Invitation for Nominations for 1970 American Institute of Nutrition Awards

Nominations are requested for the 1970 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, 1969*, to be considered for the 1970 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.

Borden Award in Nutrition [1]

The Borden Award in Nutrition, consisting of $1000 and a gold medal, is made available by the Borden Foundation Inc. The Award is given in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritional significance of any food or food component. The Award will be made primarily for the publication of specific papers during the two previous years but the Jury of Award may recommend that it be given for important contributions made over a more extended period of time. Employees of Borden Inc 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
1969 – H. P. Broquist

NOMINATING COMMITTEE:

R. T. HOLMAN, *Chairman*
R. G. HANSEN
A. L. BLACK

Send nominations to:

R. T. HOLMAN
Hormel Institute
University of Minnesota
Austin, Minnesota 55912

Osborne and Mendel Award

The Osborne and Mendel Award of $1000 and an inscribed scroll has been established by the Nutrition Foundation,

[1] Sponsors of nominees for this award should note that a change has been made in the area of research which this award recognizes.

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 a most significant recent contribution or 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.

Individuals who have received another award provided by the Nutrition Foundation are not eligible for this award, unless the new award is clearly for research and contributions different from that of the first award.

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
1969 – H. N. Munro

NOMINATING COMMITTEE:

M. K. HORWITT, *Chairman*
E. L. R. STOKSTAD
H. N. MUNRO

Send nominations to:

M. K. HORWITT
St. Louis Univ. School of Medicine
St. Louis, Missouri 63104

Mead Johnson Award for Research in Nutrition [2]

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 40th birthday at the time the award is presented. Selection by the Jury of Award will be based either on a single outstanding piece of recent research in nutrition or on a series of recent papers on the same subject.

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
1969 – R. H. Wasserman

NOMINATING COMMITTEE:

H. E. SAUBERLICH, *Chairman*
H. P. BROQUIST
H. F. DELUCA

Send nomination to:

H. E. SAUBERLICH
U. S. Army Medical Research and Nutrition Laboratory
Fitzsimons General Hospital
Denver, Colorado 80240

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.
1969 – F. J. Stare

NOMINATING COMMITTEE:

OLAF MICKELSEN, *Chairman*
O. C. JOHNSON
C. G. KING

Send nominations to:

OLAF MICKELSEN
Department of Foods & Nutrition
Michigan State University
East Lansing, Michigan 48823

[2] Sponsors of nominees for this award should note the change which has been made in the age limitation.

Invitation for Nominations for 1970 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:

L. A. Maynard, *Chairman*
T. H. Jukes
A. B. Morrison
E. E. Howe
H. M. Linkswiler

Send nominations to:

L. A. Maynard
Cornell University
Ithaca, New York 14850

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)
S. Kon (1969)
Samuel Lepkovsky (1966)
Karl E. Mason (1969)
Leonard A. Maynard (1960)
Elmer V. McCollum (1958)
Harold H. Mitchell (1958)
Agnes Fay Morgan (1959)
John R. Murlin (1958)
Leo C. Norris (1963)
Bernard L. Oser (1969)
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:

L. C. NORRIS, *Chairman*
A. E. SCHAEFER
P. L. DAY

Send nominations to:

L. C. NORRIS
Department of Poultry Husbandry
University of California
Davis, California 95616

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
Egon H. Kodicek
Joachim Kühnau
Josef Masek
Thomas Moore
Hiroshi Morimoto
R. A. Morton
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
M. Swaminathan
Emile F. Terroine
Jean Tremolieres
Eric John Underwood
Artturi I. Virtanen

Study of One-carbon Metabolism in Neonatal Vitamin B_{12}-deficient Rats [1,2]

JAMES C. WOODARD
Department of Pathology, College of Medicine, University of Florida, Gainesville, Florida

ABSTRACT By feeding diets deficient in vitamin B_{12} and other lipotropic substances, anomalies such as hydrocephalus, umbilical hernia and spina bifida were produced in neonatal rats. The incidence of congenital hydrocephalus was increased when 2.1 or 4.2 mmoles/100 g diet of DL-methionine was added to diets deficient in choline and vitamin B_{12}. Measurements of DNA, RNA, protein, proteolipid, and total lipid showed no quantitative differences between embryos born to dams maintained on deficient or control rations. Likewise, there were no differences found in brain phospholipids or in the incorporation of L-methionine-methyl-^{14}C into various chemical constituents. It was concluded that alterations in brain phospholipids did not play a significant role in the pathogenesis of the nutritionally induced neural developmental anomalies, and it is suggested that dams furnish one-carbon fragments to the developing embryos at the expense of their own tissue requirements.

Deficiencies of vitamin B_{12} or folic acid during gestation result in congenital hydrocephalus in neonatal rats (1, 2). Likewise, rats born to dams fed a ration low in folic acid produce offspring which, if fed this same ration until maturity, show inferior maze-learning abilities. It has been suggested that brain alterations occur before birth and subsequent nutritional deficiency has little if any effect on maze learning (3). The nutritional quality of the mother's diet may have a direct effect upon the biochemical content of the fetus. For example, female rats fed an 8% protein diet for 1 month before mating and throughout gestation gave birth to young whose brains contained significantly less deoxyribonucleic acid (DNA) and protein than did progeny from dams fed a 27% protein diet (4). It has been suggested that the quantitative biochemical changes may constitute the basis for the frequently reported impaired behavior of the offspring from protein-deprived mothers. Bruemmer et al. (5) reported that 1-day-old offspring from vitamin B_{12}-deficient rats had higher DNA concentrations per gram of brain tissue, and ribonucleic acid (RNA) concentration per cell was lower in B_{12}-deficient offspring (5).

Morphologic alterations in neonatal animals indicate that hydrocephalus induced by vitamin B_{12} and that induced by folic acid deficiency are similar (6, 7). Hydrocephalus is caused by stenosis of the cerebral aqueduct and is associated with aplasia of the subcommissural organ, pineal gland and other neural structures. The basic defect is thought to be concerned with the multiplication, migration or maturation of primitive neural elements. A number of dietary factors other than vitamin B_{12} have been shown to influence the incidence or severity of lesions. The deletion of choline from a vitamin B_{12}-deficient diet increased the incidence of abnormalities in neonatal rats, and methionine in amounts equivalent to choline in methyl groups did not offer the same protective effects (8). A synergistic action was also found when X-methyl folic acid was added to a vitamin B_{12}-deficient diet (8). This interrelation between developmental neural abnormalities and the role of vitamin B_{12} in one-carbon metabolism has been confirmed by O'Dell (9).

Because it has been shown that depleting the dam of lipotropic agents increases the incidence of developmental anomalies (8), and because liver and plasma phospholipids are known to be reduced by a deficiency of choline (10), this experiment was designed to investigate the role

Received for publication December 27, 1968.

[1] Supported in part by National Institutes of Health Grant nos. GM-1142 and ES00266.

[2] This material was presented, in part, at the 52nd Annual Meeting of the Federation of American Societies for Experimental Biology, April, 1968.

of one-carbon metabolism in the pathogenesis of nutritionally induced hydrocephalus. The purposes of this experiment were: 1) to determine whether any biochemical alterations could be detected in brains or livers from neonatal rats born to dams maintained on diets deficient in vitamin B_{12} or other lipotropic agents, and 2) to determine if methionine can prevent the enhancing effects choline deficiency has on the induction of hydrocephalus.

METHODS

Female weanling albino rats of the Caesarean Derived (CD), Sprague-Dawley strain were obtained commercially.[3] The animals were housed in air-conditioned animal quarters; food and water were supplied ad libitum. Three female animals were housed together in large, raised, screen-wire cages and fed either a vitamin B_{12}-deficient diet or a vitamin B_{12}-supplemented diet during the growth period. The 20% protein diet shown in table 1 was supplemented with 0.18% choline bitartrate and 0.32% DL-methionine during the growth period; vitamin B_{12} was added to the control diet at the rate of 50 µg/kg of diet. At 11 weeks of age, the rats were started on the various experimental rations.

TABLE 1
Composition of basal diet

	% of diet
Sucrose	65
Soybean protein [1]	20
Salts [2]	5
Cottonseed oil [3]	10
Vitamins [4]	*mg/100 g diet*
Thiamine·HCl	1.6
Pyridoxine·HCl	1.6
Riboflavin	1.6
Ca pantothenate	4.0
Nicotinic acid amide	5.0
Folic acid	0.5
Inositol (meso)	10.0
dl-α-Tocopheryl acetate	10.0
Menadione	1.0
	IU/100 g diet
Vitamin A	2,500
Vitamin D_2	600

[1] Promine D, Central Soya Company, Chicago (1.0% methionine).
[2] Hegsted, D. M., R. C. Mills, C. A. Elvehjem and E. B. Hart 1941 J. Biol. Chem., *138*: 459.
[3] Wesson Oil, Wesson Sales Company, Fullerton, Calif.
[4] Vitamin B_{12} (50 µg/kg of diet) was added to control diets.

Females were fed the experimental ration for 4 weeks prior to breeding. Male animals, fed a commercial ration, were housed separately until the time of breeding when they were placed with the females, one male per cage. Vaginal smears were checked daily for the presence of sperm. Females which were found to be positive were moved to single cages and fed the experimental diets throughout gestation.

The neonatal offspring from the eight groups of animals, consisting of six females each, were used. Control animals which were supplemented with vitamin B_{12} during the growth period were placed on diets 1 and 2, whereas vitamin B_{12}-deficient animals were given diets 3 through 8 (table 2). The weights of the litters, the number of embryos per litter, and the number of hydrocephalic animals were recorded. Various litters were chosen consecutively for the isolation of brain phospholipids, or for the determination of the incorporation of ^{14}C-methyl-labeled methionine into various chemical fractions of the liver and brain. Hydrocephalic animals were excluded from the litters on which chemical analyses were performed, with the exception of litters containing all hydrocephalic individuals which were analyzed separately.

The results presented in the tables represent the mean values obtained from individual samples within each dietary treatment. The data in tables 4 and 5 were treated statistically using an analysis of variance. An F value was computed to determine differences between dietary treat-

TABLE 2
Experimental diets

Diet no.	Addition to basal diet
1	0.18% choline bitartrate + 0.32% DL-methionine + vitamin B_{12}[1]
2	vitamin B_{12}[1]
3	0.18% choline bitartrate + 0.32% DL-methionine
4	0.18% choline bitartrate
5	0.32% DL-methionine
6	0.64% DL-methionine
7	0.96% DL-methionine
8	None

[1] Vitamin B_{12} added at the rate of 50 µg/kg diet.

[3] Charles River Laboratories, Inc., Wilmington, Mass.

ments. In tables 4 and 6 each dietary group was then compared with the vitamin B_{12}-supplemented control group.

Brain phospholipids. The analysis of phospholipids was conducted on neonatal rats whose mothers were fed experimental diets 1, 3 and 5 (table 2). Each analysis was performed on a sample consisting of brains from two littermates, and each sample was run in triplicate. In this way the phospholipids from a total number of 32 litters were examined. The lipids were extracted by the method of Folch et al. (11), and analyzed quantitatively by the procedure outlined by DeBohner et al. (12). The extract was evaporated under nitrogen and adjusted to a constant volume so that 4 μg of phosphorus would be present in 25 μl of lipid extract.

Chromatoplates were prepared using silica-gel G according to the method of Stahl (13). The sample was applied, and the plates were developed in the alkaline solvent mixture described by Müldner et al. (14). After drying, detection of spots was carried out with iodine vapors. The silica containing each spot was removed and the phospholipids eluted twice using the method employed by Marinetti et al. (15). The eluted phospholipids were ashed and analyzed for phosphorus according to the method of Chen et al. (16).

Incorporation of L-methionine-methyl-^{14}C. Six individual embryos from a litter received subcutaneous injections of L-methionine-methyl-^{14}C (17.0 mCi/mmole) at the rate of 0.1 μCi/g embryo. Litters were injected with radioisotope within 4 hours of birth and only viable active embryos were chosen for biochemical determinations. Care was taken that the subcutaneous injection of isotope did not escape from the needle puncture hole. An "incorporation time" of 1 hour showed maximum uptake of methionine into serum proteins; therefore, this time period was used throughout these experiments. Embryos were exsanguinated after 1 hour by decapitation at the atlanto-occipital joint. Livers and brains were removed; the different organs from six animals of one litter were placed in separate beakers containing cold 0.25 M sucrose. The total weight of the liver and brain was determined. A 20% homogenate was obtained, and duplicate samples were then separated into acid-soluble, lipid, proteolipid, protein, and nucleic acid components using a modification of the extraction techniques of Schneider (17).

The chloroform-methanol extract outlined by Schneider was separated into two components by adding distilled water to the lipid solvent. The buffy coat layer (proteolipid) which formed at the aqueous methanol-chloroform interphase was removed from the chloroform-soluble lipid by differential freezing. The amount of lipid was determined by weight after evaporating the chloroform with nitrogen. The Folin-Ciocalteu method was used to quantitate the amount of protein and proteolipid (18). RNA was determined using orcinol reagent, and DNA was determined by the diphenylamine reaction as outlined by Schneider (19). Equal volumes of each fraction were used for the chemical and radioactive determinations. Proteinaceous materials were dissolved using hydroxide of Hyamine.[4] Dissolved fractions were added to scintillation fluid (20), stored in the dark for 24 hours to prevent chemiluminescence, and counted with a liquid scintillation counter.[5] Efficiency and the degree of quenching were determined by the external standard ratio method.

RESULTS

The incidence of hydrocephalus was highest in animals whose dams were fed a vitamin B_{12}- and choline-deficient diet and supplemented with varying quantities of methionine (diets 5, 6 and 7, table 3). These results are similar to those obtained in previous experiments (8). When methionine was added in amounts equivalent to choline in methyl groups (diet 5), hydrocephalus was not prevented. Twice the number of moles of methionine did not reduce the incidence; however, depression was observed when the level of methionine was increased to 6.3 mmoles/100 g diet. When comparisons of brain, liver or total embryo weights were made, a statistical difference was noted; however, within groups comparisons showed that these dif-

[4] *p*(Diisobutyl-cresoxyethoxyethyl) dimethylbenzyl ammonium hydroxide, Packard Instrument Company, Downers Grove, Ill.

[5] Beckman model DPM 100, Beckman Instruments, Inc., Fullerton, Calif.

TABLE 3
Incidence of hydrocephalus

Diet no.	Supplement to basal diet	No. of litters	Litters hydrocephalic	No. of embryos	Embryos hydrocephalic
	mmoles/100 g diet		%		%
1	Choline, 0.7 + methionine, 2.1 + vitamin B_{12}[1]	23	0	143	0
2	Vitamin B_{12}[1]	33	0	196	0
3	Choline, 0.7 + methionine, 2.1	22	0	180	0
4	Choline, 0.7	13	0	95	0
5	Methionine, 2.1	17	24	132	7
6	Methionine, 4.2	14	36	110	14
7	Methionine, 6.3	13	7	107	1
8	None	13	0	80	0

[1] Vitamin B_{12} added at the rate of 50 μg/kg diet.

ferences were not the result of vitamin B_{12} deficiency (table 4).

Four different phospholipid components were isolated from the brains of neonatal rats. The identification of the phospholipids was based upon R_F values (12) and the use of various spot tests as outlined by Skidmore and Entenman (21). The phospholipids identified were phosphatidyl serine, sphingomyelin, lecithin and phosphatidyl ethanolamine (fig. 1). With the system employed phosphatidyl inositol should migrate with phosphatidyl serine; however, it could not be detected in this experiment by means of spot tests. The results of the quantitative measurements of the four phospholipids indicate that there was no significant difference between any of the experimental groups; however, some variation among the triplicate samples was encountered (table 5).

The results of the isolation and quantitation of the various biochemical constituents from embryonic livers and brains are shown in table 6. The results represent the means obtained from six litters containing six neonates each. Each litter was obtained from a separate female rat on the dietary treatment. Differences between duplicate samples were found to be relatively small; however, very large differences were found between individual litters. Statistically significant differences were found between some mean values, but, when comparisons were made between dietary treatments these differences became nonsignificant. There were insufficient data obtained from completely hydrocephalic litters for statistical evaluation, but it was obvious by inspection that no differences existed. Fluctuations observed in the incorporation of methionine or its methyl group into the various chemical constituents were related to endogenous differences in neonatal metabolism, and could not be correlated with a deficiency of vitamin B_{12} or methyl groups during embryonic development.

DISCUSSION

The dietary experiments indicated that a higher incidence of congenital abnormalities is produced when choline- and vitamin B_{12}-deficient diets are supplemented with methionine. In our experience it has not been unusual to find that dams fed diets deficient in vitamin B_{12}, but supplemented with choline, give birth to relatively few anomalous young, although the mothers have low liver levels of vitamin B_{12}. It is, therefore, not surprising that no congenital anomalies were observed in the groups fed diets 3 and 4. The addition of 4.2 mmoles of DL-methionine/100 g diet (diet 6, table 3) did not reduce the incidence of congenital anomalies although the dams received twice the number of methyl groups furnished by choline (diet 4). The highest levels of methionine (diet 7) also failed to totally prevent hydrocephalus. These results are in contradistinction to the sparing action that methionine exerts in the choline-deficient hemorrhagic kidney syndrome of weanling rats (22). The dichotomy of the methionine action in these two models could be explained

TABLE 4

Weight comparisons

Diet no.	Supplement to basal diet	Avg embryo wt	Brain wt per six embryos	Liver wt per six embryos
	mmoles/100 g diet	*g*	*g*	*g*
1	Choline, 0.7 + methionine, 2.1 + vitamin B_{12}[1]	6.3	1.5	1.9
2	Vitamin B_{12}[1]	5.4	1.4	1.3
3	Choline, 0.7 + methionine, 2.1	6.0	1.4	1.6
4	Choline, 0.7	5.0	1.2	1.2
5	Methionine, 2.1	5.0	1.3	1.4
6	Methionine, 4.2	5.7	1.4	1.5
7	Methionine, 6.3	6.0	1.5	1.5
8	None	5.5	1.4	1.6
Significance of dietary group comparisons		0.05	0.01	0.05
Significance of pairwise comparisons		nsc[2]	nsc	5 versus 1 (0.01) 4 versus 1 (0.05) 2 versus 1 (0.01)

[1] Vitamin B_{12} added at the rate of 50 μg/kg diet.
[2] nsc = no significant comparison.

TABLE 5

Analysis of brain phospholipids

Phospholipids	Dietary treatment 1	3	5
μg P/g fresh tissue			
Phosphatidyl serine	467	391	310
Phosphatidyl ethanolamine	753	638	704
Lecithin	1148	944	926
Sphingomyelin	327	268	338
Significance	ns[1]	ns	ns

[1] ns = not significant.

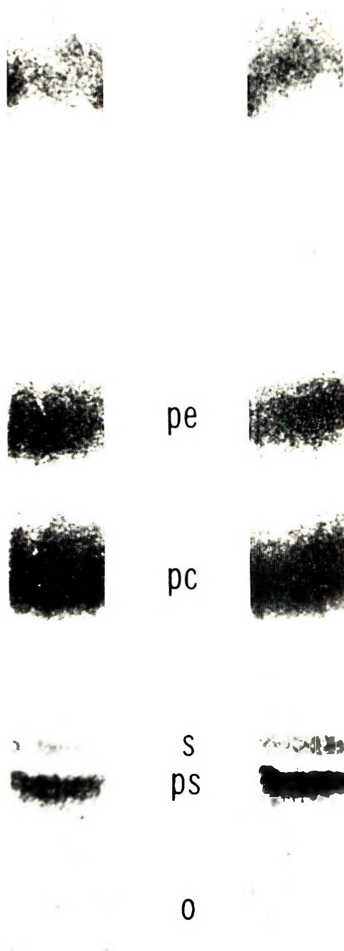

Fig. 1 Typical thin-layer chromatograms of phospholipids isolated from fetal brains. o = origin, ps = phosphatidyl serine, s = sphingomyelin, pc = phosphatidyl choline or lecithin, and pe = phosphatidyl ethanolamine.

if a minimal dietary level of methyl groups was necessary for embryonic development and fetal growth. Since methionine is an essential amino acid, it would serve a dual function in protein synthesis and methyl neogenesis.

Inhibition of phospholipid synthesis plays a direct role in the pathogenesis of choline-deficiency fatty liver and is of questionable significance in the hemorrhagic kidney syndrome of weanling rats. The role phospholipids play in the pathogenesis of nutritionally induced neural developmental anomalies was investigated by isolating and quantitating the various phospholipid components. No differences in the

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TABLE 6

Biochemical analysis and incorporation of L-methionine-methyl-^{14}C

Diet no.	Supplement to basal diet	DNA			RNA		Protein		Lipid		Proteolipid		Acid soluble
		Avg mg DNA/ animal	mg DNA/ g wet wt	dpm/ mg DNA	mg RNA/ g wet wt	dpm/ mg RNA	mg protein /g wet wt	dpm/ mg protein	mg lipid /g wet wt	dpm/ mg lipid	mg protein /g wet wt	dpm/ mg protein	dpm/ g wet wt
	mmoles/100 g diet												
							Liver						
1	Choline, 0.7+ methionine, 2.1+ vitamin B_{12}[1]	0.62	1.9	512	104	42	79	688	18.1	3998	26.2	1071	138,552
2	Vitamin, B_{12}[2]	0.57	2.7	308	123	64	81	1133	22.8	5313	29.7	1426	225,207
3	Choline, 0.7+ methionine, 2.1	0.60	2.4	742	88	76	78	1582	22.6	11136	38.7	1741	97,938
4	Choline, 0.7	0.59	3.1	608	121	107	84	1114	17.2	8325	49.8	812	125,196
5	Methionine, 2.1	0.51	2.5	724	115	68	89	1906	25.0	5548	26.2	1953	121,054
6	Methionine, 4.2	0.49	2.1	661	96	76	82	1436	18.9	8594	30.5	1339	117,598
7	Methionine, 6.3	0.38	1.5	723	121	67	83	1097	21.0	5964	33.5	1539	120,614
8	None	0.66	2.3	235	95	77	70	853	26.1	5090	29.3	1271	119,035
Significance between diets		ns[3]	ns	ns	ns	ns	ns	0.05	ns	0.01	ns	ns	ns
Significance of pairwise comparisons		nsc[4]	nsc	nsc	nsc	nsc	nsc	5 versus 1 (0.05)	nsc	5 versus 3 (0.10) 2 versus 3 (0.10) 8 versus 3 (0.10) 1 versus 3 (0.05)	nsc	nsc	nsc
							Brain						
1	Choline, 0.7+ methionine, 2.1+ vitamin B_{12}[1]	0.26	1.0	296	32.0	37	48.0	366	23.0	106	7.9	343	84,092
2	Vitamin B_{12}[2]	0.58	2.6	129	38.8	91	51.0	514	21.9	113	7.4	479	81,360
3	Choline, 0.7+ methionine, 2.1	0.32	1.4	528	30.1	110	37.0	1207	21.0	161	12.3	826	67,060
4	Choline, 0.7	0.40	1.8	690	27.0	231	43.0	638	21.9	155	14.5	614	62,325
5	Methionine, 2.1	0.39	1.9	708	35.1	137	50.0	1354	22.4	184	9.2	1050	87,874
6	Methionine, 4.2	0.30	1.3	514	27.2	95	47.0	706	21.2	150	9.5	482	67,294
7	Methionine, 6.3	0.40	1.7	536	33.5	101	49.0	570	21.9	143	6.8	1314	59,013
8	None	0.37	1.5	247	37.8	67	43.0	453	21.7	97	8.3	430	85,213
Significance between diets		ns	ns	ns	0.01	0.10	0.10	0.01	ns	ns	0.05	ns	ns
Significance of pairwise comparisons		nsc	nsc	nsc	nsc	nsc	nsc	5 versus 1 (0.10)	nsc	nsc	nsc	nsc	nsc

[1] Vitamin B_{12} (50 μg/kg diet) was fed throughout growth period.
[2] Animals raised on a vitamin B_{12}-deficient diet throughout growth period and then supplemented with vitamin B_{12} prior to breeding.
[3] ns = not significant.
[4] nsc = no significant comparison.

brain phospholipid patterns could be detected between neonates whose dams were raised on vitamin B_{12}-supplemented or deficient diets, or between offspring whose mothers were fed diets severely deficient in lipotropic agents. Likewise, the incorporation of methyl groups from methionine into brain and liver lipids was not significantly different. These results indicated that the congenital neural abnormalities induced by vitamin B_{12} deficiency cannot be attributed to major alterations in the synthesis of phospholipids. Total lipid analysis of neonatal livers showed that the animals whose dams were fed diets deficient in lipotropic substances did not have increased amounts of liver fat although the livers from their mothers had microscopic evidence of severe fatty metamorphosis. The incorporation of L-methionine-methyl-^{14}C into various chemical components indicated that there were no major shifts in the methyl requirements of the various tissue constituents. The constant specific activities found in the various biochemical substances might be attributed to the fact that dams are furnishing methyl groups for embryonic growth at the expense of regulating their own methyl homeostasis.

The total amounts of DNA and RNA per gram tissue were not found to be changed by a deficiency of vitamin B_{12}. These results differ from those previously reported by other workers (5, 23) who found a decrease in the amount of RNA and an increased amount of DNA within the brain. As explained by O'Dell and Bruemmer (23) the differences in the content of nucleic acids previously reported might result because deficient offspring failed to feed as well as control animals. Care was taken in our experiments to ensure that newborn animals did not nurse; this was verified by examining the stomach contents at necropsy. It should be noted, however, that the tissues in our experiment were obtained within 5 hours of birth, whereas the tissues in the previous experiments were obtained from 1-day-old neonates. Since maternal nutrition has been severed there is sufficient time difference between the two experiments to allow major metabolic alterations to occur.

ACKNOWLEDGMENTS

The assistance in the analysis of brain phospholipids by B. Carlton Lynn and the technical assistance of Dennis D. Short are gratefully appreciated. Statistical evaluations were performed with the aid of Dr. J. I. Thornby and the University of Florida computer center.

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Effects of Forced-feeding on Blood Levels of Pyruvate, Glucocorticoids and Glucose and on Adrenal Weight in Thiamine-deprived and Thiamine Antagonist-treated Rats [1,2]

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ABSTRACT To study the factors involved in the anorexia and hyperpyruvicemia of thiamine deficiency, rats were force-fed a liquefied thiamine-deficient diet by stomach tube. Force-feeding decreased the life expectancy of thiamine-deprived and oxythiamine-treated (OTh) rats by 0.33 to 0.50%, compared with similar rats fed ad libitum, but had no effect on life expectancy of pyrithiamine-treated (PTh) rats. Bloating was observed in all force-fed deficient groups and appeared to correlate well with the development of anorexia in rats fed ad libitum. It was so severe in force-fed OTh-treated rats that the daily food ration had to be cut by one-half. Thiamine-deprived and OTh-treated rats still showed significant increases in blood pyruvate and plasma corticosterone levels, and in gut and adrenal weights despite force-feeding to reduce inanition. Longer treatment with OTh appeared to exhaust the ability of the adrenals to put out corticosterone. PTh treatment resulted in increases in plasma corticosterone and adrenal weight in the ataxic but preconvulsive state, which increased further after convulsions had started, along with a significant increase in blood pyruvate. Because the animals are unable to use the food these results suggest that the anorexia is the result of some local biochemical disturbance in the gut, and that elevations of blood pyruvate in thiamine-deficient states are possibly due to two factors: a hyperactivity of the hypophyseal-adrenal system, and specific disturbances in pyruvate metabolism.

