo, Illinois.
[4] Mazola, Corn Products Company.
[5] Johnson et al. (15).
[6] In corn oil, provided /kg diet: vitamin A acetate, 15,000 IU; vitamin D, 2000 IU; and α-tocopheryl acetate, 50 mg.
[7] In glucose, provided in mg/kg diet: thiamine·HCl, 15; riboflavin, 15; nicotinic acid, 50; pyridoxine·HCl, 15; Ca pantothenate, 50; menadione, 10; folic acid, 5; biotin, 2; vitamin B_{12} (when added), 0.05.
[8] Nutritional Biochemicals Corporation, Cleveland.

Urinary AIC was determined by color development with Bratton and Marshall (16) reagents for diazotizable amines and subsequent spectrophotometric analysis. Since AIC differs from many aromatic amines present in urine in that it can not be acetylated, pretreatment of urine with acetic anhydride achieves greater specificity (17). Indican also interferes with AIC determination, and this can be quantitatively removed by an anion-exchange resin (18). Urine specimens were collected through screened funnels into flasks containing 2 ml of 1 N H_2SO_4 as a preservative. A 24-hour sample was diluted to 25 ml with distilled water, adjusted to pH 2 with concentrated H_2SO_4 or NaOH, and filtered by gravity through Whatman no. 1 filter paper. Filtered urine (10 ml) was applied to a column (I.D., 8 mm) which had been fitted with a glass wool plug and contained 1.5 g of anion-exchange resin (AG2-X8).[6] Urine was eluted by gravity and the resin was washed with 2 ml of 0.01 N H_2SO_4, bringing the total eluate volume to 12 ml. An aliquot of the eluate (4 ml) was treated with 0.1 ml of acetic anhydride and, after 30 minutes of occasional vigorous shaking, acetylation was stopped with 3 ml of 0.36 N H_2SO_4. One milliliter of each of the following reagents was then added with automatic syringe-type pipettes: 1) 0.1% sodium nitrite; 2) 2 minutes later, 0.5% ammonium sulfamate; and 3) 30 seconds later, 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride (color reagent). The identical procedure (after column treatment) was followed with 4-ml aliquots of standard solutions that contained from 0.1 to 15 μg of AIC (linear range). Distilled water replaced the color reagent in urine blanks. After 20 to 60 minutes, samples were read in one-centimeter cuvettes at 540 mμ with a Beckman-DB spectrophotometer. The timing and reagent concentrations reported here are those which were found to yield maximal color development and stability under our laboratory conditions. Duplicate analyses agreed within 5% and recovery of AIC

[5] Berkeley Pacific Laboratories, Berkeley, California.
[6] Bio-Rad Laboratories, Richmond, California.

that was added to the urine before column treatment was greater than 95%.

A preliminary growth study with 8 rats per group was conducted to assess the ability of the basal vitamin B_{12}- and methionine-deficient diet to support nearly normal growth while still allowing a growth response to both vitamin B_{12} and methionine. In the second experiment, the basal diet alone and the basal diet plus vitamin B_{12}, thyroid powder, or both, were fed. Growth, food intake and AIC excretion were measured and the influences of vitamin B_{12} injection and methionine feeding on AIC excretion were studied. Food intake was recorded for the first 10 weeks of the study.

RESULTS

In the preliminary growth study, 4 groups of 8 rats were fed the basal diet with modifications as shown in table 2. The group average weights at the end of 10 weeks and the average daily gain during the 10-week feeding trial are also presented. Vitamin B_{12} supplementation elicited a significant growth response ($P < 0.05$) [7] in the absence of, but not in the presence of, the methionine supplement. Methionine supplementation supported a highly significant increase ($P < 0.01$) in body weight as compared with that of rats fed either of the diets without added methionine.

The diet modifications and the 10-week weight gain, food intake and feed efficiency data for rats whose AIC excretion was later studied are presented in table 3. Vitamin B_{12}-deficient animals (groups 1 and 3) gained less weight and ate less food than their respective vitamin B_{12}-supplemented controls (groups 2 and 4). The retarded growth of vitamin B_{12}-deficient rats cannot be totally accounted for by depressed appetite since their feed efficiency ratios are lower as well. Diets containing thyroid powder supported poorer growth and enhanced food consumption as compared with these measurements of rats fed the respective diets without added thyroid powder. All differences in weight gain, food intake and feed efficiency attributable either to vitamin B_{12} status (1 vs. 2 and 3 vs. 4) or to thyroid powder status (1 vs. 3 and 2 vs. 4) are significant at the 5% level.

TABLE 2

Effect of vitamin B_{12} and methionine on growth

Group	Supplements		Body wt, 13 weeks [1]	Daily gain [1]
	Methionine	Vitamin B_{12}		
	g/kg diet	*μg/kg diet*	*g*	*g*
G1	—	—	305 ± 40 [2]	3.6
G2	—	50	353 ± 26	4.3
G3	5	—	424 ± 36	5.3
G4	5	50	421 ± 27	5.3

[1] Experimental diets fed 10 weeks.
[2] SD.

After several weeks (as early as 6 weeks, but only representative data at 9 and 11 weeks are reported), the average daily AIC excretion of vitamin B_{12}-deficient rats was approximately twice that of the vitamin B_{12}-supplemented controls (table 4). This was true whether or not thyroid powder was present in the ration. If the AIC excretion data were adjusted for differences in body weight, this would further spread the already significant differences between supplemented and deficient groups. However, in control rats, AIC excretion is unrelated to age (5–20 weeks) or to body weight (100–500 g) and ranges from 15 to 25 μg/day. Therefore, experimental AIC excretion is most appropriately reported as micrograms per day, not corrected for differences in body sizes among dietary groups.

Groups 1 and 2 were then subdivided to study the effects of vitamin B_{12} injection (1a and 2a) and methionine feeding (1b and 2b) on AIC excretion. During the second day after injection with vitamin B_{12}, most of the previously vitamin-deficient rats of group 1a excreted similar amounts of AIC as the continuously supplemented rats of group 2a. All were within control range 5 days after, and the 10 μg dose of vitamin B_{12} still effectively prevented an increase in urinary AIC even 10 days after injection. Reduction of AIC excretion by vitamin B_{12} injection was somewhat delayed in the vitamin-deficient, thyroid-fed animals of group 3. However, 3 days after injection, groups 3 and 4 experienced similar AIC excretion rates and, again, this ef-

[7] Significance of differences between means assessed by Student's *t* test.

fect was still in evidence at 10 days post-injection.

The vitamin B_{12}-deficient and sufficient rats of groups 1 and 2, respectively, that had not been injected with vitamin B_{12} were fed diets containing 2% added methionine for 3 days. Urinary AIC was determined daily during methionine feeding and for 3 days after return to the original diets (table 5). During the second and

TABLE 3

Effect of vitamin B_{12} and thyroid powder on weight gain and food intake

Group	Thyroid supplement	Vitamin B_{12} supplement	Wt [1] gain	Food [1] intake	Feed [1] efficiency
	g/kg diet	*μg/kg diet*	*g*	*g*	*g/kg food*
1 (9) [2]	—	—	220 ± 38 [3]	1101 ± 115	199 ± 21
2 (10)	—	50	278 ± 24	1256 ± 98	222 ± 13
3 (5)	3	—	162 ± 24	1268 ± 86	127 ± 12
4 (5)	3	50	230 ± 24	1481 ± 131	155 ± 9

[1] During 10 weeks of feeding.
[2] Numbers in parentheses indicate number of rats/group.
[3] SD.

TABLE 4

Urinary AIC of vitamin B_{12} supplemented and deficient rats fed diets with or without thyroid powder and the effect of injection of 10 μg of vitamin B_{12} on aminoimidazolecarboxamide (AIC) excretion

Group [2]	Supplements		Weeks fed diets		Days after vitamin B_{12} injection [1]				
	Vitamin B_{12}	Thyroid	9	11	1	2	3	5	10
	μg/kg	*g/kg*	*μg AIC/24 hr*		*μg AIC/24 hr*				
1a	—	—	42 ± 14 [3]	52 ± 26	40 ± 17	24 ± 12	—	16 ± 2	23 ± 5
2a	50	—	20 ± 2	22 ± 2	24 ± 4	19 ± 4	—	17 ± 1	19 ± 2
	P value [4]		**	**	*	ns	—	ns	ns
3	—	3	40 ± 9	46 ± 11	34 ± 10	31 ± 8	22 ± 4	—	23 ± 3
4	50	3	23 ± 3	24 ± 4	26 ± 7	22 ± 1	22 ± 1	—	25 ± 4
	P value [4]		**	**	*	**	ns	—	ns

[1] Groups 1a and 2a were injected during week 13; groups 3 and 4 were injected during week 12.
[2] Five rats/group. Groups 1a and 2a are part of groups 1 and 2 (table 3).
[3] SD.
[4] Probability that AIC excretions of vitamin B_{12} supplemented and deficient groups are not significantly different. Calculated by Student's *t* test. ** = significant ($P < 0.05$); * = borderline significance ($0.05 < P < 0.1$); ns = not significant ($P > 0.1$).

TABLE 5

Temporary depression in aminoimidazolecarboxamide (AIC) excretion of vitamin B_{12}-deficient rats by methionine supplementation

Group [1]	Vitamin B_{12}	Weeks fed diets before methionine feeding [2]		Days during methionine feeding [3]			Days after methionine feeding [2]		
		12	14	1	2	3	1	2	3
	μg/kg	*μg AIC/24 hr*		*μg AIC/24 hr*			*μg AIC/24 hr*		
1b	—	40 ± 12 [4]	40 ± 11	31 ± 12	24 ± 2	26 ± 4	35 ± 11	38 ± 11	37 ± 8
2b	50	23 ± 2	20 ± 3	21 ± 2	21 ± 5	23 ± 2	21 ± 1	22 ± 2	22 ± 3
	P value [5]	**	**	*	ns	ns	**	**	**

[1] Groups 1b (n = 5) and 2b (n = 4) were those animals from groups 1 and 2 (table 3) that had not been injected with vitamin B_{12} (table 4).
[2] For 14 weeks before and 3 days after methionine feeding, rats were fed the basal, low methionine diet (table 1) with or without vitamin B_{12} as indicated.
[3] During methionine feeding, 20 g of DL-methionine replaced 20 g of soy protein/kg diet.
[4] SD.
[5] Probability that AIC excretion of vitamin B_{12} supplemented and deficient groups is not significantly different. Calculated by Student's *t* test. ** = significant ($P < 0.05$); * = borderline significance ($0.05 < P < 0.1$); ns = not significant ($P > 0.1$).

third days of methionine feeding, AIC excretion of vitamin B_{12}-deficient animals (group 1b) decreased to that of the vitamin-supplemented controls (group 2b). Immediately upon removal of the methionine supplement, the urinary AIC of group 1b animals returned to its elevated pre-methionine level. AIC excretion of vitamin B_{12}-supplemented rats was unaffected by methionine feeding.

DISCUSSION

In formulation of the basal diet, 2 factors were considered. First, the diet had to be low in methionine as well as vitamin B_{12} to demonstrate the effects of vitamin B_{12} deficiency on folic acid metabolism (6). Second, the diet should be adequate in other nutrients (as indicated by good growth rates) so that measurements of control animals are representative of a normal metabolic situation. Silverman and Pitney (3) limited methionine by using a low protein (9% casein) diet. However, even their vitamin-sufficient ration resulted in very poor growth rates, frequently less than one gram per day. Soy protein, which is low in methionine, can be incorporated as 20% of a diet and still provide less methionine than a 9% casein diet. By using soy protein, then, the basal diet satisfied the requirement of the laboratory rat for crude protein, as well as for fat, vitamins (except vitamin B_{12}) and minerals as established by the NRC (19).

Male, Sprague-Dawley rats, when fed diets adequate in all known nutrients, are expected to attain a weight of about 350 g at 10 weeks after weaning (19). This represents an average gain of approximately 4.4 g/day. The data in table 2 show that our basal diet fulfills the requirements for this study. It is deficient in methionine and vitamin B_{12} (as evidenced by a growth response to these nutrients when added) and also supports very good growth.

Whether the hyperthyroid state had any specific effect on the severity of vitamin B_{12} deficiency is equivocal. In the presence of thyroid powder, vitamin B_{12} stimulated growth to approximately the same absolute amount (68 g in 10 weeks) as it did in rats fed diets containing thyroid powder (58 g in 10 weeks). Still, owing to their smaller body size, this absolute growth response represents a much greater percentage benefit from vitamin B_{12} in hyperthyroid rats, i.e., vitamin B_{12}-supplemented thyroid-fed rats gained 42%, whereas the non-thyroid gained only 26% more weight than their deficient counterparts, respectively. Perhaps this indicates greater depletion of vitamin B_{12} in hyperthyroid rats. If this is so, however, it is not manifest in AIC excretion. Vitamin B_{12} deficiency doubled urinary AIC, regardless of the presence of thyroid powder. The average AIC excretions of hyper- and euthyroid rats of similar vitamin B_{12} status were not significantly different, even when expressed on a body weight basis. However, injection of vitamin B_{12} lowered the AIC excretion rate more slowly in thyroid-fed, vitamin B_{12}-deficient rats than in rats fed the basal diet without thyroid powder. The poor health (as evidenced by diarrhea, watery eyes and nose and dull, matted fur) of the thyroid-fed animals as compared with the apparently good health of the euthyroid rats, also prevents any definitive evaluation of their respective vitamin B_{12} status.

In our experiments, methionine supplementation at 2% of the diet was as effective in lowering AIC excretion of vitamin B_{12}-deficient rats as injection of vitamin B_{12} itself. This parallels the changes in FIGLU excretion with these dietary manipulations in rats (3) and chicks (4).

While our investigation was in progress, McGeer et al. (20) reported that methionine supplementation did not lower AIC excretion of vitamin B_{12}-deficient rats. Their conrtol diet consisted of grains and other natural foodstuffs, and their vitamin B_{12}-deficient ration was a commercially prepared [8] defined diet which differed from the control diet in numerous aspects other than their respective vitamin B_{12} content. Elevated AIC excretion could have been due to a dietary factor other than lack of vitamin B_{12}. This contention is supported by the fact that even addition of vitamin B_{12} to their deficient ration did not significantly reduce AIC excretion. Since our investigation deals with the effect of methionine on urinary AIC which is responsive to vitamin B_{12} status, the report of

[8] Nutritional Biochemicals Corporation, Cleveland.

McGeer et al. does not constitute contradictory evidence.

Recent biochemical studies offer an explanation at the enzyme level for our observations on the influence of dietary vitamin B_{12} and methionine on AIC excretion. The tissue concentration of S-adenosylmethionine increases after injection of methionine (21). This compound is a potent inhibitor of methylenetetrahydrofolate reductase (22), the enzyme which catalyzes the irreversible (23) reduction of methylene-THF to methyl-THF. Vitamin B_{12} deficiency greatly depresses the level of cobamide-containing methyltetrahydrofolate-homocysteine transmethylase (24), the enzyme which catalyzes the release of THF from methyl-THF during methionine biosynthesis. Thus, methionine feeding, by elevating S-adenosylmethionine concentration, would inhibit formation of methyl-THF, whereas vitamin B_{12} supplementation would enhance utilization of methyl-THF. Either way, THF is released (from "trapped" methyl-THF) and the metabolic blocks causing the elevated excretion of AIC and FIGLU would be abolished.

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Effects of Dietary Lipid and Protein on Growth and Nutrient Utilization by Dairy Calves at Ages 8 to 18 Weeks [1]

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ABSTRACT Relationships between dietary lipid and protein were investigated with Holstein calves, 8 to 18 weeks of age. A 2-dimensional, central composite experimental design was used, with 5 levels each of corn oil and soy protein. Nine experimental rations, formulated by substituting combinations of corn oil from zero to 8% and soy protein from zero to 28% in a basal ration, were evaluated in a 10-week feeding period. Growth and feed consumption were recorded and ration digestibility was determined at 8, 13, and 18 weeks by indicator-ratio techniques. Multiple regression techniques, applied to the observed results, predicted that an optimal combination of 1.9% corn oil and 16.2% soy protein substituted in the basal ration would have resulted in maximal growth for the 10-week feeding period. Considering the contributions of the basal ration, maximal growth was predicted to occur with a ration whose dry matter contained 4.54 kcal/g of gross energy and 24.8% crude protein. When 18-week predicted nitrogen and energy digestion coefficients, body weight gains, and dry matter intakes were combined, 0.75 kg of digestible protein and 12.49 megcal of digestible energy/day were predicted to result in maximal growth of the 146-kg calf. Additions of corn oil up to 4% resulted in only a slight reduction in growth. At levels of oil above 4%, digestibilities of dry matter, energy and lipid were reduced, feed intake declined, and growth was inferior.

Optimal dietary ratios of protein and energy have been established or investigated for some animal species (1-4). The level of ingested energy can be increased most readily by additions of fats. Normally, the intake of lipid by ruminants is low except during the milk-consuming period of early life. Research attempts to increase dietary fat for cattle generally have resulted in inefficient utilization of the lipids (5, 6). It therefore seemed worthwhile to search for means of improving the process in order that truly high-energy rations might be fed.

The present investigation was undertaken to determine the effects of dietary protein and lipid upon the growth and nutrient utilization of dairy calves. The 8- to 18-week-old calf was chosen because there appears to be little research information on this age group, in contrast with that for the younger, and older, bovine.

PROCEDURE

A two-dimensional central composite block design as described by Box (7) was used to study the effect of protein and lipid upon growth and related processes. This design was chosen for its efficiency in that a large experimental area could be covered with a minimum of treatments. Purified soy protein and corn oil were used as the dietary source of protein and lipid. A basal ration was formulated to be low in these components. Corn oil and soy protein were substituted for cornstarch to form the 9 rations [4] shown in table 1. This resulted in only minor alterations in the general make-up of the rations. Only the basal ration and added soy protein contributed to the total nitrogen of the experimental feeds; and, only basal plus added oil accounted for the fatty acids. The gross energy of a ration was a result of the combinations of basal mixture, corn

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TABLE 1

Dietary variables and composition of the dry matter of the rations

Ration no.	Dietary variables [1]				Composition of dry matter [2,3]		
	Corn oil		Soy protein		Nitrogen × 6.25	Total fatty acids	Gross energy
	Code		Code				
		%		%	%	%	*cal/g*
1	−1	2	−1	7	15.2	3.78	4407
2	1	6	−1	7	15.0	8.07	4676
3	−1	2	1	21	29.7	4.19	4637
4	1	6	1	21	29.9	8.19	4866
5	0	4	0	14	22.5	5.92	4626
6	0	4	−2	—	7.9	5.90	4473
7	0	4	2	28	37.3	5.81	4786
8	−2	—	0	14	22.6	2.17	4406
9	2	8	0	14	22.6	9.78	4903

[1] Corn oil and soy protein were substituted in the basal ration for cornstarch. Composition of the basal ration was: (in percent) ground timothy hay, 25; ground corn, 15; ground oats, 10; dried molasses, 10; dicalcium phosphate, 2; trace mineralized salt, 1; cornstarch, 32; and ground polyethylene, 5. (The trace mineralized salt contained in percent: NaCl, 96.7; Mn, 0.2; Fe, 0.16; Cu, 0.033; Co, 0.01; I, 0.007; Zn, 0.005.) Vitamin A (palmitate), vitamin D (irradiated dry yeast) and antioxidant (Santoquin, Monsanto Chemical Company, St. Louis) were added at rates of 1,840 IU, 587 IU and 0.125 g, respectively, /kg of ration. Polyethylene was included in the ration only during digestion trials (8).

[2] Based on a polyethylene-free basis.

[3] Acid detergent fiber and acid detergent lignin content were 14.09 and 2.45%, respectively, for all rations.

oil, and soy protein. Adding protein and oil in a 7:2 ratio resulted in some of the high protein rations being almost equal in energy to that of the high oil ration. Polyethylene was added to the basal mixture for use as a nonabsorbed marker, but it was present only during the time of digestibility measurements. The additions of corn oil and soy protein listed in table 1 are percentages of the basal. At times when polyethylene was removed, these additions were 1.053 times the values listed in table 1.

Thirty-six calves were used in this trial. Ration 5 was fed along with milk replacer during the first 42 days. After the time of replacer feeding, all calves continued to be fed ration 5 for two additional weeks. They then were divided into 9 groups of four each and assigned to one of the rations in table 1. A 10-week feeding trial (8-18 weeks) was conducted. Rations were fed ad libitum at all times. Growth was recorded as increase in body weight, in heart girth, and in height at withers. The calves were measured and weighed on 3 consecutive days at the beginning and end of the trial.