Ever since thiamine was shown to be an essential cofactor in pyruvate metabolism (1–4) elevated levels of blood pyruvate have consistently been associated with thiamine deficiency states (5–7). Although much work has been done in this area, the role of the disturbances in α-ketoacid metabolism as contributing factors in the development of the various symptoms of thiamine deficiency has still not been adequately elucidated. It has been suggested by DeCaro and Rindi (8) that the increased levels of blood pyruvate found in thiamine deficiency states may largely result from the associated stress of inanition, and hence, to hyperfunction of the hypophyseal-adrenal system.

This study was designed to investigate this hypothesis and the factors involved in the anorexia of thiamine deficiency by eliminating the stress of inanition in thiamine-deficient rats with force-feeding.

EXPERIMENTAL PROCEDURES

Adult male rats of the Sprague-Dawley strain, weighing between 160 to 200 g, were used throughout the experiments. The animals were housed in separate galvanized cages, maintained between 20 and 30°, and given free access to water and commercial laboratory pellet feed [3] until the experimental regimen was started.

The basal thiamine-deficient diet was prepared in 10-kg batches as described earlier (6) and stored at 0 to 4°. The liquid diet was prepared by mixing 200 g of the basal thiamine-deficient diet with 100 ml tap water and liquefying it in a blender [4] for 3 to 5 minutes. This liquid diet was also stored at 0 to 4° and warmed to room temperature before feeding.

Received for publication November 22, 1968.

[1] Part of this work was done in partial fulfillment of the requirements for the degree of Master of Science by R. A. Bitter. This is no. VI of the series: Studies on the Physiological Functions of Thiamine.

[2] Supported by Public Health Service Research Grant no. AM02448 from the National Institute of Arthritis and Metabolic Diseases, and a grant from the Research Foundation, National Association for Mental Health.

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

[4] Waring Blendor, Waring Products Company, Win sted, Conn.

Eight groups of rats were assigned to one of the following treatments as described in table 1.

Injections of vitamin, plus antagonists and saline were given subcutaneously under the foreleg, using a 26-gauge hypodermic needle, to the respective groups between 5 and 6 PM daily.

Prior to treatment, all groups were fasted from 20 to 24 hours to remove excess food from their digestive systems which might contain thiamine; then they were placed on the basal thiamine-deficient diet as described previously. For the first 2 days of the experiment, the diet was given to all groups ad libitum. At the beginning of day 3, group 1 was fed the ad libitum diet; groups 2 through 8 were taken from the ad libitum diet and force-fed the liquefied thiamine-deficient diet twice daily at 7 to 8 AM and 5 to 6 PM with a stomach tube (9). A plastic infant tube (size 8 French, 38.1 cm long) with the outlet openings enlarged to twice the normal size, was used on a 20-ml syringe to administer the diet. The rats received 4 g diet/100 g body weight twice daily.

All animals were killed between 11 AM and 12 noon on the day when the deficient animals showed terminal symptoms of deficiency, i.e., emaciation, bloating, immobility, ataxia or convulsions. Because most of the deficient animals were bloated and had food retained in the gut at killing, the force-fed control group was divided into two groups, 2a and 2b, to test the effects of food in the stomach on blood pyruvate, plasma glucose and gut weight. Rats of group 2a were fed as usual in the morning and killed at 11 AM while rats from group 2b were not given the morning feeding on the day of killing. Rats were anesthetized with ether, the dorsal aorta exposed by abdominal incision and blood withdrawn from the dorsal aorta with a 21-gauge needle into a heparinized 10-ml syringe. Four milliliters of the blood sample were mixed thoroughly in a centrifuge tube with an equal volume of ice-cold 0.6 M perchloric acid solution, according to a method from Boehringer and Soehne (10); the remainder of the collected sample was placed in a heparinized centrifuge tube. The two aliquots were then centrifuged

TABLE 1
Dietary treatments

Group no.	Diet	Supplements by injection
Control (1)	Solid, thiamine deficient, ad libitum	10 μg thiamine in 0.2 ml 0.9% saline/100 g body wt
Control (2)	Liquid, thiamine deficient, force-fed	10 μg thiamine in 0.2 ml 0.9% saline/100 g body wt
Thiamine deprived (3)	Liquid, thiamine deficient, force-fed	0.2 ml 0.9% saline/100 g body wt
Oxythiamine (OTh)-[1] treated, 1 week (4)	Liquid, thiamine deficient, force-fed	2.0 mg OTh and 10 μg thiamine in 0.2 ml 0.9% saline/100 g body wt
OTh-treated, 2 weeks (5)	Liquid, thiamine deficient, force-fed	2.0 g OTh and 10 μg thiamine in 0.2 ml 0.9% saline/100 g body wt
OTh-treated, 3 weeks (6)	Liquid, thiamine deficient, force-fed	2.0 mg OTh and 10 μg thiamine in 0.2 ml 0.9% saline/100 g body wt
Pyrithiamine (PTh)-[1] treated, before convulsions (7)	Liquid, thiamine deficient, force-fed	50 μg PTh and 10 μg thiamine in 0.2 ml 0.9% saline/100 g body wt
PTh-treated, after convulsions (8)	Liquid, thiamine deficient, force-fed	50 μg PTh and 10 μg thiamine in 0.2 ml 0.9% saline/100 g body wt

[1] Oxythiamine (OTh) and pyrithiamine (PTh) obtained from Sigma Chemical Company, St. Louis, Mo., or Calbiochem, Los Angeles, Calif.

at 3000 rpm (1085 × g) for 10 minutes. The supernatant solution was decanted from the precipitated protein and the plasma from the red cells was poured into glass test tubes. Both preparations were then stored at − 20° until used.

The adrenal glands were carefully removed, cleaned of excess fat and weighed. The stomach and intestines were also removed, stripped of excess tissue and weighed with their contents.

A modification of the method of Boehringer and Soehne (10) was used for the pyruvic acid assay. The following modifications were made: the recommended concentration of reduced β-nicotinamide adenine dinucleotide (NADH)[5] was found to be insufficient for the elevated levels of pyruvate found in the rats; therefore, the concentration was increased from 3×10^{-3} M to 8×10^{-3} M. It was also necessary to use 2.5 ml of the final solution rather than 2.0 ml for the optical density measurements. At the time of assay, the frozen supernatant solutions were thawed and 3.0-ml aliquots were added to 1.0 ml of 1.1 M K_2HPO_4 solution. This solution was mixed and allowed to stand 10 minutes in an ice bath. The solution was filtered from the precipitated potassium perchlorate through a fluted filter paper. After the temperature of the solution was equilibrated at approximately 25°, 2.50 ml of the solution, which was buffered to approximately pH 7 with K_2HPO_4, were used for the assay. This 2.50-ml sample was pipetted into a 3.0-ml cuvette along with 0.05 ml of 8×10^{-3} M NADH solution. The optical density was read in a spectrophotometer[6] at 340 mμ. Then 0.05 ml lactate dehydrogenase solution containing 0.75 mg protein/ml was added with thorough mixing. After 2 minutes, the optical density was again read at 340 mμ. The method was checked using a suitable amount of a standard pyruvate solution in place of the blood extract.

The levels of plasma glucose were determined by the commercially prepared glucose oxidase reagent and the experimental procedures of Somogyi (11) and Saifer and Gerstenfeld (12). Plasma levels of corticosterone were determined by the method of Guillemin et al. (13).

RESULTS AND DISCUSSION

The effects on body weight gain of force-feeding compared with feeding ad libitum are illustrated in figure 1. Force-fed control rats receiving 8 g of the dry diet per day in liquefied form, grew at a rate strictly comparable with the control rats allowed ad libitum access to the diet. Both thiamine-deprived and PTh-treated force-fed rats continued to grow at a somewhat sub-

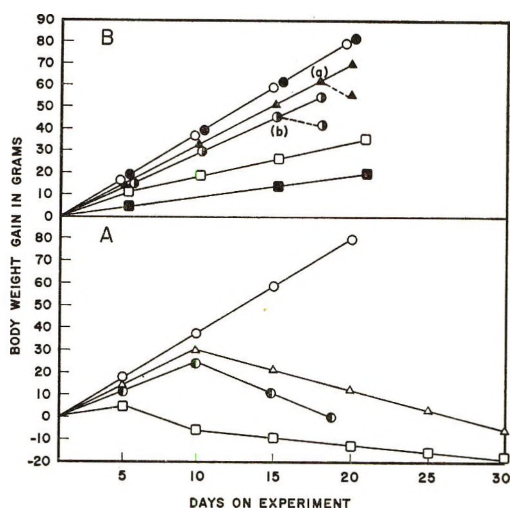

Fig. 1 Group growth curves for rats fed ad libitum (A) (data for these curves were taken from Gubler (6)) and force-fed (B) the basal thiamine-deficient diet with supplements injected subcutaneously as indicated below. The force-fed rats received 8 g diet/day in two portions by stomach tube, with the exception of the OTh-treated rats; the ration for the OTh-treated rats had to be cut to 4 g/day after day 4 to prevent bloating. Control group (10 μg thiamine/100 g body wt per day): ad lib. ○——○, force-fed ●——●; thiamine deprived: ad lib. △——△, force-fed ▲——▲; OTh-treated (10 μg thiamine + 2000 μg OTh/100 g per day): ad lib. □——□, force-fed ■——■; and PTh-treated (10 μg thiamine + 50 μg PTh/100 g per day): ad lib. ◑——◐, force-fed ◑ ◑. Weight gain after subtraction of weight of gut contents - - - - - -. OTh-treated rats showed bloating from the beginning, whereas thiamine-deprived and PTh-treated rats did not until deficiency symptoms appeared at (a) and (b), respectively. The growth curves in B were derived from the rats for which data are reported in table 2.

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[5] NADH (β-DPNH), grade III, lactic dehydrogenase (LDH), type II, and sodium pyruvate, type II, were purchased from the Sigma Chemical Company, St. Louis, Mo.; glucose oxidase reagents were obtained from Dade Reagent, Inc., Miami, Florida.

[6] Beckman DU, Beckman Instruments, Inc., Fullerton, Calif.

normal rate instead of leveling off and beginning to lose weight at around 10 to 12 days, as was always the case with similar rats fed ad libitum. However, after anorexia and intestinal bloating appeared in these rats, about 2 days before death (points a and b, fig. 1), the continued weight gain was due largely to accumulation of food in the gut as shown by the dotted lines. This would suggest that as soon as the deficiency progresses to that point where anorexia becomes severe, the animal is almost totally unable to utilize the food available in the gut. Since anorexia and weight loss were evident from the beginning in OTh-treated rats fed ad libitum, it was not surprising to find that these rats gained weight much more slowly when force-fed than any of the other groups, and most of the weight gain represented unused food accumulation in the gut. These results suggest a specific local biochemical disturbance in the gut rather than a central mechanism as the basis for the anorexia and inability to utilize the food. It is of interest to note that force-feeding led to earlier death by an average of 10 days in the thiamine-deprived and OTh-treated groups, but had no effect on mortality in the PTh-treated group. This is shown by comparison of the curves in A with those in B (fig. 1). Although the rats were killed, they would have survived less than 24 hours at this stage if allowed to die. It should be noted that the OTh-treated rats began bloating near the beginning of the experimental treatment and the diet ration had to be reduced to 4 g/day or they would have died of the effects of the bloating. According to Veen (14) gastric emptying time is retarded in the terminal stages of thiamine deficiency, but it is not responsible for initiating the marked anorexia. It is not clear, however, whether gastric atonia precedes and plays a role in induction of anorexia or not. Work is now in progress to study the biochemical and histological changes in the gut in relation to the onset of anorexia.

Because the control rats in groups 2a and 2b showed significant differences in blood pyruvate and plasma glucose levels (table 2), it was felt that group 2a, with food present in the gut, would serve as a more realistic control for the deficient groups than would group 2b. For statistical comparisons, group 2a has been used as the control for all the other groups. Groups 1 and 2b are included in table 2, however, for the sake of comparison. As can be seen, force-feeding, per se, had no effect on blood pyruvate or plasma glucose levels, nor on gut or adrenal weights when compared with animals fed ad libitum. Hence, it would appear that the force-feeding itself imposed no significant stress on the animals.

As shown in table 2, the thiamine-deprived rats (group 3) showed highly significant increases in blood pyruvate and adrenal weights, with more moderate but still significant increases in plasma corticosterone and gut weight, compared with group 2a. The plasma glucose level, on the other hand, was somewhat decreased. Oxythiamine treatment resulted in significant increases in all the parameters studied after only 1 week. Blood pyruvate and adrenal weight increased further after 2 and 3 weeks of treatment; the plasma glucose returned to even a subnormal level, when compared with the nonfasted controls (2a), but normal if compared with the fasted force-fed or ad libitum controls. The plasma corticosterone level returned to a normal level also, despite the hypertrophy of the adrenal gland. This would suggest that partial exhaustion of the adrenal cortex may have occurred after week 1 of OTh-treatment. Shinozaki (15) also noted a hyperfunction of the adrenal cortex in cells of the zona fasciculata in the earlier phases of thiamine deficiency, but a functional depression in the terminal phases. PTh-treated rats, killed when neurological symptoms appeared (ataxia and incoordination), but before frank convulsions developed (group 7), showed highly significant increases in plasma corticosterone levels and adrenal weight, but no elevation of blood pyruvate or plasma glucose. When they were killed, 24 to 48 hours after convulsions started, there were further significant increases in plasma corticosterone and adrenal weights and a moderately significant increase in the blood pyruvate level. Plasma glucose levels were not affected even though these animals had food retained in the gut.

TABLE 2

Blood pyruvate and plasma glucose and corticosterone levels and gut and adrenal weights of control, thiamine-deprived and antagonist-treated force-fed rats [1]

Group no.	No. of rats	Blood pyruvate	Plasma glucose	Plasma cortico-sterone	Gut wt	Adrenal wt
		mg/100 ml	*mg/100 ml*	*µg/100 ml*	*g/100 g*	*mg/100 g*
Ad libitum, control (1)	10	1.70 ± 0.080 [2]	151.3 ± 6.84 [2]	11.02 ± 0.653	6.8 ± 0.29 [3]	15.74 ± 0.476
Force-fed groups, control (2b) [4]	10	1.72 ± 0.051	153.8 ± 8.16	11.54 ± 0.660	5.9 ± 0.14	15.58 ± 0.281
Control (2a) [4]	10	2.53 ± 0.101	167.5 ± 5.17	11.54 ± 0.663	12.3 ± 0.47	14.37 ± 0.415
Thiamine-deprived (3)	10	3.18 ± 0.185 [3]	135.5 ± 6.99 [2]	18.61 ± 0.592 [2]	14.8 ± 1.34 [2]	22.08 ± 0.596 [3]
OTh-treated						
1 week (4)	10	2.88 ± 0.162 [2]	212.8 ± 13.34 [3]	19.29 ± 0.871 [3]	18.3 ± 2.53 [3]	23.41 ± 0.974 [3]
2 weeks (5)	10	3.45 ± 0.137 [3]	185.3 ± 13.34 [2]	9.51 ± 0.371	21.9 ± 0.78 [3]	23.73 ± 1.389 [3]
3 weeks (6)	10	3.44 ± 0.146 [3]	148.6 ± 8.21 [2]	11.23 ± 1.169	17.8 ± 1.14 [3]	27.80 ± 0.828 [3]
PTh-treated						
Before convulsions (7)	10	2.56 ± 0.124	163.6 ± 7.83	16.88 ± 1.288 [3]	12.5 ± 0.52	18.51 ± 0.476 [3]
After convulsions (8) [5]	10	2.81 ± 0.107 [2]	171.3 ± 8.77	21.64 ± 2.265 [3]	9.9 ± 0.46 [3]	25.52 ± 0.911 [3]

[1] All values represent mean ± SE.
[2] Significant at level of $P < 0.05$.
[3] Significant at level of $P < 0.005$.
[4] Animals not fed on morning of killing (b); animals fed before killing (a).
[5] Animals in convulsions 24 to 48 hours before killing.

The nonparallel nature of the responses of blood pyruvate and adrenalcortical activity in the three OTh-treated and the two PTh-treated groups would suggest that the stress-induced increases in adrenalcortical activity make some contribution to the increases in blood pyruvate in the thiamine-deficiency states, but also there must be a significant, more specific contribution from the disturbance in pyruvate metabolism associated with a deficiency of active coenzyme ThDP. Although some investigators (16–18) have reported no alteration in total pyruvate metabolism in thiamine-deficient animals, other studies have shown changes in blood pyruvate levels and in activity of pyruvate dehydrogenase complex in the tissues in thiamine-deficiency states (6, 19–21).

The next step in elucidating this problem would be to study these parameters in adrenalectomized rats in these three deficiency states. Rindi and co-workers (22, 23) have made such a study in thiamine-deprived rats previously adrenalectomized or hypophysectomized. He also reported a better correlation between elevations of blood pyruvate and adrenal hypertrophy than between blood pyruvate and tissue thiamine levels in thiamine-deprived rats. Such a study is being carried out to include OTh- and PTh-treated rats as well.

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Nutritional Evidence for the Absence of the Complete Ornithine-Urea Cycle in the Insect, *Argyrotaenia velutinana* (Lepidoptera: Tortricidae)[1]

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ABSTRACT Studies were made of the ornithine-urea cycle in the plant-feeding insect, *Argyrotaenia velutinana* (Walker), by replacing dietary arginine with intermediaries involved in the cycle. Axenic rearing on graded concentrations of arginine showed that arginine is a dietary requirement. This was in agreement with previous studies using the radioactive tracer technique which demonstrated that arginine was not synthesized by the insect. Likewise, the optimum dietary concentration of arginine found in this study was between 0.10 and 0.15% at a 2.0% dietary amino acid mixture level, which agreed with previous studies of the dietary quantitative amino acid requirements based on the pattern of amino acids found in the carcass of the insect. The dietary arginine requirement could be satisfied by substituting twice the equimolecular amount of citrulline: ornithine, creatine, or guanidoacetic acid could not replace arginine. Biological studies resulting from axenic rearing for three successive generations on a diet in which citrulline replaced arginine indicated that the conversion of citrulline to arginine was nutritionally adequate to satisfy the requirements for growth, development and reproduction of the insect.

Uric acid is the major end product of nitrogen metabolism in most terrestrial insects studied. Urea is also a common nitrogenous constituent of insect excreta, but in most cases has been reported in only small amounts (1). Urea is the main nitrogenous waste product of mammals, amphibians and chelonian reptiles, and the principal method of urea formation from protein break-down in these animals is by the ornithine-urea cycle of Krebs and Henseleit (2). Direct biochemical evidence in favor of the existence of this cycle in insects is very inconclusive, and the results of relevant nutritional studies are hardly less equivocal (3). Arginine, citrulline and ornithine occur as intermediaries in the Krebs-Henseleit cycle and only arginine has been shown to be an indispensable dietary component for insects (4). Citrulline can partially replace arginine in the diet of the yellow-fever mosquito, *Aedes aegypti* (5), the honey bee, *Aphis mellifera* (6), the vinegar fly, *Drosophila melanogaster* (7) and the saw-tooth grain beetle, *Oryzaephilus surinamensis* (8). Ornithine cannot replace arginine in the nutrition of the honey bee (6), vinegar fly (7), or the saw-tooth grain beetle (8), and only slightly in the yellow-fever mosquito (5).

The present study was undertaken to investigate by nutritional methods the presence or absence of the ornithine-urea cycle in the plant-feeding insect *Argyrotaenia velutinana* (Walker). The study includes biological observations of insects which developed and reproduced on diets containing intermediaries of the cycle.

EXPERIMENTAL

The methods for continuous rearing of the insect in the laboratory on a semipurified diet were the same as those reported in a previous study (9). The composition of the control diet used for axenic rearing of the insect is shown in table 1, and is similar to that previously reported by Rock and King (10). The dietary constituents were obtained commercially.[2] The pattern of the dietary amino acid mixture was similar to the amino acid pattern of the insect's carcass; feeding tests showed this

Received for publication December 23, 1968.
[1] Paper no. 2782 of the Journal Series of the North Carolina State University Agricultural Experiment Station, Raleigh, North Carolina.
[2] Nutritional Biochemicals Corporation, Cleveland, Ohio.

TABLE 1

Composition of the control diet

Constituents	*g/100 g diet*
Dextrose	5.000
Safflower oil	0.300
Cholesterol	0.200
Salt mixture W [1]	1.200
ZnCl	0.002
$FeSO_4 \cdot 7H_2O$	0.040
$CuSO_4 \cdot 5H_2O$	0.002
Amino acids (L-allo free) [2]	2.067
Vitamin mixture [3]	0.142
Tween 80 [4]	0.300
Agar	3.000
2 M KOH added to give pH 6.2	
Distilled 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 amino acid mixture: (in milligrams per 100 g diet) arginine, 125; histidine, 100; isoleucine, 92; leucine, 156; lysine·HCl, 190; methionine, 72; phenylalanine, 107; threonine, 100; tryptophan, 90; valine, 95; alanine, 125; aspartic acid, 180; glutamic acid, 220; glycine, 97; proline, 99; serine, 57; cystine, 60; and tyrosine, 102.

[3] Composition of vitamin mixture: (in milligrams per 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 alpha-tocopherol, 15.

[4] Polyoxyethylene sorbitan monooleate, Atlas Powder Company, Wilmington, Del

amino acid pattern to support maximum larval growth (10).

Graded amounts of the intermediaries in the Krebs-Henseleit cycle were added individually or in combination to diets to evaluate their ability to support growth, development and reproduction. Aseptic rearing techniques and methods for preparing the diets were generally the same as reported previously (11). Egg masses were surface sterilized and one newly hatched larva was transferred aseptically to each 20-mm by 90-mm specimen vial containing 4 to 5 g heat-sterilized medium; the vials were stoppered with sterilized cotton. The insects were reared at 27 ± 0.5° with continuous illumination. Each experiment had two to four replicates with each replicate using from 25 to 115 individual insects. The nutritional adequacy of the diets was evaluated by comparing larval growth rates until pupation, 1-day-old pupal weights, pupal maturation periods, survival to the moth stage and egg deposition. Observations for larval growth to the pupal stage were terminated at 40 days, and the larvae living, but not pupated at 40 days were counted as dead. For either the egg-deposition studies or the continuous rearing on a diet, at least 25 male and 25 female pupae were placed in a box where the moths emerged, mated and laid egg masses on waxed paper provided for that purpose. Egg deposition was not conducted under aseptic conditions; however, the egg masses deposited were surface sterilized if aseptic larvae were required for continuous rearing.

RESULTS

The effects of different dietary concentrations of arginine, citrulline and ornithine on development and survival are shown in table 2. The effective concentrations of arginine tested covered a range from 0.1 to 0.2% with the optimum level between 0.10 and 0.15%. Based on larval growth rates, pupal weights, and survival to the moth stage, the optimum dietary level of citrulline was about 0.2%. These results suggested that the optimum level of citrulline (about 0.2%) was twice the equimolecular amount required for the minimum effective concentration of arginine (about 0.10%), indicating that the enzymatic conversion of citrulline to arginine is not 100% efficient. Although the growth rates for both male and female on the control diet (arginine at 0.16%) were accelerated over that for citrulline at the 0.2% level, the difference was not significant ($P > 0.05$). Ornithine at two dietary concentrations resulted in 100% larval mortality when substituted for arginine (table 2). There was evidence of feeding on the ornithine diet; however, no larval development occurred beyond the first larval instar.

Table 3 outlines the results of replacing arginine in the diet by related amino compounds. Ornithine in the presence of citrulline had neither a stimulating nor inhibitory effect on growth and development of *A. velutinana*. Guanidoacetic acid alone, or in combination with ornithine, could not replace arginine in the diet. Likewise, creatine alone or in combination with guanidoacetic acid, was incapable of replacing arginine.

Table 4 shows the results of axenic rearing from the egg stage to moth emergence for three successive generations on a diet in which citrulline (0.2% level) replaced

TABLE 2

Growth and survival of Argyrotaenia velutinana fed diets containing graded amounts of arginine, citrulline and ornithine·HCl

Dietary concentration	Total no. of larvae	Male (avg)			Female (avg)			Survival [1]
		No. pupated	Days to pupation	Pupal wt	No. pupated	Days to pupation	Pupal wt	
%				*mg*			*mg*	%
				Arginine				
0.00	92	0	—	—	0	—	—	0
0.05	88	2	39.0 [a] [2]	nd [3]	4	36.0 [a]	nd [3]	5
0.10	85	43	25.9 [b]	nd	35	26.8 [b]	nd	85
0.15	74	31	24.5 [b]	nd	31	27.0 [b]	nd	74
0.20	93	46	24.2 [b]	nd	37	26.1 [b]	nd	80
				Citrulline				
0.05	105	0	—	—	0	—	—	0
0.10	60	8	37.7 [a]	15.4 [a]	4	34.5 [a]	27.0 [a]	20
0.15	105	44	26.9 [b]	19.2 [b]	22	28.0 [b]	30.2 [b]	49
0.20	109	51	24.5 [c]	19.5 [b]	38	27.8 [b]	29.6 [b]	72
0.30	104	45	24.5 [c]	19.6 [b]	37	27.1 [b]	29.0 [b]	70
Control [4]	101	56	24.2 [c]	20.1 [b]	34	26.9 [b]	28.6 [b]	76
				Ornithine·HCl				
0.15	110	0	—	—	—	—	—	0
0.20	101	0	—	—	—	—	—	0
Control [4]	92	37	24.8	24.1	33	28.3	26.7	70

[1] Percentage of the initial number of larvae to develop to moths.
[2] Values not followed by same letter are significantly different ($P < 0.05$).
[3] nd = not determined.
[4] Arginine at 0.16% dietary level.

TABLE 3

Growth and survival of Argyrotaenia velutinana fed diets in which arginine was replaced by related amino compounds [1]

Arginine replaced by	Total no. of larvae	Male (avg)			Female (avg)			Survival [2]
		No. pupated	Days to pupation	Pupal wt	No. pupated	Days to pupation	Pupal wt	
				mg			*mg*	%
Citrulline	103	49	26.2 [a] [3]	18.5 [a]	35	26.8 [a]	29.6 [a]	70
Citrulline + ornithine·HCl	94	37	26.7 [a]	19.6 [a]	37	27.4 [a]	30.5 [a]	68
Creatine	46	0	—	—	0	—	—	0
Creatine + guanidoacetic acid	43	0	—	—	0	—	—	0
Guanidoacetic acid	51	0	—	—	0	—	—	0
Ornithine·HCl + guanidoacetic acid	38	0	—	—	0	—	—	0

[1] Each compound was added at 0.2% dietary level, whether added alone or in combination.
[2] Percentage of initial number of larvae to develop to moths.
[3] Values not followed by same letter are significantly different ($P < 0.05$).

arginine. To check for the possibility of an experimental error in the preparation of citrulline diets used for continuous rearing, larvae from the laboratory strain (control) were reared on portions of the citrulline diets for the F_1 and F_2 generations. Larval growth rates, pupal maturation periods and survival to the moth stage

TABLE 4

Growth and survival of Argyrotaenia velutinana fed one generation on arginine diet (0.16% level), an alfalfa leaf meal diet, and three successive generations on a diet in which arginine was replaced by citrulline (0.2% level)

Diet	Generation	Total no. of larvae	Male (avg)			Female (avg)			Survival [2]
			No. pupated	Days to pupation	Pupal maturation period	No. pupated	Days to pupation	Pupal maturation period	
					days [1]			*days* [1]	%
Citrulline	P_1	109	51	24.6 [a 3]	nd [4]	38	27.8 [a]	nd [4]	72
Citrulline	F_1	318	143	25.4 [a]	8.1 [a]	98	28.1 [a]	8.6 [a]	74
F_1 control [5]	P_1	85	38	25.3 [a]	8.0 [a]	34	26.4 [a]	8.3 [a]	75
Citrulline	F_2	191	70	24.9 [a]	8.6 [a]	75	26.7 [a]	9.0 [a]	76
F_2 control [5]	P_1	112	49	24.7 [a]	8.7 [a]	34	28.0 [a]	8.9 [a]	75
Arginine	P_1	88	33	24.1 [a]	8.2 [a]	49	26.9 [a]	8.2 [a]	73
Alfalfa meal [6]	P_1	117	46	20.5 [b]	7.6 [a]	51	22.7 [b]	7.8 [a]	85

[1] Time interval from larval pupation to moth emergence.
[2] Percentage initial number of larvae to develop to moths.
[3] Values not followed by same letter are significantly different ($P < 0.05$).
[4] nd = not determined.
[5] Larvae from laboratory strain reared on portions of citrulline diet for F_1 and F_2 generations.
[6] Semipurified diet on which the insect is routinely reared in hte laboratory (9).

for both male and female showed no significant difference ($P > 0.05$) between the P_1, F_1 and F_2 generations reared on the citrulline diets. Likewise, the growth and survival results on the citrulline diet for the P_1, F_1 and F_2 generations were not significantly different ($P > 0.05$) than those for the F_1 and F_2 control diets (indicating absence of experimental error in diet preparation) or the P_1 arginine diet. Larval growth rates, however, for the three generations on the citrulline diet and the one generation on the arginine diet were significantly slower ($P < 0.01$) than those obtained for the one generation evaluated on an alfalfa leaf meal diet.

Table 5 summarizes the results of egg deposition studies of moths from the alfalfa leaf meal diet and moths from the citrulline diet for P_1 and F_2 generations. Because the alfalfa leaf meal diet has satisfied the dietary requirements for reproduction of *A. velutinana* after 2 years of continuous rearing, the reproductive potential of moths from the citrulline diet was compared with that of moths from the alfalfa diet. Moths from the citrulline diet for both the P_1 and F_2 generations showed a similar number of eggs per egg mass, incubation period and percentage hatch of egg masses. The percentage hatch of egg masses from the citrulline diets, however, was less and egg masses were on the average smaller than egg masses obtained from the alfalfa leaf meal diet. The egg incubation period was comparable for the two diets.

DISCUSSION

The results of this study agree with previous studies using both the single amino acid deletion technique (11), and the radioactive tracer technique (12), which showed that arginine was a dietary requirement for larval growth of *A. velutinana*. Also, previous studies established the quantitative dietary arginine requirement based on the quantitative pattern of amino acids found in the carcass of 1-day-old pupae (10), and the optimum dietary level of arginine established in the present study (between 0.1 and 0.15%) approximates that obtained by the carcass study (0.16%).

Two important physiological functions of arginine in insects and other invertebrates include the use of arginine in protein synthesis and arginine to act as an energy store in muscle as the phosphagen, arginine phosphate (1). Arginine phosphate is the only phosphagen that has been found in insect muscle (13). In mammals, amphibians and chelonian reptiles arginine is important in nitrogen metabolism by functioning in the ornithine-urea cycle whereby urea is excreted as the main nitrogenous end product (14). Terrestrial insects, birds, nonchelonian reptiles and some snails excrete uric acid as their primary nitrogenous end product. Uricotelism

TABLE 5

Comparison of oviposition by Argyrotaenia velutinana fed on citrulline diet and on alfalfa leaf meal diet

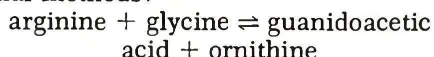
Criteria	Citrulline (generation)		Alfalfa meal [1]
	P_1	F_2	P_1
No. of egg masses observed	44	40	45
Avg no. of eggs per egg mass	42 ± 23 [2]	36 ± 19	52 ± 27
Percentage hatch	76 ± 9	71 ± 8	82 ± 7
Incubation period, days [3]	6.6 ± 0.8	6.4 ± 0.7	6.4 ± 0.7

[1] Semipurified diet on which the insect is routinely reared in the laboratory (9).
[2] Mean value ± SD of the mean.
[3] Observed at 3- to 10-hour intervals.

in insects, however, may not preclude the operation of the ornithine-urea cycle, as this cycle is functional in other uricotelic groups like reptiles (14). Direct biochemical evidence demonstrating the presence in insects of the five enzymes involved in the ornithine-urea cycle is lacking (3). Arginase is the only enzyme whose presence has been definitely confirmed (15). Arginine, citrulline and ornithine, the amino acids involved in the cycle, have been detected in tissues of certain insects. However, Porembska and Mochnacka (15) showed by enzymatic studies that the ornithine-urea cycle is inoperative in the insect *Celerio euphoisbiae* despite the presence of its three constituent amino acids.

As in the chick (16) citrulline, but not ornithine, can replace dietary arginine for larvae of *A. velutinana*. The results of biological studies of *A. velutinana* reared axenically for three successive generations on a citrulline diet would suggest a ready citrulline-arginine enzymatic conversion in the insect. Aspartic acid, which is involved in this conversion, is known to be synthesized by *A. velutinana* larvae (12) and was not added to the diet. The lack of ornithine-citrulline conversion indicated the absence or low activity of the enzyme system involved. Golberg and De Meillon (5) reported that mosquito larvae could utilize ornithine to a small extent since larval growth occurred to fourth instar. Because the mosquito diet contained yeast autolysate, the limited growth on the ornithine diet was probably due to suboptimal levels of arginine in the yeast autolysate. Ornithine had no deleterious effect on growth of *A. velutinana* when ornithine was a dietary constituent along with citrulline (table 3). This precluded the possibility that ornithine was toxic to *A. velutinana* larvae, as was suggested for the vinegar fly larvae (7). Results of the present study indicated that the complete ornithine-urea cycle is not present in *A. velutinana*. The only part shown to be operative by nutritional studies was the conversion of citrulline to arginine. Results of the biological studies showed this conversion to be nutritionally adequate to satisfy growth, development and reproduction requirements.

Because creatine has been shown to be excreted by some insects (3), the following biosynthetic pathway of arginine and creatine (17) was investigated by nutritional methods:



arginine + glycine ⇌ guanidoacetic acid + ornithine

Guanidoacetic acid is methylated to creatine by methionine. Arginine could not be replaced by guanidoacetic acid alone or in combination with ornithine (table 3). Glycine is known to be synthesized by *A. velutinana* (12) and was not added to the diet. Similar results were obtained by Davis (8) with the beetle *O. surinamensis* which suggests that both insects are incapable of synthesizing arginine by the above pathway. Davis (8) reported 35% pupation on a diet in which creatine replaced arginine. However, with *A. velutinana* arginine replaced by creatine alone or in combination with guanidoacetic acid results in 100% mortality at first larval instar (table 2). The above would suggest that if creatine is an end product of arginine metabolism in this organism by the above pathway, the pathway is not reversible. Creatine or guanidine could not re-

place arginine in the diet of a mosquito larva (5) and the chick (16).

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Amino Acid Retention and Balance in the Young Rat Fed Varying Levels of Lactalbumin [1]

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ABSTRACT The effect of the level of dietary protein intake upon amino acid balance and retention efficiency was determined over the range of 3.6 to 25.0% dietary protein using high purity lactalbumin as the protein source. A total of 12 diets was prepared and fed to groups consisting of six weanling rats each for a period of 3 weeks. Carcass retention efficiency of essential amino acids was found generally to be inversely proportional to the dietary adequacy of each amino acid although some exceptions were noted. Amino acid retention efficiency and protein efficiency ratio (PER) declined sharply below 6% dietary protein despite the achievement of weight gain. This decline was accompanied by a reduction in apparent synthesis of nonessential amino acids and an alteration in the composition of the carcass gain.

The carcass retention efficiency for an amino acid is defined as the ratio of the amount of an amino acid recovered from the carcass gain to the amount of that amino acid consumed. Such data, when combined with an analysis of the fecal and urinary content of each amino acid, can provide detailed information concerning the effect of various nutrient modifications on the overall pattern of amino acid metabolism.

This technique does not appear to have been applied extensively in the study of protein nutrition although its potential usefulness in this regard has been established. Grau (1) and Grau and Steele (2) and Schweigert et al. (3–6) were among the first to use retention efficiency in this manner. Most retention studies to date, however, have dealt with individual essential amino acids, with the exception of those by Price et al. (7). Our study reports on the effect of variation in dietary protein over a wide range upon the carcass retention and balance of both essential and nonessential amino acids using a high quality, highly digestible protein.

EXPERIMENTAL

Twelve isocaloric diets ranging in protein content from 3.6 to 25.0% were prepared using high purity lactalbumin as the sole protein source. The composition of the 25% protein diet is given in table 1. Reduction in protein content was achieved by substitution of sucrose for lactalbumin.

Seventy-eight male weanling rats of the Sprague-Dawley strain were assigned to 13 groups so that the mean initial weights of each group were approximately equal. One group was then killed by ether inhalation to serve as the zero time carcass control. The remaining rats were housed separately in balance cages for 3 weeks during which time they received the experimental diets ad libitum. Daily collections of urine and feces were made and the samples were stored in the freezer. Prior to freezing, the urine samples were kept under toluene and acidified with 6 N HCl.

At the conclusion of the balance period, all animals were fasted overnight and then killed. The intact carcasses were frozen and stored at − 2° until hydrolysis could be accomplished. The hydrolysis procedure consisted of cutting the carcasses from all rats in each diet group into about 6 to 10 pieces while frozen, and transferring them to a large round-bottom flask which contained sufficient 6 N HCl to maintain a ratio of approximately 100 volumes/g dry matter in order to minimize humin formation. The mixture was heated with a mantle under reflux for exactly 24 hours, and

Received for publication March 6, 1969.
[1] Supported in part by National Dairy Council Grant no. 71-C and by National Institutes of Health Grant no. AM 06881.
[2] Research Corporation, 405 Lexington Avenue, New York, N. Y. 10017.

TABLE 1

Composition of diet

	g/kg
Lactalbumin [1]	490
Sucrose	400
Salt mixture [2]	50
Vitamin mixture [3]	10
Crisco [4]	25
Cottonseed oil [5]	25

[1] Donated by Sheffield.
[2] USP XVI, Official Methods of Analysis, ed. 9 1960 Association of Official Agricultural Chemists, Washington, D. C.
[3] In grams per kilogram: Ca pantothenate, 2.0; niacin, 1.0; inositol, 1.0; thiamine·HCl, 0.5; menadione, 0.4; riboflavin, 0.3; pyridoxine·HCl, 0.3; folic acid, 0.02; biotin, 0.01; vitamin B_{12}, 0.001; and sucrose, 994.5. After mixing dietary ingredients, 5 ml of 20% aqueous choline chloride were added per kilogram of diet.
[4] Procter and Gamble, Cincinnati, Ohio.
[5] Six drops of percomorph oil were added to each 100 g of cottonseed oil.

nitrogen gas was bubbled through the solution throughout the hydrolysis to minimize oxidative losses.

This treatment yielded a dark brown aqueous solution topped by a layer of lipid. Despite the precautions taken some humin formation was evident. The hydrolysate was allowed to cool to room temperature and was then transferred to a volumetric cylinder for determination of the volume of the aqueous portion. Aliquots of the nonlipid segment of the hydrolysate were taken for duplicate analysis both of total nitrogen by a micro-Kjeldahl procedure and for amino acid composition with an amino acid analyzer [3] using the method of Spackman et al. (8). The amino acid values were corrected to zero time hydrolysis by application of factors derived from kinetic studies of 24, 48 and 72 hours on control samples. A further correction was made by use of norleucine and guanidinopropionic acid as internal standards. No attempt was made to analyze for tryptophan which was totally destroyed by the acid hydrolysis, or for the nitrogen or amino acid content of the lipid fraction of the mixture. The individual fecal collections from each dietary group were combined and treated in a similar fashion as were the urine specimens. The feces and urine from the few animals which died during the course of the balance study were discarded.

RESULTS

The basic growth data are summarized graphically in figure 1. Maximum weight

[3] Beckman model 120, Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif.

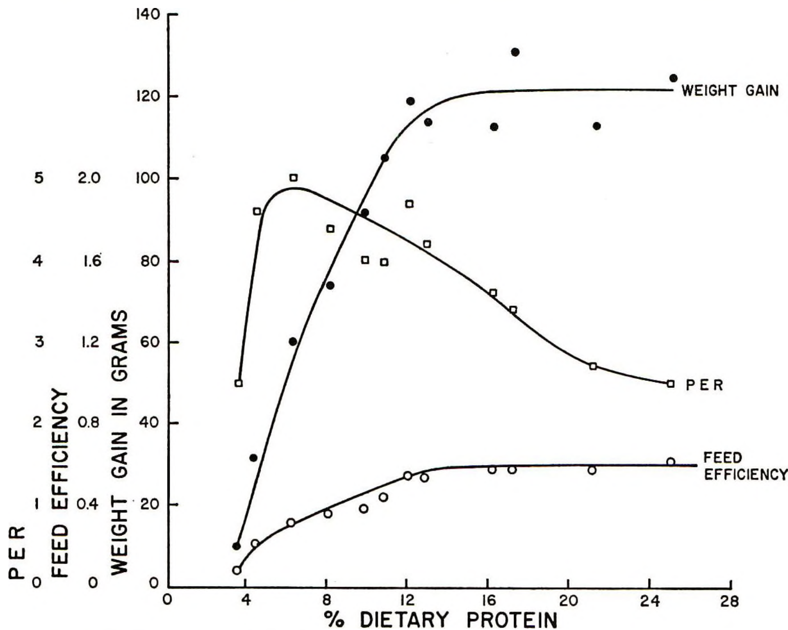

Fig. 1 Basic growth data as affected by level of dietary protein. Each point represents the average of a six-rat pool.

gain was achieved with a dietary protein level of about 12%; a slight weight gain was achieved even at 3.6% dietary protein. Feed efficiency (grams gain per gram food consumed) also reached a peak at 12% protein which was maintained with increasing levels of protein through 25%. The protein efficiency ratio (grams gain per gram protein consumed) reached its maximum in the vicinity of 4 to 8% dietary protein and then declined coincident with a decline in the slope of the weight gain curve. The efficiency of utilization of protein was greatly reduced at the 3.6% level of dietary protein. The rats were apparently not capable of efficient utilization of available amino acids at this plane of protein intake.

The effect of the level of dietary protein on the nitrogen content of urine, feces and carcass and on carcass amino acids is summarized graphically in figure 2. The ratio between millimoles of carcass nitrogen and millimoles of carcass amino acids remained constant over the range of protein intakes employed. The nitrogen balance data are presented in table 2.

Table 3 lists the amino acid balance data as a function of dietary protein. Net catabolism or anabolism was estimated by difference. The average total amino acid content of the feces was never high although it did rise slightly from 4 mmoles/rat per 3 weeks on diet 1 to 14.9 mmoles/rat per 3 weeks with diet 12. The loss of amino acids via the urine was also quite limited, never exceeding a total of 7.1 mmoles/rat per 3 weeks. Calculation of the mole percentages of the individual amino acids in the feces and urine showed a relatively constant pattern with the exception of urinary alanine which ranged between 6 and 13 moles % on the first 11 diets but was 31 moles % on diet 12. This suggests that a condition of overload aminoaciduria was arising for this particular amino acid as the dietary protein reached 25%. The average intake at this point was 30 mmoles/rat per 3 weeks.

Comparison of the estimated net catabolism or anabolism of the various amino acids is useful in considering the efficient use of these substances. At the two lowest protein intakes, net catabolism was considerably in excess of apparent synthesis. These data support the results of the PER and feed efficiency calculations: namely, that protein synthesis cannot proceed efficiently below a certain minimal level of protein. Estimated net catabolism and net anabolism were comparable in the groups fed either 6.3, 8.1, 8.9 or 10.7% protein. These results show that the net utilization of nitrogen for synthesis of nonessential

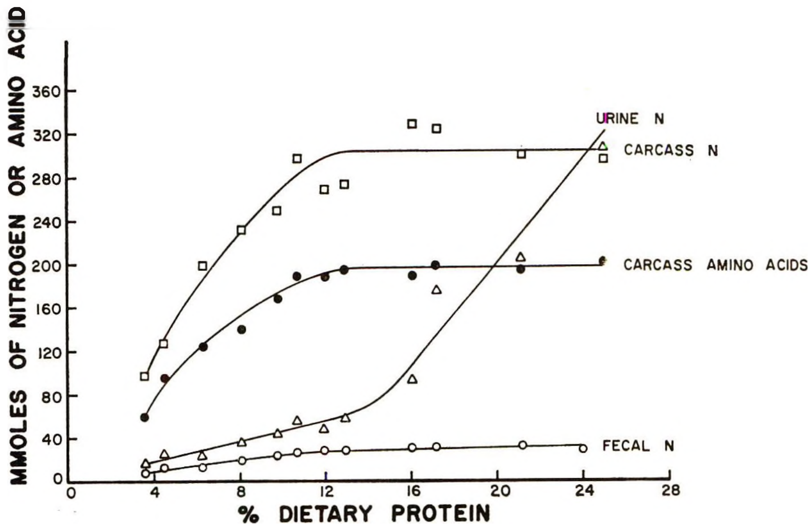

Fig. 2 Effect of dietary protein on total nitrogen of urine, feces and carcass and on carcass total amino acids. Each point represents the average of a six-rat pool.

TABLE 2
Nitrogen balance

Diet no.	1	2	3	4	5	6	7	8	9	10	11	12
Dietary protein, %	3.6	4.5	6.8	8.1	8.9	10.7	12.0	12.9	16.2	17.2	21.2	25.0
					avg mmole N/rat per 3 weeks							
Carcass gain	32	62	133	166	186	227	199	205	258	254	231	225
Feces	8	13	14	19	24	27	28	29	30	31	32	29
Urine	16	25	24	37	44	56	48	58	94	175	203	307
Total recovered	56	100	171	222	254	310	275	292	382	460	466	561
Consumed	46	78	138	192	259	290	298	312	379	451	478	592
Recovery, %	123	128	124	115	98	107	92	94	101	102	98	95

amino acids was extremely efficient with this particular dietary pattern. At the next protein level, 12% , and at all higher levels, apparent catabolism of amino acids greatly exceeded apparent synthesis as the dietary supply of amino acids was in considerable excess of the maximum capacity of the rat for production of body protein.

Estimated net synthesis was evident primarily with only five amino acids: glycine, alanine, serine, arginine and methionine. The first three amino acids can be derived from a number of different precursors and thus the comparison of product production versus precursor disappearance is of little value. In the case of arginine, however, proline is probably the principal precursor when it is available in sufficient quantity. The balance data in table 3 indicate that with one exception (10.7% protein) the net disappearance of proline matched or exceeded the net increase in arginine. The situation with methionine and its probable precursors, cysteine and aspartic acid, is more difficult to explain. The analytical data indicate a net increase in methionine of from 0.5 to 0.8 mmole/rat per 3 weeks in each of the diet groups fed the five lowest levels of protein. The net disappearance of half-cystine is comparable to the estimated increase in methionine at the two lowest levels of protein, but is considerably less in the remaining three diets. The charts from the amino acid analyzer were reexamined for evidence of abnormalities but the methionine peak was at its expected position and exhibited a symmetrical shape indicative of minimal contamination. Additional analyses of the hydrolysate confirmed the original figures. Methionine biosynthesis has been observed in certain microorganisms but efforts to find appreciable activity in mammalian tissue have failed (9). If the analytical results are indeed correct, this may indicate a limited capacity for induction of the necessary enzymes only under appropriate dietary conditions.

Carcass retention efficiency was calculated for each amino acid from the data of table 3. Carcass retention efficiency is equal to the quotient times 100 of the total amino acids of the carcass gain divided by the total amino acid consumed. Carcass gain is determined by correcting the carcass values at the end of 3 weeks for the amount of amino acid present in the rats killed at the beginning of the study. Figure 3 shows the best smooth lines for a plot of the retention efficiency of each of the essential amino acids, except sulfur amino acids and arginine, as a function of percentage dietary protein. The dots on each line represent the point at which the intake of an amino acid satisfied the National Academy of Science — National Research Council (NAS–NRC) recommended allowance for growth for the rat (10).

The essential amino acids shown in figure 3 exhibited low retention efficiencies at the two lowest protein intakes despite

TABLE 3

Balance of amino acids as affected by dietary protein level (lactalbumin)

Amino acid	Intake	Feces	Urine	Carcass gain	Net catab-olism	Net syn-thesis	Intake	Feces	Urine	Carcass gain	Net catab-olism	Net syn-thesis
	avg mmoles of amino acid/rat per 3 weeks											
	3.6% protein						4.5% protein					
Lys	2.4	0.28	0.06	0.11	2.0		4.4	0.5	0.09	1.6	1.8	
His	0.6	0.06	0.02	—	0.5		0.9	0.1	0.02	0.2	0.6	
Arg	0.6	0.18	0.02	—	0.4		0.9	0.3	0.03	0.5	0.1	
Asp	2.8	0.46	0.10	1.01	1.2		5.0	0.8	0.14	3.2	0.9	
Thr	1.6	0.27	0.04	0.61	0.7		2.7	0.4	0.05	1.8	0.4	
Ser	1.7	0.42	0.05	0.76	0.5		3.0	0.7	0.06	2.2	0.0	
Glu	4.7	0.58	0.13	1.30	2.7		8.4	1.0	0.16	4.5	2.7	
Pro	2.4	0.23	0.08	0.18	1.9		4.4	0.4	0.08	1.6	2.3	
Gly	1.0	0.37	0.23	2.12		1.7	1.8	0.6	0.23	5.6		4.6
Ala	1.9	0.37	0.09	1.60		0.2	3.3	0.6	0.12	4.0		1.4
Half-cys	0.3	—	0.02	—	0.3		0.6	—	—	0.1	0.5	
Val	1.9	0.25	0.03	1.18	0.4		3.3	0.5	0.04	2.6	0.2	
Met	0.4	0.05	0.01	0.86		0.5	0.9	0.1	0.01	1.4		0.6
Ile	1.6	0.22	0.02	0.49	0.9		2.9	0.4	0.04	1.6	0.9	
Leu	3.3	0.29	0.05	0.88	2.1		6.0	0.5	0.07	2.9	2.5	
Tyr	0.7	0.10	0.01	0.16	0.4		1.2	0.2	0.01	0.8	0.2	
Phe	0.9	0.12	0.02	0.52	0.2		1.7	0.2	0.03	1.3	0.2	
Totals	28.8	4.26	0.98	11.78	14.2	2.4	51.4	7.3	1.18	35.9	13.3	6.6
	6.3% protein						8.1% protein					
Lys	6.9	0.5	0.10	5.1	1.2		9.2	0.6	0.14	5.9	2.6	
His	1.5	0.1	0.03	1.5		0.1	1.9	0.2	0.03	1.7		
Arg	1.5	0.2	0.02	3.8		2.5	1.9	0.3	0.02	4.3		2.7
Asp	7.8	0.9	0.20	6.2	0.5		10.4	1.1	0.22	7.2	1.9	
Thr	4.4	0.5	0.06	3.7	0.1		5.8	0.6	0.07	4.2	0.9	
Ser	4.8	0.8	0.08	4.3		0.4	6.2	1.0	0.09	6.0		0.9
Glu	13.2	1.2	0.17	9.4	2.4		17.3	1.6	0.21	10.8	4.7	
Pro	6.9	0.4	0.17	3.8	2.5		9.2	0.5	0.14	5.7	2.9	
Gly	2.7	0.7	0.43	11.6		10.0	3.5	0.8	0.44	12.7		10.4
Ala	5.3	0.7	0.10	7.4		2.9	7.1	0.9	0.15	8.7		2.6
Half-cys	0.8	0.1	0.05	0.6	0.1		1.0	0.1	0.04	0.8	0.1	
Val	5.3	0.5	0.03	4.6	0.2		7.1	0.7	0.04	5.4	1.1	
Met	1.5	0.2	0.01	2.1		0.8	1.9	0.2	0.01	2.4		0.7
Ile	4.6	0.5	0.03	3.0	1.1		5.8	0.6	0.03	3.6	1.6	
Leu	9.6	0.6	0.06	5.7	3.2		12.5	0.7	0.08	6.9	4.8	
Tyr	1.6	0.2	0.01	1.6		0.2	2.5	0.2	0.02	2.0	0.3	
Phe	2.7	0.2	0.02	2.2	0.3		3.3	0.3	0.03	3.0		
Totals	81.1	8.3	1.57	76.6	11.6	16.9	106.6	10.6	1.76	91.3	20.9	17.3
	8.9% protein						10.7% protein					
Lys	11.9	0.8	0.17	7.8	3.1		13.9	0.9	0.18	9.3	3.5	
His	2.5	0.2	0.04	2.3			2.8	0.2	0.04	2.7		0.1
Arg	2.5	0.4	0.03	5.7		3.6	2.8	0.4	0.03	7.2		4.8
Asp	13.4	1.5	0.27	9.6	2.0		15.6	1.6	0.30	11.9	1.8	
Thr	7.4	0.8	0.09	6.0	0.5		8.7	0.8	0.12	6.8	1.0	
Ser	8.1	1.3	0.11	7.1		0.4	9.4	1.5	0.13	8.2		0.4
Glu	22.4	2.0	0.26	14.5	5.6		26.2	2.2	0.34	17.1	6.6	
Pro	11.9	0.6	0.14	7.2	4.0		13.7	1.1	0.12	9.9	2.6	
Gly	4.6	1.0	0.57	16.9		13.9	5.4	1.2	0.56	19.4		20.6
Ala	9.0	1.1	0.22	11.4		3.7	10.6	1.1	0.27	12.7		3.5
Half-cys	1.4	0.1	0.06	1.1	0.1		1.7	0.1	0.06	1.5		
Val	9.0	0.8	0.06	7.2	0.9		10.6	0.7	0.08	8.1	1.7	
Met	2.3	0.3	0.02	2.8		0.8	2.8	0.2	0.03	2.6		
Ile	7.6	0.8	0.06	5.0	1.7		9.0	0.4	0.06	5.6	2.9	
Leu	16.1	1.0	0.12	9.2	5.8		18.9	1.0	0.13	11.5	6.3	
Tyr	3.2	0.4	0.03	2.7	0.1		3.8	0.3	0.04	2.6	0.9	
Phe	4.4	0.4	0.05	3.9	0.1		5.2	0.4	0.04	5.1		0.3
Total	137.7	13.5	2.30	120.4	23.9	22.4	161.1	14.1	2.53	142.2	27.3	29.7

TABLE 3 (*Continued*)

Balance of amino acids as affected by dietary protein level (lactalbumin)