Apparent digestibility estimates were made, using 10 calves that were receiving ration 5 at 8 weeks of age and all calves at 13 and 18 weeks. The indicator-ratio technique was used, with polyethylene as the nonabsorbed marker (8). One week before the time of grab sample collection, polyethylene was incorporated into the rations. Fecal grab samples were obtained at 8:00 AM and 2:00 PM of the first day, and at 2:00 AM and 8:00 PM of the second day of each digestion period. This plan was followed to minimize any diurnal variations in polyethylene excretion. Fecal samples from each calf were composited and stored for later analysis. Feed samples were obtained for each digestibility period.

Feces samples were dried in a forced-air oven at 60°. After drying, the feces samples were allowed to equilibrate with the atmosphere, dried, and ground in a Wiley mill (2-mm screen).

Nitrogen analyses were run on fresh feces and feeds by the Kjeldahl procedure (9). Oxygen bomb calorimetry was used for measuring the gross energy of dried samples. Whenever polyethylene was present in samples, the gross energy was corrected for the contribution due to polyethylene. It had a gross energy value of 10,292 cal/g.

Total fatty acids (representing free and esterified forms) were determined on the dry feed and feces samples. A modification of the official extraction procedure for baked dog food was used for lipid ex-

traction (9). After extraction with a mixture of petroleum and ethyl ether, the solvent was evaporated to about 10 ml with the aid of the steam bath and nitrogen. The sample then was saponified for 1 hour using alcoholic KOH and evaporated to dryness on a steam bath under nitrogen. Two milliliters of H_2O were added and the sample was acidified with HCl, and then extracted with three 50-ml portions of petroleum ether. The extracts were combined and evaporated to about 5 ml. The fatty acids were recovered from the extract with the aid of a silicic acid column as described by McCarthy and Duthie (10). The fatty acid fraction was transferred to tared, weighed flasks, dried under nitrogen, and weighed.

Analysis of variance and multiple regression techniques were used in trial 3 (7, 11–13). The X variables were coded from -2 to $+2$ (table 1) so that the mathematical solution would be simplified. The statistical breakout accounted for treatment, linear and quadric response, and deviation from regression. The following regression equation was used in describing the varability due to dietary treatments: $\hat{Y} = b_0 + b_1 X_1 + b_2 X_2 + b_{11} X_1^2 + b_{22} X_2^2 + b_{12} X_1 X_2$; where $\hat{Y}$ equals the predicted response; X_1 and X_2 equal the coded value for the percentage substitution of corn oil and soy protein, respectively; and the *b*'s equal the multiple regression coefficients.

RESULTS AND DISCUSSION

The effects of ration upon growth, feed consumption and digestibility are presented in tables 2 and 3. Multiple regression coefficients, described variability, and standard errors are listed in table 4. Growth was significantly affected by ration when measured as body weight gain, increased heart girth, or increased height at withers. Growth is presented as body weight gain in tables 2 and 4. Total feed consumption and feed efficiency for the 10-week trial were significantly affected ($P < 0.01$) by ration (tables 2 and 4). The most efficient feed conversion was observed with ration 3, with which 3.19 kg of feed resulted in a one-kilogram gain in body weight. The effect of corn oil in decreas-

TABLE 2

Effect of diet on growth, feed consumption, and efficiency [1]

Ration no.	1	2	3	4	5	6	7	8	9
Dietary corn oil, %	2	6	2	6	4	4	4	—	8
Dietary soy protein, %	7	7	21	21	14	—	28	14	14
Body wt at wk 8, kg	74.4	66.8	67.9	68.5	71.0	68.9	60.6	76.3	66.4
Increase to wk 18, kg	71.3	47.2	70.2	63.7	67.3	15.0	46.0	78.6	48.0
Increase over wk 8, %	96	71	103	94	95	22	76	103	71
Feed consumption from wk 8 to 18, kg	241.5	181.0	220.4	209.8	215.5	134.1	154.2	261.6	175.3
Feed efficiency, feed/gain	3.39	3.86	3.19	3.32	3.23	8.94	3.45	3.32	3.84

[1] Means of 4 calves/treatment.

TABLE 3

Effects of dietary treatment and time upon ration digestibility

Ration no.	1	2	3	4	5	6	7	8	9
Dietary corn oil, %	2	6	2	6	4	4	4	—	8
Dietary soy protein, %	7	7	21	21	14	—	28	14	14
Week 8 [1]									
Dry matter intake, kg/day					1.49				
Digestibility, %									
Dry matter					66.4 ± 1.17 [2]				
Energy					62.9 ± 1.43				
Nitrogen					66.6 ± 2.06				
Total fatty acids					54.6 ± 8.68				
Week 13 [3]									
Dry matter intake, kg/day	2.61	2.12	2.26	2.03	2.49	1.47	1.87	2.79	1.93
Digestibility, %									
Dry matter	69.5	65.5	70.5	66.0	70.5	64.2	63.8	70.5	64.5
Energy [4]					67.8 ± 1.65				
Nitrogen	61.5	59.0	77.5	75.2	74.2	43.5	79.2	70.8	69.0
Total fatty acids	72.5	37.0	64.2	53.2	63.0	74.8	48.2	55.5	42.5
Week 18 [3]									
Dry matter intake, kg/day	4.01	3.02	3.57	3.47	3.53	2.00	2.43	4.44	2.79
Digestibility, %									
Dry matter	71.0	67.2	69.2	71.0	72.0	58.0	56.0	71.0	63.5
Energy	58.0	61.2	68.0	69.0	69.0	56.5	53.2	67.2	57.2
Nitrogen	65.0	65.0	79.5	82.5	77.5	53.8	80.5	74.2	70.8
Total fatty acids	73.8	47.5	73.3	64.2	64.5	73.8	70.2	53.5	24.2

[1] All calves were fed ration 5 at 8 weeks.
[2] Mean of 10 calves ± SE of mean.
[3] Mean of 4 calves.
[4] Energy digestion coefficients were determined for ration 5 only at 13 weeks.

TABLE 4

Multiple regression coefficients, variability accounted for by regression and standard error of means for responses related to growth, feed consumption and nutrient digestibility

Regression equation [1]: $\hat{Y} = b_0 + b_1X_1 + b_2X_2 + b_{11}X_1^2 + b_{22}X_2^2 + b_{12}X_1X_2$

Predicted response ($\hat{Y}$)	Multiple regression coefficients [2]						Variability		
	b_0	b_1	b_2	b_{11}	b_{22}	b_{12}	Treatment	R^2 Treatment	SE of a mean
							%	%	
Body wt increase									
8- to 18-wk, kg	72.61	−7.65 **	6.44 **	−1.99 **	−10.20 **	4.39	71 **	97 **	6.75
Increase as % of 8-wk wt	103.17	−8.38 **	11.88 **	−3.42 **	−12.83 **	3.75	69 **	95 **	9.47
Feed consumption from 8- to 18-wk, kg	228.64	−20.31 **	3.99	−1.73 **	−20.30 **	12.46	55 **	97 **	20.3
Feed efficiency, feed/gain	2.68	0.13 *	−0.98 **	0.19 **	0.84 **	−0.09	95 **	84 **	0.22
Nutrient digestibility at wk 13, %									
Dry matter	70.28	−1.71 **	0.04	0.71 **	−1.58 **	−0.04	47 **	97 **	1.72
Nitrogen	72.81	−0.69	8.65 **	−0.82 **	−2.95 **	0.06	91 **	99 **	1.96
Total fatty acids	60.89	−6.04 *	−3.67	−3.10	0.02	6.12	39 [3]	70 [3]	8.82
Nutrient digestibility at wk 18, %									
Dry matter	74.28	−1.42	−0.17	−1.61 **	−4.18 **	1.38	59 **	93 **	2.73
Energy	71.47	−2.15 *	0.10	−2.15 **	−3.99 **	1.94	56 **	88 **	3.03
Nitrogen	76.92	−0.33	7.12 **	−1.14 **	−2.48 **	0.75	86 **	98 **	2.02
Total fatty acids	68.69	−7.81	0.77	−7.19 **	1.09 **	4.31	60 **	95 **	7.35

[1] In the equation, X_1 and X_2 are the coded values for corn oil and soy protein, respectively (table 3).
[2] The quadratic terms are given a group significance since an orthogonal breakout of individual components is not possible.
[3] Non-significant ($P > 0.05$).
* Significant ($P < 0.05$).
** Significant ($P < 0.01$).

ing the efficiency of feed conversion was noted when comparisons were made between rations 1 and 2,3 and 4, and 8 and 9. The low protein ration (no. 6) resulted in the least efficient conversion of feed.

A more complete description of the dietary effects is obtained from the multiple regression equations. Response equations describing growth and feed consumption (table 4) were plotted to show the effects of added corn oil at different levels of soy protein (fig. 1). Both dietary variables showed significant linear effects ($P < 0.01$). Increased levels of corn oil depressed the percentage increase in body weight, whereas increased levels of soy protein accelerated it. The quadratic effect also was significant ($P < 0.01$) and resulted in an optimal combination of corn oil and soy protein. Increased levels of corn oil depressed feed intake in a linear manner by about 20 kg for each 2% increase of corn oil (table 4). Soy protein affected feed intake in a quadratic manner, with maximal intakes occurring between 14 and 21% soy protein (fig. 1). At low levels of soy protein (zero and 7%) the decreased growth resulting from increasing levels of corn oil was associated with comparable decreases in feed intake. However, at higher levels of soy protein, the decreased growth resulting from increasing corn oil was greater than the decrease in feed intake. This was especially evident at 28% soy protein.

Digestibility coefficients were calculated for 10 calves at 8 weeks and for all calves at 13 and 18 weeks (table 3). The standard error of means for the 8-week coefficients suggested that fatty acid digestibility was subject to more animal variability than other constituents. The lower treatment variability and large standard errors associated with lipid digestion coefficients in table 4 also suggest that this nutrient was subject to more variability than the others at 13 and 18 weeks.

The effect of age upon nutrient digestibility of ration 5 is shown in table 3. A large improvement was noted in the digestion of fatty acids from 8 to 13 weeks, with only a slight increase thereafter. The digestibility of dry matter and energy improved as the animals became older, with the greatest increase occurring during the first 5 weeks. Nitrogen differed from the other 3 nutrients in that considerable improvement was noted from 13 to 18 weeks.

Multiple regression equations for the digestibility of dry matter and energy at 18 weeks (table 4) were plotted in figure 2. Dietary corn oil and soy protein affected dry matter and energy digestibility in a manner similar to that observed for growth

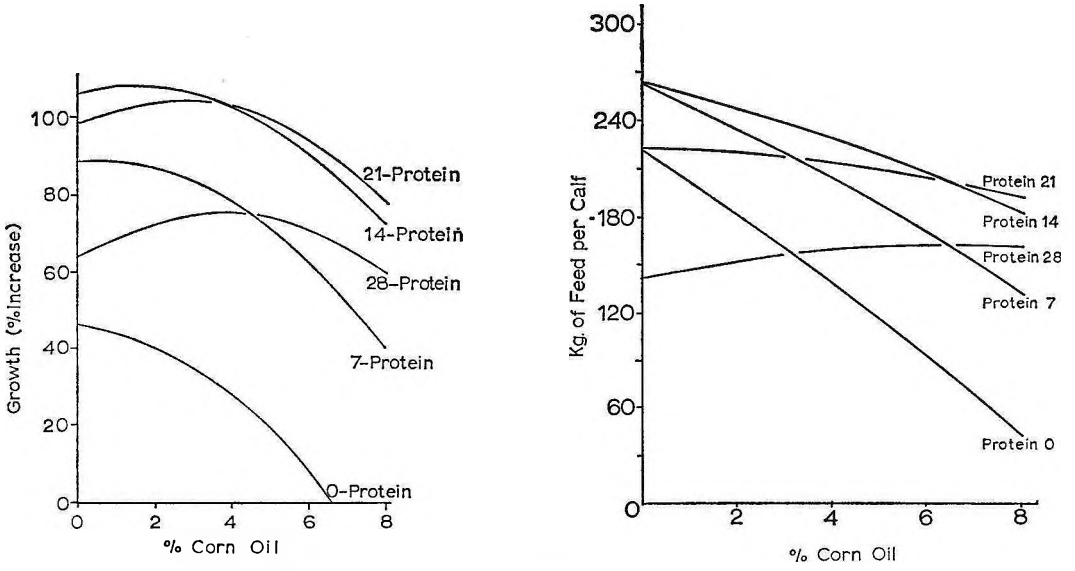

Fig. 1 Effects of dietary corn oil and soy protein upon growth and feed consumption.

(fig. 1). Corn oil expressed a negative linear effect ($P < 0.05$) upon energy digestion, and both dietary variables expressed significant quadratic effects ($P < 0.01$) upon dry matter and energy digestion. The linear effect of corn oil upon energy was most obvious at low levels of soy protein (zero and 7%).

The relationship between the dietary variables and the digestibility of nitrogen is presented in figure 3. Response equations described 99 and 98% of the variability due to treatment for nitrogen digestibility at 13 and 18 weeks, respectively (table 4). This high degree of fit to the mathematical model can be attributed to low animal variability in nitrogen digestion and also to the nature of the response. Soy protein was the most important factor in nitrogen digestibility. Positive linear effects ($P < 0.01$) for dietary protein, and negative quadratic effects ($P < 0.01$) were observed. Maximal apparent digestibility of nitrogen was reached at 21% soy protein.

Response equations describing total fatty acid digestion at 13 and 18 weeks were not similar (table 4). The 13-week fatty acid digestion was not significantly affected by treatment, but a significant amount of variation ($P < 0.01$) in the 18-week fatty acid digestion coefficients was described by the regression equation. To illustrate the dietary effects, it was necessary to plot the effect of increasing levels of soy protein at different levels of corn oil rather than plotting increasing levels of corn oil at different soy protein levels (fig. 3). Corn oil expressed a negative linear effect ($P < 0.01$) upon lipid digestibility which was most apparent with 2 to 8% additions at zero to 14% soy protein. The significant quadratic effects ($P < 0.01$) are due to the negative coefficient of corn oil which resulted in a slight increase in lipid digestibility at levels of corn oil up to 4% and contributed to the sharp decrease at levels above 4%.

Using the observed results, the optimal ration for maximal growth response was calculated. Since both quadratic terms of the response equation for percentage increase in body weight were negative, maximal body weight increases would be predicted to occur at the stationary point (7). Partial differentiation of the equation with respect to X_1 (corn oil) and X_2 (soy protein) and a simultaneous solution resulted

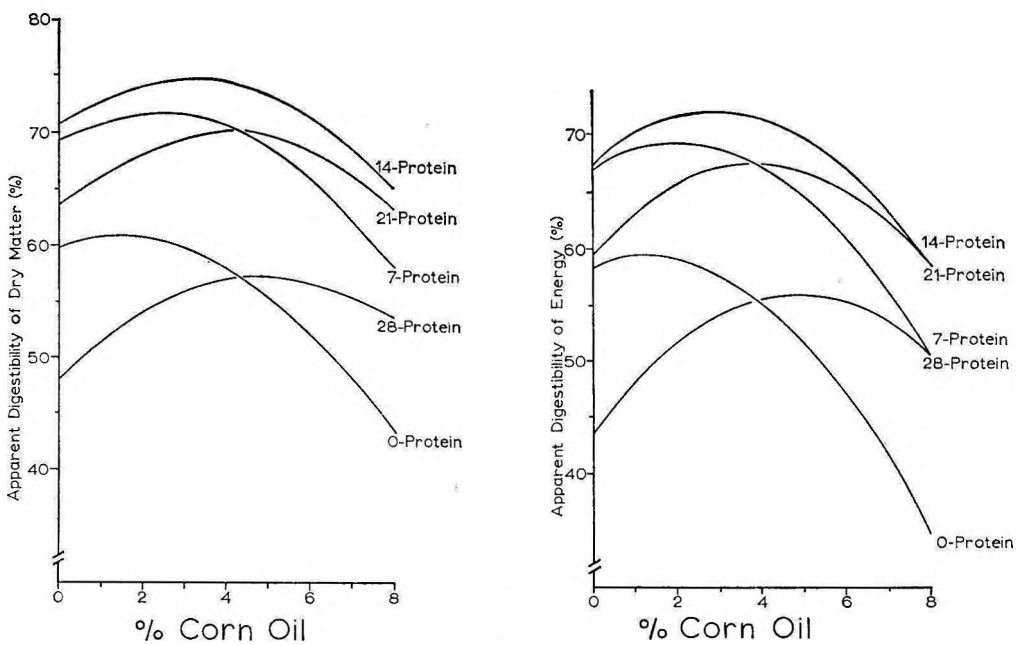

Fig. 2 Effects of dietary corn oil and soy protein upon the digestibility of dry matter and energy at 18 weeks.

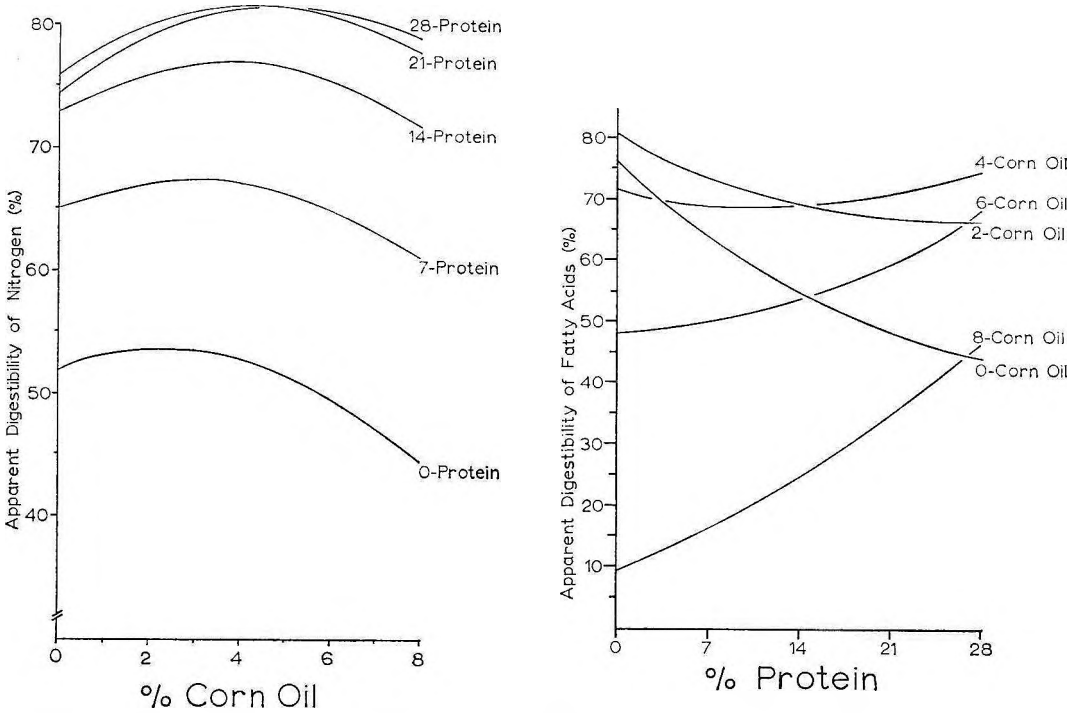

Fig. 3 Effects of dietary corn oil and soy protein upon the digestibility of nitrogen and fatty acids at 18 weeks.

in a prediction of maximal growth at 1.9% corn oil and 16.2% soy protein additions to the basal ration. Substitution of these values in the equation gave a predicted maximum of 109% increase in body weight. The observed values of 103% for rations 3 and 8 (table 2) supported this predicted value of 109%. When the contributions of the basal ration and the optimal combination of 1.9% corn oil and 16.2% soy protein were combined, maximal growth was predicted to occur with a ration whose dry matter contained 4,541 cal/g of gross energy and 24.8% protein.