Amino acid	Intake	Feces	Urine	Carcass gain	Net catabolism	Net synthesis	Intake	Feces	Urine	Carcass gain	Net catabolism	Net synthesis
	avg mmoles of amino acid/rat per 3 weeks											
	12.0% protein						12.9% protein					
Lys	18.2	1.0	0.21	11.1	5.9		17.9	1.1	0.22	11.4	5.2	
His	4.3	0.3	0.05	3.9			4.2	0.2	0.05	4.2		0.2
Arg	4.8	0.4	0.03	8.7		4.3	4.6	0.5	0.03	8.7		4.6
Asp	22.6	1.8	0.32	11.6	8.9		20.9	1.7	0.33	11.9	7.0	
Thr	12.4	0.9	0.12	6.1	5.3		11.8	0.9	0.13	6.8	4.0	
Ser	12.8	1.4	0.11	8.0	3.3		12.7	1.4	0.12	8.9	2.3	
Glu	36.0	2.2	0.35	16.0	17.4		34.8	2.1	0.36	17.0	15.3	
Pro	16.3	0.7	0.12	8.9	6.6		15.8	0.7	0.12	8.9	6.1	
Gly	8.9	1.3	0.56	22.1		15.1	9.3	1.3	0.52	21.1		13.6
Ala	15.6	1.2	0.32	13.9	0.2		15.2	1.3	0.30	13.4	0.2	
Half-cys	4.1	0.3	0.09	1.6	2.1		3.8	0.3	0.11	1.6	1.8	
Val	14.8	0.9	0.08	6.4	7.4		14.3	0.9	0.07	8.2	5.1	
Met	4.1	0.2	0.03	1.8	2.1		3.6	0.2	0.04	1.7	1.7	
Ile	12.8	0.8	0.05	4.3	7.6		12.2	0.8	0.05	5.1	6.2	
Leu	26.5	1.1	0.10	9.6	15.7		25.3	1.0	0.10	10.7	13.5	
Tyr	5.4	0.4	0.02	3.0	2.0		5.1	0.4	0.05	4.0	0.6	
Phe	7.2	0.4	0.03	4.2	2.6		7.2	0.4	0.03	4.2	2.6	
Total	226.8	15.3	2.59	141.2	87.1	19.4	218.7	15.2	2.63	147.8	71.6	18.4
	16.2% protein						17.2% protein					
Lys	22.8	1.1	0.23	9.8	11.7		31.6	1.0	0.33	11.5	18.8	
His	5.5	0.2	0.05	3.4	1.8		7.8	0.2	0.07	4.2	3.3	
Arg	5.9	0.4	0.04	8.2		2.7	8.5	0.4	0.05	8.7		0.6
Asp	27.7	1.7	0.34	12.1	13.6		37.3	1.6	0.55	11.7	23.4	
Thr	15.3	0.9	0.13	6.6	7.7		20.2	0.8	0.20	7.0	12.2	
Ser	15.5	1.4	0.12	8.6	5.4		20.8	1.3	0.18	9.1	10.2	
Glu	45.1	2.2	0.40	15.7	26.8		59.1	2.0	0.49	17.4	39.2	
Pro	20.2	0.7	0.19	9.2	10.1		26.8	0.7	0.21	9.7	16.2	
Gly	10.4	1.3	0.63	21.7		13.2	14.0	1.1	0.81	22.3		10.2
Ala	19.0	1.3	0.33	13.6	3.8		25.0	1.2	0.54	14.0	9.3	
Half-cys	4.1	0.3	0.18	2.5	1.1		6.2 [1]	0.2	0.23	2.1	3.7	
Val	17.9	0.9	0.08	6.7	10.2		22.9	0.9	0.10	7.0	14.9	
Met	4.9	0.2	0.04	2.1	2.6		6.2	0.3	0.06	2.7	3.1	
Ile	15.3	0.8	0.06	4.7	9.7		20.2	0.7	0.08	4.9	15.0	
Leu	32.2	1.0	0.11	10.2	20.9		43.5	1.0	0.16	10.9	31.4	
Tyr	6.7	0.4	0.04	2.7	3.6		9.4	0.4	0.06	3.8	5.1	
Phe	9.2	0.4	0.04	4.5	4.3		11.9	0.4	0.05	4.3	7.2	
Total	277.7	15.2	3.01	142.3	133.3	15.9	371.4	14.2	4.17	151.3	213.0	10.8
	21.2% protein						25.0% protein					
Lys	31.9	1.1	0.39	11.4	19.0		40.2	1.1	0.45	12.6	26.0	
His	8.1	0.2	0.08	3.9	3.9		9.7	0.2	0.08	4.5	4.9	
Arg	8.5	0.4	0.05	8.7		0.6	10.6	0.4	0.04	9.2	1.0	
Asp	38.0	1.8	0.65	11.7	23.8		44.5	1.6	0.85	12.0	30.0	
Thr	20.5	0.9	0.23	6.7	12.7		23.8	0.8	0.24	6.8	16.0	
Ser	21.3	1.4	0.20	8.6	11.1		25.0	1.5	0.24	8.8	14.5	
Glu	61.5	2.2	0.49	16.7	42.1		72.9	2.2	0.62	17.0	53.1	
Pro	26.2	0.7	0.25	9.4	15.8		32.3	0.7	0.24	9.4	22.0	
Gly	14.0	1.2	0.83	22.0		10.0	16.3	1.1	1.10	22.2		8.1
Ala	25.6	1.3	0.41	13.6	10.3		30.0	1.3	2.23	13.6	12.9	
Half-cys	6.9	0.3	0.24	2.7	3.7		7.4	0.2	0.30	2.3	4.6	
Val	23.8	0.9	0.11	6.7	16.1		28.4	1.0	0.14	8.6	18.7	
Met	5.5	0.3	0.06	2.8	2.3		7.5	0.2	0.08	2.6	4.6	
Ile	20.9	0.8	0.08	4.7	15.3		24.8	0.8	0.10	5.5	18.4	
Leu	44.8	1.0	0.17	10.5	33.1		50.9	1.0	0.22	11.1	38.6	
Tyr	8.3	0.4	0.06	3.3	4.5		10.1	0.4	0.09	3.9	5.7	
Phe	13.2	0.4	0.06	4.4	8.3		14.9	0.4	0.06	4.5	9.9	
Total	379.0	15.3	4.36	147.8	222.0	10.6	449.3	14.9	7.08	154.6	280.9	8.1

[1] Estimated.

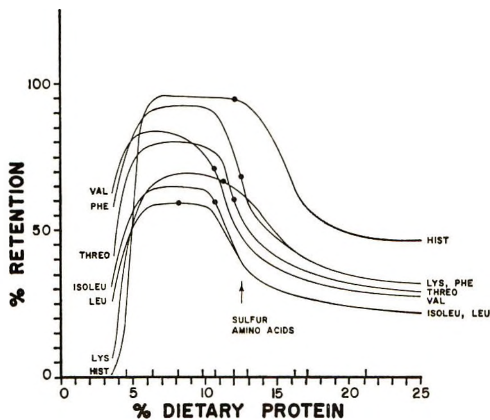

Fig. 3 Effect of dietary protein on retention efficiency of several essential amino acids. The dots on each line represent the point at which the actual intake of an amino acid satisfied the NAS-NRC recommended allowance for growth for the rat. The arrow designates this point for the sulfur amino acids. The lines are the best smooth curves drawn from the average of a six-rat pool at each level of dietary protein.

positive weight gain and nitrogen balance. All these amino acids achieved a maximum retention efficiency at about 6% dietary protein where weight gain was approximately one-half of the maximum. This level of retention efficiency was maintained over the range of 6 to 10% dietary protein and all but histidine were declining in retention efficiency at the plane of 12% protein where weight gain was maximum. The two most limiting amino acids shown in this figure, histidine and phenylalanine, exhibited the highest retention efficiencies, whereas the least limiting, leucine, showed the minimum percentage retention. The remaining amino acids in this figure were grouped in the region between the extremes. A similar pattern was seen for the nonessential amino acids shown in figure 4 in that retention efficiencies were low at the lowest protein intakes, increased to a maximum between 6 and 8% dietary protein and then declined. Proline and cysteine showed a slight deviation from this pattern in that they reached their maximum retention efficiencies at a higher level of protein intake than the others of this group.

Mole percentages for each amino acid at each level of protein were calculated for the carcass gain and compared with the amino acid composition of the whole carcass of the zero day controls. These data are summarized in table 4. The values for alanine, valine, phenylalanine and methionine from both the 3.6 and 4.5% protein groups exceeded the mean + 2 SD of the remaining 10 dietary groups. Glycine was also elevated by greater than two standard deviations in the rats fed 3.6% protein,

TABLE 4

Mole percentage of amino acids in carcass gain as affected by dietary protein (lactalbumin)

Amino acid	Zero day controls	3.6% protein	4.5% protein	Ten groups fed 6.3 to 25% protein
	mole % whole carcass	*mole % of carcass gain*		
Gly	14.1	18.0	15.7	14.6 ± 0.6 [1]
Ala	8.5	13.6	11.0	9.3 ± 0.3
Val	4.7	10.1	7.4	5.3 ± 0.7
Met	0.6	7.3	4.0	1.9 ± 0.6
Phe	3.2	4.4	3.6	3.1 ± 0.2
Asp	8.1	8.6	8.8	8.1 ± 0.3
Thr	4.6	5.2	5.1	4.6 ± 0.2
Ser	6.2	6.4	6.0	5.9 ± 0.3
Glu	12.0	11.0	12.5	11.6 ± 0.4
Ile	3.6	4.1	4.3	3.6 ± 0.4
Leu	7.4	7.4	8.0	7.4 ± 0.4
Lys	6.4	0.9	4.6	7.2 ± 0.7
Hist	2.0	0.0	0.5	2.4 ± 0.4
Arg	5.4	1.5	4.9	5.5 ± 0.6
Pro	8.3	1.6	4.6	6.2 ± 0.5
Half-cys	2.4	0.0	0.2	1.2 ± 0.4
Tyr	2.5	1.3	2.3	2.2 ± 0.2

[1] Average ± 2 SD.

but not in the animals on the 4.5% protein diet. The relative concentrations of lysine, histidine, proline and cysteine, on the other hand, were reduced to a comparable extent in the carcass gain from rats fed both the lower protein intakes, and arginine and tyrosine were also depressed below the mean − 2 SD at the 3.6% level of protein. In no instance did the carcass composition of the zero day controls fall outside the range of ± 2 SD.

DISCUSSION

Dietary amino acids may encounter several possible fates in the growing animal. Though the majority may be incorporated into body proteins, a fraction will be metabolized to carbon dioxide, water and urea; nonessential amino acids; or nitrogenous derivatives such as creatine, histamine, and nucleic acids. If the dominant factor controlling the utilization of dietary amino acids is the pressure for protein synthesis, then the retention efficiency of each amino acid (millimoles in body gain × 100 ÷ millimoles consumed) should be inversely related to the extent to which the dietary supply, including both initial content and availability, approached the minimal requirement of that amino acid. Other metabolic pressures, however, might cause an alteration in this expected pattern.

The majority of the earlier publications on retention efficiency, as noted in the introduction, have been concerned only with one or two amino acids rather than the whole array. An exception to this is the study of Price et al. (7). Using chicks as the test animal, they compared the retention efficiency of the essential amino acids from a chick starter ration (21.9% protein principally from vegetable sources) with that of a ration high in animal protein sources but containing only 16.3% total protein. The latter diet provided leucine, phenylalanine, methionine, tryptophan and arginine in quantities that ranged from 9 to 32% less than the NRC recommended allowance. Contrary to expectations, only arginine was found to have a higher retention efficiency than the essential amino acids present in presumably adequate amounts. The authors calculated catabolism by subtracting the sum of the percentages retained and excreted from 100 and found this to range from 36 to 58% of the amount ingested. They concluded that factors other than needs for synthesis of body protein influenced retention efficiency, and also that the overall process of protein synthesis was fairly inefficient.

The data from this study suggest that some modification of their conclusion may be necessary. With a well-balanced protein source (lactalbumin), the rat retained essential amino acids provided in suboptimal amounts with an efficiency that ranged from 55 to 90% as long as the dietary protein was sufficient to allow at least 50% of maximum growth. In general, the more limiting amino acids were retained with the greatest efficiency. We interpret this result to mean that with this particular diet, the demand for protein synthesis is the quantitatively dominant pressure on the fate of the majority of the ingested amino acids. Two exceptions were noted. One was the response of lysine, isoleucine and valine. The marginal requirement of these three amino acids was met by the diets containing about 11% protein. The maximum retention efficiency of valine, however, was 87% compared with only 70% for lysine and 65% for isoleucine.

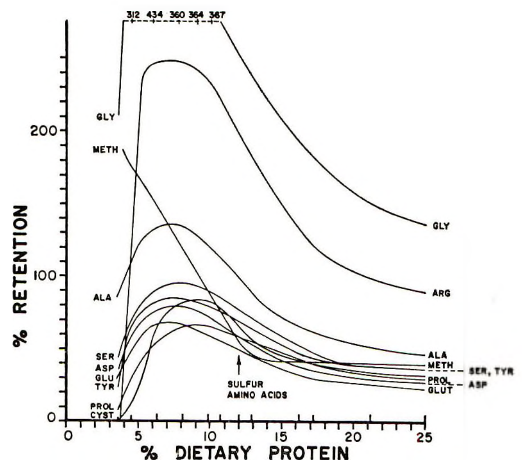

Fig. 4 Effect of dietary protein on retention efficiency of nonessential and sulfur amino acids. The arrow indicates the point at which the actual intake of sulfur amino acids satisfied the NAS-NRC recommended allowance for growth for the rat. The lines are the best smooth curves drawn from the average of a six-rat pool at each level of dietary protein.

King (11) found that when rats were fed diets containing 60 to 90% pure essential amino acids, lysine was a major contributor of nitrogen for purposes other than protein synthesis. A similar function might be in effect in this instance. An alternative suggestion is that the NRC estimated requirement for lysine and isoleucine is in error on the high side. Bressani and Mertz (12), for example, have proposed that 0.7% rather than 0.9% is the proper value for the lysine requirement of the rat. The second major departure from the anticipated pattern was the apparent net synthesis of methionine at very low levels of dietary protein. Conversion of cysteine to methionine is not believed to occur to any significant degree in animal tissues. In the absence of obvious analytical errors, however, the possibility should be entertained that under the proper conditions this can occur to a limited extent.

With moderately low protein intakes, the overall efficiency of the conversion of dietary protein to body protein was striking. Between 6 and 10% dietary protein, only 17 to 20% of the total ingested amino acid, was unrecovered and presumed catabolized, contrasted to an apparent catabolism of 36 to 58% of the essential amino acids as reported by Price et al. With diets providing only 3.6 or 4.5% total protein, however, retention efficiency declined markedly to show an apparent catabolism of about 50% of the ingested quantity.

At a dietary protein level of 6% and above, the amino acid composition of the carcass gain was very similar to the composition of the total carcass of the weanling controls. At the two lowest dietary protein levels, the carcass molar ratios of glycine, alanine, valine, methionine and phenylalanine were increased at the expense of lysine, histidine, arginine, proline, tyrosine and cysteine. Since individual proteins do not vary in their amino acid pattern, this must reflect an alteration in the proportions of body proteins. The data of this study do not allow a conclusion as to the precise changes that are taking place under these conditions. It is clear, however, that when rats are fed a reasonably well-balanced amino acid pattern at a level of total protein which results in a reduction in growth of more than 50%, the resultant animal shows a severe disruption of his usual carcass amino acid pattern. The long-term consequences of this deprivation may be profound.

ACKNOWLEDGMENTS

The authors are deeply appreciative of the efforts of Mrs. Blanche Hall, Miss B. C. Wu, and Mrs. Zella Poe in the analyses of amino acids and nitrogen and in the compilation of the data.

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Amino Acid Retention and Balance In the Young Rat Fed Varying Levels of Casein [1]

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ABSTRACT Amino acid balance and retention efficiency were determined in rats as a function of the level of dietary protein (casein). Twelve diets were prepared ranging in total protein from 4.8 to 39.1%. It was anticipated that the most limiting amino acids would show the greatest retention efficiency as long as protein synthesis was the dominant influence on utilization. The expected pattern was observed for the first three limiting amino acids but not valine and isoleucine. Protein efficiency ratio (PER) and individual amino acid retention efficiencies declined rapidly below 8% dietary protein despite continued weight gain. The decline in PER was accompanied by a change in the relative proportions of several amino acids in the carcass gain. It was proposed that the latter change resulted from a shift in the rate of protein synthesis favoring proteins essential for maintenance of life at the expense of more expandable proteins such as hair or muscle. The data are essentially in agreement with a previous study employing lactalbumin as the protein source and presenting a better balanced array of amino acids.

In a previous paper (1), we presented data describing metabolic effects on individual amino acid retention and balance by varying the dietary protein level over a range spanning both deficient and moderately excessive intakes. The protein source employed was high purity lactalbumin, a highly digestible protein providing an excellent amino acid pattern. Maximum carcass retention efficiency for the majority of essential amino acids was achieved at about 6 to 8% protein, and maximum growth occurred at 12 to 14% protein. The purpose of this paper is to compare those results with the data obtained in a study of similar design in which a protein with a less favorable amino acid pattern (casein) was employed.

EXPERIMENTAL

Twelve isocaloric diets ranging in protein content from 4.8 to 39.1% were prepared. The adjustments in level of protein were made at the expense of sucrose, all other dietary components being held equal. The composition of the 39.1% protein diet is given in table 1.

Male weanling rats of the Sprague-Dawley strain [3] were used as the experimental animal. Thirteen groups of six animals each were employed. The mean initial weights were approximately equal. One group was killed immediately by ether inhalation to serve as the zero time control. The remaining animals were housed in individual metabolism cages for 3 weeks while being offered the various diets for ad libitum consumption. Urine samples were preserved from bacterial contamination by addition of toluene. Urine and feces were collected daily and stored at − 2° in individual jars until the end of the balance period.

At the conclusion of the balance period, all animals were fasted overnight and then killed. The intact carcasses were frozen and stored at − 2° until hydrolysis could be accomplished. The hydrolysis procedure consisted of cutting the carcasses from all rats in each diet group into about 6 to 10 pieces while frozen and transferring them to a large round-bottom flask which contained sufficient 6 N HCl to maintain a ratio of approximately 100 volumes/g dry matter in order to minimize humin formation. The mixture was heated with a mantle under reflux for exactly 24 hours, and nitrogen gas was bubbled

Received for publication March 6, 1969.

[1] Supported in part by National Dairy Council Grant no. 71-C and National Institutes of Health Grant no. AM 06881.

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[3] Obtained from Dublin Animal Laboratories, Dublin, Va.

TABLE 1
Composition of diet

	g/kg
Casein [1]	490
Sucrose	400
Salt mixture [2]	50
Vitamin mixture [3]	10
Crisco [4]	25
Cottonseed oil [5]	25

[1] Donated by Sheffield.
[2] USP XVI, Official Methods of Analysis, ed. 9 1960 Association of Official Agricultural Chemists, Washington, D. C.
[3] In grams per kilogram: Ca pantothenate, 2.0; niacin, 1.0; inositol, 1.0; thiamine·HCl, 0.5; menadione, 0.4; riboflavin, 0.3; pyridoxine·HCl, 0.3; folic acid, 0.02; biotin, 0.01; vitamin B_{12}, 0.001; and sucrose, 994.5. After mixing dietary ingredients, 5 ml of 20% aqueous choline chloride was added per kilogram of diet.
[4] Procter and Gamble, Cincinnati, Ohio.
[5] Six drops of percomorph oil added to each 100 g of cottonseed oil.

through the solution throughout the hydrolysis to minimize oxidative losses.

This treatment yielded a dark brown aqueous solution topped by a layer of lipid. Despite the precautions taken some humin formation was evident. The hydrolysate was allowed to cool to room temperature and was then transferred to a volumetric cylinder for determination of the volume of the aqueous portion. Aliquots of the nonlipid segment of the hydrolysate were taken for duplicate analysis both of total nitrogen by micro-Kjeldahl procedure and for amino acid composition with an amino acid analyzer [4] using the method of Spackman et al. (2). The amino acid values were corrected to zero time hydrolysis by application of factors derived from kinetic studies of 24, 48 and 72 hours on control samples. A further correction was made by employment of norleucine and guanidinopropionic acid as internal standards. No attempt was made to analyze for tryptophan which was totally destroyed by the acid hydrolysis or for the nitrogen or amino acid content of the lipid fraction of the mixture. The individual fecal collections from each dietary group were combined and treated in a similar fashion as were the urine specimens. The feces and urine from the few animals which died during the course of the balance study were discarded.

RESULTS

The data for weight gain, feed efficiency, and PER are presented graphically in figure 1, and for carcass, urinary and fecal nitrogen and carcass total amino acids in figure 2. Nitrogen balance data are summarized in table 2. Maximum weight gain and feed efficiency were achieved at about 18 to 20% dietary protein while the highest PER was obtained between 10 and 12% protein. Our PER values of 3.5 to 4.0 for casein at this level of protein intake are higher than the usually reported values of 2.5 to 3.0. Part of this difference may be the shorter time interval (3 weeks compared with 4 weeks), but we have consistently obtained PER values after 4 weeks in excess of 3.0 from weanling rats obtained commercially [5] and fed casein diets.

The fecal nitrogen values were quite low, ranging from 5 mmoles/rat per 3 weeks at the lowest protein level to 35 mmoles/rat per 3 weeks with the highest protein intake. The almost identical range obtained for fecal N (8 to 32) in the previous study with lactalbumin suggests that there were no major differences in digestibility between the two proteins. Thus, differences in the fate of the ingested amino acids can be regarded as resulting solely from differences in the amino acid pattern offered the rats. A difference in urinary nitrogen levels was noted in that there was an increased loss of nitrogen in the animals fed casein beginning at 12 to 14% dietary protein when growth was only about one-half to two-thirds maximum. With lactalbumin, urinary nitrogen did not show a marked increase until after maximum growth had been obtained. These data indicate that the casein amino acids were not used as efficiently as those of the more complete protein, lactalbumin. The ratio of millimoles of carcass amino acid to millimoles of carcass total nitrogen was constant, and therefore, apparently independent of the levels of dietary protein employed in this study.

The amino acid balance data for each of the 12 diets are presented in table 3. Apparent net catabolism or anabolism was calculated by comparing the total ingested amino acid with the total recovery from the feces, urine and net increase in carcass (carcass gain). The millimoles of fecal

[4] Beckman model 120, Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif.
[5] See footnote 3.

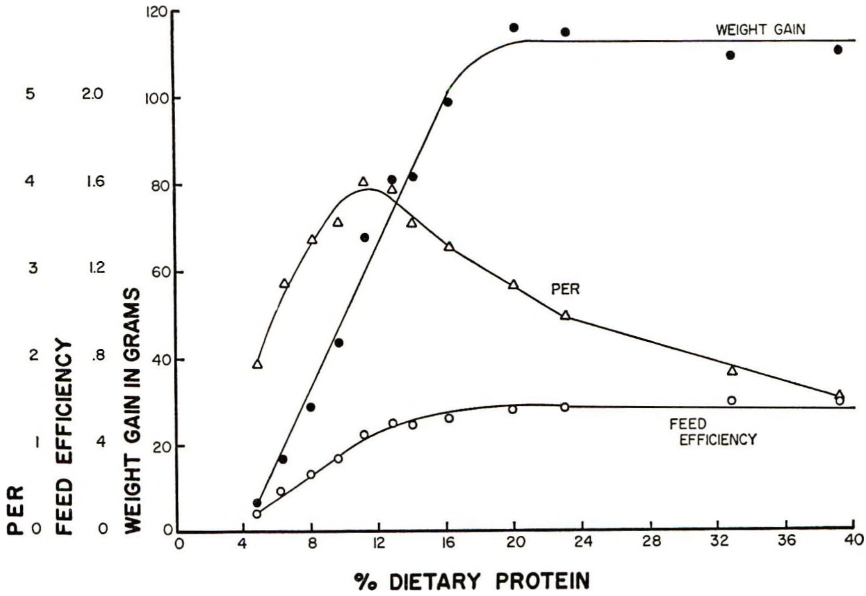

Fig. 1 Basic growth data as affected by level of dietary protein. Each point represents the average of a six-rat pool.

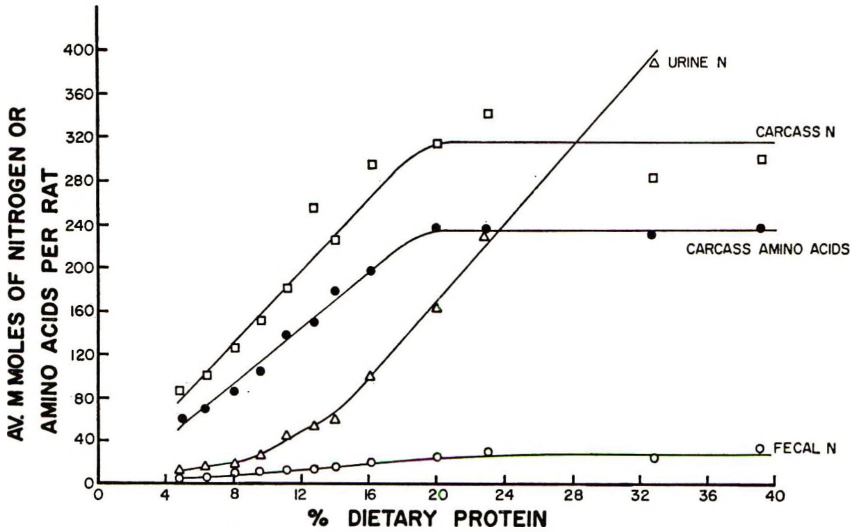

Fig. 2 Effect of dietary protein on total nitrogen of urine, feces and carcass and on carcass total amino acids. Each point represents the average of a six-rat pool.

amino acids increased from 2.8 to a maximum of about 18 with increasing dietary protein, compared with a range of 4 to 15 with lactalbumin. The mole percentage distribution of each amino acid remained constant. Urinary loss of intact amino acids was also meager and did not exceed 8.6 mmoles/rat per 3 weeks. As with the feces, the mole percentage distribution remained constant.

In the previous study with lactalbumin, about 50% of the total ingested amino

TABLE 2

Nitrogen balance

Diet no.	1	2	3	4	5	6	7	8	9	10	11	12
Dietary protein, %	4.8	6.4	8.0	9.6	11.2	12.8	14.0	16.1	20.0	23.0	32.8	39.1
	avg mmole N/rat per 3 weeks											
Carcass gain	26	41	65	90	120	194	159	228	247	275	217	232
Feces	5	7	9	11	13	14	16	20	26	30	25	35
Urine	12	16	18	26	44	54	60	100	164	229	392	497
Total	43	64	92	127	177	262	235	348	437	534	634	764
Consumed	47	65	91	125	196	243	263	343	464	524	674	823
Recovery, %	92	98	101	102	90	108	90	101	95	102	94	93

acids were apparently catabolized at the level of 3.6% dietary protein where growth was less than 50% of maximum. Between 6 and 10% dietary protein, however, only 17 to 20% of the total dietary amino acids were unrecovered and presumably metabolized. In the current study, apparent catabolic loss was also in the order of 50 to 55% when the availability of dietary protein restricted growth to below one-half the maximum. Catabolic loss dropped only to 38 to 45%, however, as protein intake was increased over the range of 10 to 16%. That this was mostly wasted is shown not only by the previously cited data on urinary nitrogen, but also by the fact that apparent net synthesis of amino acids was 18 to 21 mmoles/rat per 3 weeks in these animals, and 17 to 30 mmoles/rat per 3 weeks in rats showing comparable growth rates in the lactalbumin study (6 to 12% dietary protein).