Nutrient digestibility of the optimal ration was predicted from the response equation for 18 weeks (table 4). At the 1.9% corn oil and 16.2% soy protein combination, digestibilities for dry matter, energy, nitrogen, and fatty acids were predicted to be 71.9, 70.0, 77.7 and 67.4%, respectively. Dry matter intake at 18 weeks of age for the optimal diet was estimated from the average intakes of rations 1,3,5 and 8 (table 3). These rations are located around the optimal point in the experimental design. This estimated dry matter intake of 3.89/kg/calf/day multiplied by the gross energy and crude protein of the optimal ration resulted in a total intake of 17.66 megcal of gross energy and 0.96 kg of crude protein per calf per day. From the predicted digestion coefficients for energy and protein, it was calculated that maximal growth would occur with 12.49 megcal of digestible energy and 0.75 kg of digestible protein. The weight of the calf was estimated as being 146 kg by using an average starting weight of 70 kg at 8 weeks (table 2) and the predicted body weight increase of 109% by 18 weeks of age.

The digestible energy requirement for maximal growth of 12.49 megcal per day for the 146-kg calf is in agreement with the requirements suggested by the NRC (14). Their recommendation for a 150-kg heifer grown for herd replacement is 11.2 megcal of digestible energy and for veal calves of similar size 14.1 megcal. The calculated digestible protein requirement for maximal growth in this study for the

18-week, 146-kg calf was 0.75 kg. This value is greater than NRC recommended levels. The values also are hgher than requirements for younger calves as reported by Bryant and associates (15).

The existence of an optimal ratio of protein to energy for the young dairy calf is in agreement with the study of Brown and Lassiter (4). They suggested that the optimal ratio was 1:46 or lower when the ratio of the ration was defined as a percentage of crude protein: kilocalories of estimated net energy per pound (454 g) for calves from 6 tc 12 weeks of age, gaining up to 0.74 kg/day. Since NRC requirements for dairy cattle are expressed as grams of digestible protein and calories of digestible energy, the protein-to-energy ratio of the present study was expressed as grams of digestible protein per kilocalories of digestible energy per kilogram. In this study, maximal growth was predicted to occur when a kilogram of ration dry matter contained 193 g digestible protein and 3210 kcal digestible energy, or a protein-to-energy ratio of 1:17. Adjustment of the 1:46 ratio of Brown and Lassiter (4) into similar units resulted in a ratio of 1:22.

The response equation for growth indicated that 1.9% corn oil addition to the basal ration was optimal. However, additions up to 4% caused only a slight decline in the growth response when dietary protein was adequate (fig. 1). The digestibility values for dry matter, energy and nitrogen were predicted to be near a maximum at the 4% corn oil addition (figs. 2 and 3). Digestibility of fatty acids was predicted to be about 70% (fig. 3). At corn oil additions above 4%, however, there was a decrease in growth as well as nutrient digestibility. Thus, the lipids from corn oil could not be predicted to be utilized efficiently by calves of this age at levels above 4%.

ACKNOWLEDGMENTS

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Effect of Dietary Lipid and Protein on Serum Proteins, Lipids and Glucose in the Blood of Dairy Calves [1,2]

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ABSTRACT The effects of dietary corn oil and soy protein upon blood serum proteins, blood lipids and glucose were determined in 36 male Holstein calves at ages 8, 13, and 18 weeks. Additions of corn oil and soy protein to a basal diet were varied from zero to 8% and zero to 28%, respectively. Blood serum proteins were not affected by age of calves when sampled but were significantly affected by ration at 13 and 18 weeks. At low dietary protein levels, increased levels of corn oil lowered blood serum proteins. Of the blood serum proteins, β-globulin was the most responsive to dietary changes. Maximal values for blood glucose occurred with dietary combinations that resulted in maximal growth. Blood lipids were affected by the age of the calf as well as ration. A gradual increase was noted in the blood lipid level as the trial progressed. Phospholipids appeared to account largely for the increase observed from 8 to 13 weeks, whereas the 13- to 18-week increase was a result of increased cholesterol. A quadratic relationship existed between dietary corn oil and blood cholesterol, with maximal cholesterol values occurring at approximately 4% corn oil. Dietary protein expressed a negative linear effect upon blood lipids.

Information accumulated over the past decade has associated dietary lipids, blood cholesterol and heart disease in humans. Because of the relationship between protein and lipid in such basic structures as blood serum lipoproteins and cell membranes, it appeared desirable also to consider dietary protein. Although there is relatively little information with respect to dairy calves, results with other animals suggest an interrelationship. Lassiter et al. (1) reported that extremely high levels of protein in the diet of calves reduced the level of blood serum γ-globulin, and that dietary levels which were optimum for growth resulted in highest serum protein levels. The concentration of plasma lipids of the calf was reported to vary directly with the level of dietary fat (2). The addition of fat to the diet has been shown to result in increased plasma cholesterol in calves (3), pigs, (4, 5) and rats (6). Dupont and Lewis (6) observed that cholesterol synthesis in rats was enhanced, and non-cholesterol lipids depressed, by adding dietary fats. The experiments of Barnes et al. (5) and Greer et al. (7) with young pigs support the hypothesis that a low protein intake results in increased levels of serum cholesterol. The depressing effect of dietary protein upon plasma cholesterol was demonstrated with mice by Leveille and Sauberlich (8), who showed that added levels of dietary protein also reduced liver levels of cholesterol and other lipids.

The present investigation was undertaken to determine the interrelationships among dietary protein and lipid and blood serum proteins and blood lipid constituents in the young ruminating calf. The dietary effects upon blood glucose also were observed.

PROCEDURE

Thirty-six Holstein calves were used in a 2-dimensional central composite block design experiment, in which 5 levels each of corn oil (0, 2, 4, 6 and 8%) and soy-protein (0, 7, 14, 21 and 28%) were studied. Details of the rations, feeding

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[2] Some of the data were presented at the 1966 meeting of the American Society of Animal Science, July 31–August 4, Rutgers University, New Brunswick, New Jersey.

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and experimental design were described earlier (9).

Blood samples were collected from the jugular vein of 10 calves receiving ration 5 at 8 weeks of age and from all calves at 13 and 18 weeks. Immediately after removal of the blood, 25 ml were placed in 25 ml of ethyl alcohol for lipid analysis, 5 ml in $CdSO_4$ for glucose analysis and the remainder (50 ml) allowed to clot. The clotted sample was centrifuged and the serum removed. The serum was frozen for subsequent protein analysis.

For blood glucose determination, protein-free filtrates (1:10) of whole blood were prepared by the $CdSO_4$-NaOH technique (10). Blood glucose analyses were made on the protein-free filtrate using the idometric titration method described by Somogyi (11). Glucose was determined on samples obtained when the calves were 18 weeks old.

Blood serum protein was determined by Kjeldahl analysis for nitrogen (12). This was corrected for nonprotein nitrogen, then computed as protein by multiplying by 6.25. Serum non-protein nitrogen was estimated as Kjeldahl nitrogen on that part of serum remaining after tungstic acid precipitation (13). Serum proteins were separated by cellulose acetate electrophoresis (14). Following separation, the strips were stained, cleared, and scanned. The area under the appropriate peaks was determined and expressed as a percentage of the total area of the scan. The relative percentages of the different serum proteins were multiplied by the concentration of total serum protein to calculate the amount of the individual proteins. Purified solutions of albumin and γ-globulin were used, along with diluted serum, for the quantification of the procedure.

Blood lipids were fractionated in the samples obtained at ages 8 and 18 weeks and in part of the 13-week samples. Total lipids were extracted from 25 ml of whole blood according to the procedure of McCarthy et al. (15). Following extraction, the lipids were fractionated into phospholipids and nonpolar lipids using the method of Hirsch and Ahrens (16). The fatty acids were isolated from the nonpolar fraction on a KOH silicic acid column and then eluted with formic acid (17). Cholesterol in the neutral lipids was estimated using a standard colorimetric procedure.[4]

Analysis of variance and multiple regression techniques were used as previously described (9).

RESULTS AND DISCUSSION

The effects of dietary treatment and length of time the respective diets were fed are presented in tables 1 and 2. Multiple regression coefficients, described variability and standard error for 13- and 18-week responses are shown in table 3.

The composition of serum proteins for calves fed ration 5 exhibited only minor changes during the trial (table 1). The 8-week serum protein distribution was 48:20:14:18 for albumin, α-globulin, β-globulin and γ-globulin, respectively. At 13 weeks the overall effect of rations upon total serum protein was nonsignificant ($P > 0.05$), but the linear effect of corn oil was significant ($P < 0.05$). The β-globulin concentration was significantly affected ($P < 0.01$) by diet at 13 weeks (table 3). At the end of the trial (18 weeks), total serum protein and all the constituents except α-globulin showed significant dietary effects (table 3).

The response equation (table 3) which describes the relationship between dietary variables and serum protein is presented graphically in figure 1. Only data on the calves when 18 weeks of age were used in preparation of this figure. Dietary additions of corn oil with low protein levels (zero and 7% added soy protein) resulted in a reduction of serum protein. The effect of dietary protein was dependent upon the addition of corn oil. At low levels of corn oil, increased protein levels from zero to 28% soy protein resulted in only minor changes in serum protein concentrations. At high levels of oil, this change of dietary protein was predicted to result in as much as a 1.75 g/100 ml change in serum protein. This dietary protein-lipid interaction was significant at the 10% level of probability. Of the serum protein constituents, β-globulin was the most re-

[4] Bausch and Lomb, Clinical Methods and Calibrations Manual. Catalogue no. 33-29-46. Rochester, New York.

TABLE 1

Effects of dietary treatment and time on blood serum nitrogen

Ration no.	1	2	3	4	5	6	7	8	9
Dietary corn oil, %	2	6	2	6	4	4	4	—	8
Dietary soy protein, %	7	7	21	21	14	—	28	14	14
Week 8 [1]									
Nonprotein N, mg/ml					0.26 ± 0.013 [2]				
Protein N × 6.25, g/100 ml					6.48 ± 0.112				
albumin					3.11 ± 0.073				
α-globulin					1.30 ± 0.043				
β-globulin					0.88 ± 0.036				
γ-globulin					1.18 ± 0.067				
Week 13 [2]									
Nonprotein N, mg/ml	0.21	0.09	0.38	0.20	0.18	0.06	0.25	0.19	0.14
Protein N × 6.25, g/100 ml	6.29	6.11	6.47	6.48	6.39	6.20	6.40	6.79	6.16
albumin	3.14	3.08	3.04	3.21	3.15	3.13	3.12	3.22	3.16
α-globulin	1.10	1.24	1.16	1.12	1.26	1.21	1.17	1.27	1.17
β-globulin	0.93	0.72	0.97	0.92	0.88	0.68	0.67	1.04	0.84
γ-globulin	1.10	1.10	1.28	1.27	1.12	1.14	1.37	1.30	1.00
Week 18 [2]									
Nonprotein N, mg/ml	0.12	0.14	0.24	0.26	0.24	0.11	0.29	0.24	0.24
Protein N × 6.25, g/100 ml	6.44	5.96	6.80	6.89	6.41	6.00	6.58	6.70	6.28
albumin	3.25	3.02	3.32	3.34	3.19	3.07	3.17	3.34	3.36
α-globulin	1.19	1.24	1.24	1.30	1.28	1.24	1.16	1.07	1.13
β-globulin	0.98	0.71	0.92	0.90	0.85	0.60	0.86	1.02	0.96
γ-globulin	1.06	0.99	1.34	1.30	1.12	1.11	1.34	1.28	0.86

[1] All calves were fed ration 5 at 8 weeks.
[2] Mean of 10 calves ± SE of a mean.
[3] Mean of 4 calves.

TABLE 2

Effects of dietary treatment and time upon blood lipids and glucose

Ration no.	1	2	3	4	5	6	7	8	9
Dietary corn oil, %	2	6	2	6	4	4	4	—	8
Dietary soy protein, %	7	7	21	21	14	—	28	14	14
Week 8 [1]									
Total blood lipid, mg/100 ml					225 ± 32.3 [2]				
Phospholipids					96 ± 14.1				
Fatty acids					21 ± 3.1				
Neutral lipids					92 ± 14.4				
Total cholesterol					70 ± 9.1				
Week 13 [3]									
Total blood lipid, mg/100 ml	347	440	245	404	415	468	373	360	244
Phospholipids [4]					242	182	128	110	80
Fatty acids [4]					14	17	8	12	14
Neutral lipid [4]					146	235	205	234	148
Total cholesterol [4]					108	150	130	164	107
Week 18 [3,5]									
Total blood lipid, mg/100 ml	422	324	392	421	904	1123	290	396	506
Phospholipids	130	127	122	177	96	394	104	148	184
Fatty acids	31	35	15	33	53	28	10	13	18
Neutral lipids	234	144	227	186	708	685	139	230	296
Total cholesterol	150	97	159	126	616	599	93	186	236
Blood glucose, mg/100 ml	72	68	67	69	68	51	61	66	64

[1] All calves were fed ration 5 at 8 weeks.
[2] Mean of 10 calves ± SE of a mean.
[3] Mean of 4 calves.
[4] Blood lipids from calves on the first four rations were not fractionated at 13 weeks.
[5] Blood glucose was determined on 18-week samples only.

TABLE 3

Multiple regression coefficients, variability accounted for by regression and standard errors of means for responses related to blood serum proteins, blood lipids and glucose

Regression equation [1]: $\hat{Y} = b_0 + b_1X_1 + b_2X_2 + b_{11}X_1^2 + b_{22}X_2^2 + b_{12}X_1X_2$

Predicted response ($\hat{Y}$)	Multiple regression coefficients [2]						Variability, %		SE of a mean
	b_0	b_1	b_2	b_{11}	b_{22}	b_{12}	Treatment	R^2 Treatment	
Blood serum nitrogen									
Week 13									
Nonprotein N, mg/ml	0.225	− 0.034 *	0.056 **	− 0.013	− 0.014	− 0.014	52 **	70 **	0.05
Protein N × 6.25, g/100 ml	6.347	− 0.117 *	0.078	0.029	− 0.014	0.049	28 [3]	82 [3]	0.18
Albumin	3.118	− 0.001	0.002	0.016	− 0.000	0.058	12 [3]	71 [3]	0.09
α-Globulin	1.194	− 0.009	− 0.011	0.002	− 0.005	− 0.046	18 [3]	40 [3]	0.07
β-Globulin	0.914	− 0.054 **	0.018	0.009 **	− 0.057 **	0.039	60 **	90 **	0.06
γ-Globulin	1.140	− 0.052	0.068	0.005	0.029	− 0.002	21 [3]	85 [3]	0.13
Week 18									
Nonprotein N, mg/ml	0.202	0.004	0.051 **	0.008	− 0.003	− 0.001	81 **	91 **	0.02
Protein N × 6.25, g/100 ml	6.518	− 0.101	0.205 **	− 0.000	− 0.051	0.142	57 **	87 **	0.16
Albumin	3.207	− 0.014	0.049 *	0.037 *	− 0.020 *	0.064	40 [3]	82 *	0.08
α-Globulin	1.117	0.019	− 0.007	0.039	0.028	0.000	31 [3]	57 [3]	0.06
β-Globulin	0.872	− 0.035	0.053 **	0.031 **	− 0.035 **	0.062	57 **	91 **	0.06
γ-Globulin	1.156	− 0.079 *	− 0.088 **	− 0.019	0.019	0.008	46 *	83 **	0.10
Blood glucose at wk 18, mg/100 ml	71.86	− 0.73	1.40	− 1.44 **	− 3.66 **	1.69	65 **	75 **	2.4
Blood lipids, mg/100 ml									
Total lipids at wk 13	389.30	1.69	− 27.19	− 23.57	6.18	16.81	43 *	51 [3]	49.2
Phospholipids [4]							76 **		18.9
Fatty acids [4]							56 *		1.5
Neutral lipids [4]							49 *		23.2
Total cholesterol [4]							32 [3]		19.2
Total lipids at wk 18	591.36	12.56	− 133.19 **	− 54.69 **	9.21 **	31.69	88 **	45 **	57.2
Phospholipids	84.76	10.29	− 44.57 **	19.67 **	40.45 **	14.25	82 **	72 **	23.4
Fatty acids	47.39	2.67	− 4.50	− 8.24	− 7.46	3.38	48 *	60 *	7.7
Neutral lipids	419.10	0.06	− 88.15 **	− 57.17 **	− 19.80 **	12.56	89 **	36 **	42.9
Total cholesterol	336.77	1.23	− 81.27 **	− 48.97 **	− 15.13 **	5.06	92 **	33 **	32.8

[1] Multiple regression equation X_1 and X_2 are the coded values for corn oil and soy protein, respectively.
[2] The quadratic terms are given a group significance since an orthogonal breakout of individual components is not possible.
[3] Nonsignificant ($P > 0.05$).
[4] These lipids were not determined for all calves; thus the responses were not fitted to the regression equation.
* Significant ($P < 0.05$).
** Significant ($P < 0.01$).

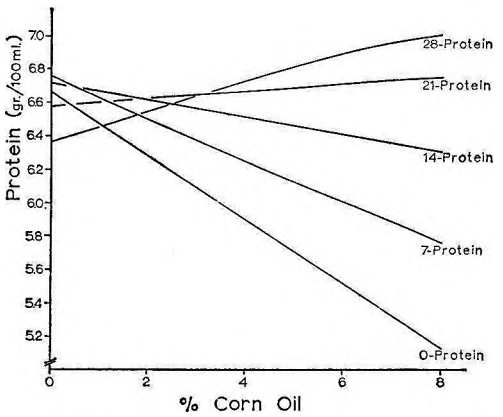

Fig. 1 Effect of dietary corn oil and soy protein upon blood serum protein at 18 weeks.

sponsive to dietary changes (tables 1 and 3). Increased levels of corn oil resulted in a reduction of β-globulin. Dietary protein showed a positive linear effect upon 18-week albumin ($P < 0.05$), β-, and γ-globulin ($P < 0.01$). Quadratic effects also were observed for albumin ($P < 0.05$) and β-globulin ($P < 0.01$).

Blood serum nonprotein nitrogen increased linearly with soy protein additions at 13 and 18 weeks ($P < 0.01$). At 13 weeks, corn oil additions reduced serum nonprotein nitrogen in a linear manner ($P < 0.05$; table 3). The effect of corn oil upon serum nonprotein nitrogen was nonsignificant at 18 weeks.

It has been reported that feeding a high protein milk replacer to calves resulted in a decline in blood serum γ-globulin (1). In the present study, γ-globulin was highest with those diets high in dietary protein (table 1). Waldroup et al. (18) reported that protein deficiency in pullets resulted in a decrease in serum α- and β-globulins. Low protein diets resulted in a decrease in β-globulin concentration for the calves but α-globulin was not affected by ration at 13 or 18 weeks. The reduction observed in blood serum protein with increasing corn oil at low dietary protein may be related to blood lipid transport. Lipids are transported in bovine blood as α- and β-lipoproteins. Increasing levels of dietary lipid will require increased synthesis, by the liver and intestinal tract wall, of serum lipoproteins for the marked increase in lipid transport. This increased demand for lipoprotein synthesis could stress the amino acid pool in a manner similar to that described for ketotic cows that are mobilizing large amounts of body lipids (19).

Blood glucose levels at 18 weeks were significantly affected by ration (tables 2 and 3). Figure 2, derived from the response equation, illustrates the relationship of dietary variables to blood glucose. The response of blood glucose to dietary variables was similar to the growth response (9). Blood glucose was related to dietary variables in a quadratic manner ($P < 0.01$) with maximal glucose values occurring for dietary combinations that resulted in maximal growth. At high levels of protein (28% soy protein) additions of oil up to 6% resulted in a 10 mg/100 ml increase of blood glucose, whereas at the low level of protein (zero percent soy protein) an 8% addition of corn oil resulted in a blood glucose decline of 15 mg/100 ml.

Examination of blood lipid values presented in table 2 suggested that changes occurred as the trial progressed. The mean

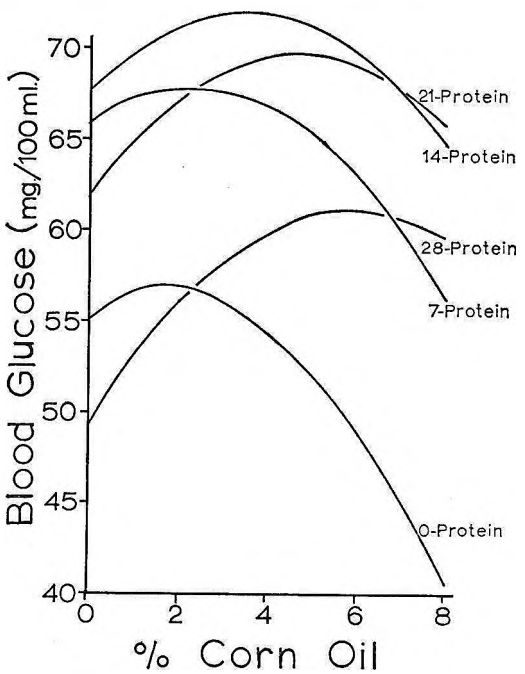

Fig. 2 Effects of dietary corn oil and soy protein upon blood glucose at 18 weeks.

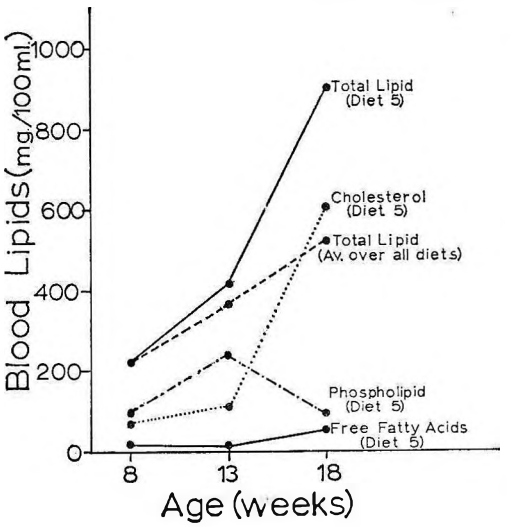

Fig. 3 Effect of age upon the blood lipids from calves fed ration 5.

values for calves fed ration 5 are presented for the 3 time-periods in figure 3. When averaged over all calves, total blood lipid showed a linear increase with age, but calves fed ration 5 showed a rapid increase in blood lipids during the latter part of the trial. This increase was the result of a sharp increase in cholesterol and a decrease in phospholipid. Free fatty acids showed only a slight increase at 18 weeks.

Excluding 13-week cholesterol, significant ration effects were noted for all lipid constituents at both time-periods (table 3). In both 13- and 18-week samples, higher blood lipid values were observed for calves fed rations medium in oil than for those fed rations high in oil (ration 5 versus 8 and 9, table 2). Phospholipids appeared to account for this response at 13 weeks and neutral lipids were responsible at 18 weeks.

Response equations for blood lipids are presented in table 3. It was apparent that a low and variable amount of treatment variation was described by the multiple regression model. This suggested that an accurate description of responses of blood lipids would require a higher degree, more complex model. However, in this experiment significant amounts ($P < 0.01$) of treatment variation were described by the model and response equations were helpful in understanding dietary effects.

Figure 4 illustrates the effects of corn oil and soy protein upon total blood lipids at 18 weeks. Corn oil additions up to 4% resulted in an increase in total lipids. Blood lipids declined with corn oil additions above those of 4%. Low protein rations produced the highest blood lipid values, with increasing levels resulting in a linear decline ($P < 0.01$). The highest and lowest blood lipid values observed at 18 weeks were for rations 6 and 7, respectively (table 2).

The effects of dietary lipid and protein upon blood phospholipid and cholesterol are illustrated in figure 5. Additions of soy protein resulted in a negative linear effect upon both blood lipid constituents ($P < 0.01$). Significant quadratic effects also were observed for both lipids. A minimal phospholipid value was predicted to occur at approximately 2% corn oil and 21% soy protein. Quadratic coefficients for cholesterol were negative, thus resulting in maximal values at about 4% corn oil. The general shape of the response curve for blood cholesterol suggested that this blood lipid largely accounted for the total blood lipid response observed in figure 4.

The negative linear effect of dietary protein upon blood lipids is in agreement with studies reported for calves (3), pigs (5, 7) and mice (8). Of the blood lipids, cholesterol appeared to be the most responsive to the level of dietary protein. This relationship indicates that cholesterol metabolism may depend upon adequate protein or that cholesterol synthesis is accelerated in protein deficiency.

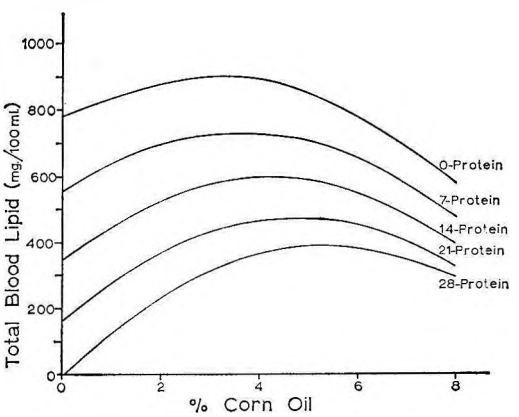

Fig. 4 Effects of dietary corn oil and soy protein upon total blood lipids at 18 weeks.

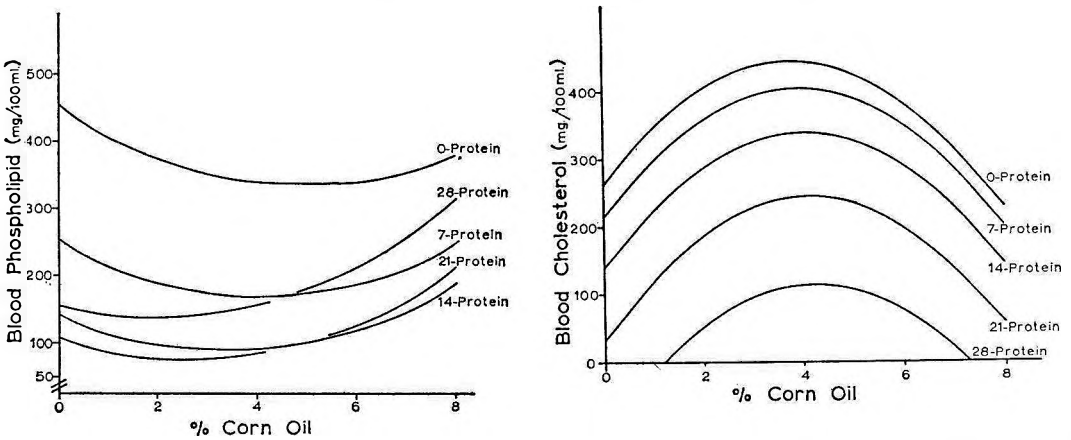

Fig. 5 Effects of dietary corn oil and soy protein upon blood phospholipid and cholesterol at 18 weeks.

ACKNOWLEDGMENTS

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Action of Bovine and Ovine α-Amylases on Various Starches [1,2]

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ABSTRACT The actions of porcine, bovine, ovine and fungal α-amylases on five solubilized starches were investigated. The substrates used were potato, corn, rice, wheat and protozoal starch. Enzyme activities were determined by measuring maltose and limit dextrin production, glucose production, and the breakdown of the starch molecule. Large differences were noted in the action of the amylolytic enzymes from different species fed different starches. The bovine and ovine enzymes appeared to be inferior to the porcine and fungal enzymes in the breakdown of the different starch molecules. When maltose and limit dextrin production was used as the measure of activity, the bovine and ovine enzymes appeared to be superior to the porcine and fungal enzymes. Glucose production was low in all cases. Corn appeared to be poorly attacked by these amylases if the breakdown of the starch molecules was the only criterion of enzyme action. However, cornstarch was second only to potato starch as a substrate for maltose and limit dextrin production. The production of maltose and limit dextrin from cornstarch was similar for the three pancreatic amylases. The Michaelis constants and activation energies were also similar for all three pancreatic enzymes.

The common assumption that almost all the starch fed to ruminants is fermented by microorganisms in the rumen has limited interest in its post-ruminal utilization. However, recent Kentucky experiments have shown that substantial amounts of starch pass out of the rumens of steers fed high concentrate diets (1) and that the pancreas from steers fed such diets contain significantly more amylase than pancreas from steers fed only roughage.[3] If the action of pancreatic amylases accounts for an important portion of starch digestion, this apparent adaptation of pancreatic amylase production to increased starch intake may be an important factor in the overall animal adaptation to high concentrate diets. Results of a previous study [4] of the effect of changes in diet on pancreatic secretion indicated that ruminants adapt to dietary changes in starch level but that the response is slower than in other animals. These observations seemed to justify further investigation of the action of ruminant pancreatic amylase. In the present studies the actions of bovine and ovine pancreatic amylases on several common starches were compared with the actions of porcine pancreatic amylase and the fungal amylase from *Aspergillus oryzae*.

EXPERIMENTAL PROCEDURE

The enzymes used in this investigation were from several sources. The two-times crystalline porcine α-amylase was prepared from hog pancreas.[5] The fungal α-amylase was prepared from *Aspergillus oryzae*.[6] The bovine amylase was an acetone powder preparation from a homogenate of pancreatic tissue. The ovine enzyme, also an acetone powder preparation, was prepared from pancreatic secretion. Protein concentration of each preparation was estimated from optical density at 280 mμ. All enzyme solutions were prepared by diluting with 0.02 M PO_4 (pH 7.2) to an optical density at 280 mμ which preliminary determinations had indicated would give approximately equal

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[3] Clary, J. J., G. E. Mitchell, Jr., C. O. Little and N. W. Bradley 1966 Dietary influence on bovine pancreatic amylase. J. Anim. Sci., 25: 899 (abstract).

[4] Clary, J. J., G. E. Mitchell, Jr. and C. O. Little 1967 Adaptation of sheep pancreatic secretion to dietary change. J. Anim. Sci., 26: 917 (abstract).

[5] Worthington Biochemical Corporation. Lot no. AA5729; maximal activity 770 units/mg.

[6] Sigma Type IV; lot no. 85B0250.

[7] Nutritional Biochemicals Corporation, Cleveland.

activity against potato starch by the starch-iodine method. Yield of bovine and ovine enzymes was variable but usually less than 1% of tissue protein. The corn, rice, wheat and potato starches were purchased commercially.[7] The protozoal starch was prepared from bovine rumen fluid by the method of Mould and Thomas (2). All 5 starches were solubilized before assay (3) and all reaction mixtures were activated with calcium chloride.

Weighed amounts of starch were boiled for 3 minutes in a 0.2 M phosphate buffer (pH 7.2). The solutions were then made to equal concentration (2 mg/ml). Into each tube 10 ml of substrate was added. The tubes with the substrate were then placed in water baths at 37°. Triplicate determinations were conducted on 2 tubes of each substrate for each enzyme. One milliliter of enzyme solution was added to each tube after temperature equilibrium was established. After exactly 10 minutes the tubes were removed from the water bath and the reaction was stopped by the addition of 1 ml of 2 N H_2SO_4.

The activity was measured by three methods. The initial attack of amylase on the starch molecules was measured by the iodine method of Howard and Yudkin (4). Maltose and limit dextrin, the normal products of amylase action, were measured by Bernfeld's method (5) modified so that the same reaction mixture used in the starch-iodine method could be used for maltose and limit dextrin determination. The modification involved the addition of 2 drops of 5 N NaOH to a 1-ml aliquot of the incubated mixture to insure a neutral pH.

The third method of measuring amylase activity was glucose production. Glucose was determined by the glucose oxidase peroxidase method (6) with the following modification: 0.2-ml aliquot from the incubation mixture used in the starch-iodine method was used instead of 0.02 ml as in the original method. Maltose incubation controls were also run to detect maltase or glucose oxidase contamination. Standards were used in all determinations.

Kinetic determinations were made using the Bernfeld (5) method. Five points were determined in triplicate. Linear regressions, which were significant ($P < 0.01$) in all cases, were then calculated.

RESULTS AND DISCUSSION

Results showing the action of the 5 amylases on 5 types of starches are presented in tables 1, 2, 3 and 4. Tables 1 and 2 show the results as measured by the starch-iodine method. All enzymes were more active against protozoal starch than they were against potato starch. Protozoal starch had an unusual color with a different absorption maximum when reacted with iodine and this starch-iodine complex may have been more sensitive to enzyme attack than those formed with other substrates. The fungal amylase appeared to be the most active enzyme when comparisons were made using the starch-iodine method to study the potato starch used to standardize enzyme concentrations. The bovine, ovine and porcine en-

TABLE 1

Substrate effect of breakdown of starches by different amylases (starch-iodine complexes)

Source of enzyme	Change in OD at 620 mμ				
Porcine	substrate	protozoal	potato	rice	wheat	corn
	% change [1]	42.0	28.0	21.2	19.0	16.7
Bovine	substrate	protozoal	potato	rice	wheat	corn
	% change [1]	34.8	28.0	19.9	17.7	13.3
Ovine	substrate	protozoal	potato	rice	wheat	corn
	% change [1]	30.7	28.0	25.6	20.6	12.1
Aspergillus	substrate	wheat	protozoal	rice	potato	corn
	% change [1]	35.2	31.3	30.8	28.0	23.4

[1] Means not underscored by the same line are significantly different ($P < 0.05$).

TABLE 2

Breakdown of different starches by porcine, bovine, ovine and Aspergillus amylases (starch-iodine complexes)

Substrate		Change in OD at 620 mμ			
Corn	enzyme	Aspergillus	porcine	bovine	ovine
	% change [1]	23.4	16.7	13.3	12.1
Wheat	enzyme	Aspergillus	ovine	porcine	bovine
	% change [1]	35.2	20.6	19.0	17.7
Rice	enzyme	Aspergillus	ovine	porcine	bovine
	% change [1]	30.8	25.6	21.2	19.9
Protozoal	enzyme	porcine	bovine	Aspergillus	ovine
	% change [1]	42.0	34.8	31.3	30.7

[1] Means not underscored by the same line are significantly different ($P < 0.05$).

TABLE 3

Substrate effect on maltose and limit dextrin production from starch by different amylases

Enzyme				*μmoles*		
Porcine	substrate	potato	corn	protozoal	rice	wheat
	maltose [1]	2.17	1.96	1.78	1.74	1.55
Bovine	substrate	potato	corn	rice	protozoal	wheat
	maltose [1]	2.30	1.86	1.81	1.69	1.58
Ovine	substrate	potato	corn	protozoal	rice	wheat
	maltose [1]	2.47	1.94	1.93	1.85	1.69
Aspergillus	substrate	potato	corn	protozoal	rice	wheat
	maltose [1]	1.42	1.24	1.15	1.10	0.97

[1] Means not underscored by the same lines are significantly different ($P < 0.05$).

TABLE 4

Maltose and limit dextrin production from different starches by porcine, bovine, ovine and Aspergillus amylases

Substrate		*μmoles*		*μmoles*	
Potato	enzyme	ovine	bovine	porcine	Aspergillus
	maltose [1]	2.47	2.30	2.17	1.42
Corn	enzyme	porcine	ovine	bovine	Aspergillus
	maltose [1]	1.96	1.94	1.86	1.24
Wheat	enzyme	ovine	bovine	porcine	Aspergillus
	maltose [1]	1.69	1.58	1.55	0.97
Rice	enzyme	ovine	bovine	porcine	Aspergillus
	maltose [1]	1.85	1.81	1.74	1.10
Protozoal	enzyme	ovine	porcine	bovine	Aspergillus
	maltose [1]	1.93	1.78	1.69	1.15

[1] Means not underscored by the same line are significantly different ($P < 0.05$).

zymes all appeared to be less active against corn, wheat and rice than the fungal amylase as measured by the starch-iodine method.

When production of maltose and limit dextrin was used to measure activity (tables 3 and 4), the results were different from those obtained using the starch-iodine method. In this case the fungal amylase gave lower activity values than the other enzymes. Maltase contamination might have occurred, but the maltose incubation tubes showed no measureable disappearance of maltose or production of glucose. Potato starch appeared to yield the most maltose and limit dextrin. Production of maltose and limit dextrin from protozoal starch was less than that from corn and rice starch. The ruminant amylases appear to be relatively more active in producing maltose and limit dextrin than in the initial breakdown of starch (starch-iodine method). In other words, the ruminant amylases appeared to be more efficient in the breakdown of starch fragments than the porcine enzyme. Since the starch that enters the small intestine in the ruminant is probably more fragmented owing to rumen action than starch in the diet of a hog, this might be expected. Species differences in breakdown have been reported previously (7). The determination of glucose showed that very little glucose was produced and no trends were noted.

Analysis of variance procedures and Duncan's new multiple range test (8) were used to evaluate differences in results obtained by the starch-iodine method and the Bernfeld method (5). The enzyme effect, substrate effect and enzyme-substrate interaction were all highly significant. When individual substrate responses are compared, there appears to be a significant difference between the 2 measurements of enzyme activity. Cornstarch, for example, ranked lowest in all cases using starch-iodine complex breakdown but was second only to potato starch as a substrate for limit dextrin and maltose production. These differences may be due to differences in enzyme action and in the physical composition of the starch.

The basis of the reaction of the iodine with the starch is not well-understood, but the degree of α-1 $\rightarrow$ 6 linkages in the starch is in part responsible for the degree of binding. The degree of α-1 $\rightarrow$ 6 linkages is also important to the amylases as they cannot hydrolyze α-1 $\rightarrow$ 6 linkages (10). β-Amylase, for example, starts at the non-reducing end and hydrolyzes maltose units off the amylase molecule until it comes to an α-1 $\rightarrow$ 6 linkage. α-Amylase acts in random fashion to give a mixture of glucose, maltose, dextrin and other oligosaccharides. When the starch-iodine activity measurement is used to evaluate the enzyme effect, the ruminant amylase appears to be less active against all substrates. The reverse is true when production of maltose and dextrin is used as a measure of enzyme activity. The ruminant amylase probably can be considered to be as efficient as other amylases in the production of maltose and dextrin, or even more efficient. Since maltose and dextrin are normal products of amylase action this is important. The production of maltose and limit dextrin from corn is not significantly different for the three pancreatic enzymes investigated. This suggests that these amylases can digest cornstarch, a major source of carbohydrates in practical diets for all 3 species, with similar efficiencies.

The Michaelis constants, basically substrate concentrations at half maximal velocity, and activation energies for these three pancreatic amylases provide further comparisons (table 5). The Michaelis constants are similar to each other and to other published values (10). The activation energies of these three pancreatic amylases are also similar, indicating that the amounts of energy required to place the substrate molecule in the reactive state are about the same. Thus, differences in the action of these enzymes on the sub-

TABLE 5

Kinetic comparisons of bovine, ovine and porcine amylases

	K_M [1]	Activation energy
		kcal/mole
Bovine	2.8 – 3.8	10,600
Ovine	2.6 – 3.2	11,700
Porcine	1.9 – 2.5	9,800

[1] $\times 10^{-3}$ moles of glycosidic bonds/liter.

strates studied cannot be attributed to differences in these kinetic parameters.

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Effect of Amino Acid Imbalance in Rats Fed Ad Libitum, Interval-fed, or Force-fed [1]

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ABSTRACT Since food intake has been shown to be of utmost importance in the response of animals fed amino acid imbalanced diets, a study was made in which rats were fed once a day or were force-fed and the food intake and growth were followed and compared with ad libitum-fed rats. Amino acid imbalances were created by adding 2 to 12% of amino acid mixture lacking one indispensable amino acid to low protein diets. The severity of the adverse effect on food intake and growth increased with increasing concentration of the imbalancing amino acid mixture added to the diet. Supplementation of the imbalanced diets with the limiting amino acid (0.45% of DL-threonine) completely prevented the adverse effects caused by the addition of 5.4% of an amino acid mixture lacking threonine to a 6% casein diet. The food intake and growth of interval-fed rats (trained to eat a single 2-hour meal daily) fed the imbalanced diet was not depressed until the concentration of the imbalancing amino acid mixture was greatly increased. Force-feeding stimulated growth of rats receiving different imbalanced diets without causing obvious abnormalities.

Depression in food intake and growth are characteristic signs in amino acid balances whether the imbalance is created by the addition of relatively small amounts of one or two amino acids or by the addition of a large amount of a mixture of indispensable amino acids devoid of growth-limiting amino acid (1). The decrease in food intake (and therefore growth) is usually more severe with the latter type and can be consistently observed in 3 to 6 hours. The depression in food intake and growth can be induced most readily in weanling rats (2).

Since reduced efficiency of nitrogen utilization appears to be secondary to depression in food intake (3, 5–7), and since food intake and growth can be stimulated by the administration of insulin (4), or by exposing rats to a cold environment (8, 9), it has been postulated that the depression in food intake is the primary effect causing the growth depressions of rats ingesting the imbalanced diets.

The present studies were undertaken in an effort to further assess the validity of the concept that the retardation of the growth of rats fed an amino acid imbalanced diet is a consequence of depression in food intake.