Carcass retention efficiency was calculated for each amino acid from the data of table 3. Retention efficiency is equal to the quotient times 100 of the average content of amino acid of the carcass gain divided by the average amount of that amino acid consumed by the rats during the test period (3 weeks). Carcass gain is determined by correcting the carcass values at the end of the test period for the amount of each amino acid present in the rats killed at the beginning of the study. Figure 3 shows the best smooth lines for a plot of retention efficiency for each of the essential amino acids except sulfur amino acids and arginine as a function of percentage dietary protein. The dots on each line represent the point at which the intake of an amino acid satisfied the National Academy of Sciences — National Research Council (NAS–NRC) recommended allowance for the rat. The values for the nonessential amino acids and methionine, half-cystine and arginine are shown in figure 4.

Among the amino acids in figure 3 (those not synthesized to any appreciable extent), maximum retention efficiency was obtained with threonine and lysine, the two most limiting amino acids in this figure. The remaining five amino acids, however, did not show equal retention efficiency despite the fact that the minimum requirement of each was satisfied at approximately the same level of dietary protein. Specifically, the maximum retention efficiency obtained by either valine or isoleucine was of the order of 35 to 40% whereas leucine, phenylalanine and histidine were retained with maximum efficiencies which ranged from 50 to 53%. The nonessential amino acids (fig. 4) were retained with maximum efficiency between 8 and 14% dietary protein whether they were present in the diet in excess or deficit with respect to carcass amino acid composition.

Mole percentages for each amino acid at each level of protein were calculated for the carcass gain and compared with the amino acid composition of the whole carcass of the zero day controls. These data are summarized in table 4. The values for the whole carcass at day zero were within ± 2 SD from the mean of the carcass gain values of the groups fed the 10 highest levels of protein with the sole exception of methionine. When the pattern

TABLE 3

Balance of amino acids as affected by dietary protein level (casein)

Amino acid	Intake	Feces	Urine	Carcass gain	Net catab-olism	Net syn-thesis	Intake	Feces	Urine	Carcass gain	Net catab-olism	Net syn-thesis
	avg mmoles of amino acid/rat per 3 weeks											
	4.8% protein						6.4% protein					
Lys	2.4	0.16	0.02	0.64	1.6		3.6	0.2	0.03	1.6	1.8	
His	0.8	0.04	0.02	0.26	0.5		1.3	0.1	0.02	0.5	0.7	
Arg	0.6	0.06	0.01	0.48	0.1		1.1	0.1	0.01	1.1		0.1
Asp	2.1	0.25	0.04	1.09	0.7		3.2	0.3	0.07	2.0	0.8	
Thr	1.5	0.17	0.02	0.50	0.8		2.1	0.2	0.03	1.0	0.9	
Ser	2.1	0.34	0.03	0.85	0.9		3.0	0.5	0.04	1.3	1.2	
Glu	6.2	0.45	0.11	1.64	4.0		9.3	0.7	0.16	2.2	6.3	
Pro	4.2	0.20	0.06	1.00	2.9		6.7	0.2	0.08	1.6	4.8	
Gly	1.0	0.22	0.17	2.52		1.9	1.4	0.3	0.24	3.6		2.7
Ala	1.4	0.22	0.15	1.40		0.4	2.0	0.3	0.53	2.3		1.1
Half-cys	0.1	0.02	—	0.11			0.1	—	—	0.2		0.1
Val	2.2	0.18	0.02	0.62	1.4		3.3	0.2	0.03	1.1	1.9	
Met	0.7	0.02	0.01	0.26	0.4		0.7	—	0.01	0.4	0.3	
Ile	1.7	0.16	0.01	0.42	1.1		2.5	0.2	0.02	0.8	1.5	
Leu	2.7	0.18	0.02	0.93	1.6		4.2	0.2	0.02	1.6	2.3	
Tyr	0.9	0.06	0.01	0.21	0.6		1.5	0.1	0.01	0.5	0.9	
Phe	1.1	0.09	0.01	0.40	0.6		1.8	0.1	0.01	0.7	1.0	
Totals	31.7	2.81	0.71	13.33	17.2	2.3	47.8	3.7	1.31	22.5	24.4	4.0
	8.0% protein						9.6% protein					
Lys	5.2	0.3	0.05	3.0	1.9		7.4	0.3	0.06	4.3	2.7	
His	1.8	0.1	0.02	0.9	0.8		2.8	0.1	0.03	1.4	1.3	
Arg	1.6	0.1	0.02	2.0		0.6	2.6	0.2	0.02	3.0		0.6
Asp	4.7	0.5	0.09	3.1	1.1		6.8	0.6	0.12	5.0	1.1	
Thr	3.2	0.3	0.05	1.6	1.3		4.5	0.4	0.06	2.7	1.4	
Ser	4.6	0.7	0.07	2.2	1.7		6.8	0.9	0.08	3.5	2.3	
Glu	13.4	0.9	0.22	4.5	7.8		19.7	1.2	0.28	6.7	11.5	
Pro	9.6	0.3	0.10	3.2	6.0		14.6	0.5	0.12	3.8	10.2	
Gly	2.0	0.4	0.27	6.2		4.9	3.0	0.5	0.33	8.8		6.6
Ala	2.9	0.4	0.80	3.6		1.9	4.1	0.5	0.86	5.4		2.7
Half-cys	0.1	0.1	—	0.2			0.1	—	—	0.3		0.2
Val	5.0	0.3	0.04	1.9	2.7		7.2	0.5	0.05	2.6	4.1	
Met	1.3	—	0.01	0.6	0.7		2.0	—	0.01	0.9	1.1	
Ile	3.6	0.3	0.03	1.3	2.0		5.2	0.4	0.04	1.9	2.8	
Leu	6.0	0.3	0.04	2.8	2.9		8.8	0.4	0.06	4.1	4.2	
Tyr	2.0	0.1	0.03	0.7	1.1		3.1	0.1	0.02	1.0	2.0	
Phe	2.6	0.1	0.03	1.1	1.4		3.7	0.2	0.05	1.7	1.8	
Totals	69.6	5.2	1.87	38.9	31.4	7.4	102.4	6.8	2.19	57.1	46.5	10.1
	11.6% protein						12.8% protein					
Lys	10.1	0.4	0.07	6.0	3.7		13.0	0.4	0.08	6.8	5.7	
His	3.6	0.1	0.03	1.9	1.6		4.6	0.1	0.04	2.4	2.0	
Arg	3.3	0.2	0.03	4.0		0.9	3.8	0.2	0.04	5.3		1.7
Asp	8.7	0.7	0.14	8.0		0.1	10.9	0.7	0.18	9.1	0.8	
Thr	5.9	0.4	0.08	4.1	1.3		6.7	0.4	0.10	4.9	1.3	
Ser	8.4	1.2	0.11	5.5	1.6		9.4	1.0	0.11	6.3	2.0	
Glu	25.7	1.4	0.35	11.1	12.8		32.3	1.4	0.40	12.2	18.3	
Pro	18.6	0.5	0.17	6.0	12.0		23.2	0.5	0.18	6.5	16.1	
Gly	3.9	0.5	0.43	14.5		11.6	4.8	0.5	0.48	15.2		11.4
Ala	5.4	0.60	0.75	8.7		4.7	6.7	0.6	0.84	9.7		4.4
Half-cys	0.3	—	—	0.9		0.6	0.3	0.1	—	1.2		1.0
Val	9.8	0.6	0.07	4.0	5.2		11.8	0.5	0.07	4.8		
Met	2.6	—	0.01	1.5	1.1		2.9	—	0.01	1.7	1.2	
Ile	6.9	0.5	0.04	3.0	3.4		8.6	0.5	0.05	3.5	4.6	
Leu	11.7	0.5	0.07	6.6	4.6		14.6	0.5	0.08	7.6	6.5	
Tyr	4.1	0.2	0.03	1.8	2.1		4.8	0.2	0.04	2.6	2.0	
Phe	5.0	0.2	0.06	3.0	1.8		6.1	0.2	0.06	3.2	2.6	
Totals	134.0	8.0	2.44	90.6	51.2	17.9	164.5	7.8	2.76	103.0	63.1	18.5

TABLE 3 (*Continued*)

Balance of amino acids as affected by dietary protein level (casein)

Amino acid	Intake	Feces	Urine	Carcass gain	Net catab-olism	Net syn-thesis	Intake	Feces	Urine	Carcass gain	Net catab-olism	Net syn-thesis
	avg mmoles of amino acid/rat per 3 weeks											
	14.0% protein						16.1% protein					
Lys	13.9	0.5	0.20	8.2	5.0		18.3	0.5	0.19	9.6	8.0	
His	5.3	0.2	0.07	2.3	2.8		6.9	0.2	0.07	2.9	3.8	
Arg	5.1	0.2	0.06	5.5		0.7	6.7	0.3	0.07	6.8		0.4
Asp	13.7	1.1	0.31	10.0	2.3		17.2	1.0	0.36	12.2	3.6	
Thr	9.4	0.8	0.20	5.6	2.8		12.0	0.6	0.22	6.6	4.6	
Ser	13.2	1.7	0.27	7.8	3.4		16.6	1.8	0.32	8.8	5.7	
Glu	42.6	2.2	0.78	14.9	24.7		54.6	2.3	0.88	16.1	35.3	
Pro	29.2	0.9	0.37	9.8	18.1		34.4	0.7	0.44	9.9	23.4	
Gly	6.4	1.0	0.63	18.5		13.7	8.0	0.9	0.73	21.1		14.7
Ala	8.9	1.0	0.98	11.4		4.5	11.2	1.7	1.52	13.1		5.1
Half-cys	0.3	0.1	—	1.1		0.9	0.6	—	—	1.7		1.1
Val	14.4	0.8	0.18	5.7	7.7		17.6	0.8	0.22	6.6	10.0	
Met	4.0	0.1	0.04	1.7	2.2		4.9	0.1	0.04	1.9	2.9	
Ile	11.1	0.8	0.13	3.9	6.3		14.0	0.8	0.16	4.6	8.5	
Leu	18.5	0.8	0.20	8.8	8.7		23.2	0.7	0.24	10.4	11.9	
Tyr	7.1	0.3	0.10	2.6	4.1		8.2	0.2	0.08	3.2	4.7	
Phe	8.1	0.4	0.09	3.3	4.4		10.3	0.3	0.10	4.3	5.6	
Totals	211.2	12.9	4.61	121.1	92.5	19.8	264.7	12.9	5.64	139.8	128.0	21.3
	20.0% protein						23.0% protein					
Lys	21.9	0.8	0.20	11.4	9.5		27.7	0.7	0.25	12.1	14.6	
His	8.3	0.2	0.08	3.3	4.7		10.5	0.2	0.09	3.5	6.7	
Arg	8.1	0.4	0.06	8.1		0.4	10.3	0.3	0.09	8.6	1.3	
Asp	21.5	1.4	0.35	15.4	4.3		26.3	1.3	0.41	14.8	9.8	
Thr	15.6	1.0	0.26	8.3	6.0		18.3	0.9	0.30	8.0	9.1	
Ser	21.0	2.1	0.32	11.7	6.7		25.5	2.2	0.40	11.3	11.7	
Glu	65.0	3.0	0.85	20.7	40.5		81.2	2.9	1.01	21.7	55.6	
Pro	48.0	1.0	0.48	14.6	31.9		55.3	1.0	0.54	15.0	38.8	
Gly	10.2	1.3	0.83	29.4		21.4	12.5	1.2	0.90	26.8		16.4
Ala	14.0	2.7	0.73	16.7		6.1	17.3	2.2	0.92	16.3		2.1
Half-cys	0.8	0.2	—	2.7		2.1	0.8	—	—	2.4		1.6
Val	21.6	1.2	0.22	8.3	11.9		26.9	1.1	0.27	8.7	16.9	
Met	6.7	0.1	0.03	2.2	4.4		7.8	0.1	0.04	1.7	6.0	
Ile	17.7	1.0	0.16	5.2	11.3		21.1	1.0	0.19	5.8	14.1	
Leu	29.0	1.0	0.24	12.9	14.8		36.0	1.0	0.29	13.0	21.7	
Tyr	10.8	0.6	0.06	3.9	6.3		13.1	0.4	0.08	3.8	8.9	
Phe	13.6	0.4	0.13	5.0	8.0		15.5	0.4	0.15	4.9	10.1	
Totals	333.8	18.4	5.00	179.8	160.3	30.0	406.1	16.9	5.93	178.4	225.3	20.07
	32.8% protein						39.1% protein					
Lys	36.0	0.6	0.26	11.3	23.8		43.2	0.8	0.33	9.3	32.8	
His	13.1	0.2	0.09	3.0	9.8		17.3	0.2	0.12	3.1	13.9	
Arg	13.3	0.2	0.10	7.7	5.3		17.3	0.4	0.09	8.4	8.5	
Asp	32.6	1.2	0.48	14.2	16.7		43.4	1.5	0.62	14.1	27.2	
Thr	21.8	0.9	0.35	7.7	12.9		30.0	1.0	0.52	7.7	20.7	
Ser	31.0	1.8	0.45	11.0	17.8		44.0	2.3	0.62	11.2	29.9	
Glu	110.3	2.5	1.10	21.7	85.0		144.1	3.2	1.52	22.1	117.3	
Pro	63.0	0.8	0.61	14.8	46.8		87.0	1.2	1.09	15.8	68.9	
Gly	14.6	1.0	1.00	28.4		15.8	19.3	1.3	1.14	28.4		11.6
Ala	20.7	1.4	0.69	14.5	4.1		27.6	1.9	0.84	16.5	8.4	
Half-cys	1.1	0.1	0.04	2.5		1.6	1.3	—	0.04	2.8		1.5
Val	32.7	1.0	0.31	8.6	22.8		42.5	1.2	0.44	9.6	31.3	
Met	9.9	0.1	0.02	2.2	7.6		12.9	0.1	0.08	2.2	10.6	
Ile	25.6	0.8	0.22	5.9	18.6		35.3	1.1	0.32	6.4	27.5	
Leu	49.0	0.8	0.38	12.7	35.1		62.2	1.1	0.48	13.3	47.3	
Tyr	16.2	0.3	0.09	3.9	11.9		22.1	0.5	0.10	3.7	18.0	
Phe	18.7	0.5	0.21	4.7	13.3		24.7	0.4	0.21	5.1	19.0	
Totals	509.6	14.2	6.40	174.8	331.5	17.4	674.2	18.2	8.56	179.7	481.3	13.1

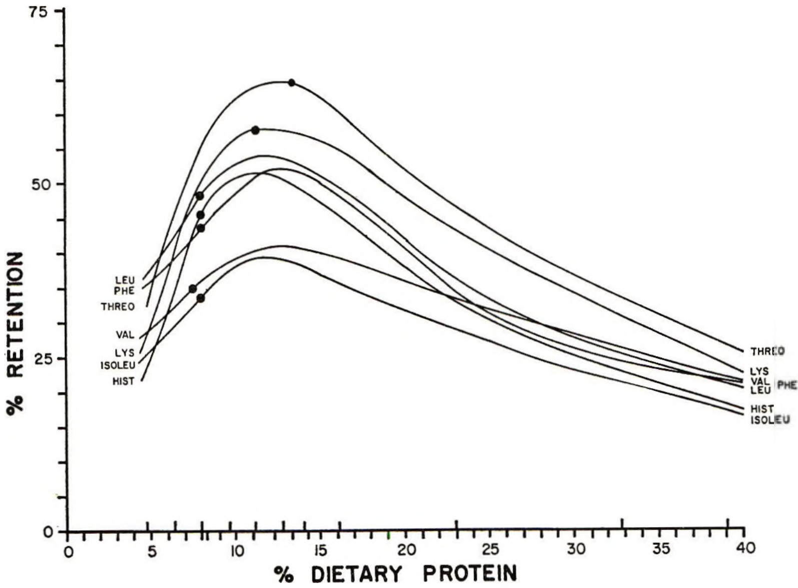

Fig. 3 Effect of dietary protein on retention efficiency of several essential amino acids. The dots on each line represent the point at which the actual intake of an amino acid satisfied the NAS–NRC recommended allowance for growth for the rat. The lines are the best smooth curves drawn from the average of a six-rat pool at each level of dietary protein.

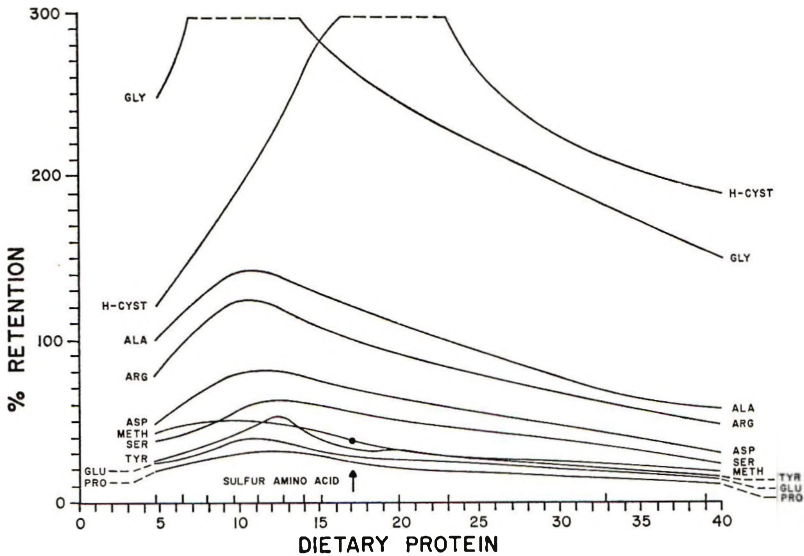

Fig. 4 Effect of dietary protein on retention efficiency of nonessential and sulfur amino acids. The dot and arrow indicate the point at which the actual intake of sulfur amino acids satisfied the NAS–NRC recommended allowance for growth for the rat. The lines are the best smooth curves drawn from the average of a six-rat pool at each level of dietary protein.

TABLE 4

Mole percentage of amino acids in carcass gain as affected by dietary protein (casein)

Amino acid	Zero day controls	4.8% protein	6.4% protein	Ten remaining groups
	mole % whole carcass	*mole % of carcass gain*		
Gly	14.6	18.9	16.0	15.6 ± 1.1 [1]
Ala	8.7	10.5	10.2	9.3 ± 0.7
Val	5.1	4.7	5.0	4.8 ± 0.5
Met	1.1	2.0	1.7	1.4 ± 0.2
Phe	3.1	3.0	3.1	2.9 ± 0.4
Asp	8.4	8.2	9.0	8.4 ± 0.7
Thr	4.6	3.7	4.4	4.5 ± 0.4
Ser	6.3	6.4	5.8	6.2 ± 0.4
Glu	12.3	12.3	10.1	12.0 ± 0.7
Ile	3.5	3.1	3.4	3.3 ± 0.3
Leu	7.2	7.0	7.2	7.3 ± 0.2
Lys	6.5	4.8	7.2	6.7 ± 1.4
His	2.0	1.9	2.3	2.0 ± 0.5
Arg	5.2	3.6	4.8	4.8 ± 0.6
Pro	6.8	7.5	7.0	7.7 ± 1.8
Half-cys	1.9	0.8	0.7	1.1 ± 0.8
Tyr	2.7	1.6	2.1	2.1 ± 0.4

[1] Average ± 2 SD.

from the carcass gain of rats consuming the lowest level of protein (4.8%) was compared with that from the 10 diets highest in protein, however, the mole percentages of glycine, alanine and methionine were elevated above the mean in excess of 2 SD at the expense of lysine, arginine, tyrosine and threonine.

DISCUSSION

In a rapidly growing animal consuming a diet containing less than optimum quantity of a well-digested protein, the dominant influence upon the essential amino acid requirement is probably the amount of each amino acid required for the synthesis of new tissue. Under these circumstances, one would expect that the most limiting amino acids would be retained most efficiently. Amino acids supplied in amounts which exceeded the ability of the animal for protein synthesis would probably be catabolized rather than retained in a free amino acid pool. Amino acids do, however, give rise to a variety of essential intermediates in addition to their incorporation into body protein. The degree to which these other metabolic pressures affect the amino acid requirement, and hence retention efficiency, has not been studied extensively.

Price et al. (3) using chicks fed a 16.3% protein ration that had deficits in leucine, phenylalanine, methionine, tryptophan and arginine which ranged from 9 to 32% less than the NRC recommended allowances. Contrary to expectations, only arginine was found to have a higher retention efficiency than the essential amino acids provided in excess of their minimum requirements. They concluded that metabolic needs other than the need for synthesis of body protein influenced retention efficiency. Bunce and King (1) using 12 diets ranging in protein (lactalbumin) content from 3.6 to 25.0% found that, in general, the more limiting amino acids were retained with the greatest efficiency. Two possible exceptions were lysine and isoleucine which were retained less efficiently than valine despite being equally limiting according to the NAS–NRC recommended allowance.

The data of this study provide additional support to the view that the quantitatively most important factor affecting the dietary requirement of an amino acid is the need for that amino acid as a component of a tissue protein. There is also evidence of some deviation from this general rule in that valine and isoleucine were not recovered from the carcass as efficiently as leucine, phenylalanine and histidine at a

level of casein consumption where all five amino acids were provided at their recommended minimum intake. Since this discrepancy cannot be accounted for by changes in body composition or in fecal or urinary loss of intact amino acid, these data suggest that some of the valine and isoleucine was converted to metabolites of an essential nature.

The pattern of dietary amino acids may be of importance as well. In the lactalbumin study (1), it was suggested that the somewhat inefficient utilization of lysine might reflect a preferential use of its nitrogen in the synthesis of nonessential amino acids. In this study, where nonessential amino acids provided a greater proportion of the dietary amino acid pattern, lysine was retained with improved efficiency. The reason for the failure of isoleucine to be retained as expected might be that its true requirement is less than that listed in the NAS–NRC table.

The effect of a total protein intake that allows only less than 50% of optimal growth deserves comment. Under these conditions weight increase does occur, but retention efficiency of all amino acids is greatly reduced and there appear to be changes in the amino acid composition of the carcass gain. This was observed with either lactalbumin or casein as the protein source although the changes with the former seemed more pronounced. These effects can most likely be attributed either to preferential synthesis of organs and enzymes essential to the maintenance of life at the expense of less essential areas such as muscle or hair, or to a shift in the proportion of collagen to other tissue components. In any case, the animal produced under these circumstances is not just a small animal but is an abnormal one which cannot make efficient use of the meager supply of amino acids which is available. These data lend support to the mounting evidence that severe protein deprivation in the young growing animal may have severe and extended sequelae.

ACKNOWLEDGMENTS

The authors are deeply appreciative of the efforts of Mrs. Blanche Hall, Miss B. C. Wu, and Mrs. Zella Poe in the analyses of amino acids and nitrogen and in the compilation of the data.

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Effect of Spontaneous Activity on Response to Intermittent Feeding [1]

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ABSTRACT In earlier studies, weanling rats fed intermittently were found to ingest less food and accumulate more body fat but less nitrogen than control animals fed ad libitum. In adult animals, only food intake differences developed. The response of animals housed in cages with attached running wheels to these feeding schedules was examined and compared with that of conventionally housed animals. When activity was allowed, intermittently fed weanling, but not adult, rats were significantly more active than control animals. The food intake and body compositional differences characteristic of conventionally housed weanling rats, did not develop. In adult animals housed in activity cages, also unlike findings with conventional housing, food intake was not modified by the feeding schedule. Intermittently fed animals housed in activity cages increased food intake significantly more during periods of unrestricted feeding than did those housed conventionally. When food intake equaled that of control animals body compositional differences did not develop in the weanling animals.

In earlier studies, weanling rats alternated between satiety and partial caloric restriction were found to differ from animals fed ad libitum in several physiologic characteristics (1, 2). During the first 60 days of feeding, experimental rats ingested less food and accumulated more fat but less protein than controls animals. Body compositional, but not intake, differences gradually disappeared after this time. In adult animals, differences in intake alone occurred.

The experiments reported here were conducted to determine whether spontaneous activity was also influenced by this feeding schedule and whether activity would, in turn, modify the intake and compositional differences previously observed.

METHODS

Male rats of the Holtzman strain [3] were maintained at 22° with 12-hour photoperiods. One group of weanling animals was housed individually in conventional cages; others were kept in similar sized cages with attached running wheels connected to a counter which registered each revolution. Weanling animals were maintained in cages with attached running wheels for 60 and 120 days. Responses of 80-day-old animals were similarly studied for a 60-day period in the activity cages.

Water was available ad libitum to all animals. The diet had the following percentage composition: dextrose, 60; casein, 21; fat,[4] 15; salt mixture, 4 (3); plus a complete vitamin supplement.[5] Control rats had food present continuously; experimental animals were alternated between 3 days of unrestricted intake and 3 days with food sufficient only to maintain body weight. Food during periods of caloric restriction was increased from 3 to 10 g/day as the animal increased in size. This amount of food was approximately 65% of ad libitum intake in both growing and adult animals.

Food intake, body weight, and, where indicated, wheel revolutions were recorded at 3-day intervals. Animals were killed with ether fumes upon completion of the

Received for publication January 13, 1969.

[1] Supported in part by Public Health Service Grant no. HD00009 from the National Institutes of Health. This work was carried out, in part, during Z. Al Nejjar's tenure as an Atomic Energy Commission Fellow and during F. W. Heggeness' tenure as National Institutes of Health Career Development Awardee no. 5K 3HB 14904.

[2] Current address: National Institutes of Nutrition, Baghdad, Iraq.

[3] Holtzman Company, Madison, Wis.

[4] Crisco, Procter and Gamble, Cincinnati, Ohio.

[5] Each kilogram of diet contained: vitamin A, 25,000 IU; vitamin D, 3000 IU; choline chloride, 2.25 g; and (in milligrams) α-tocopherol, 150; inositol, 150; riboflavin, 30; menadione, 68; p-aminobenzoic acid, 150; niacin, 130; pyridoxine·HCl, 30; thiamine·HCl, 30; Ca pantothenate, 90; biotin, 0.60; folic acid, 2.50; and vitamin B_{12}, 0.40.

feeding studies and the gut contents discarded. Carcasses were desiccated, weighed and pulverized in a laboratory blender.[6] Aliquots of the dry powder were used for analyses. Nitrogen was determined by the micro-Kjeldahl method. Fat was extracted in a Soxhlet apparatus with methanol–chloroform (1:1). Solvents were removed by evaporation and petroleum ether–chloroform (6:1) soluble fraction was weighed. Values were recorded as total amount per animal.

RESULTS

Intermittently fed weanling animals exercised slightly but significantly more than did continuously (ad libitum) fed rats (fig. 1). They accomplished 60% of their total activity during the periods of restricted feeding. The variations in the spontaneous activities of similarly treated adult animals were large and coefficients of variations were high. Activities of adults placed in activity cages at 2 months of age were not significantly modified by the feeding schedule (fig. 1). Similarly, running activities of continuously and intermittently fed animals housed in activity cages from weaning were not significantly different between days 62 and 122. Wheel revolutions over the 60-day period were 244 ± 61 × 10³ and 170 ± 50 × 10³ for intermit-

[6] Waring Products Company, Winsted, Conn.