EXPERIMENTAL

Young male rats of the Holzman strain were used, with water being offered ad libitum at all times. All rats were individually caged and were fed the basal diet as described below for at least 3 days to allow them to adjust to the environment before each experiment was begun. The basal or control diet contained either 6% of casein supplemented with 0.3% DL-methionine or 6% of fibrin plus the following: (in per cent) salt mixture,[4] 4; corn oil,[5] 5; vitamin

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[4] The salt mixture contained: (in percent) $CaCO_3$, 29.29; $CaHPO_4 \cdot 2H_2O$, 0.43; KH_2PO_4, 34.31; NaCl, 25.06; $MgSO_4 \cdot 7H_2O$, 9.98; Fe ($C_6H_5O_7 \cdot 6H_2O$, 0.623; $CuSO_4$, 0.156; $MnSO_4 \cdot H_2O$, 0.121; $ZnCl_2$, 0.020; KI, 0.0005; $(NH_4)_6MO_7O_{24} \cdot 4H_2O$, 0.0025; and $Na_2SeO_3 \cdot 5H_2O$, 0.0015. The salt mix was prepared by and purchased from General Biochemicals, Inc., Chagrin Falls, Ohio. The salts at 5% of the diet provided in percent of element; Ca, 0.592; P, 0.394; K, 0.493; Na, 0.493; Cl, 0.760; Mg, 0.049; Fe, 0.0049; Cu, 0.0019; Mn, 0.00195; Zn, 0.0004; I, 0.000019; Mo, 0.000005; and Se, 0.0000025.

[5] Mazola, Corn Products Company, New York.

mixture,[6] 0.5; choline chloride, 0.2; and a dextrin-starch mixture (1:2) as the carbohydrate to make up to 100%. Imbalances were created by adding a mixture of amino acids devoid of either threonine (threonine-imbalanced) or histidine (histidine-imbalanced) to the casein or fibrin basal diet, respectively (table 1). The corrected diets were prepared by adding enough threonine or histidine to the respective imbalanced diets to prevent the adverse effects caused by the amino acid imbalance. All additions of amino acids were compensated for by adjusting the percentage of the carbohydrate. The basal and the imbalanced diets contained the same amount of threonine or histidine which was derived entirely from the 6% of casein or fibrin. Threonine and histidine are the most-limiting amino acids, respectively, in each basal diet.

Measurement of rate of food intake. Groups of 10 rats each were fed, individually, a weighed amount of the basal, the threonine-imbalanced or the corrected diet and food intake was recorded at certain time-intervals as indicated. Corrections were made for spillage throughout each experiment.

Interval-feeding. Male rats weighing about 110 g were fed a stock diet [7] for 2 to 3 days to allow them to adjust to the new environment. They were then offered an adequate purified diet (15% casein) for a 2-hour period once a day for one or two weeks until they had adapted to the new feeding regimen. The rats were then interval-fed the basal diet for another one or two weeks. At the end of the training period, rats that had gained approximately the same amount of weight and were consuming about the same amount of food were selected for the experiments. Groups of 9 rats each were fed the basal or the threonine-imbalanced diet for 2 hours. Daily food intake and weight gains of the animals were recorded.

[6] Five-tenths percent of the vitamin mixture in the diet provided the rats with 0.44% sucrose plus the following vitamins in mg/kg diet: thiamine·HCl, 5; riboflavin, 5; niacinamide, 25.0; Ca D-pantothenate, 20; pyridoxine·HCl, 5; folic acid, 0.5; menadione, 0.5; *d*-biotin, 0.2; vitamin B_{12} (0.1% in mannitol), 30; ascorbic acid, 50 (added to prevent thiamine destruction); vitamin E acetate (25% in a mixture of gelatin, sugar and starch), 400; vitamin A acetate and vitamin D_2 (325,000 USP units of A/g and 32.500 USP units of D_2/g in a mixture of gelatin, sugar and starch), 12.31. (The thiamine, niacinamide, folic acid, menadione and vitamin B_{12} were purchased from Nutritional Biochemicals Corporation, Cleveland, and the rest were purchased from Hoffmann-La Roche, Inc., Nutley, New Jersey.

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

TABLE 1

Composition of control and imbalanced diets

	Casein control diet	Fibrin control diet	Threonine-imbalanced diets			Histidine-imbalanced diet
			1	2	3	
	%	%	%	%	%	%
Casein	6	—	6	6	6	—
Fibrin	—	6	—	—	—	6
Corn oil [1]	5	5	5	5	5	5
Salt mixture [2]	4	4	4	4	4	4
Vitamins [2]	0.5	0.5	0.5	0.5	0.5	0.5
Choline chloride	0.2	0.2	0.2	0.2	0.2	0.2
DL-Threonine	—	—	—	—	—	0.4
DL-Methionine	0.3	—	0.3	0.3	0.3	0.4
DL-Tryptophan	—	—	0.15	0.24	0.32	0.2
L-Leucine	—	—	0.54	0.84	1.12	0.6
DL-Isoleucine	—	—	0.80	1.20	1.60	0.4
DL-Valine	—	—	0.82	1.30	1.68	0.4
L-Histidine·HCl	—	—	0.22	0.36	0.48	—
DL-Phenylalanine	—	—	0.34	0.54	0.72	0.6
L-Lysine·HCl	—	—	0.58	0.90	1.20	0.6
L-Arginine·HCl	—	—	—	—	—	0.2
Dextrin:starch (1:2)	84	83.7	80.55	78.62	76.88	80.8
Total	100	100	100	100	100	100

[1] Mazola, Corn Products Company, New York.
[2] See Experimental section for composition.

Force-feeding. The diets used in the force-feeding experiments were the same as those used in the ad libitum feeding experiments except that the casein was micro-pulverized to render it suitable for tube feeding. The diets were blended with distilled water so that each milliliter of the liquid diet contained approximately 0.6 g of dry diet. The liquid diet was prepared just before feeding. Moisture determination was carried out for each batch of liquid diet prepared and the amounts of dry diet consumed were calculated. For intubation, a 16-gauge curved metal hypodermic needle with a smooth rounded tip was used with a hypodermic syringe.

Ninety 100-g rats were force-fed the basal diet in increasing quantities each day for 6 days to allow them to adapt to the tube-feeding regimen. At the end of the adaptation period, animals that showed progressive weight gains were selected and divided into 3 groups as indicated. They were then force-fed quantities of the basal, the imbalanced and the corrected diets approximately equal to the amount that rats fed the basal diet consumed voluntarily. The diets were administered 3 times daily: at 9 AM, 5 PM and at midnight. The animals were weighed just before the morning feeding. In a preliminary experiment no food remained in the stomach of the rat at the time of weighing. All rats again had free access to water.

Another 3 groups of animals (6 rats/group) of similar weight were fed the same basal and experimental diets ad libitum to serve as controls.

RESULTS

The food intake of rats fed the imbalanced diets containing 3.5% (6 rats/group), 5.4% and 7.1% (7 rats/group) of the amino acid mixture lacking threonine was depressed (fig. 1). Note the gradual increase in food intake after the fifth day. The depressions in growth rate were 46%, 64% and 74%, respectively, for the groups fed the imbalanced diets for 14 days (fig. 2), which indicated that the severity of the depression in food intake and growth increased with each increase in the quantity of the imbalancing amino acid mixture added to the low protein basal diet. As threonine was added to the imbalanced diet in increasing quantities (from 0.1% to 0.45%, 6 rats/group), the rate of growth and food intake progressively increased (figs. 3 and 4). Although a level of 0.2% of DL-threonine added to the imbalanced diet alleviated completely the food and growth depression

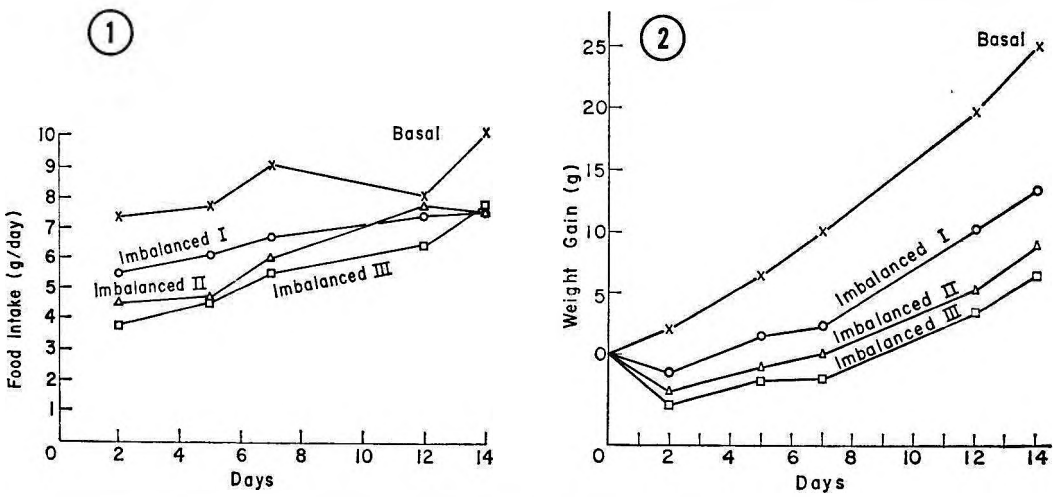

Fig. 1 Food intake of rats fed basal or imbalanced diets containing different concentrations of imbalancing amino acid mixtures. Basal: 6% casein plus 0.3% DL-methionine; imbalanced I: basal plus 3.5% amino acid mixture lacking threonine; imbalanced II: basal plus 5.4% amino acid mixture lacking threonine; and imbalanced III: basal plus 7.1% amino acid mixture lacking threonine.

Fig. 2 Changes in body weight of rats fed basal or imbalanced diets containing different concentrations of imbalancing amino acid mixture.

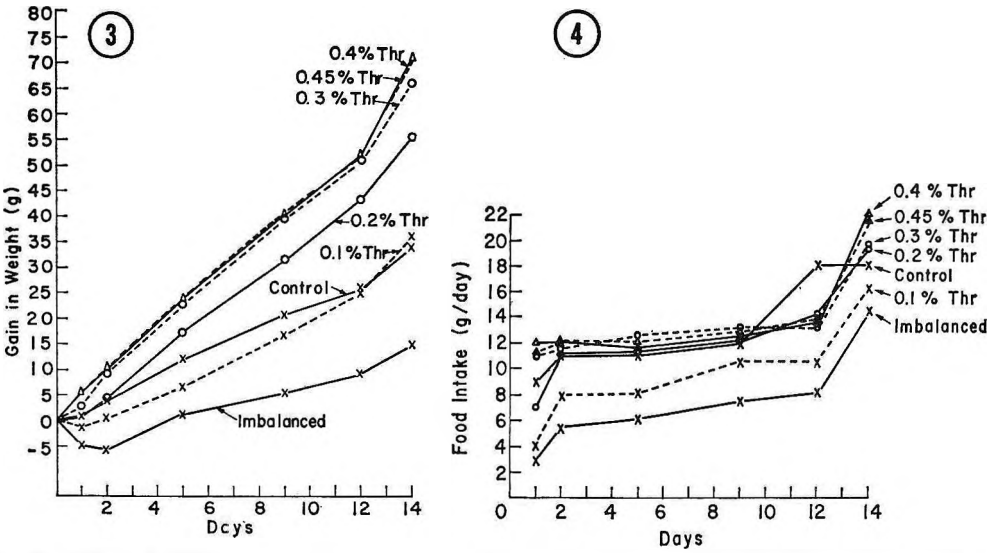

Fig. 3 Effect of addition of graded amounts of threonine (from 0.1%–0.45%) on growth of rats fed a 6% casein diet containing 5.4% of an amino acid mixture lacking threonine.

Fig. 4 Effect of addition of graded amounts of threonine (from 0.1%–0.45%) on food intake of rats fed a 6% casein diet containing 5.4% of an amino acid mixture lacking threonine.

as compared with the controls, 0.4% of threonine was necessary to stimulate growth maximally.

Protein-depleted rats (depleted for 7 days and starved for 12 hours), receiving the imbalanced diet containing 5.4% of the amino acid mixture lacking threonine, showed depression in food intake within 12 hours. The food intake was depressed continuously for the entire 72 hour experimental period (fig. 5). The rapid decrease in food intake prompted a more detailed study of the early part of the feeding period. The food intake of starved, non-protein-depleted rats ingesting the same imbalanced diet was also rapidly depressed compared with that of rats consuming either the basal or the corrected diet supplemented with 0.45% DL-threonine (fig. 6). By 4 hours' time the rats ingesting the imbalanced diet had consumed only about half the quantity of food eaten by those fed the basal or the corrected diet. The food intake pattern of the group fed the corrected diet was similar to that of the control animals throughout the entire 24-hour experimental period.

Effect on interval-fed rats. The food intake and weight gain of rats fed for 2 hours daily the imbalanced diet (5.4% amino acid mixture lacking threonine) were not markedly depressed until the concentration of the imbalancing amino acid mixture was increased to 12.4% (figs. 7 and 8). A depression in food intake of 22% was observed by the end of the experimental period.

Effect on force-fed rats. The change in body weight of rats fed ad libitum, the casein basal diet, the imbalanced diet containing 5.4% of the imbalancing amino acid mixture lacking threonine, or the corrected diet are shown in (fig. 9). There was the usual depression in food intake of rats consuming the imbalanced diet (fig. 10). Rats in the imbalanced group lost weight for 3 days before they began to gain. The control group did not lose weight, whereas rats ingesting the corrected diet gained much more than the control. There was a severe depression (44%) in the food intake of rats fed the imbalanced diet. The food intakes of rats fed the control or the corrected diet were similar (fig. 10).

When rats (6 rats/group) were force-fed the imbalanced diet (threonine-imbalanced) in approximately the amounts consumed by control rats fed ad libitum, their growth was stimulated and they gained as much as, or even slightly more than, control rats (4 rats/group) force-fed the same amount of basal diet. The growth rate of

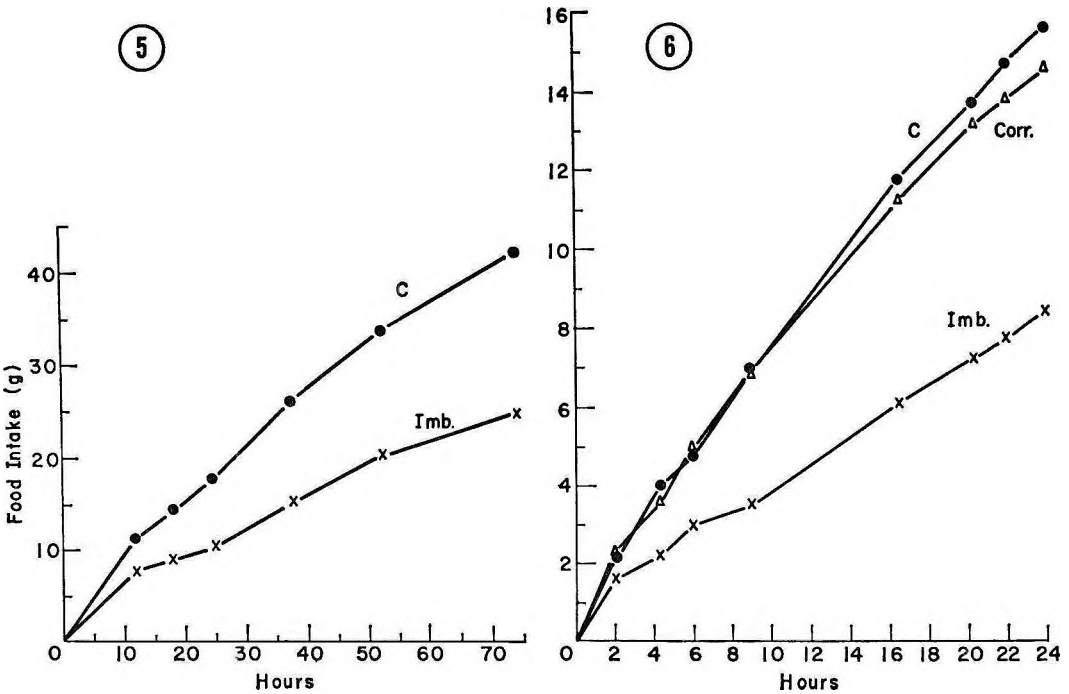

Fig. 5 Rate of food intake of protein-depleted rats fed the control or the imbalanced diet containing 5.4% of an amino acid mixture lacking threonine.

Fig. 6 Rate of food intake of nondepleted rats fed the basal diet, the imbalanced diet or the corrected diet. C: control (6% casein plus 0.3% DL-methionine); Imb: control plus 5.4% amino acid mixture lacking threonine; and Corr: corrected (imbalanced plus 0.45% DL-threonine).

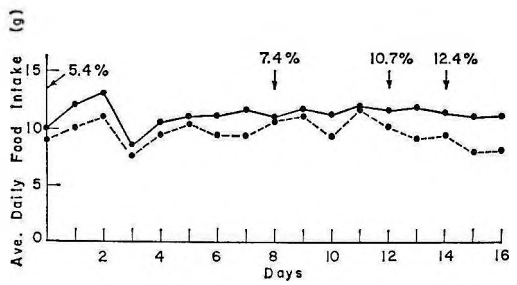

Fig. 7 Average daily food intake of rats trained to eat a single 2-hour meal daily and fed the basal or the imbalanced diets containing different concentrations of amino acid mixtures (from 5.4%–12.4%) lacking threonine; · ——— · control, · – – – – – · imbalanced.

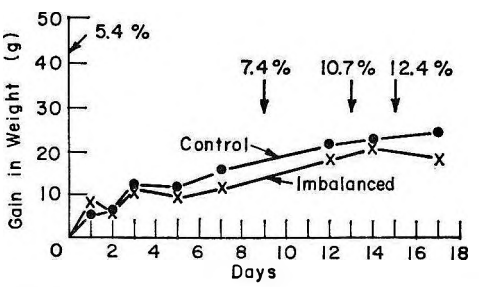

Fig. 8 Changes in body weight of rats trained to eat a single 2-hour meal daily and fed the basal or the imbalanced diets containing different concentrations of amino acid mixtures (from 5.4%–12.4%) lacking threonine.

the group (5 rats) force-fed the corrected diet (supplemented with 0.45% threonine) was higher than that of those force-fed the same amount of the imbalanced or the basal diet (fig. 11).

When 3.8% of the imbalancing amino acid mixture lacking histidine was added to a low fibrin diet the usual food intake and growth depression was observed in rats fed ad libitum. Supplementation of the imbalanced diet with 0.1% histidine improved growth above that of the controls (fig. 12). Food consumption of the control and the corrected groups was similar, whereas the imbalanced group showed a 52% depression in food intake (fig. 13).

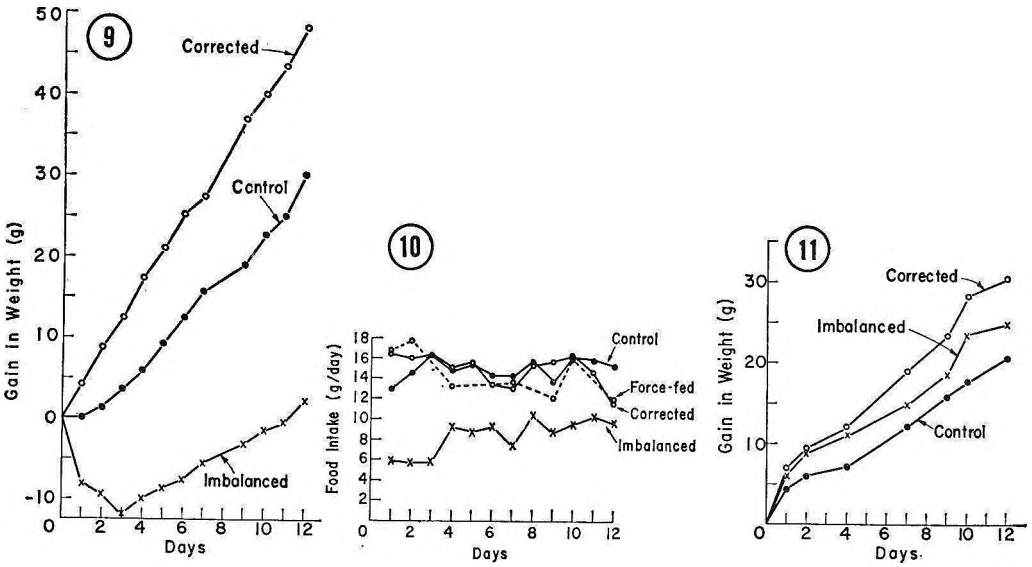

Fig. 9 Changes in body weight of rats fed ad libitum the control diet, the threonine-imbalanced diet or the corrected diet.