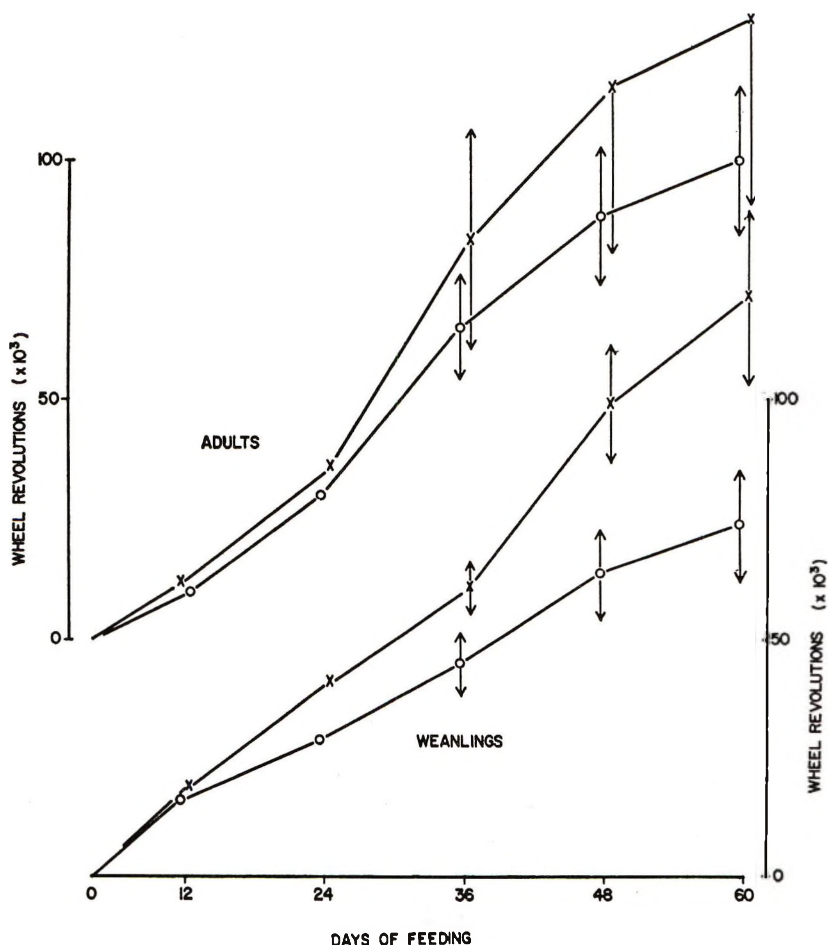

Fig. 1 Running activity (wheel revolutions (cumulative, × 10³)) in ad libitum (O) or intermittently fed (X) weanling and adult animals. Vertical arrow indicates standard error of the mean.

TABLE 1

Food intake and body composition of control and experimental animals

Treatment	Body wt	Food intake	Fat content	Nitrogen content
	g	g	g	g
A. Weanling rats, conventional housing (22 to 62 days)				
Continuously fed (6) [1]	294 ± 8 [2]	789 ± 10	36.5 ± 1.4	9.3 ± 0.3
Intermittently fed (7)	280 ± 8	680 ± 16 [3]	49.4 ± 2.5 [3]	7.9 ± 0.3 [3]
B. Weanling rats, caged with running wheel (22 to 62 days)				
Continuously fed (8)	304 ± 6	783 ± 18	31.3 ± 1.4	9.1 ± 0.3
Intermittently fed (8)	285 ± 4 [3]	745 ± 11	31.4 ± 3.3	9.0 ± 0.4
C. Weanling rats, caged with running wheel (62 to 122 days)				
Continuously fed (8)	398 ± 5	1025 ± 10 [4]	39.6 ± 3.1	11.8 ± 0.4
Intermittently fed (8)	357 ± 7 [3]	964 ± 30 [4]	37.6 ± 1.6	11.2 ± 0.3
D. Adult rats, caged with running wheel (60 days)				
Continuously fed (6)	379 ± 13	939 ± 45	36.2 ± 2.6	12.4 ± 0.2
Intermittently fed (6)	362 ± 10	872 ± 14	36.3 ± 3.8	11.5 ± 0.4

[1] Number of animals per group.
[2] Mean ± SE.
[3] Value significantly different from continuously fed (control) in that group ($P < 0.05$).
[4] Food intake between days 62 to122.

tently and continuously fed animals, respectively.

Response of conventionally housed weanling animals was the same as observed earlier (1). Namely, food intake was decreased, fat content increased, and total carcass nitrogen slightly decreased in intermittently, as compared with continuously, fed animals (table 1 A). In contrast, food intake, fat and nitrogen content of control and experimental weanling animals caged with activity wheels did not differ (table 1 B). Body weights of intermittently fed exercising animals were slightly but significantly below that of continuously fed rats similarly housed.

Food intakes of adult control and experimental animals reared from weaning in activity cages (table 1 C) or introduced to these cages at 2 months of age (table 1 D) were not different. At 122 days, body weights of intermittently fed rats maintained in activity cages from weaning were slightly but significantly lower than rats fed ad libitum, but fat and nitrogen contents were not different.

Food intake of all weanling animals permitted spontaneous exercise was the same as that of control animals that were not. Nitrogen content was unaffected by activity but body fat content was lower in all active weanling animals than in nonexercising, intermittently fed ones (table 1 A and B). Food intake of exercising adult animals was similar to that previously reported for sedentary adult rats fed ad libitum (1).

DISCUSSION

The transient alterations in body composition observed in conventionally housed weanling rats fed on a 3-day feeding schedule are similar to those observed by other investigators (4–7) in older animals force-fed or allowed access to food only briefly each day. The reported changes in body composition have been associated with alterations of thyroid function (6) and physical activity (8). Despite similar body compositional changes the feeding schedules employed in the studies reported here were different and compositional changes induced were confined to the period of rapid growth.

The responses to intermittent feeding previously observed (1) in weanling and adult rats did not develop when they were spontaneously active. Running activity did not alter the food intake or body nitrogen content of animals fed ad libitum.

In all intermittently fed animals the quantity of food fed during the periods of partial caloric restriction was approximately 65% of ad libitum intake. Adult and weanling experimental animals housed

conventionally increased intakes during the periods of unrestricted feeding to 112 ± 3%, of that ingested by control rats, an amount insufficient by 20% or more to compensate for the deficit incurred during restricted feeding. Adaptation to this feeding schedule appeared to involve a modification in satiety regulation and, as a result, total caloric intake was reduced below that which could be considered usual.

In contrast, experimental weanling and adult rats allowed spontaneous activity increased their intakes during the periods of ad libitum feeding to 129 ± 4% and 125 ± 4%, respectively, of that ingested by control animals. This occurred whether activity was modified (weanling animals) or not (adult animals) by the feeding schedule. This increased food consumption during ad libitum feeding compensated within 10% or less for the deficit acquired during caloric restriction, and as a result, the total intake of the experimental animals was about that of the control rats.

In conventionally housed weanling rats, the intermittent feeding schedule was also associated with a metabolic adaptation that favored deposition of fat but not protein (1). Certain earlier observations suggested that this involved the activity of the thyroid (2). The mechanisms involved and exact interrelations between intake and body composition remain to be established. Body composition as well as intake changes did not develop when weanling animals were permitted running activity. These findings suggest that the two adaptations are interrelated, as neither has been observed to appear singly in weanling rats. This metabolic adaptation either does not occur in the intermittently fed adult or it does not become evident; rapid growth may be necessary for its appearance.

Spontaneous activity appeared to modify the behavioral and metabolic adaptations to these feeding schedules. This, in turn, altered the intake and compositional changes that developed without running activity. The observations reported here indicate that the examination of the effect of any feeding schedule on activity must consider that running behavior can, in turn, markedly modify the adaptations elicited by the nutritional stress.

ACKNOWLEDGMENTS

The authors acknowledge the technical assistance of Kathleen Srokose and Kenneth Williams. The authors also thank Dr. E. F. Adolph for discussions.

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Preconception Irradiation of Dam and Composition of Offspring [1]

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ABSTRACT Whole-body X-irradiation of the female rat alters the ovulation response at next estrus. Number of ova shed can be predictably varied from 10 to 35 depending upon the dose and time in the estrous cycle it is administered. If bred, pregnancy is uneventful but litter size ranges from 2 to 18 pups, depending upon dose of radiation. The purpose of these experiments was to examine the effect of preconceptional irradiation and the number of pups per litter on the growth of the offspring. Fat content of newborn pups was not influenced by litter size or preconception irradiation. Total body nitrogen was 80% of control value in offspring of mothers given over 300 r with a maximal effect seen at 400 r. Nitrogen content was not related to litter size. Additional young were cross-fostered to unirradiated dams and then fed ad libitum. At 100 days nitrogen content of animals born of mothers administered up to 400 r was not different from control value, but was decreased to 90% of control value in offspring of females receiving 600 r. Growth potential appeared to be slightly but permanently modified by 600 r of preconceptional radiation.

In the adult female mouse (1), hamster,[3] and rat (2–5) whole-body irradiation alters the ovulatory response at the next estrus. In the rat, the number of ova shed can be predictably varied between about 10 and 35 depending upon the dose of irradiation and time prior to estrus that it is administered (4, 5). If such animals mate, pregnancies are uneventful but litter sizes range from 2 up to 18 pups. Offspring appear normal in size and appearance, and survival and reproductive capacities are unchanged (6). It has not been established whether litter size or preconceptional irradiation modifies other pre- and postnatal physiologic maturation processes.

The purpose of these experiments was to determine the effects of preconceptional radiation and litter size on the development of the offspring. An effect on growth, if present, could be mediated directly by radiation-induced injury, indirectly via litter size, or both. Radiation exposures were selected that would permit these factors to be dissociated. Body composition, and in particular nitrogen content, was selected as the index of growth.

METHODS

Virgin, female rats of the Long-Evans strain,[4] weighing 180 to 200 g, were housed at 22° with 12-hour photoperiods. Water and a commercial laboratory ration [5] were provided ad libitum. Estrous cycles were monitored by daily cytological examination of the vaginal lavage and, at specified times, females were subjected to whole-body irradiation. Exposures of zero to 600 r were administered at intervals of 0.5 to 4.0 days prior to the next expected estrus to establish litter sizes between 2 and 18 in the subsequent pregnancy (table 1). A sufficient number of animals were

Received for publication January 24, 1969.

[1] Supported in part by National Institutes of Health Grant no. HD00009 and a contract with the U. S. Atomic Commission at the University of Rochester Atomic Energy Project. This report has been assigned no. UR 49-1027. The work was carried out during the tenure of F. W. Heggeness under Career Development Award no. 5K 3HD14907 from the Public Health Service.

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[3] Hahn, E. W. 1968 Federation Proc., 27: 739 (abstract).

[4] Obtained from Blue Spruce Farms, Altamont, N. Y.

[5] Purina Laboratory Chow, Ralston Purina Company, St. Louis, Mo. Diet contained not less than 23% crude protein, 4.5% fat, and no more than 6% crude fiber and 9% ash.

TABLE 1

Litter size following whole-body irradiation [1]

Whole-body radiation dose	Time of irradiation	Litter size [2]
r	*days before mating*	*no. of pups*
0	3.5–4.5	10–12
200	3.5–4.5	14–16
300	0.5	1–3
400	1.5	10–12
400	3.5–4.5	17–20
600	3.5–4.5	14–16

[1] Hahn, E. W., and W. F. Ward (5).
[2] Only litters within these limits were used.

treated so that each treatment group contained 15 or more females.

Animals were irradiated in individual radial compartments of a slowly rotating plastic cage. Desired dose was delivered by an X-ray unit [6] operating at 250 kvp and 15 ma. Filtration was via an aluminum parabolic filter plus 0.5 mm Cu; the half-value layer was 2.15 mm Cu. Target-skin distance was 63.5 cm and the dose rate about 19 r/minute. Before each day's exposure, the intensity of radiation was measured with a 100 Victoreen condenser R chamber [7] and exposure time corrected for temperature and barometric pressure.

After irradiation, females were placed with fertile, unirradiated males. The appearance of a copulatory plug was considered indicative of mating and pregnancy was dated from this point. Bred animals were individually housed and food intake and weight gains were measured in 10 representative animals from each treatment.

Five controls and seven animals that had received 400 r whole-body radiation were killed on day 14 of pregnancy and mean dry weights of fetuses and placentas were determined. On day 22, all remaining young carried to term were delivered by caesarian section. Only litters with the number of offspring within the limits outlined in table 1 were used. After weighing, sexing, and surgically coding to identify maternal treatment, randomly selected male offspring were cross-fostered to unirradiated females 25 hours or less postpartum. Remaining young were killed for analysis. Each foster dam was provided with a litter of eight young with no more than four pups from any one natural mother. Foster mothers received the commercial laboratory ration [8] and water ad libitum. Pups were weaned at 21 days of age and fed the maternal diet ad libitum until killed at 100 days of age. Body weights were recorded weekly; food intake was not measured.

Twenty newborns of both sexes were randomly selected from each group for analysis. Individual carcasses were lyophilized to constant weight and used intact for determination of either fat or nitrogen content. One hundred day-old animals were killed with ether fumes. After removal of gut contents, carcasses were lyophilized until dry; they were weighed and then pulverized in a blender [9] with acetone. Acetone was not discarded but removed by evaporation, and aliquots of the dry powder were used for analysis. Nitrogen content was determined by the micro-Kjeldahl method. Fat was extracted with methanol – chloroform (1:1) in a Soxhlet apparatus. Solvent was evaporated and the petroleum ether–chloroform (6:1) soluble fraction determined gravimetrically. Values were recorded as total fat and nitrogen content per animal.

A second group of virgin females of the same age and strain were exposed to zero, 200, 400, or 600 r at 4 $\pm$ 0.5 days prior to estrus. Young were delivered as before and only the newborn males in each litter were pooled for analysis for dry weight and nitrogen content. Values were recorded as the mean content per male pup per litter. No offspring of this second group were reared.

Fetal growth was examined in offspring of one group of females irradiated with 400 r one month before breeding. At term, offspring were delivered by caesarian section. Males from each litter were pooled, and body weight, dry solids and nitrogen were determined.

The effect of irradiation on the nitrogen content of the uterus during pseudopreg-

[6] Picker Industrial X-ray, Picker Nuclear, White Plains, N. Y.
[7] Victoreen Instrument Division, Cleveland, Ohio.
[8] See footnote 5.
[9] Waring Products Company, Winsted, Conn.

nancy with and without deciduomata reaction was also determined. Pseudopregnancy was induced by mechanical stimulation of the cervix during estrus and animals immediately exposed to zero, 200, 400, or 600 r of whole-body irradiation. On day 4 postirradiation, a group of animals was anesthetized with ether and the endometrium of both uterine horns traumatized with a blunt needle. On day 8 postirradiation all animals were killed and the entire uterus removed and analyzed for nitrogen.

Statistical evaluation employed was the *t* test for small samples of unequal size (7).

RESULTS

Following mating, no deaths occurred in animals receiving zero, 200, or 300 r, but mortality in females receiving 400 or 600 r was 5 and 10%, respectively. Whereas 95% of the bred animals administered zero or 200 r were pregnant at term, 15% of the surviving mated females that had been exposed to 300 to 600 r were not.

Animals that did not conceive showed a modest weight gain (table 2). Food intake of animals receiving 300 r and having 2 ± 1 (mean ± SE) offspring was not significantly increased above that of nonpregnant animals. Animals with litter sizes between 10 and 18 had significantly larger food intakes than nonpregnant animals, but no relationship to size of litter was observed.

Fetal and placental weights 14 days after conception were not altered by 400 r preconceptional irradiation. Fetal dry weights averaged 17.0 ± 1.2 mg in control animals and 16.6 ± 1.3 mg in irradiated animals. Placental dry weights were 26.1 ± 1.4 and 26.0 ± 2.3 mg in control and experimental animals, respectively.

At 22 days, body fat content of offspring was unaffected by litter size or by irradiation of mother (fig. 1). Total carcass nitrogen was decreased in newborns from mothers that were exposed to 300 r or more of whole-body irradiation. Exposure to 400 r enhanced this effect but 600 r produced no further reduction (fig. 1).

In trial 2 (table 3), dry weight and nitrogen content of male offspring showed the same relationships to maternal irradiation as was observed in group 1 animals. Although the water content (body weight minus dry weight) of fetuses from females irradiated 30 days prior to mating was significantly greater than in controls, dry weight and nitrogen content were not.

Uterine nitrogen content in pseudopregnancy without deciduomatal reaction was decreased after 600 r whole-body irradiation. During pseudopregnancy, with stimulation to form decidua, exposures of 200 and 400 r reduced deciduomata responses. Deciduomata were induced in less than 10% of the animals receiving 600 r; the value was not statistically reliable and was not included (table 4).

Postnatal growth of male offspring of group 1 control and irradiated animals is shown in figure 2. No treatment significantly affected the rate of total body weight gain. Carcass nitrogen content,

TABLE 2

Food intake and weight gain during pregnancy

Treatment dose [1]	Litter size	Food intake	Body wt gain
		g	g
Not pregnant	—	356 ± 13 [2]	40 ± 4
Control	10–12	412 ± 13 [3]	120 ± 7 [3]
200 r	14–16	418 ± 13 [3]	123 ± 8 [3]
300 r	1–3	368 ± 11	103 ± 9 [3]
400 r	10–12	417 ± 12 [3]	133 ± 8 [3]
400 r	17–20	409 ± 13 [3]	142 ± 7 [3]
600 r	14–16	395 ± 12 [3]	103 ± 7 [3]

[1] Ten animals per group.
[2] Value ± SE.
[3] Value significantly different from that for nonpregnant animals ($P < 0.05$).

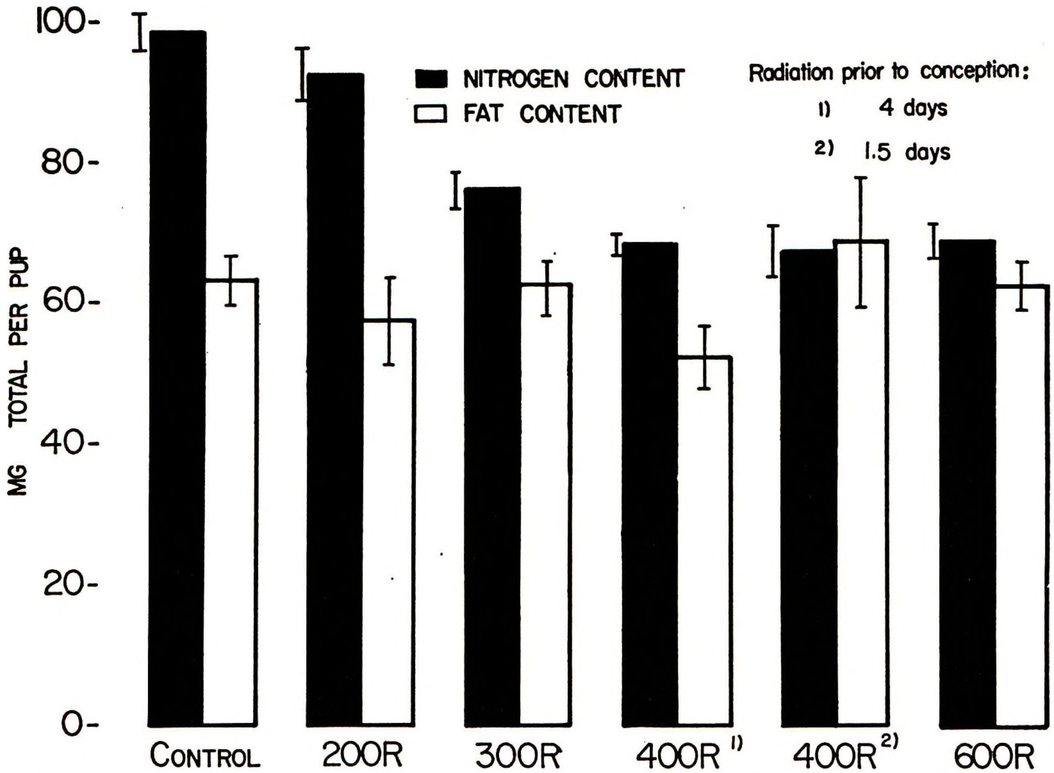

Fig. 1 Nitrogen and fat content of offspring of unirradiated and X-irradiated females. Vertical bars indicate standard error of the mean.

TABLE 3

Body weight and nitrogen content of male offspring of control and irradiated animals

Treatment [1]	Control	200 r [2]	400 r [2]	600 r [2]	400 r [3]
Litter size, no. of pups	10–12	14–16	17–20	14–16	10–12
Fetus wt, g	4.85 ± 0.17 [4]	4.68 ± 0.10	4.48 ± 0.18	4.65 ± 0.07	5.58 ± 0.06 [5]
Fetus dry wt, g	0.753 ± 0.037	0.737 ± 0.024	0.624 ± 0.032 [6]	0.630 ± 0.019 [6]	0.761 ± 0.038
Total fetus nitrogen, mg	84.2 ± 4.1	76.2 ± 2.0	68.0 ± 3.5 [6]	68.5 ± 0.8 [6]	90.9 ± 1.8 [7]

[1] Nine litters per group.
[2] Administered 4 days prior to conception.
[3] Administered 30 days prior to conception.
[4] Mean ± SE.
[5] Value significantly different from control ($P < 0.01$).
[6] Value significantly different from control ($P < 0.05$).
[7] Value significantly different from that for 400 and 600 r animals ($P < 0.01$).

body fat and dry weight at 100 days were slightly but significantly reduced only in young born of females given 600 r. Fat content of the offspring of mothers receiving 200 r was significantly greater than that of controls (table 5).

DISCUSSION

Irradiation prior to mating altered the nitrogen content of the conceptus in two groups of animals. Whether the nitrogen deficit was general, or localized to specific sites or organ systems of fetus, was not

TABLE 4

Nitrogen content of the uterus in pseudopregnancy following irradiation with and without deciduomata

Treatment [1]	Control	200 r	400 r	600 r
	mg	*mg*	*mg*	*mg*
Pseudopregnancy [2]	9.30 ± 0.67 [3]	9.84 ± 0.09	7.52 ± 0.72	6.27 ± 0.18 [5]
Pseudopregnancy with deciduomata [2,4]	38.4 ± 2.7	29.0 ± 2.5 [5]	23.4 ± 4.4 [5]	—

[1] Eight animals per group.
[2] Determined 8 days after irradiation.
[3] Mean ± SE.
[4] Uterus traumatized on day 5 following irradiation.
[5] Value significantly lower than control ($P < 0.05$).

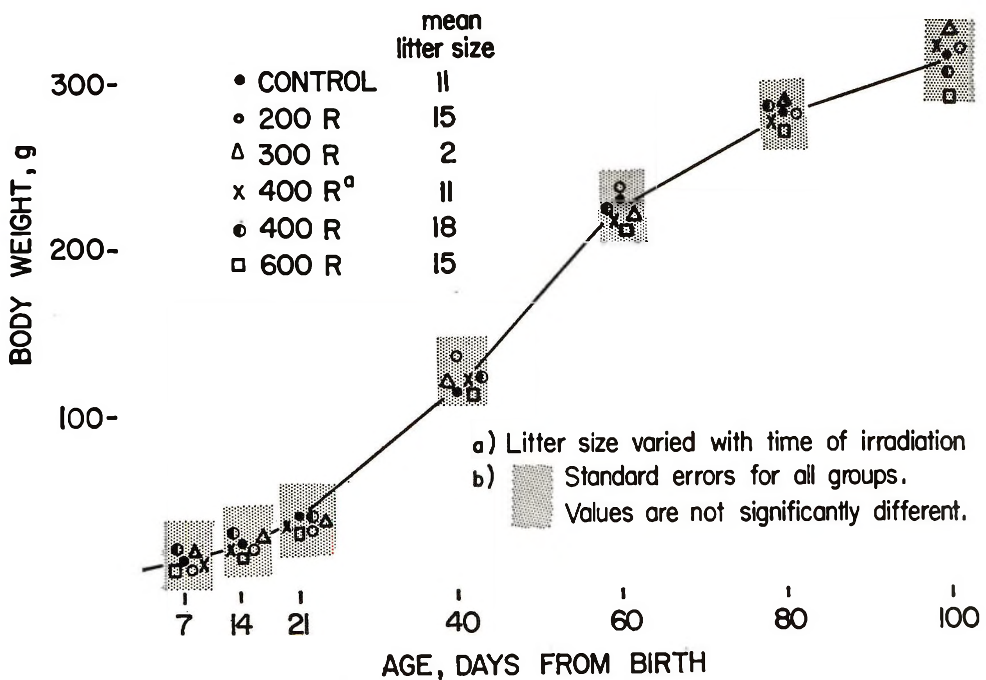

Fig. 2 Postnatal growth of male pups from unirradiated and X-irradiated females.

TABLE 5

Body composition of male offspring at 100 days of age

Preconception whole-body irradiation [1]	Dry wt	Body fat	Body nitrogen
	g	*g*	*g*
Control	116.9 ± 2.0 [2]	34.4 ± 1.9	10.77 ± 0.08
200 r	126.7 ± 3.2	48.3 ± 3.4 [3]	10.28 ± 0.39
300 r	112.8 ± 4.7	36.1 ± 3.3	10.13 ± 0.28
400 r [4]	121.9 ± 3.6	41.1 ± 2.7	10.24 ± 0.23
400 r [5]	123.6 ± 2.3	36.3 ± 2.0	10.90 ± 0.34
600 r	102.9 ± 2.1 [3]	28.0 ± 1.0 [3]	9.83 ± 0.14 [3]

[1] Ten animals per group.
[2] Value ± SE.
[3] Value significantly different from control ($P < 0.05$).
[4] Irradiation 1.5 days prior to conception.
[5] Irradiation 4 days prior to conception.

determined. Differences in body composition were not present in the 14-day-old fetuses but may be undetectable at this time.

Radiation per se, rather than the size of the litter, appeared to be the factor responsible for the reduction in fetal nitrogen accumulation. Radiation doses of 400 r given at times that yielded litters of either 10 or 18 exerted the same effect on fetal nitrogen content. In addition, control animals receiving zero and those administered 400 r 2 days prior to estrus, and those receiving 200 and 600 r had comparable litter sizes (table 1), but nitrogen contents of newborns were related to the radiation dose administered to the mother and not to litter size. A threshold for this effect on fetal growth appeared to exist above 200 r; at exposures of 400 r, the effect was maximal.

Preconceptional irradiation stunting of fetal growth could be produced via genetic changes in the oocyte, modification of maternal environment, or both. Investigations by others provide support for all of these possibilities. Maternal irradiation induced morphological changes in the oocyte (3, 8–11). The appearance of micronuclei, assumed to represent chromosomal fragmentation, supports the contention that genetic damage, sometimes leading to lethal mutations, results. Inhibition of mitosis is known to follow irradiation (11, 12), and uterine responses to pseudopregnancy have been shown by data presented here and by work of others [10] (13) to be depressed. In the hamster, changes in the oviducts and uterus persist several months following irradiation (14). Transplantation of mammalian ova revealed both oocyte damage (15–17) and maternal dysfunction [11] (18, 19) as contributing factors in the embryonal mortality associated with irradiation.

The data presented here, plus that of others (14) of the effect of irradiation on reproductive structures, suggests that maternal damage must be a major factor in fetal stunting.

The nitrogen content of offspring of animals conceiving 30 days after irradiation was not different from that of controls. The effect of irradiation on fetal nitrogen accumulation persisted for less than 4 weeks. The increased water content (total body weight − dry weight) (table 3) of these offspring may reflect a different and more permanent change induced by irradiation.