Fig. 10 Food intake of rats fed ad libitum the control diet, the threonine-imbalanced diet or the corrected diet.

Fig. 11 Changes in body weight of rats force-fed the control diet, the threonine-imbalanced diet or the corrected diet. Control: 6% casein plus 0.3% DL-methionine; imbalanced: control plus 5.4% amino acid mixture lacking threonine; and corrected: imbalanced plus 0.45% of DL-threonine.

In the force-feeding experiment using the histidine-imbalanced diet, rats force-fed either the control (7 rats/group) or the imbalanced diets (5 rats/group), grew at the same rate. Rats (6 rats/group) force-fed the corrected diet gained more weight than the control or the imbalanced group (fig. 14). All rats in the experiment were force-fed the same quantity of their respective diets as the control rats consumed ad libitum. To establish that the residual food left in the stomach of the force-fed rats did not contribute to the weight gains of the animals, the extent of stomach-emptying of rats force-fed the various experimental diets was determined 8 hours after the animals had been force-fed equivalent quantities of the liquid diets used in the experiments, and the results show that only about 2% of the amounts force-fed remained in the stomach at the time the animals were weighed.

DISCUSSION

The method used in these experiments to create an amino acid imbalance consisted of adding a quantity of an indispensable amino acid mixture devoid of one indispensable amino acid to a diet containing a suboptimal amount of protein. This has proved to be a consistent and effective method of creating amino acid imbalances (2, 11). Using 3.5% of an indispensable amino acid mixture lacking threonine, Harper (12) observed that the magnitude of the food intake and growth depression caused by this imbalancing amino acid mixture was most pronounced when the diet contained 6% casein and was less if the level of casein in the diet was higher. The results presented here indicate that by adding various concentrations of the indispensable amino acid mixture lacking threonine to a 6% casein diet supplemented with 0.3% methionine, the severity of depression in food intake and growth increased proportionately as the concentration of the imbalancing amino acid mixture added to the basal diet was raised.

The palatability of the added imbalancing amino acid mixture, the rate of stomach-emptying of animals ingesting the imbalanced diet, or the rate of absorption and transport of the limiting amino acid do not appear to contribute to the adverse effects

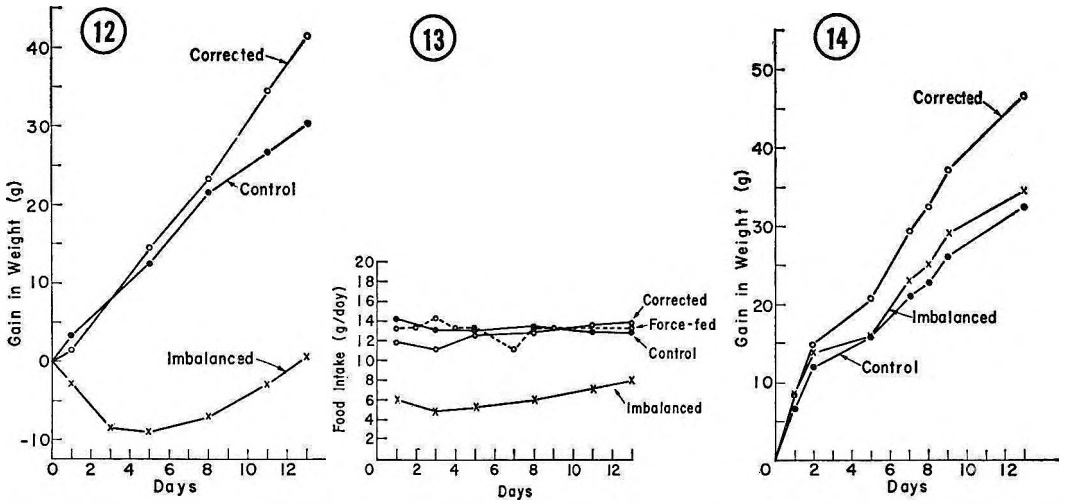

Fig. 12 Changes in body weight of rats fed ad libitum the control diet, the histidine-imbalanced diet or the corrected diet.

Fig. 13 Food intake of rats fed ad libitum the control diet, the histidine-imbalanced diet or the corrected diet.

Fig. 14 Changes in body weight of rats force-fed the control diet, the histidine-imbalanced diet or the corrected diet. Control: 6% fibrin; imbalanced: control plus 3.8% amino acid mixture lacking histidine; and corrected: imbalanced plus 0.1% L-histidine·HCl.

of amino acid imbalance (1). The response of the animals to the ingested amino acid imbalanced diets was rapid. The depression in food intake occurred within 24 hours concomitantly with a reduction in the rate of growth. The onset of the adverse effect on food intake was more rapid when the concentration of the imbalancing amino acid mixture was increased.

This leads to the idea that amino acid imbalances probably bring about some physiological or biochemical changes which affect the feeding behavior of the animals. Rats trained to consume their daily rations in 2 hours did not suffer a severe initial reduction of food intake (fig. 8). This experiment was repeated with another group of 8 rats, using autoclaved starch [8] as the carbohydrate instead of a mixture of dextrin and starch. Although both the control and the imbalanced groups consumed slightly more food in the 2-hour feeding period than those fed the diets containing the mixed carbohydrate, no appreciable adverse effect on food intake was observed initially in rats fed the imbalanced diet containing 5.4% of the amino acid mixture lacking threonine. Increasing the concentration of the imbalancing amino acid mixture to 7.4% and 12.4% on days 9 and 13 of the experiment caused only moderate food intake depressions of 12% and 27%, respectively. The growth rate of rats ingesting the imbalanced diet was not initially depressed. Growth retardation was observed, however, when the concentration of the amino acid mixture in the imbalanced diet was increased and food intake was depressed.

In another experiment, no food intake depression was observed in rats trained to eat for a shorter period of time (one hour) and fed an imbalanced diet containing 5.4% amino acid mixture lacking threonine, (dextrin: starch (1:2) as carbohydrate) even though the amount of the imbalancing amino acid mixture was raised to 7.8%, 10.7% and 12.4% on days 9, 13 and 15, respectively, of the 16-day experimental period. The control animals did not gain any weight throughout the experiment because of the relatively low food consumption in the one-hour feeding period. The rats in the imbalanced group, although consuming nearly the same amount of food daily as the controls, lost

[8] Moist starch heated in an autoclave at 121° for 2 to 3 hours, then dried and ground.

weight slightly, particularly when the concentration of the imbalancing amino acid mixture in the imbalanced diet was increased, due probably to insufficient caloric intake.

These observations, together with the observation that animals force-fed the imbalanced diets gained as much as the controls or even slightly more; and those of Kumta and Harper (4) on the stimulation of food intake of rats fed an imbalanced diet by insulin injections; and of Klain et al. (8) and Harper and Rogers (9) on alleviation of the adverse effect on food intake and growth by cold exposure of rats fed an imbalanced diet, indicate that imbalanced diets can, in fact, support growth if food intake, and thus the intake of the most limiting amino acid in the diet, is increased. However, the observations that rats trained to eat for only 2 hours daily suffered a depression in food intake when the level of the imbalancing amino acid mixture was raised from 5.4 to 12.4% and that insulin injection (1) and cold exposure of rats (9) could not prevent the deleterious effects of amino acid imbalance in rats fed severely imbalanced diets, indicate there is a point at which the balance between the concentration of the most limiting amino acid and that of the surplus of indispensable amino acids is such that the food intake of rats ingesting an imbalanced diet cannot be stimulated.

Through plasma amino acid studies, some clear-cut positive evidence of altered amino acid metabolism due to amino acid imbalance has been obtained. Changes in plasma amino acid pattern in amino acid imbalance have been reported by Kumta and Harper (13). The most-limiting amino acid, histidine, in the plasma of animals ingesting a histidine-imbalanced diet decreased rapidly below that of the control rats shortly after the ingestion of the imbalanced diet, whereas the concentrations of those added to create the imbalance rose. Using the same imbalanced diet, Sanahuja and Harper (14) were able to demonstrate an association between the decrease of the most-limiting amino acid in the plasma and the food intake depression of rats fed the imbalanced diet.

Amino acid imbalance created by adding a surplus of all but one of the indispensable amino acids to a low protein diet did not increase catabolism or enhance excretion of the most-limiting amino acid in urine or feces (15). Competition among amino acids for transport sites would not appear to explain the effects of ingestion of an imbalanced diet (16). Incorporation of the most-limiting amino acid into liver protein, however, was enhanced in rats fed amino acid imbalanced diets (15, 17). Provision of an excess of the second most-limiting amino acid, as occurs when an imbalanced diet is fed, should lead to more efficient utilization of the most-limiting amino acid for protein synthesis and this, together with the low activities of enzymes of amino acid degradation in rats fed a low protein diet, appears to be the basis for the changes in blood amino acid pattern as a result of feeding an imbalanced diet. The results of the force-feeding experiments and of the experiments on rats fed for only a short interval daily indicate that rats can utilize the limiting amino acid in an imbalanced diet as efficiently, if not more so, than the same amino acid in an appropriate control diet, provided food intake can be maintained.

Thus, the depression in growth caused by an amino acid imbalance is a secondary rather than a primary effect. All of these observations tend to indicate that some basic mechanism regulating food intake is affected by an amino acid imbalance. It is known that the depression in food intake and the change in plasma amino acid pattern in amino acid imbalance resemble that observed in animals fed a much more deficient diet; a diet, which, when force-fed, causes the development of pathologic lesions and other abnormalites (18–21). The similarity between the initial response of rats fed ad libitum a diet devoid of a single indispensable amino acid and that of rats fed an imbalanced diet suggests that the inhibition of food intake might be the result of some protective mechanism involved in homeostatic control within the animal body.

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Effect of Amino Acid Imbalance on Dietary Choice in the Rat [1]

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ABSTRACT A study was undertaken in an effort to determine more precisely the influence of the severity of an amino acid imbalanced diet and the importance of the quantity of the limiting amino acid on the choice of the rats for or against the imbalanced diets. When rats were allowed to choose between an imbalanced diet (which would support a slow rate of growth) and a protein-free diet (which would not support growth), they selected the latter almost exclusively. They also selected the imbalanced diet supplemented with the most-limiting amino acid over the imbalanced diet. Rats offered a choice between a protein-free diet and one of a series of imbalanced diets containing increasing increments of the amino acid mixture lacking threonine (from 1% to 5.4%) showed a decreasing preference for the imbalanced diet and an increasing preference for the protein-free diet as the quantity of amino acid mixture in the imbalanced diet was increased. When increasing levels of threonine (from 0.05% to 0.45%) were added to the imbalanced diet, the rats showed a gradually decreasing preference for the protein-free diet. Rats pre-fed a high protein diet consumed greater quantities of the imbalanced diet than those pre-fed diets with lower concentration of protein.

Depression in food intake and growth and changes in food preference are some of the early effects of dietary amino acid imbalances. When rats that had previously been protein-depleted, starved or fed ad libitum were offered a choice between an imbalanced diet containing an amino acid mixture lacking histidine and a protein-free diet, the rats invariably rejected the imbalanced diet (1). The time required for the distinctive preference to become evident varied with the previous treatment of the animals. Also rats showed no clear-cut choice between two balanced diets (the basal and the corrected histidine-imbalanced diets) even though the diets differed in nutritive value. Rats were, however, able to discriminate between the imbalanced diet and the corrected diet. These 2 diets were nearly identical in texture and presumably in palatability since they differed only by 0.1% of L-histidine·HCl. Furthermore, Harper and Rogers (2) have demonstrated that rats kept in the cold, and offered a choice between a protein-free diet and an imbalanced diet show a decided preference for the protein-free diet even though they show no depression in food intake or weight gain when offered the imbalanced diet alone. Thus, it is apparent that some physiological or biochemical change must occur in rats fed an amino acid imbalanced diet to cause them to select a nutritionally inferior diet over one of higher nutritive value.

The present studies were undertaken to determine the effects of graded levels of the imbalancing amino acid mixture, the effects of graded levels of the limiting amino acid, and the influence of the previous diet on food preferences of the rat.

EXPERIMENTAL

Male weanling or young rats of Holtzman or Sprague-Dawley strain were used in these experiments. They were housed in individual, suspended cages with screen bottoms in which were placed two identical spillage-proof food containers. The food cups were placed daily in the cage at random to prevent any position preference. All rats were fed the basal diets for 3 days to allow them to adjust to the new environment. The animals were fed as

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described below, and food intake was measured and recorded daily. Water was offered ad libitum. Groups of 4 to 12 rats were used as indicated.

Diets. The basal diet contained 6% casein supplemented with 0.3% DL-methionine (for the threonine-imbalance studies) or 5% casein supplemented with 0.3% L-methionine and 0.2% L-threonine (for the histidine-imbalance experiments); salt mixture,[4] 4%; corn oil,[5] 5%; vitamin mixture,[6] 0.5%; choline chloride, 0.2% and dextrin-starch (1:2) mixture as the carbohydrate to make up 100%. The protein-free diet was similar to the basal diet except that the mixed carbohydrate mentioned above replaced the casein and amino acids. Amino acids were added at the expense of the mixed carbohydrate.

Amino acid imbalances were created by adding a mixture of indispensable amino acids devoid of one to the basal or control diet. The threonine-imbalanced diet contained 5.4% of a mixture of amino acids devoid of threonine (table 1). The histidine-imbalanced diet differed from that used by Sanahuja and Harper (3) in that casein was used as the protein instead of fibrin. To make histidine more nearly limiting, 0.3% L-methionine and 0.2% L-threonine were included in the casein diet. To create the histidine imbalance then, 5.4% of an amino acid mixture devoid of histidine (table 1) was added to the casein basal diet. The corrected diet

[4] The salt mixture contained: (in percent) $CaCO_3$, 29.29; $CaHPO_4 \cdot 2H_2O$, 0.43; KH_2PO_4, 34.31; NaCl, 25.06; $MgSO_4 \cdot 7H_2O$, 9.98; Fe ($C_6H_5O_7 \cdot 6H_2O$, 0.623; $CuSO_4$, 0.156; $MnSO_4 \cdot H_2O$, 0.121; $ZnCl_2$, 0.020; KI, 0.0005; $(NH_4)_6MO_7O_{24} \cdot 4H_2O$, 0.0025; and $Na_2SeO_3 \cdot 5H_2O$, 0.0015. The salt mix was prepared by and purchased from General Biochemicals, Inc., Chagrin Falls, Ohio. The salts at 5% of the diet provided in percent of element; Ca, 0.592; P, 0.394; K, 0.493; Na, 0.493; Cl, 0.760; Mg, 0.049; Fe, 0.0049; Cu, 0.0019; Mn, 0.00195; Zn, 0.0004; I, 0.000019; Mo, 0.000005; and Se, 0.0000025.

[5] Mazola, Corn Products Company, New York.

[6] Five-tenths percent of the vitamin mixture in the diet provided the rats with 0.44% sucrose plus the following vitamins in mg/kg diet: thiamine·HCl, 5; riboflavin, 5; niacinamide, 25.0; Ca D-pantothenate, 20; pyridoxine·HCl, 5; folic acid, 0.5; menadione, 0.5; *d*-biotin, 0.2; vitamin B_{12} (0.1% in mannitol), 30; ascorbic acid, 50 (added to prevent thiamine destruction); vitamin E acetate (25% in a mixture of gelatin, sugar and starch), 400; vitamin A acetate and vitamin D_2 (325,000 USP units of A/g and 32,500 USP units of D_2/g in a mixture of gelatin, sugar and starch), 12.31. (The thiamine, niacinamide, folic acid, menadione and vitamin B_{12} were purchased from Nutritional Biochemicals Corporation, Cleveland, and the rest were purchased from Hoffmann-La Roche, Inc., Nutley, New Jersey.

TABLE 1

Composition of control and imbalanced diets

	Threonine imbalanced experiment diets		Histidine imbalanced experiment diets	
	Control	Imbalanced	Control	Imbalanced
	%	%	%	%
Casein	6	6	5	5
Corn oil [1]	5	5	5	5
Salt mixture [2]	4	4	4	4
Vitamins [2]	0.5	0.5	0.5	0.5
Choline chloride	0.2	0.2	0.2	0.2
L-Threonine	—	—	0.2	0.45
DL-Methionine	0.3	0.3	—	—
L-Methionine	—	—	0.3	0.3
DL-Tryptophan	—	0.24	—	—
L-Tryptophan	—	—	—	0.15
L-Leucine	—	0.84	—	0.9
DL-Isoleucine	—	1.20	—	—
L-Isoleucine	—	—	—	0.6
DL-Valine	—	1.30	—	—
L-Valine	—	—	—	0.6
L-Histidine·HCl	—	0.36	—	—
DL-Phenylalanine	—	0.54	—	—
L-Phenylalanine	—	—	—	0.9
L-Lysine·HCl	—	0.9	—	0.9
L-Arginine·HCl	—	—	—	0.6
Dextrin:starch (1:2)	84	78.62	84.8	79.9
Total	100	100	100	100

[1] Mazola, Corn Products Company, New York.
[2] See Experimental section for composition.

used in the threonine-imbalance study was the imbalanced diet supplemented with 0.45% DL-threonine. In some experiments, as indicated below, corrected diets containing 0.3%, 0.25%, 0.2%, 0.1% or only 0.05% DL-threonine were used. In one experiment, 1% and 2% of the imbalancing amino acid mixture devoid of threonine were used. Addition or deletion of amino acids was compensated for by adjusting the concentration of the mixed carbohydrate. The 25% and 80% casein diets used in the pre-feeding studies were similar to the basal diet, but no methionine was added.

RESULTS

Food preference of rats previously fed basal diet. When rats (4 rats/group) were given a choice between the imbalanced diet containing 5.4% of an amino acid mixture lacking threonine and the protein-free diet, they ate the protein-free diet almost exclusively throughout and consumed a little of the imbalanced diet in the beginning and toward the end of the first and the second weeks. In contrast, the groups offered a choice between the protein-free diet and the corrected or the basal diet rejected the protein-free diet after the first 2 days of the experiment (figs. 1, 2 and 3). The feeding pattern of rats offered a choice between the threonine-imbalanced and the corrected diets is shown in figure 4. Both diets were similar in texture and palatability since the corrected diet contained only 0.45% of additional threonine. Nevertheless, the rats were able to discriminate between the 2 diets, and although they did not show an exclusive preference for the corrected diet, they consumed only very small quantities of the imbalanced diet. The same type of study was carried out with the basal diet in place of the corrected diet, as shown in figure 5. A clear preference for the basal diet was evident on the first day. Rejection of the imbalanced diet was nearly immediate and this persisted throughout the 14-day experimental period.

To establish whether rats rejected the imbalanced diet because of a difference between the palatability of the basal diet and the imbalanced diet owing to the relatively large quantity of free amino acids in the latter, rats were offered a choice between the corrected and the basal diet. The results are shown in figure 6. Twelve rats were used per group in this experiment. There was no evidence of a distinctive preference for either of the two balanced diets in the 2-week study.

In a reversal study (fig. 7), rats (4 rats/group) were offered a choice between the threonine-imbalanced diet containing 5.4% of an amino acid mixture lacking threonine and the protein-free diet. As usual, the animals rejected completely the imbalanced diet. At the end of the seventh day, the imbalanced diet was replaced by the corrected diet, the animals continued to avoid the corrected diet. At the end of the second week, the animals still maintained their preference for the protein-free diet. The protein-free diet was then withdrawn for 1 day so that the rats were forced to eat the corrected diet. Thereafter, they continued to eat the corrected diet even when the protein-free diet was returned to the cage.

To determine how different degrees of amino acid imbalance affected the preference of rats for the protein-free diet, 6 rats were offered a choice between a protein-free diet and successively imbalanced diets containing 5.4%, 2% or 1% of amino acid mixture lacking threonine. The average consumption of the protein-free diet decreased as the imbalance became less severe. The diet containing 2% of the amino acid mixture devoid of threonine made up more than 50% of the total food consumed, whereas the one containing only 1% of the imbalancing mixture constituted almost the entire food intake (fig. 8). In another experiment, 5 groups of 4 rats each were offered a choice between a protein-free diet and one of a series of corrected diets containing from 0% to 0.45% of threonine for 7 days. The results are summarized in figure 9. The rats were highly sensitive to the concentrations of threonine in the corrected diet and consumed a large amount of the protein-free diet when the threonine supplement was reduced to 0.1%. The average consumption of the protein-free diet increased with decreasing concentrations of threonine in the corrected diets.

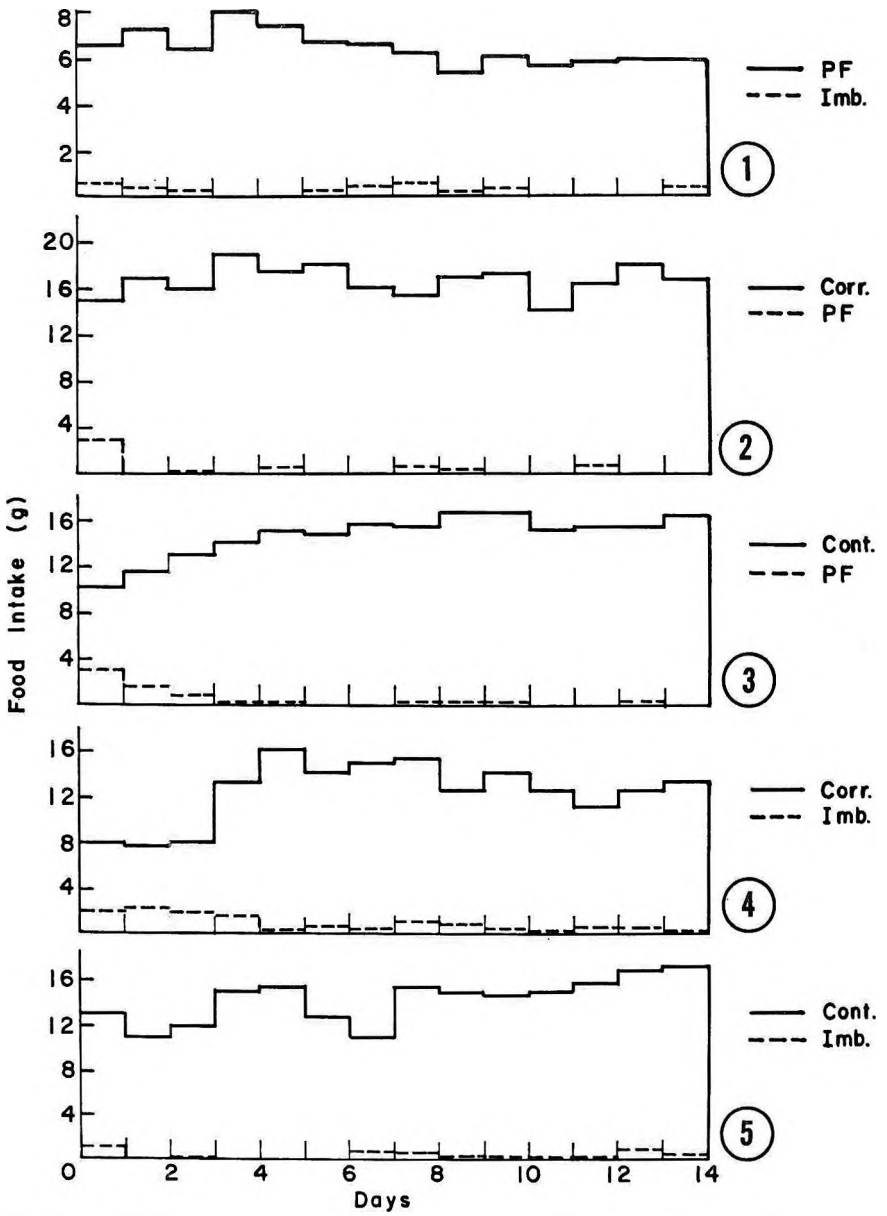

Fig. 1 Food selection by rats fed imbalanced (Imb.) diet low in threonine and protein-free (PF) diet.

Fig. 2 Food selection by rats fed the corrected (Corr.) diet and protein-free (PF) diet.

Fig. 3 Food selection by rats fed the control (Cont.) diet and protein free (PF) diet.

Fig. 4 Food selection by rats fed imbalanced (Imb.) diet low in threonine and corrected (Corr.) diet.

Fig. 5 Food selection by rats fed control (Cont.) diet and the threonine imbalanced (Imb.) diet. Control diet: 6% casein plus 0.3% DL-methionine; imbalanced diet: control plus 5.4% amino acid mixture lacking threonine; corrected diet: imbalanced plus 0.45% DL-threonine; and protein-free diet: same composition as the control diet except that protein and methionine were replaced by mixed carbohydrate (Dextrin-starch 1:2).

The feeding behavior of rats depleted of protein for 2 weeks and offered a choice between a protein-free diet and the imbalanced diet containing 5.4% of an amino acid mixture lacking threonine is shown in figure 10. On the first day one rat ate mainly the imbalanced diet and another ate equal amounts of both. All 4 rats rejected the imbalanced diet on the second day and consumed exclusively the protein-free diet.

Influence of previous diet on food preference. To determine the effect of prefeeding an imbalanced diet on the subsequent choice between a protein-free diet and an imbalanced diet, animals were fed in a preliminary period a threonine-imbalanced diet containing 5.4% of an amino acid mixture lacking threonine for 4 weeks and then were offered a choice between a protein-free diet and an imbalanced diet. Individual food intake patterns of 4 rats are shown in figure 11. Two of the four rats selected the protein-free diet almost immediately. The other two continued to eat the imbalanced diet and some of the protein-free diet at the beginning of the experiment, and thereafter, they consumed the imbalanced diet only intermittently. To determine how the feeding patterns of the rats persisted, the experiment was continued for 5 weeks. Two rats rejected almost entirely the imbalanced diet throughout, and toward the conclusion of the experiment, they curtailed even the consumption of protein-free diet and died subsequently of continuous protein depletion and probably starvation, even though they had before them at all times an imbalanced diet that would support some growth. Animals that consumed the imbalanced diet intermittently survived the experimental period.

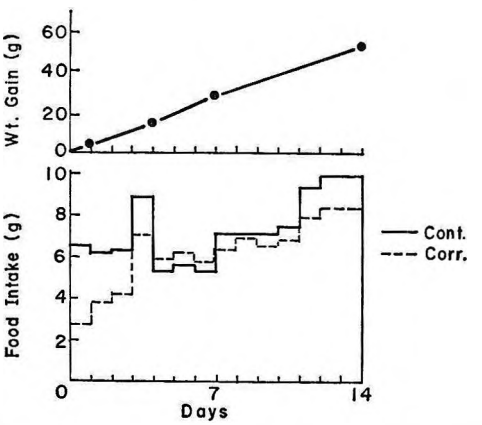

Fig. 6 Changes in body weight and food preference of rats offered control (Cont.) and correct (Corr.) diets simultaneously.

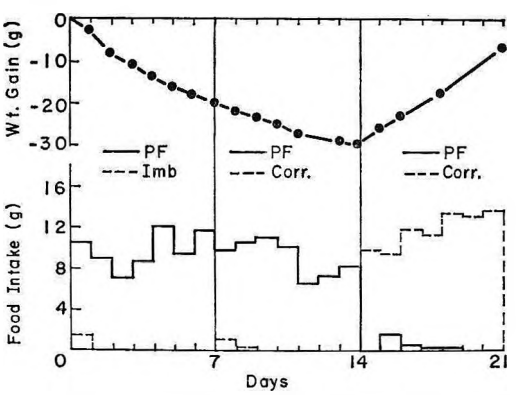

Fig. 7 Changes in body weight and food preference of rats offered protein-free (PF) diet and imbalanced (Imb.) diet or protein-free (PF) diet and corrected (Corr.) diet together.

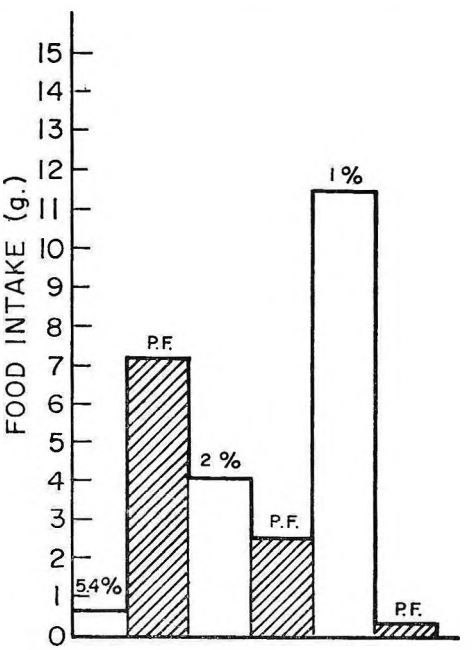

Fig. 8 Food selection by rats fed protein-free (PF) diet and imbalanced diets containing different levels of amino acid mixtures lacking threonine.

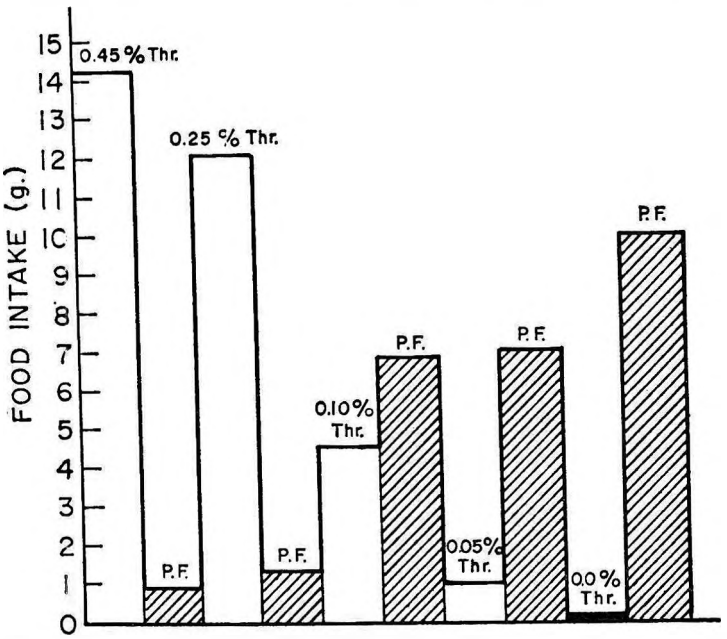

Fig. 9 Food selection by rats fed protein-free (PF) diet and imbalanced diets containing different levels of threonine with the imbalancing amino acid mixture.

The effect on subsequent consumption of a protein-free and an imbalanced diet (containing 5.4% of an amino acid mixture lacking threonine) of rats pre-fed for 5 weeks a low protein (6% casein), a "normal" protein (25% casein), or a high protein (80% casein) diet are shown in figure 12. Rats previously fed the high protein diet consumed more of the imbalanced diet than rats pre-fed "normal" or low protein diets. Seven of the ten rats pre-fed the low protein diet rejected the imbalanced diet after the first day. Three of the ten rats from the low protein pre-fed group consumed some of the imbalanced diet after the eighth day. Animals pre-fed the "normal" protein diet consumed mainly the protein-free diet for 4 days and thereafter, their consumption of the imbalanced diet increased markedly. Rats pre-fed the high protein diet ingested mostly the imbalanced diet immediately, and the intake of the protein-free diet remained low throughout the experimental period. The proportion of total food ingested from the imbalanced diet appeared to be directly associated with the previous dietary protein load.

In an attempt to establish that the effect of a high protein intake on the subsequent consumption of an imbalanced diet was a

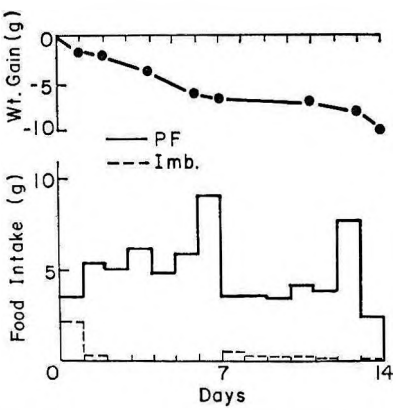

Fig. 10 Changes in body weight and food preference of protein-depleted rats fed imbalanced (Imb.) diet and protein-free (PF) diet simultaneously. Control diet: 6% casein plus 0.3% DL-methionine; imbalanced diet: control plus 5.4% amino acid mixture lacking threonine; corrected diet: imbalanced plus 0.45% DL-threonine; and protein-free diet: same composition as the control diet except that protein and methionine were replaced by mixed carbohydrate (Dextrin-starch 1:2).

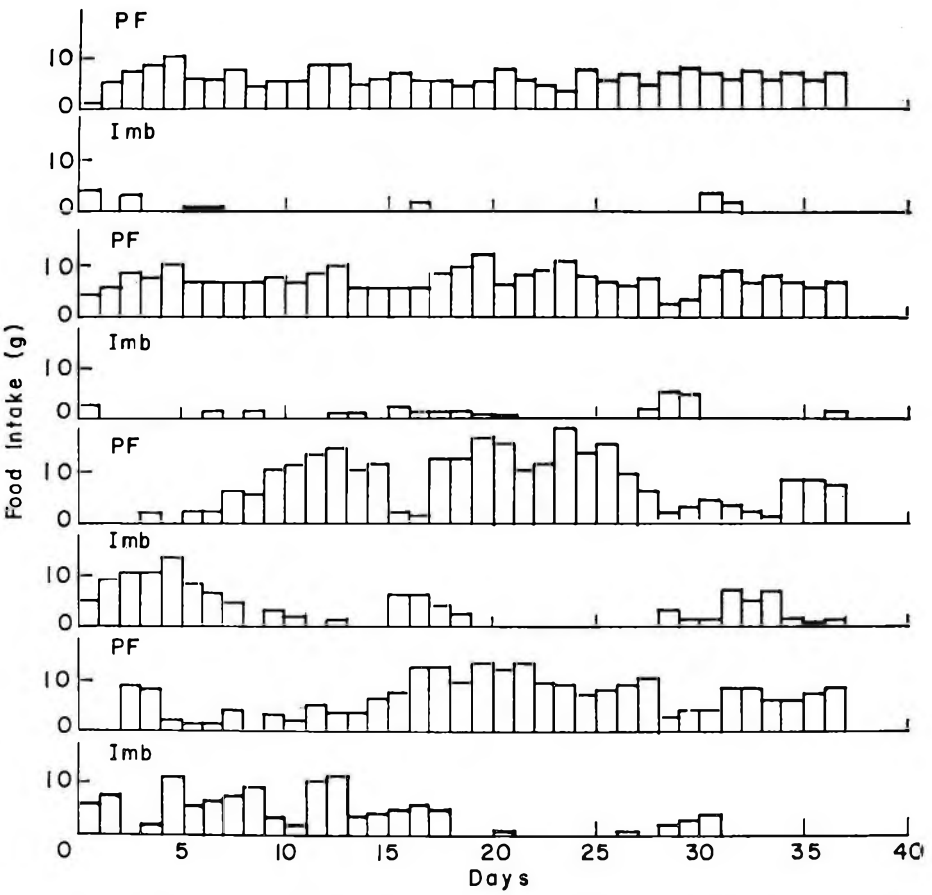

Fig. 11 Food selection by individual rats pre-fed imbalanced (Imb.) diet and subsequently fed protein-free (PF) diet and imbalanced diet simultaneously. Imbalanced diet: 6% casein plus 0.3% DL-methionine; and protein-free diet: contained all the necessary nutrients except the protein. The deleted protein was compensated by adjusting the level of the mixed carbohydrate (Dextrin-starch 1:2).

general phenomenon, a group of 6 rats pre-fed an 80% casein diet for 4 weeks was offered a choice between a protein-free diet and an imbalanced diet containing 5% casein and an amino acid mixture lacking histidine. After 5 days (fig. 13) all animals selected the imbalanced diet over the protein-free diet.

DISCUSSION

The results obtained with non-depleted rats agree well with the observation of Sanahuja and Harper (3). All rats preferred the protein-free, the basal, or the corrected diet over the imbalanced diet. Also, the rats selected the balanced diets (the basal and the corrected diet) over the protein-free diet. Since the protein-free diet did not support growth and the imbalanced diet did, the anomalous behavior of the rats offered a choice between these 2 diets obviously involved factors other than nutritive value. The addition of 0.25% of DL-threonine (fig. 9) does not enhance the taste or texture of a diet that already contains 5.4% of free amino acids. The rejection of the imbalanced diet by rats shortly after they were offered the choice between a protein-free diet and an imbalanced diet was probably due to some undesirable physiological or biochemical change following the ingestion of the imbalanced diet. The contin-

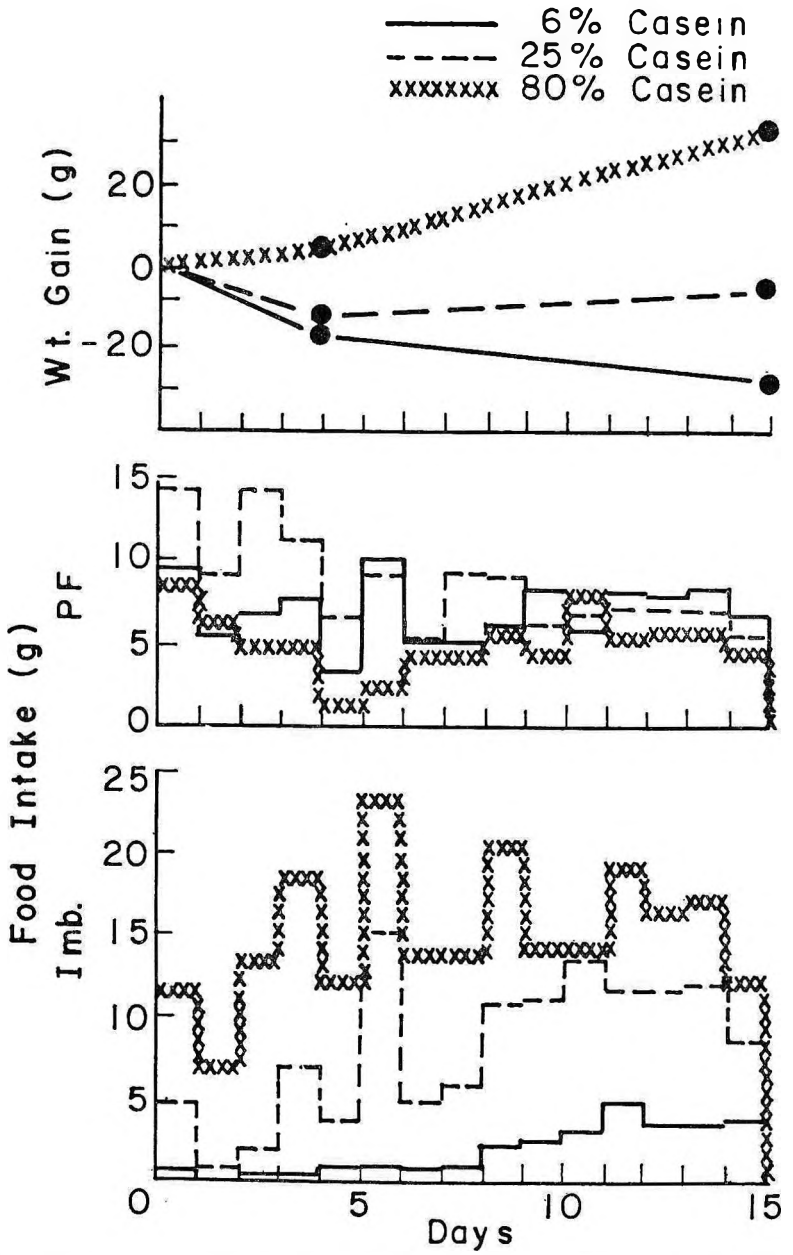

Fig. 12 Changes in body weight and food selection of rate pre-fed diets containing different levels of casein (from 6%–80%) and subsequently offered a choice between a protein-free (PF) diet and an imbalanced (Imb.) diet containing 6% casein and 5.4% of an amino acid mixture lacking threonine. ·———·, Pre-fed 6% casein diet; ·– – – – –·, pre-fed 25% casein diet; and . xxxxx ., pre-fed 80% casein diet.

uous preference for the protein-free diet over the imbalanced one was probably the result of a "learned response" since, once the preference becomes established the rats even rejected the corrected diet in favor of the protein-free (fig. 7). This is probably a result of the inability of the rat to distinguish between the imbalanced diet

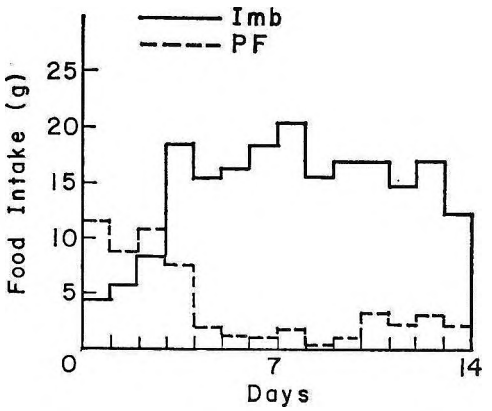

Fig. 13 Food selection of rats pre-fed 80% casein diet and subsequently offered a choice between a protein-free diet and an imbalanced diet containing 5% casein plus 0.3% L-methionine, 0.2% L-threonine, and 5.4% amino acid mixture lacking histidine.

and the corrected diet solely on the basis of smell, taste or texture.