The observation that body solids, nitrogen and fat content at 100 days are slightly but significantly lower in offspring of animals that received 600 r suggests that this dosage of irradiation may permanently affect the growth potential of the embryo. The LD_{50} for rats under experimental conditions employed is about 750 r and 600 r approaches this value. As all animals employed in the postnatal growth study were fostered by unirradiated controls, this effect is probably attributable to the effects of irradiation of the natural mother prior to conception. It would be of interest to determine whether stunting induced by preconception radiation is similar to that produced by other means (20, 21).

In the rat, unlike other species (22–24), an inverse relationship between litter size and birth weight is uncertain (25), but Huggett and Widdas (26) found no significant relation between these two factors. The experiments reported here indicate that litter size as modified by irradiation does not influence fat accumulation by the fetus. When litter size was modified by preconceptional irradiation, nitrogen accretion also appeared to be independent of the number of offspring present in the uterus.

Fetal growth is influenced by many genetic and environmental factors. Among others, maternal size, parity and nutrition have all been shown to influence birth weight (27). These studies show that fetal growth can even be interfered with by an environmental factor applied prior to conception itself. It is of special interest that at lower doses the fetal stunting is reversible, above this it is not, at least up to 100 days after birth. Species specificity, time that irradiation effect persists, recovery time postnatally, and effect of additional environmental factors on irradiation-induced fetal stunting remain to be established.

[10] Ketchel, M. M., M. Wilson and E. Babas 1968 personal communication.

[11] Iaschenko, Z. F. 1961 Nucl. Sci. Abstr., *15*: 2521.

ACKNOWLEDGMENTS

The authors thank Edward Moore for animal care, Kathleen Srokose, Barbara General and Kenneth Williams for technical assistance, and Florence Van Slyke for supervision of irradiation. The authors express their appreciation to Dr. E. F. Adolph for discussions and reading of the manuscript.

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Hepatic ATP and Triglyceride Levels in Choline-deficient Rats with and without Dietary Orotic Acid Supplementation [1]

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ABSTRACT Hepatic ATP depletion is probably important in the pathogenesis of the experimental fatty livers produced by orotic acid and several other agents that inhibit hepatic lipoprotein production. Because choline deficiency also impairs lipoprotein production, we have investigated the role of ATP depletion in this condition. Rats fed a choline-deficient diet for 5 days had no change in hepatic ATP despite a 30-fold increase in triglycerides. Furthermore, adenine sulfate, an ATP precursor, failed to influence the degree of lipid accumulation when added to the diet. In contrast, adding 1% orotic acid to a choline-supplemented diet for 5 days produced a 50% fall in ATP along with a sevenfold increase in hepatic triglycerides, and 0.25% dietary adenine sulfate completely prevented both these effects. A previously reported protective effect of orotic acid in choline deficiency was confirmed by the fact that choline-deficient rats fed orotic acid had about 50% lower hepatic triglyceride levels than those not given this agent. Despite this, orotic acid lowered ATP in choline-deficient animals equally as well as in choline-supplemented animals. These results indicate that unlike the lipid accumulation induced by orotic acid, the fatty liver of choline deficiency is not related to hepatic ATP depletion. The paradoxical protective effect of orotic acid in choline deficiency appears to be independent of its influence on ATP.

Low hepatic adenosine triphosphate (ATP) levels occur in the experimental fatty livers produced by a variety of agents, including orotic acid (1, 2), ethionine (3, 4), CCl_4 (4, 5), azaserine (4), phosphorus (6) and L-tryptophan (7). After orotic acid or ethionine, the drop in ATP precedes triglyceride accumulation (2, 3), implying a pathogenetic relationship. Furthermore, giving either ATP or its precursor, adenine sulfate, prevents not only the fall in ATP (1–4) but the fatty liver as well (3, 4, 8, 9). With the other agents it has not been established if low hepatic ATP precedes fat accumulation or merely accompanies it. The fatty livers produced by orotic acid, ethionine, CCl_4 and phosphorus are also each associated with defective formation or release of lipoproteins by the liver (9–14). Thus, the common link in these conditions could be a lack of high energy phosphate associated with the depletion of ATP, resulting in impaired lipoprotein production.

Current evidence indicates that the fatty liver of choline deficiency also is due to impaired lipoprotein synthesis or release (15–17), but the possible role of ATP depletion in its pathogenesis is not known. Therefore, we have measured the changes in hepatic ATP and triglyceride levels produced by feeding a choline-deficient diet, and have assessed the influence of adenine sulfate on these changes. In addition, because orotic acid has recently been shown to paradoxically diminish the accumulation of hepatic fat in choline-deficient animals (18, 19), we have also studied the relationship between this effect of orotic acid and its ability to deplete ATP.

MATERIALS AND METHODS

The choline-deficient diet consisted of 6% vitamin-free casein, 25% alcohol-extracted peanut meal, 43% sucrose, 20% lard, 5% salt mix and 1% vitamin mix. Details of its composition have previously been published (20).

We used eight experimental diets, as follows: *CD diet*, choline-deficient diet alone; *CS diet*, CD diet supplemented with

Received for publication February 13, 1969.
[1] Supported by National Institutes of Health Research Grant no. AM 05966-06 and Training Grant no. AM 05180-09.

0.5% choline chloride; *CD + Ad diet,* CD diet supplemented with 0.25% adenine sulfate; *CS + Ad diet,* CS diet supplemented with 0.25% adenine sulfate; *CD + OA diet,* CD diet supplemented with 1% orotic acid; *CS + OA diet,* CS diet supplemented with 1% orotic acid; *CD + OA + Ad diet,* CD diet supplemented with both 1% orotic acid and 0.25% adenine sulfate; and *CS + OA + Ad diet,* CS diet supplemented with both 1% orotic acid and 0.25% adenine sulfate.

Male rats of the Sprague-Dawley strain,[2] weighing 38 to 50 g, were fed a laboratory ration [3] for 3 days before being placed on the experimental diets. They were housed individually and allowed food and water ad libitum. Body weight and food intake of each animal were recorded. All rats were killed after being fed the experimental diets for 5 days; earlier experiments have shown that if longer periods are used hepatic triglyceride levels may be influenced by the development of acute renal necrosis (20).

We first measured hepatic ATP levels in animals fed the CD diet; rats given the CS diet and others fed the laboratory ration served as controls. To study the influence of orotic acid and adenine sulfate on ATP, other groups of animals were fed the CD + OA, CD + OA + Ad, CS + OA and CS + OA + Ad diets, respectively. Livers were prepared for ATP assay by a modification of the method of Hyams et al. (4). The animals were decapitated and about 0.6 to 0.8 g of liver was removed; it was weighed and homogenized for 30 seconds with 5 ml ice-cold 0.1 M NaF in a microblender.[4] The homogenate was then transferred into approximately 30 ml boiling water. Total time from removal of the liver to transfer of the homogenate was about 75 seconds. The mixture was boiled for 20 minutes, cooled, and brought to a volume in milliliters equal to 50 times the original weight of the liver specimen in grams. After centrifugation, the supernatant solution was assayed for ATP using the luciferin-luciferase method (21). In preliminary experiments this technique yielded approximately 95% recovery of known amounts of ATP. Duplicates usually agreed within 3%.

Other animals were used to study the effects of adenine sulfate and orotic acid on the triglyceride accumulation of choline deficiency. Weight-matched groups of rats were each fed one of the eight experimental diets for 5 days. The animals were decapitated and their livers homogenized with distilled water (1:3, w/v) in a microblender.[5] Duplicate aliquots of the fresh homogenate were assayed for triglycerides by the method of Butler et al. (22). Results were compared with those obtained in animals fed the laboratory ration and killed at the beginning of the experimental period.

The statistical significance of differences between groups was calculated by the paired *t* test (23).

RESULTS

As indicated in table 1, the choline-deficient diet had no effect on hepatic ATP concentration after 5 days of feeding (CD group). In contrast, 1% dietary orotic acid produced a marked fall in ATP in both choline-supplemented (CS + OA group) and choline-deficient (CD + OA group) rats. Equivalent decreases of about 50% occurred in both these groups of animals, indicating that choline deficiency did not influence the ability of orotic acid to depress ATP concentration. Dietary supplementation with 0.25% adenine sulfate completely prevented this effect of orotic acid, both in animals that were not cho-

TABLE 1

Effect of choline deficiency, orotic acid and adenine sulfate on hepatic ATP levels after 5 days of feeding

Dietary group [1]	Hepatic ATP [2]
	μmoles/g wet wt liver
Laboratory chow	1.228 ± 0.144
CS	1.306 ± 0.070
CD	1.235 ± 0.051
CS + OA	0.612 ± 0.076 [3]
CD + OA	0.642 ± 0.105 [4]
CS + OA + Ad	1.115 ± 0.152
CD + OA + Ad	1.265 ± 0.071

[1] See Materials and Methods for explanation. Each group consisted of six animals.
[2] Mean ± SD.
[3] $P < 0.001$ compared with CS group.
[4] $P < 0.001$ compared with CD group.

[2] Charles River Breeding Laboratories, Wilmington, Mass.
[3] Purina Laboratory Chow, Ralston Purina Company, St. Louis, Mo.
[4] Waring Blendor, Waring Products Company, Winsted, Conn.
[5] See footnote 4.

line-deficient (CS + OA + Ad group) and in those that were (CD + OA + Ad group).

Table 2 summarizes the hepatic triglyceride concentrations. Food intake and weight gain over the 5-day period were similar in each group of animals, agreeing within 10%. Rats on the choline-supplemented diet (CS group) maintained hepatic triglycerides at the same level as controls fed laboratory ration. Choline deficiency (CD group) caused about a 30-fold increase in triglycerides, and the degree of lipid accumulation was not influenced by adenine sulfate (CD + Ad group). On the other hand, orotic acid (CS + OA group) produced only a sevenfold increase in triglyceride levels, but this was completely prevented by adenine sulfate (CS + OA + Ad group). When orotic acid was added to the choline-deficient diet (CD + OA group), triglycerides rose to only about one-half the level produced by choline deficiency alone; this effect of orotic acid was partially nullified by adenine sulfate (CD + OA + Ad group).

DISCUSSION

This study indicates that unlike orotic acid-induced fatty liver, the lipid accumulation of choline deficiency is not associated with ATP depletion. Orotic acid produced a low hepatic ATP level and an increase in hepatic triglycerides; dietary adenine sulfate prevented both these effects, confirming previous observations (1, 2, 8, 9). In contrast, 5 days of choline deficiency had no effect on hepatic ATP concentration despite the fact that it produced a much greater increase in triglycerides than orotic acid, nor did adenine sulfate influence the degree of lipid accumulation. In the only previous study on this point, Dianzani (6) found a modest decrease in hepatic ATP after 1 week of choline deficiency and a greater decrease after 5 to 6 weeks. ATP depletion is clearly not of primary pathogenetic importance in choline deficiency, however, since it does not *precede* an increase in triglycerides.

This finding is somewhat unexpected since deficiency of ATP accompanies several other experimental fatty livers due to impaired lipoprotein formation or release. Low ATP levels occur in the fatty livers produced by ethionine (3, 4), CCl_4 (4, 5) and phosphorus (6) as well as orotic acid, suggesting that depletion of high energy phosphate may be responsible for the defective lipoprotein production in each of these conditions. This possibility is strengthened by the fact that, at least in the case of orotic acid and ethionine, ATP depletion occurs before triglycerides begin to accumulate (2, 3), and giving ATP or adenine sulfate prevents the fatty infiltration as well as the fall in ATP (1–4, 8, 9).

Production of lipoproteins by the liver is a complex process, however, involving synthesis of the protein moiety (apoprotein); coupling of apoprotein with triglyc-

TABLE 2

Effect of choline deficiency, orotic acid and adenine sulfate on hepatic triglyceride levels after 5 days of feeding

Dietary group [1]	Food intake	Body wt		Hepatic triglycerides [2]
		Initial	Gain	
	g	*g*	*g*	*µmoles/g wet wt liver*
Base-line chow	—	56	—	7.3 ± 1.0
CS	49	57	25	7.4 ± 1.7
CS+Ad	47	56	26	11.5 ± 4.3
CD	48	56	26	210.1 ± 49.3 [3]
CD+Ad	48	57	24	211.7 ± 34.5
CS+OA	47	55	27	49.9 ± 29.2 [4]
CS+OA+Ad	45	56	26	7.1 ± 1.5
CD+OA	47	55	25	101.6 ± 14.2 [5]
CD+OA+Ad	45	55	26	159.2 ± 43.7 [6]

[1] See Materials and Methods for explanation. Each group consisted of six animals.
[2] Mean ± SD.
[3] $P < 0.001$ compared with CS group.
[4] $P < 0.01$ compared with CS group.
[5] $P < 0.001$ compared with CD group
[6] $P < 0.05$ compared with CD + OA group, but not significantly different from CD group.

erides, phospholipids and cholesterol; and transfer of the assembled lipoprotein into plasma. Little is known about how this process is accomplished, and it is possible that ATP is necessary at some stages but not at others. If so, then choline deficiency might impair a step not requiring high energy phosphate. Choline deficiency, moreover, probably curtails phospholipid synthesis (24), which could prevent lipoprotein production by causing general injury to phospholipid-containing organelles as well as by impairing a specific step in lipoprotein formation. Lombardi and Oler (16) have recently found that choline-deficient rats injected with ^{14}C-leucine have a low specific activity of not only their plasma lipoproteins but also of albumin and total nonlipoprotein proteins, indicating that the defect in choline deficiency may indeed not specifically involve lipoprotein production.

The present study also suggests that the beneficial influence of orotic acid on the fatty liver of choline deficiency is not due to its effect on hepatic ATP. A specific interrelationship between orotic acid and choline was not suspected until Porta et al. (18) recently discovered that orotic acid paradoxically decreased hepatic lipid accumulation in choline-deficient rats. In the present study 5 days of orotic acid feeding diminished hepatic triglycerides in choline-deficient animals by approximately 50%, even though it increased triglycerides sevenfold in choline-supplemented rats; yet in both groups of animals orotic acid caused equivalent decreases in hepatic ATP. Moreover, in choline-deficient rats fed orotic acid, adenine sulfate prevented the fall in ATP even though triglyceride levels actually increased. These effects of orotic acid cannot be due to a "pseudolipotropic" artifact from reduced calorie intake (25) because the weight gain and food intake of our various experimental groups were similar. The data suggest that the protective effect of orotic acid in choline deficiency is unrelated to its influence on ATP, and also provide further evidence that lipid accumulation in choline deficiency is not due to ATP depletion. Orotic acid may somehow spare the total body choline requirement rather than have a specific action on the liver, since it also protects against the kidney lesion of choline deficiency (19).

ACKNOWLEDGMENTS

The authors thank Dr. Nicholas Alexander for helpful advice in the methodology of ATP measurement, and Miss Rose Moquin for technical assistance.

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Dietary-induced Depletion and Repletion of Avian Liver Xanthine Dehydrogenase [1,2]

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ABSTRACT Changes in body weight, liver weight, liver nitrogen, liver xanthine dehydrogenase (XDH) and plasma uric acid levels were studied in chicks previously fed a diet containing 25% (control) or 75% isolated soybean protein, and subsequently starved or fed a protein-free diet from 1 to 4 days. The influence of realimentation of the two diets following periods of starvation or the feeding of the protein-free diet was also studied. A close relationship between the increases in XDH activity and plasma uric acid was observed during adaptation of chicks to the high protein diet. A 70% increase in total liver XDH activity was observed 12 hours after the initiation of the high protein diet. The increase in liver XDH activity was essentially linear the first 2 days, at which time a maximal response to the high level of dietary protein was observed. Body weight, liver weight and liver nitrogen decreased in control chicks from the beginning of the fasting period, whereas liver XDH activity did not begin to decrease until after 24 hours. Plasma uric acid levels gradually increased throughout the fasting period. These results were in contrast to the concomitant depressions in liver XDH activity and plasma uric acid observed during the first 24 hours of the fast in chicks adapted to the high protein diet. Both groups of chicks exhibited increased liver weights, and decreased liver nitrogen, XDH activity and plasma uric acid following the consumption of the protein-free diet from 1 to 4 days. A larger increase in XDH activity was noted in chicks fed the protein-free diet than in fasted chicks following the realimentation of the high protein diet, whereas the increases were of a similar magnitude when the control diet was refed. The different metabolic influences exerted by the consumption of a protein-free diet and by starvation on the parameters studied are discussed.

The effect of alterations in dietary protein intake for limited periods on avian liver xanthine dehydrogenase (XDH) activity has been previously reported (1). Feeding a diet devoid of protein for 24 hours resulted in a significant reduction in total liver xanthine dehydrogenase activity. Feeding a diet containing 75% isolated soybean protein greatly enhanced total liver xanthine dehydrogenase activity as compared with control chicks fed a diet containing 25% isolated soybean protein. A 24-hour fast resulted in either the maintenance or reduction of total liver xanthine dehydrogenase levels depending on the previous level of dietary protein consumed by the chicks. In addition, a direct relationship was observed between liver xanthine dehydrogenase activity measured in vitro and total uric acid excretion (2).

The results of these studies stimulated further interest in the time-course response of avian liver xanthine dehydrogenase to the effects of alterations in dietary protein intake. In the present series of experiments, chicks previously fed the control or high protein diets were starved or fed a protein-free diet from 1 to 4 days. Changes in chick weight, liver weight, liver nitrogen, liver xanthine dehydrogenase, and plasma uric acid levels were observed during this time, and also following progressive realimentation of the control or high protein diets. In addition, a detailed study is presented on the time-course changes in liver xanthine dehydrogenase activity during a 10-day adaptation to the high protein diet.

EXPERIMENTAL

Animals. In each of the experiments reported, 1-day-old White Rock cockerels were maintained in electrically heated batteries with raised wire floors and fed a diet containing 25% isolated soybean pro-

Received for publication December 5, 1968.
[1] Journal Paper no. 3541, Purdue University Agricultural Experimental Station.
[2] Supported in part by Public Health Service Research Grant no. AM-11487-01 from the National Institute of Arthritis and Metabolic Diseases, and by a National Aeronautics and Space Administration traineeship to the senior author.
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tein for 10 days. At this time, one-half the chicks were transferred to a high protein diet containing 75% isolated soybean protein for periods of 4 to 10 days before initiation of the protein-free or starvation regimens; the remaining one-half were continued with the 25% isolated soybean protein diet. Details of each experiment will be described further in the text. Deionized water was provided ad libitum to all treatment groups.

Diets. The 25% isolated soybean protein diet contained the following ingredients: (in grams per kilogram) isolated soybean protein, 250.0; glucose monohydrate, 587.39; soybean oil, 50.0; cellulose, 30.0; mineral mixture (1), 60.48; vitamin mixture (1), 15.0; DL-methionine, 5.0; choline chloride (70%), 2.0; and butylated hydroxytoluene, 0.13. The 75% isolated soybean protein diet contained three times the levels of isolated soybean protein and DL-methionine used in the 25% isolated soybean protein diet added at the expense of glucose monohydrate. The protein-free diet was identical to the 25% isolated soybean protein diet with the exception that all the protein and DL-methionine were replaced by glucose monohydrate. The protein content (N × 6.25) of the 25 and 75% isolated soybean protein diets was 21.0 and 63.9%, respectively.

Xanthine dehydrogenase assay. The preparation of liver samples and spectrophotometric measurements of XDH activity have been described previously (1). It was assumed that activators or inhibitors of the enzyme were not present since the measured activity of added amounts of enzyme preparation from any two treatments whose initial activities differed greatly gave additive values.

Plasma uric acid assay. Blood was collected in heparinized syringes by cardiac puncture before killing the animals. The plasma was separated by centrifugation and uric acid levels determined by the uricase method as described in a technical bulletin.[4]

Nitrogen determinations and statistical treatment of data. Liver nitrogen was determined by the Kjeldahl method on duplicate samples of fresh liver. All data were analyzed by the analysis of variance with the Newman-Keuls test criterion serving as the basis for mean comparisons (3).

RESULTS

Trial 1. This study was designed to investigate the changes in avian liver xanthine dehydrogenase and plasma uric acid levels during a 10-day adaptation to a high protein diet. The results are illustrated in figure 1 and are in general agreement with those described previously (2). In the present study, however, more time intervals were used to characterize the early response of xanthine dehydrogenase to a high level of dietary protein intake. Day zero of the experiment refers to 10-day-old chicks fed the control diet containing 25% isolated soybean protein. At this time, one-half the chicks were changed from the control diet to the high protein diet. Liver xanthine dehydrogenase activity and plasma uric acid levels were determined from six chicks fed either the high protein or control diets on each of the days indicated in figure 1. Compared with the control value at day zero, a 70% increase in total liver xanthine dehydrogenase activity was observed 12 hours after the initiation of the high protein diet. The increase in enzyme activity in response to the high level of dietary protein was linear for the

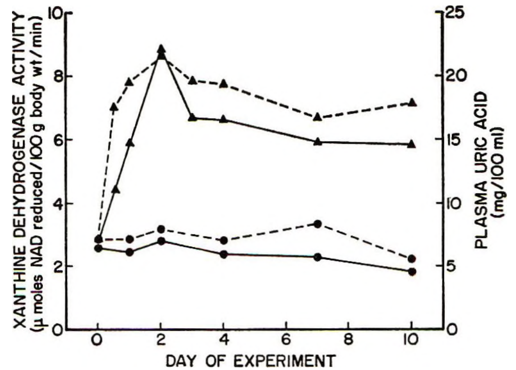

Fig. 1 Changes in liver xanthine dehydrogenase and plasma uric acid in chicks during adaptation to a high protein diet. ●——● and ●– – – –● represent enzyme and plasma uric acid values for chicks fed the control diet containing 25% isolated soybean protein, respectively. ▲——▲ and ▲– – – –▲ represent enzyme and plasma uric acid values for chicks fed the high protein diet, respectively.

[4] Sigma Chemical Company, St. Louis, Tech. Bull. no. 680, 1965.

first 2 days, at which time a maximal response was observed. Enzyme activity expressed on a body weight basis decreased at day 3 but remained at levels approximately threefold higher than those for control chicks during the remainder of the experiment.

The changes in plasma uric acid levels generally corresponded to the changes in liver xanthine dehydrogenase activity, although the increase in plasma uric acid after feeding the high protein diet for 12 hours was proportionally greater than the increase in enzyme activity during this time. The peak in plasma uric acid corresponded with the peak in liver xanthine dehydrogenase activity observed at day 2 of the experiment. As was observed for liver xanthine dehydrogenase, plasma uric acid levels did not differ appreciably throughout the experiment for chicks fed the control diet containing 25% isolated soybean protein.

Trial 2. In this study, changes in chick weight, liver weight, liver nitrogen, liver xanthine dehydrogenase, and plasma uric acid were observed in 1) chicks starved for 24 or 48 hours, 2) chicks fed a diet devoid of protein for 24 or 48 hours, 3) chicks starved or fed the protein-free diet for 48 hours and subsequently refed the high protein diet for 1, 2 or 4 days, and 4) chicks fed the protein-free diet for 48 hours and subsequently starved for 1, 2 or 4 days. The control data (treatment 1) are for chicks fed the high protein diet ad libitum for 4, 7 and 10 days. These time intervals correspond, within 1 day, to the 24, 48 and 96 "hours of treatment" listed for treatments 2 through 6, respectively. All 1-day-old chicks were fed initially a diet containing 25% isolated soybean protein for 10 days and then offered the high protein diet ad libitum for 4 days prior to starvation or feeding the protein-free diet. The results of this study are presented in tables 1 and 2.

The changes observed in liver weight and liver nitrogen (table 1) resulting from starvation or the consumption of a protein-free diet are similar to those observed previously (1). Compared with ad libitum values, significantly ($P < 0.05$) smaller relative liver weights were observed in starved chicks, whereas significantly ($P < 0.05$) larger weights were noted in chicks fed the protein-free diet. The loss of total liver nitrogen following starvation compared with that following the consumption of the protein-free diet was not statistically significant ($P > 0.05$). For the refed chicks previously starved or fed the protein-free diet for 48 hours, relative liver weight and liver nitrogen were not significantly different from the control values within 48 hours of dietary protein repletion. There was additional liver nitrogen depletion in starved chicks previously fed the protein-free diet (treatment 6).

The effects of the treatments described on liver xanthine dehydrogenase and plasma uric acid are shown in table 2. Compared with control values, starving or feeding chicks a diet devoid of protein for 1 or 2 days significantly reduced total liver xanthine dehydrogenase levels. The reduction in xanthine dehydrogenase observed for chicks fed the protein-free diet was significantly greater than that observed for the starved chicks. The response of liver xanthine dehydrogenase to dietary protein repletion was greater for chicks that were previously fed the protein-free diet (treatment 5) than for chicks that were previously starved (treatment 4). Data for the starved chicks previously fed the protein-free diet (treatment 6) indicated a further loss of xanthine dehydrogenase activity compared with chicks fed the protein-free diet for 48 hours.

The changes in plasma uric acid levels as a result of the dietary alterations in protein intake generally reflect the changes in liver xanthine dehydrogenase activity. This statement, however, is subject to certain limitations. As shown in table 2, an inverse relationship between plasma uric acid levels and enzyme activity was observed for chicks starved for 24 hours versus chicks starved for 48 hours. A similar inverse relationship was observed for chicks fed the protein-free diet for 24 and 48 hours. It is suggested that these differences represent alterations in the degree of endogenous amino acid catabolism, and that physiological levels of xanthine dehydrogenase are normally present in excess of those required from a functional standpoint. For the situations described this would be independent of activation of enzyme already present since absolute

TABLE 1

Effect of dietary protein depletion and repletion on chick weight, liver weight, and liver nitrogen (trial 2)

Treatment [1,2]	Hours of treatment	Chick wt			Liver wt	Relative liver wt	Liver N/g liver	Liver N/100 g body wt [3]
		Initial	Diet change	Killed				
		g	*g*	*g*	*g*	*g/100 g body wt*	*mg*	*mg*
1 (Controls, 75% isolated soy protein ad libitum)	Day 4 [4]	—	—	199 ± 14	8.5 ± 0.4	4.3 ± 0.2	33.2 ± 1.1	142.3 ± 5.8
	Day 7 [4]	—	—	266 ± 8	10.7 ± 0.4	4.0 ± 0.1	33.9 ± 0.3	136.3 ± 3.8
	Day 10 [4]	—	—	333 ± 8	12.3 ± 0.5	3.7 ± 0.1	34.4 ± 0.2	126.2 ± 2.6
2 (Starved groups)	24	226 ± 11	—	204 ± 10	6.3 ± 0.2	3.1 ± 0.1 *	33.9 ± 0.6	95.2 ± 1.5 *
	48	205 ± 19	—	171 ± 17	4.9 ± 0.4	2.9 ± 0.1 *	34.3 ± 1.0	84.8 ± 4.7 *
3 (Protein-free groups)	24	222 ± 12	—	219 ± 13	11.2 ± 1.0	5.1 ± 0.2 *	20.8 ± 1.2 *	103.7 ± 4.9 *
	48	216 ± 10	—	211 ± 10	10.6 ± 0.5	5.0 ± 0.2 *	18.9 ± 0.6 *	92.2 ± 2.2 *
4 (Starved groups (48 hours) refed high protein diet)	24	237 ± 13	198 ± 12	232 ± 13	9.2 ± 0.5	4.0 ± 0.1	29.9 ± 0.3 *	119.4 ± 3.1 *
	48	205 ± 8	170 ± 9	221 ± 12	8.6 ± 0.6	3.9 ± 0.1	34.2 ± 0.6	132.4 ± 3.6
	96	207 ± 6	170 ± 4	276 ± 5	10.5 ± 0.5	3.8 ± 0.1	33.8 ± 0.4	127.8 ± 4.5
5 (Protein-free group (48 hours) refed high protein diet)	24	214 ± 10	210 ± 11	222 ± 10	9.9 ± 0.7	4.4 ± 0.3	28.9 ± 0.3 *	128.0 ± 7.0 *
	48	232 ± 14	225 ± 14	278 ± 16	12.3 ± 0.7	4.5 ± 0.2	33.1 ± 0.7	147.3 ± 7.6
	96	236 ± 10	232 ± 9	314 ± 23	12.6 ± 1.1	4.0 ± 0.1	34.7 ± 0.5	138.2 ± 4.0
6 (Protein-free group (48 hours) starved)	24	229 ± 6	224 ± 8	200 ± 7	6.4 ± 0.3	3.2 ± 0.1 *	29.5 ± 0.5 *	85.0 ± 2.2 *
	48	225 ± 6	222 ± 6	187 ± 5	5.7 ± 0.3	3.1 ± 0.1 *	31.9 ± 0.5 *	82.2 ± 2.8 *
	96	234 ± 15	231 ± 16	172 ± 11	5.2 ± 0.3	3.0 ± 0.1 *	31.7 ± 0.4 *	71.5 ± 1.5 *

[1] Ten-day-old chicks previously fed a diet containing 25% isolated soybean protein were adapted to the 75% isolated soybean protein diet for 4 days prior to starvation or feeding the protein-free diet.