The choice between the imbalanced and the corrected diets was not achieved as readily or completely as the other choices. The similarities in smell, taste and texture of the 2 diets apparently compelled the rats to sample enough of each diet to facilitate their recognition and permit subsequent preference for the corrected diet. When the diets were similar in amino acid balance even though they differed in physical properties or nutritive value, as in the choice between the basal and the corrected diet, no clear preference developed, due apparently to the absence of the adverse physiological or biochemical effects associated with either diet.

Information about the critical point at which rats discriminate between protein-free and imbalanced diets was obtained through the experiment in which groups were offered a protein-free diet and one of a series of diets containing increasing amounts of the imbalancing amino acid mixture or one of a series of imbalanced diets containing increasing amounts of the most limiting amino acid. The results indicate that the intake of the imbalanced diet is regulated by some mechanism that is quite sensitive to quantitative relationships among the dietary amino acids, and that the animals presumably seek relief by selecting the protein-free diet in proportion to the degree of the undesirable physiological or biochemical effect. Thus the degree of preference for the protein-free diet provides a rather sensitive index for the extent of "imbalance" of the diet.

In another type of dietary choice experiment involving the presence of a relatively large amount of a single amino acid, Rogers et al. (4) have shown that rats fed a high leucine diet (5% leucine) and a protein-free diet selected the protein-free which would not support growth to the high leucine diet, which, although it caused depression in food intake, would support some growth. Rats preferred also the protein-free diet over the high leucine diet (L diet), which had been improved nutritionally with isoleucine and valine supplements (LIV diet). Further improvement of the LIV diet with tryptophan, threonine, and phenylalanine improved the choice but still several rats chose the protein-free diet. However, the rats selected the LIV diet over the L diet when they were allowed a choice, but they again rejected the LIV diet in favor of the protein-free diet when the former was substituted for the L diet. This also indicates that food preference measurements may provide a highly sensitive test for other adverse effects of dietary amino acids.

The choice experiment with the protein-depleted rats agreed in general with that reported by Sanahuja and Harper (3) in that some rats seek temporary repletion with the imbalanced diet on the first day and one rat consumed the imbalanced diet intermittently for repletion after the seventh day. All rats, however, rejected the imbalanced diet on the second day. The adverse effect occurred earlier in this study than in that reported by Sanahuja and Harper (3).

The behavior of rats pre-fed the imbalanced diet (fig. 11) for one month indicates something of the variability in the responses of rats offered choices involving an imbalanced diet. Two of the four rats did not seek even temporary protein repletion, i.e., the rats refused the imbalanced diet throughout the entire experimental period. They apparently developed an intensive abhorrence for the imbalanced diet and thereafter died of severe

protein depletion. The other 2 rats ate enough imbalanced diet to survive.

The "learned response" in these dietary preference experiments is undoubtedly caused by some biochemical change that results from ingestion of the imbalanced diet. Some alteration in the metabolic state of the animals—perhaps triggered by the change in the plasma amino acid pattern, especially the rapid fall in the concentration of the growth limiting amino acid in the plasma shortly after the ingestion of the imbalanced diet together with elevations in the amino acids added to create the imbalance (5–7), could provide an adequate signal. Both the basal and the corrected diets produced a more balanced amino acid pattern and the rats failed to select distinctly between these two. The initial rejection of the imbalanced diet, therefore, is due apparently to an undesirable physiological or biochemical disturbance, whereas the continued avoidance of the imbalanced diet may be attributed to the recollection of the previous unfavorable dietary experience.

The feeding behavior of rats pre-fed diets containing higher concentrations of protein may be explained through the concept of "protein reserves" as defined by Allison and Bird (8). The labile protein, presumably laid down owing to a high protein intake, may contribute to the plasma amino acids thus modifying the plasma and tissue amino acid patterns of rats ingesting the imbalanced diet, thus preventing the unfavorable response of the rats to the imbalanced diet. However, many of the enzymes of amino acid catabolism are induced by a high protein intake. This could result in an increased rate of catabolism of the excess of amino acids added to create the imbalance and, hence, of more rapid restoration of the plasma amino acid pattern toward normal. Shapiro and Fisher [7] and Fisher et al. (9) have demonstrated the beneficial effect of feeding a high protein diet to chicks before feeding them amino acid imbalanced diets. It is well-known that food intake and growth depression is much less in adult animals ingesting the imbalanced diet (10). The previous dietary history, particularly protein intake in relation to amino acid requirements and the level of the amino acid catabolic enzymes of the adult, rather than age per se, may account for the less severe adverse effects of amino acid imbalance in the adult animal.

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Influence of Dietary Protein Levels and Hydrocortisone Administration on the Branched-chain Amino Acid Transaminase Activity in Rat Tissues [1]

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ABSTRACT To determine whether the branched-chain amino acid transaminase (BATase) could be regulated by changes in dietary protein and glucocorticoid treatment, the activity of this enzyme was measured in tissues of 200-g rats receiving diets containing zero, 18 or 75% protein and in tissues of rats given hydrocortisone injections. It was found that BATase increased significantly in liver, kidney and muscle, but not in intestinal tissue of rats receiving 75% or zero per cent casein diets for 10-day periods, as compared with enzyme activities of tissues from rats given an 18% casein diet. Some increases in BATase were also noted in tissues of rats given an 18% casein diet when the animals were injected with hydrocortisone. After multiple doses of this hormone, BATase increased in liver and muscle, but not in kidney. All 3 branched-chain amino acids were transaminated at the same rate by BATase from liver, kidney and intestine, but the BATase from muscle transaminated leucine at a faster rate than either isoleucine or valine.

Reports have indicated that in mammalian tissue the branched-chain amino acids (BATase) are transaminated by a single enzyme specific for these compounds (1–3). The tissue distribution of BATase is different from most other transaminases in that there is a higher activity for this enzyme in muscle and kidney than in liver. For this reason, the dietary and hormonal control of BATase is of particular interest. In the present study, the effects of dietary protein levels and hydrocortisone administration on BATase in various tissues were measured concurrently with some enzymes of amino acid catabolism in liver known to be induced by dietary protein and cortisone treatment.

EXPERIMENTAL

Male rats (200 to 230 g) of the Sprague-Dawley strain were allotted at random to the treatments in each experiment. The basal diet consisted of the following: (in %) casein, 18.0; glucose, 63.8; corn oil, 10.0; mineral mixture, 4.0; vitamin mixture, 2.2; and cellulose, 2.0. The mineral and vitamin mixtures [3] have been described previously (4). All animals were allowed the basal diet ad libitum for several days before the experimental period.

In the first experiment (table 1), the dietary protein level was varied. One group of rats received the basal diet containing 18% casein; another group received a diet containing zero per cent casein, in which all the casein in the basal diet was replaced by glucose; the third group of animals received a 75% casein diet, made by supplementing the protein in the basal diet with additional casein and removing an equivalent amount of glucose. All animals received the diets for a 10-day period. They were killed 8 hours after food had been removed from the cages.

In the experiment with hydrocortisone administration, all rats received the basal diet. The rats in group 1 were given a single dose of hydrocortisone acetate [4] (1 mg/100 g body weight) intraperitoneally. Rats in group 2 were treated as those in group 1 daily for 3 days. Rats in group 3 were given 2 mg of hydrocortisone acetate/100 g body weight intraperitoneally for 7 days. For each group of animals, some rats received the same amount of a saline solution intraperitoneally. All animals were

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[3] Obtained from Nutritional Biochemicals Corporation, Cleveland.

[4] Cortef Acetate, The Upjohn Company, Kalamazoo, Michigan.

TABLE 1

Branched-chain amino acid transaminase (BATase) activity of tissues from rats fed diets varying in protein content

Tissue	Casein in diet	BATase activity [2] with indicated substrate		
		Leucine	Isoleucine	Valine
	%			
Liver	18	18.4± 2.0	17.0± 2.9	18.7± 1.5
	75	29.5± 3.1 **	26.6± 4.6 *	28.9± 2.9 **
	—	33.4± 6.9 *	30.0± 5.5 *	33.9± 6.0 *
Kidney	18	303.4±15.9	303.2±35.1	348.1±15.7
	75	422.5±60.3 *	414.4±50.1 *	479.3±78.4 *
	—	241.7± 8.0 ***	238.8± 1.7 *	270.6± 5.7 ***
Muscle	18	125.4± 8.8	83.3± 9.8	72.0± 7.1
	75	191.2±13.6 ****	175.5±12.7 ***	148.0±14.9 ***
	—	215.3±14.0 ****	200.0±11.5 ****	190.6±13.8 ****
Intestine	18	46.2± 6.1	47.9± 2.7	46.3± 1.3
	75	91.1±36.2	94.5±43.5	84.1±38.1
	—	58.0±11.0	56.3± 9.1	56.3± 7.9

[1] Mean value ± SD of 3 samples each representing a pool from 2 rats. Values are expressed as mμmoles of keto acid formed from the amino acid substrate/10 minutes/mg of protein.
*, **, ***, **** Significant difference from the mean value with 18% casein at the 5%, 1%, 0.5% and 0.1% levels, respectively, by t test.

killed 6 hours after the last injection of hormone. No food was given during this interval.

For each experiment, the animals were anesthetized with ether. The blood was removed by cardiac puncture, and liver, kidney, muscle and intestine were rapidly cut out and frozen over dry ice. The tissues were then stored at − 20° until assayed for enzyme activity. Preliminary experiments showed that tissues could be maintained under these conditions for a week with no detectable changes in enzyme activity. Within this time-period, one gram of each frozen tissue was homogenized in 9 ml of 0.1 M sodium phosphate buffer (pH 7.4) in the Virtis homogenizer and the homogenates were centrifuged at 5000 × *g* for 15 minutes. The supernatant was assayed for BATase according to the procedure of Ichihara and Koyama (1) by measuring the amount of keto acid formed on incubation with a branched-chain amino acid and α-ketoglutarate. Tyrosine transaminase, threonine dehydratase and tryptophan pyrrolase activities of liver tissue were determined by the methods of Kenney (5), Ishikawa et al. (6) and Knox (7), respectively. Protein concentrations in all tissues were measured by the biuret method (8), with bovine serum albumin as the standard.

In some experiments, measurements of amino acid concentrations were made using the procedures of ion exchange chromatography (9, 4).

RESULTS

Dietary protein levels and liver enzymes. It is shown in tables 1 and 2 that, for animals fed the 75% casein diet, the activities of all liver enzymes tested (BATase, tryptophan pyrrolase, tyrosine transaminase and threonine dehydratase) were significantly higher than those of rats fed the 18% casein diet.

In animals fed the protein-free diet, the BATase and tyrosine transaminase were again increased above those found in the group receiving the 18% casein diet, but tryptophan pyrrolase and threonine dehydratase were lower.

The BATase activities with leucine, isoleucine or valine as substrates were similar with all diets tested.

Dietary protein levels and BATase in other tissues. For kidney tissue, BATase was higher in animals receiving the 75% casein diet than in the group given the basal 18% casein diet, whereas it was significantly lower for rats in the non-protein group. The activity of this enzyme in the intestinal tissue of the rats fed the high protein diet was twice as high as that of

the basal diet group, but this difference was not significant. Both in kidney and intestinal tissue, the enzyme activities with the 3 substrates were similar.

In muscle, both the zero per cent and the 75% casein diets were associated with increased BATase, and the non-protein diet had the more pronounced effect of the two. Muscle was the only tissue studied in which leucine was transaminated at a faster rate than the other 2 amino acids, and the difference was significant ($P < 0.005$ by t test).

Hydrocortisone and BATase. The effects of hydrocortisone administration on BATase are summarized in table 3. With a single dose of 1 mg of hydrocortisone/100 g of body weight (group 1), no change in BATase was found in any tissue. In the same experimental animals, tryptophan pyrrolase, tyrosine transaminase and threonine dehydratase in liver tissue were found to be sensitive to this dose of hydrocortisone, and their activities were all increased (table 2).

After the injection of 1 mg of hydrocortisone/100 g body weight for 3 days (group 2, table 3), the BATase in liver tissue was elevated significantly with all 3 substrates, but these increases were less than 100%, and less than those found with the other enzymes tested in the same experimental animals (tables 2, 3). In muscle tissue, BATase was increased specifically with isoleucine as a substrate. The enzyme activity in kidney tissue was not altered.

In group 3, a greater amount (2 mg/100 g body weight) of hydrocortisone was injected for a longer period (7 days), but this treatment failed to raise the enzyme levels above those found in group 2.

Although the BATase activity in liver was increased significantly in the animals receiving hydrocortisone for 3 days, this increase was not associated with any change in the concentration of the branched-chain amino acids in liver tissue. The mean values ± SD for control animals in μmoles/100 g of tissue were: Val, 51 ± 5; Ileu, 30 ± 9; and Leu, 64 ± 11. Comparable values for animals injected with hydrocortisone were: Val, 44 ± 10; Ileu, 30 ± 6; and Leu, 63 ± 10.

DISCUSSION

These experiments show that BATase is an adaptive enzyme, and that its activity in liver and muscle tissue is influenced by both dietary protein levels and hydrocortisone administration. However, the BATase in kidney responds only to dietary protein.

As reported previously by others (1), kidney showed the highest BATase activity, followed by skeletal muscle, with liver having the lowest activity. The liver activity in this experiment was approximately

TABLE 2

Effect of dietary protein content and hydrocortisone administration on other liver enzymes catabolizing amino acids

Treatment	Liver enzyme activity [1]		
	Trp-pyrrolase [2]	Tyr-transaminase [3]	Thr-dehydratase [4]
Diet treatment			
18% casein	8.8 ± 1.9	43.1 ± 5.9	826.6 ± 248.3
75% casein	19.1 ± 2.0 ***	65.6 ± 12.3 *	4693.0 ± 256.3 ****
0% casein	2.7 ± 1.1 **	51.9 ± 9.9	49.0 ± 8.5 **
Hydrocortisone administration			
None injected	6.5 ± 1.0	24.6 ± 3.4	377.0 ± 47.5
Injected 1 day	43.5 ± 8.1 ***	118.3 ± 17.5 ***	734.3 ± 226.0
None injected	6.1 ± 0.5	27.8 ± 6.9	454.5 ± 143.8
Injected 3 days	42.8 ± 11.7 **	81.1 ± 29.3 *	1085.4 ± 68.3 ***

[1] Mean value ± SD of 3 samples each representing a pool from 2 rats.
[2] Values for enzyme activity in μmole of kynurenine formed from Trp/60 min/g of liver (dry wt).
[3] Values for enzyme activity in μmole of *p*-hydroxyphenylpyruvate formed from Tyr/10 min/mg of protein.
[4] Values for enzyme activity in μmoles of α-ketobutyric acid/60 min/g liver (fresh).
*, **, ***, **** Indicates significant difference from controls of 18% casein or saline at the 5%, 1%, 0.5% and 0.1% level, respectively, by t test.

TABLE 3

Branched-chain amino acid transaminase (BATase) activity of tissues from rats injected with hydrocortisone

Tissue	Group [1] no.	HC [2] injection	BATase activity [3] with indicated substrate		
			Leucine	Isoleucine	Valine
Liver	1	—	13.2 ± 1.3	11.0 ± 1.4	15.3 ± 1.0
		+	13.3 ± 0.6	11.4 ± 1.3	13.5 ± 0.7
	2	—	13.1 ± 1.4	9.6 ± 1.9	11.3 ± 1.7
		+	17.1 ± 1.8 *	17.4 ± 1.2 ***	19.0 ± 1.3 ***
	3	—	12.8 ± 2.6	11.0 ± 1.0	13.3 ± 1.6
		+	23.2 ± 2.7 **	18.3 ± 0.9 ****	22.6 ± 1.0 ***
Kidney	1	—	153.8 ± 8.0	140.3 ± 31.7	82.6 ± 26.2
		+	164.0 ± 9.8	140.6 ± 22.1	81.3 ± 18.1
	2	—	168.4 ± 4.0	182.0 ± 21.1	114.8 ± 20.5
		+	179.7 ± 6.5	177.1 ± 7.1	98.5 ± 26.9
	3	—	148.1 ± 12.7	144.1 ± 16.6	89.5 ± 3.4
		+	159.8 ± 5.7	136.0 ± 3.0	98.3 ± 5.8
Muscle	1	—	121.3 ± 13.7	84.0 ± 14.6	58.8 ± 3.3
		+	109.7 ± 10.5	93.4 ± 11.1	59.1 ± 3.0
	2	—	116.8 ± 13.7	99.5 ± 7.1	69.3 ± 11.4
		+	130.8 ± 6.1	146.0 ± 7.4 ***	73.0 ± 9.2
	3	—	91.3 ± 2.7	72.0 ± 9.6	38.3 ± 4.2
		+	112.2 ± 11.6 *	102.6 ± 16.0 *	57.7 ± 5.0 *

[1] Group 1 was given a single dose of HC (1 mg/100 g of body wt/day). Group 2 was given the same dose of HC or saline daily for 3 days. Group 3 was given HC (2 mg/100 g body wt/day) or saline daily for 7 days.

[2] Hydrocortisone acetate in saline (HC).

[3] Values are expressed as mμmoles of keto acid formed from amino acid substrate/10 minutes/mg protein. Mean value ± SD of 3 samples, each representing a pool from 2 rats.

*, **, ***, **** Significant difference from mean value of saline controls at the 5%, 1%, 0.5% and 0.1% level, respectively, by *t* test.

twenty-fold higher than that reported by Ichihara and Koyama (1), possibly because of a difference in the strain of rats used.

According to Ichihara and Aki,[5] the increase in liver BATase of rats fed a high protein diet is detectable only with leucine as a substrate. In our experiment, the increase in BATase activity with a high protein diet was noted with all 3 substrates. These differences in results may have been caused by differences in experimental conditions. The elevation of BATase observed in the rats fed a high protein diet might be due to the enzyme induction by substrates (leucine, isoleucine and valine), the levels of which may be increased in concentration in the tissues with this dietary procedure. The effectiveness of these substrates in inducing BATase is being studied. The BATase activities in liver and muscle were also enhanced by a protein-free diet, when enzyme activity was reported per milligram of tissue protein. Since the total protein of tissues is reduced by feeding protein-free diets, this finding may not represent an absolute increase in BATase in the tissues. Other experiments [6] have shown that the plasma levels of the branched-chain amino acids, particularly valine and isoleucine, are reduced when a protein-free diet was fed. It is possible that these changes in plasma amino acid levels are in some way related to alterations in BATase activity.

As to the effect of a single dose of hydrocortisone, BATase was not elevated when the activity of other amino acid-catabolyzing enzymes were increased significantly (table 3, group 1, vs. table 2). The enhancement of BATase activity found in groups 2 and 3 with multiple doses of hormone might not be a direct effect of hydrocortisone. Peraino (10) has suggested that cortisone might influence adaptive enzymes through interaction with other regulators such as amino acids.

According to Knox (11), there are 2 types of enzyme induction, a hormone type

[5] Ichihara, A., and K. Aki 1967 Branched chain amino acid transaminase and its change in activity under various physiological conditions in rats. Abstr. International Congress of Biochemistry, V: 837, G-3.

[6] Authors' unpublished results.

and a substrate type. Hormone-type induction was prevented when actinomycin inhibited RNA synthesis, as well as when puromycin inhibited protein synthesis, whereas substrate-type induction was not prevented by actinomycin, but was prevented by puromycin. In a preliminary experiment [7] with actinomycin D, kidney BATase was inhibited significantly, whereas liver BATase was increased.

It has been suggested from studies on the purified enzyme that BATase in hog heart is a single protein.[8] However, the variable responses of BATase in different tissues to dietary protein level, hydrocortisone and actinomycin D suggest the possibility that BATase in different tissues may not represent a single protein in all instances. Another possible explanation is that the various tissues have different control mechanisms for BATase.

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[7] See footnote 6.
[8] See footnote 5.