[2] Values represent means ± SE of six observations each.

[3] Initial body weight for treatments 2, 3 and 6; body weight at killing for treatments 1, 4 and 5.

[4] Chicks in these groups remained on ad libitum feeding of the high protein diet for 4, 7 and 10 days and were killed at time intervals corresponding, within 1 day, to the 24, 48 and 96 "hours of treatment" listed for treatments 2 through 6, respectively.

* Significantly differs from appropriate control value, $P < 0.05$.

TABLE 2

Effect of dietary protein depletion and repletion on avian liver xanthine dehydrogenase and plasma uric acid (trial 2)

Treatment [1,2]	Hours of treatment	Xanthine dehydrogenase activity [3]			Plasma uric acid
		units/g liver	*units/100 g body wt*	*units/100 mg liver N*	*mg/100 ml*
1 (Control, 75% isolated soy protein ad libitum)	Day 4 [4]	1.57 ± 0.13	6.6 ± 0.36	4.7 ± 0.33	19.3 ± 1.8
	Day 7 [4]	1.48 ± 0.08	5.9 ± 0.28	4.4 ± 0.26	16.6 ± 0.9
	Day 10 [4]	1.58 ± 0.04	5.8 ± 0.17	4.6 ± 0.10	17.8 ± 0.9
2 (Starved groups)	24	1.23 ± 0.05	3.5 ± 0.19 *	3.6 ± 0.18 *	9.7 ± 0.8 *
	48	1.15 ± 0.07	2.7 ± 0.15 *	3.2 ± 0.18 *	13.6 ± 1.2
3 (Protein-free groups)	24	0.57 ± 0.07 *	2.8 ± 0.24 *	2.7 ± 0.28 *	2.2 ± 0.2 *
	48	0.30 ± 0.01 *	1.5 ± 0.09 *	1.6 ± 0.08 *	2.7 ± 0.2 *
4 (Starved group (48 hours) refed high protein diet)	24	0.88 ± 0.08 *	3.5 ± 0.39 *	2.9 ± 0.29 *	22.8 ± 0.3
	48	1.39 ± 0.12	5.4 ± 0.51	4.0 ± 0.31	16.4 ± 1.1
	96	1.32 ± 0.07	5.0 ± 0.43	3.9 ± 0.23	15.9 ± 1.3
5 (Protein-free group (48 hours) refed high protein diet)	24	0.68 ± 0.05 *	3.0 ± 0.36 *	2.3 ± 0.19 *	14.2 ± 1.1
	48	1.36 ± 0.07	6.0 ± 0.34	4.1 ± 0.26	17.8 ± 0.5
	96	1.76 ± 0.11	7.1 ± $.58 *	5.1 ± 0.34	17.1 ± 0.8
6 (Protein-free group (48 hours) starved)	24	0.30 ± 0.03 *	0.9 ± 0.08 *	1.0 ± 0.09 *	9.0 ± 1.3 *
	48	0.29 ± 0.01 *	0.7 ± 0.05 *	0.9 ± 0.06 *	5.5 ± 0.5 *
	96	0.31 ± 0.02 *	0.7 ± 0.04 *	1.0 ± 0.05 *	5.5 ± 0.6 *

[1] Ten-day-old chicks previously fed a diet containing 25% isolated soybean protein were adapted to the 75% isolated soybean protein diet for 4 days prior to starvation or feeding the protein-free diet.
[2] Values represent means ± SE of six observations each.
[3] One unit represents enzyme activity catalyzing the reduction of 1 μmole NAD/minute at 25°.
[4] Chicks in these groups remained on ad libitum feeding of the high protein diet for 4, 7 and 10 days and were killed at time intervals corresponding, within 1 day, to the 24, 48 and 96 "hours of treatment" listed for treatments 2 through 6, respectively.
* Significantly differs from appropriate control value. $P < 0.05$.

levels of liver xanthine dehydrogenase were reduced.

Trial 3. This experiment was similar to trial 2 with the exception that chicks adapted to the control diet were studied rather than chicks adapted to the high protein diet. In this study, 10-day-old chicks that were fed the 25% isolated soybean protein diet were also 1) starved or fed a diet devoid of protein for 1 to 4 days, and 2) refed the control diet for 1 to 6 days following 2 days of starvation or protein-free intake. The results of this study are shown in tables 3 and 4. Treatment means were compared with the control values for the same day of the experiment.

As was shown previously in trial 2 with chicks adapted to a high protein diet, starving chicks that were originally fed a diet containing 25% isolated soybean protein resulted in a significant decrease in relative liver weight (table 3). The decrease in liver nitrogen on a body weight basis was proportionally not as great, however, as that observed in trial 2. Compared with control values, feeding a protein-free diet generally resulted in an increase in relative liver weight and a decrease in total liver nitrogen. Chicks that were starved for 48 hours and then refed the control diet exhibited increases in relative liver weights after 24 hours which were double those of chicks that had been starved for 48 hours. This represented an absolute increase of approximately 9 g.

The response of liver xanthine dehydrogenase and plasma uric acid to the treatments described is presented in table 4. Unlike the effect of a 24-hour starvation period in chicks previously adapted to the high protein diet (table 2), in which case total xanthine dehydrogenase levels were decreased significantly, absolute levels of this enzyme were not changed during a

TABLE 3

Body weight, liver weight, and liver nitrogen changes in chicks following periods of dietary protein depletion and repletion (trial 3)

Treatment [1,2]	Day of exp.	Chick wt			Liver wt	Relative liver wt	Liver N/g liver	Liver N/100 g body wt [3]
		Initial	Diet change	Killed				
		g	*g*	*g*	*g*	*g/100 g body wt*	*mg*	*mg*
1 (Controls, 25%	0	—	—	163 ± 3	7.2 ± 0.17	4.4 ± 0.2	25.4 ± 0.7	112.0 ± 4.6
isolated soybean	1	—	—	176 ± 2	7.9 ± 0.26	4.5 ± 0.1	26.0 ± 0.4	117.1 ± 3.7
protein ad libitum)	2	—	—	223 ± 7	10.3 ± 0.64	4.6 ± 0.2	26.0 ± 0.6	120.2 ± 4.1
	3	—	—	227 ± 11	9.7 ± 0.67	4.3 ± 0.2	26.8 ± 0.8	113.7 ± 4.4
	4	—	—	268 ± 10	10.7 ± 1.27	4.0 ± 0.3	27.1 ± 1.4	106.6 ± 4.5
	6	—	—	296 ± 20	12.4 ± 0.75	4.2 ± 0.3	28.0 ± 0.4	118.5 ± 7.2
	8	—	—	295 ± 26	11.8 ± 1.50	4.0 ± 0.3	29.9 ± 0.9	119.0 ± 9.2
2 (Starved groups)	1	174 ± 3	—	157 ± 3	5.8 ± 0.28	3.7 ± 0.2 *	32.3 ± 0.6 *	106.9 ± 5.8
	2	181 ± 10	—	155 ± 9	5.2 ± 0.29	3.4 ± 0.1 *	32.9 ± 0.3 *	94.3 ± 2.8 *
	3	177 ± 6	—	142 ± 5	4.3 ± 0.23	3.0 ± 0.1 *	33.7 ± 0.3 *	82.4 ± 3.0 *
	4	163 ± 4	—	123 ± 4	3.6 ± 0.18	2.9 ± 0.1 *	34.2 ± 0.5 *	75.4 ± 2.6 *
3 (Protein-free groups)	1	196 ± 5	—	193 ± 5	8.3 ± 0.37	4.3 ± 0.2	21.6 ± 0.9 *	91.1 ± 3.3 *
	2	177 ± 3	—	173 ± 3	10.0 ± 0.73	5.8 ± 0.4 *	17.4 ± 1.1 *	96.6 ± 3.2 *
	3	178 ± 7	—	177 ± 6	10.3 ± 0.58	5.8 ± 0.2 *	17.7 ± 0.2 *	102.9 ± 3.4 *
	4	178 ± 5	—	179 ± 5	9.9 ± 0.92	5.5 ± 0.5 *	17.6 ± 1.3 *	94.8 ± 3.1
4 (Starved group	3	182 ± 4	153 ± 3	202 ± 5	14.4 ± 1.06	7.1 ± 0.5 *	18.1 ± 1.3 *	126.3 ± 1.8
(48 hours) refed	4	182 ± 6	154 ± 6	221 ± 9	11.1 ± 0.91	5.0 ± 0.2 *	27.7 ± 0.8	138.1 ± 4.9 *
control diet)	6	181 ± 5	152 ± 6	269 ± 7	10.9 ± 0.18	4.1 ± 0.1	28.9 ± 0.4	118.3 ± 4.4
	8	181 ± 8	156 ± 8	322 ± 14	11.6 ± 0.44	3.6 ± 0.1	28.4 ± 0.4	101.8 ± 2.5 *
5 (Protein-free group	3	170 ± 4	167 ± 4	200 ± 5	9.9 ± 1.03	4.9 ± 0.4	24.4 ± 0.6 *	118.7 ± 9.0
(48 hours) refed	4	167 ± 3	163 ± 3	213 ± 4	9.0 ± 0.61	4.3 ± 0.3	27.9 ± 0.7	118.4 ± 7.0 *
control diet)	6	174 ± 4	173 ± 5	267 ± 12	9.9 ± 0.46	3.7 ± 0.1	28.5 ± 0.6	105.9 ± 2.1
	8	150 ± 6	144 ± 5	288 ± 12	10.2 ± 0.44	3.6 ± 0.1	27.9 ± 0.4	99.0 ± 3.9 *

[1] One-day-old chicks were fed a diet containing 25% isolated soybean protein for 10 days (experimental day zero, before starving or feeding the protein-free diet).

[2] Values represent means ± SE of five observations each.

[3] Body weight at time of killing for treatments 1, 4 and 5; initial body weight for treatments 2 and 3.

* Significantly differs from appropriate control value, $P < 0.05$.

TABLE 4

Changes in avian liver xanthine dehydrogenase and plasma uric acid following periods of dietary protein depletion and repletion (trial 3)

Treatment [1,2]	Day of exp.	Xanthine dehydrogenase activity [3]			Plasma uric acid
		units/g liver	*units/100 g body wt*	*units/100 mg liver N*	*mg/100 ml*
1 (Controls, 25% isolated soybean protein ad libitum	0	0.66 ± 0.07	2.9 ± 0.3	2.6 ± 0.3	—
	1	0.64 ± 0.07	2.9 ± 0.3	2.5 ± 0.3	5.5 ± 0.7
	2	0.62 ± 0.05	2.9 ± 0.3	2.4 ± 0.2	5.2 ± 0.7
	3	0.68 ± 0.02	2.9 ± 0.2	2.6 ± 0.1	7.5 ± 0.6
	4	0.72 ± 0.02	2.8 ± 0.2	2.7 ± 0.1	7.7 ± 0.5
	6	0.64 ± 0.06	2.7 ± 0.2	2.3 ± 0.2	6.8 ± 0.6
	8	0.54 ± 0.07	2.2 ± 0.2	1.8 ± 0.3	7.0 ± 1.1
2 (Starved groups)	1	0.93 ± 0.07 *	3.0 ± 0.1	2.9 ± 0.2	5.1 ± 0.3
	2	0.69 ± 0.04	2.0 ± 0.1 *	2.1 ± 0.1	6.0 ± 0.3
	3	0.61 ± 0.06	1.5 ± 0.2 *	1.8 ± 0.2 *	6.2 ± 0.5
	4	0.47 ± 0.04 *	1.0 ± 0.1 *	1.4 ± 0.1 *	8.8 ± 1.0 *
3 (Protein-free groups)	1	0.38 ± 0.04 *	1.6 ± 0.2 *	1.7 ± 0.2 *	2.4 ± 0.6 *
	2	0.17 ± 0.02 *	1.0 ± 0.2 *	1.0 ± 0.2 *	2.5 ± 0.2 *
	3	0.08 ± 0.01 *	0.5 ± 0 *	0.5 ± 0 *	3.0 ± 0.4 *
	4	0.05 ± 0.01 *	0.3 ± 0 *	0.3 ± 0 *	2.8 ± 0.5 *
4 (Starved group (48 hours) refed control diet)	3	0.29 ± 0.04 *	2.0 ± 0.3 *	1.6 ± 0.2 *	9.0 ± 0.4 *
	4	0.53 ± 0.06 *	2.8 ± 0.4	2.0 ± 0.3 *	7.8 ± 0.3 *
	6	0.57 ± 0.05	2.3 ± 0.2	2.0 ± 0.2	5.5 ± 0.7
	8	0.54 ± 0.03	1.9 ± 0.1	1.9 ± 0.1	5.4 ± 0.5
5 (Protein-free group (48 hours) refed control diet)	3	0.16 ± 0.03 *	0.8 ± 0.2 *	0.6 ± 0.1 *	6.6 ± 0.6
	4	0.38 ± 0.04 *	1.7 ± 0.3 *	1.4 ± 0.2 *	6.0 ± 0.9
	6	0.41 ± 0.01 *	1.5 ± 0.1 *	1.4 ± 0.4 *	5.5 ± 0.4
	8	0.40 ± 0.03 *	1.4 ± 0.1 *	1.4 ± 0.1	5.8 ± 0.6

[1,2] See table 3, footnotes 1 and 2.
[3] One unit represents enzyme activity catalyzing the reduction of 1 μmole NAD/minute at 25°.
* Significantly differs from appropriate control value, $P < 0.05$.

similar starvation period in chicks originally fed a diet containing 25% isolated soybean protein. After the initial 24 hours of starvation, however, total enzyme levels were reduced. These changes generally coincide with the loss of total liver nitrogen. Compared with control values, liver xanthine dehydrogenase activity was significantly lower on each day of the experiment for chicks fed the protein-free diet. These data suggest that during periods of protein-free intake, liver xanthine dehydrogenase protein is depleted at a rate greater than the loss of total liver protein. Refeeding the control diet to chicks that were previously starved or fed the protein-free diet resulted in increases in total xanthine dehydrogenase levels which were of much smaller magnitude than observed previously when a higher level of dietary protein was fed (trial 2).

In general, plasma uric acid levels were not changed appreciably in the starved chicks compared with chicks fed the 25% isolated soybean protein diet ad libitum. Plasma uric acid levels of chicks fed the protein-free diet were significantly lower than the corresponding control values throughout the experiment. Plasma uric acid levels were significantly higher than the control values the first 2 days of refeeding the starved chicks, whereas they were similar to control values throughout the refeeding period in chicks previously fed the protein-free diet.

DISCUSSION

Starvation or the consumption of protein-free or low protein diets results in a decrease in tissue protein, although each method of protein depletion may affect the tissue levels of specific enzymes in different ways. In the present studies with chicks, feeding a protein-free diet for 1 to 4 days resulted in a greater loss of liver xanthine dehydrogenase activity than did fasting for similar periods. Unlike the response of urea cycle enzymes in rat liver (4), it

appeared that avian liver xanthine dehydrogenase was not maintained, or increased, during progressive starvation. During the first 24 hours of starvation, however, total xanthine dehydrogenase levels of chicks previously fed a diet containing 25% isolated soybean protein were not changed. Similar results have been reported by Stirpe and Della Corte (5) and Della Corte and Stirpe (6) for starved chicks originally fed an enriched 25% casein diet. This effect is in contrast to a significant reduction in total xanthine dehydrogenase after 1 day of starvation in chicks previously fed a diet containing 75% isolated soybean protein.

Two explanations may be suggested for the difference in response of liver xanthine dehydrogenase to starvation, depending upon whether the starved chicks were initially fed a normal or high level of protein. One explanation is related to the existing levels of xanthine dehydrogenase in both groups of chicks prior to starvation. Chicks adapted to the high protein diet have approximately three to four times the levels of liver xanthine dehydrogenase compared with chicks fed the control diet containing 25% isolated soybean protein. The other explanation may be based upon a relative comparison of the levels of amino acid catabolism in both groups of starved chicks. It is suggested that the rate of amino acid catabolism, and hence uric acid production, in starved chicks previously fed a diet containing 25% isolated soybean protein is comparable to the rate when this diet is fed ad libitum. Schimke (4) has reported net increases in liver arginase activity and urea excretion in rats during a 7-day fast compared with rats fed a 15% casein diet ad libitum. For starved chicks previously fed a diet containing 75% isolated soybean protein, however, it is suggested that absolute rates of amino acid catabolism are lower than when the high protein diet is fed ad libitum. The lower xanthine dehydrogenase activity and plasma uric acid levels in starved chicks originally fed the high protein diet, compared with the ad libitum levels, support this suggestion.

In studies with rats, where absolute levels of liver arginase were determined by immunochemical procedures and enzyme turnover by isotope labeling techniques, Schimke (7) related the changes in activity of this enzyme during starvation and low protein intake to alterations in the rates of synthesis and degradation. During starvation it was demonstrated that arginase degradation ceased while synthesis continued at a constant rate. The net effect was an increase in total arginase levels during a 6-day starvation period. Switching rats from a 70% casein diet to an 8% casein diet was shown to increase the rate of arginase degradation and decrease its rate of synthesis.

Studies reported by Rosenthal and Vars (8) indicated a net increase of 58% in the liver arginase content of protein-depleted rats during a 4-day starvation period in rats. In their studies, rats were initially fed a protein-free diet for 2 weeks prior to starvation. In addition to the enhanced arginase activity, liver protein was also shown to increase during starvation in the protein-depleted rats. It was suggested that the increase in liver arginase activity was a specific response to an increased rate of protein catabolism. In the present studies, however, liver xanthine dehydrogenase and total liver nitrogen were reduced in starved chicks originally fed a protein-free diet for 48 hours.

Litwack and Fisher (9) reported that a deficiency of a single essential amino acid reduced liver xanthine dehydrogenase levels in the chick. Della Corte and Stirpe (6) suggested that the 5% casein diet used in their studies was inadequate to permit normal rates of xanthine dehydrogenase synthesis to occur. From the results of the present studies, and those presented earlier (1, 2), it appears that the response of avian liver xanthine dehydrogenase was similar to the response of the urea cycle enzymes in rats during periods of high protein intake. Progressive starvation, however, decreased total levels of xanthine dehydrogenase in chicks, but resulted in either the maintenance or enhancement of urea cycle enzyme activity in rats. A possible explanation for this difference may be found from results of studies recently reported by Krakoff and Meyer (10). In their studies with allopurinol, an inhibitor of xanthine oxidase, uric acid production ceased, yet chick growth was not greatly affected. Nitrogen arising from the catabolism of amino acids was not ex-

creted in the form of uric acid, but as unidentified nitrogen compounds. The possibility exists that xanthine dehydrogenase is not an indispensable enzyme for the chick, whereas the opposite appears to be true for mammalian arginase.

When chicks were changed from the control diet containing 25% isolated soybean protein to a diet containing 75% isolated soybean protein a significant increase in liver xanthine dehydrogenase activity was observed within 12 hours. The maximal response of this enzyme to the high protein diet was observed after 48 hours, after which time enzyme activity, expressed on a body weight basis, decreased to a level approximately threefold higher than that observed for chicks fed a diet containing 25% isolated soybean protein. The changes in plasma uric acid levels generally reflected the changes in liver xanthine dehydrogenase activity, although the increase in plasma uric acid after feeding the high protein diet for 12 hours was proportionally greater than the increase in enzyme activity during this time. It is emphasized that to suggest a direct relationship between plasma uric acid levels and xanthine dehydrogenase activity could be misleading. The activity of xanthine dehydrogenase measured during various intervals in these experiments represents cumulative effects of the dietary treatments, whereas plasma uric acid levels do not. A better estimate of a relationship between liver xanthine dehydrogenase activity and uric acid formation might be obtained by determining total uric acid excretion. Evidence of direct relationships between liver arginase activity and urea excretion in rats (11), and liver xanthine dehydrogenase activity and uric acid excretion in chicks (2) have been reported.

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Development of Spleen and Thymus in Offspring of Protein-deficient Rats [1]

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ABSTRACT Maternal protein deficiency was induced by feeding a diet containing 5% protein (methionine-supplemented casein) from conception to day 18 of pregnancy, conception to parturition, or 1 day postpartum to weaning. Control diets containing 15% protein were fed to the females, except at the times indicated, and to the offspring after weaning. Growth of the thymus and spleen in progeny was examined from gestational day 18 to 24 weeks of age, and response to antigenic stimulus was measured during adulthood. Fetal growth of thymus and spleen was retarded when the mother was depleted throughout gestation, often to a greater degree than was body weight. Differences in organ weights between depleted and control rats at birth increased during the first 3 days postpartum, although females in both groups received the control diet after delivery. Speed of recovery of the retarded organs depended upon the timing and duration of protein restriction. Effects of the maternal deficiency during gestation or lactation were reversible to the extent that at 24 weeks of age organ weights per unit body weight were not different from those of controls. Immune response of the progeny after they had been fed an adequate diet was not impaired as a result of the maternal protein deficiency.

Lymphoid tissues are particularly sensitive to protein malnutrition. In the spleen, the weight, DNA, RNA, number of specific antibody-forming cells and output of antibody decreased when adult rats were fed a low protein diet (1). Splenic weight and nucleic acids were similarly reduced when large numbers of pups were left with a single female in lactation (2). Some of these effects have been overcome by refeeding protein [2] (3) or by reducing litter size in lactation (2). Such reversibility, however, generally was minimal when nutritional deprivation occurred early in life (4). Though thymectomy in the adult may be of little consequence, neonatal removal impaired production of immunoglobulins by other tissues later in life (5) and often led to wasting and early death (6). The weight of the thymus and the thymus-to-body weight ratio were decreased by undernutrition for 9 days after birth (2), although the thymus-to-body weight ratio at birth was not lowered after feeding a 6% protein diet to the mother in gestation (7). The thymus atrophied in protein-calorie malnourished pigs (8) and in rats force-fed diets deficient in single amino acids (9). Growth of the spleen and thymus, two tissues related to immune response, is reported here for offspring of rats fed a low protein diet during gestation or lactation. To determine whether immunological function of these organs was permanently impaired by the restricted protein intake of the mother, primary immune response was measured in the progeny after they had reached adulthood and while their protein intake was adequate.

PROCEDURE

Three studies were conducted to compare low and moderate intakes of protein during gestation or lactation. Rats of the Sprague-Dawley strain were fed the laboratory stock diet [3] initially. Males used for breeding were fed this diet throughout the study except when housed overnight with a female fed another diet. Daily vaginal smears were begun when females weighed 200 g and were about 9 weeks of age. A male was introduced during each estrus

Received for publication December 17, 1968.

[1] Journal Paper no. J-6126, Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa. Project no. 1717.

[2] Kenney, M. A., unpublished observations.

[3] Containing: (in percent) cornmeal, 42.1; dried skim milk, 18.4; linseed meal, 12.0; wheat germ, 7.5; yeast (part irradiated, containing 0.6% Ca pantothenate), 7.5; cottonseed oil, 6.4; alfalfa meal, 1.5; casein, 3.8; $CaCO_3$, NaCl and trace elements, 0.8. Supplemented weekly with 6 mg α-tocopherol and 400 mg cod liver oil/rat.

cycle until sperm or a vaginal plug indicated mating had occurred. The day following was designated as day zero, and the female was given a purified diet providing 15 or 5% protein. The low protein diet was fed for the first 18 days, or throughout gestation, or for 20 days of lactation. The rest of the time, including the interval of at least 2 weeks between loss or weaning of a litter and remating, females and their offspring were fed 15% protein.

Diets were formulated to provide approximately 100% of the mineral requirements and 150% of needs for vitamins, as summarized by the Committee on Animal Nutrition of the National Research Council (10). Although absolute requirements for reproduction often exceed those for growth, a diet meeting growth needs may also provide amounts of most nutrients sufficient for reproduction because of increased total food intake at that time. From the Committee's tabulation, exceptions are vitamin A, thiamine, riboflavin, sodium and potassium. Increased vitamin A and sodium requirements for reproduction were met in this study by inclusion of sodium chloride and additional vitamin A in a diet based on growth requirements. Potassium content of the salt mix in all diets exceeded the requirement for growth and approximated that for reproduction. Intakes of thiamine and riboflavin were not increased until needed, near the end of gestation (10). Thus, the control diet for females, which provided 15% protein, contained the following: (in percent) vitamin-free test casein (13.40% N), 17.75; DL-methionine, 0.200; sodium chloride, 1.0; salt mix,[4] 4.0; vitamin mix,[5] 5.0; cottonseed oil, 5.0; nonnutritive fiber, 2.0; and cornstarch, 65.0. This diet provided 18,000 IU vitamin A/kg diet and was supplemented with an aqueous solution of 100 μg thiamine·HCl and 50 μg riboflavin/day from day 18 of pregnancy through lactation. The low (5%) protein diet for females was similar to the control diet except that it contained 5.92% casein, 0.067% methionine and 77.0% cornstarch. The 15% protein diet fed to the progeny after weaning was similar to the control diet for females, but 1% sodium chloride was replaced with cornstarch and vitamin A was reduced to 3000 IU/kg. All diets were fed ad libitum.

Females in study A were permitted to raise three litters. Controls were given 15% protein through the reproductive cycle; others were fed the low protein diet from conception to delivery in pregnancy 1 or 2, followed by the control diet in lactation. Because these deficient pups did not survive to weaning, the depletion period was shortened to include only the first 18 days in pregnancy 3.

In study B, foster-mothering was introduced in an attempt to increase survival of depleted pups. Whenever two females became pregnant on the same day, one was fed 5% protein until parturition and the other, 15%. Litters were reduced to 10, and one-half the control litter was exchanged with one-half the depleted litter within 24 hours of birth. Both females were then fed the control diet. Additional females in study B were fed the control diet in gestation and the low protein diet from 1 day postpartum to weaning at 21 days. Only two litters were produced by each female in this study.

In studies A and B females were weighed daily. Individual pups were weighed at weekly intervals, after identification by earclip when 7 days old. Litters were reduced to 10 at parturition. Due to variations in litter size, the actual number killed from a litter varied; litter means are reported, to avoid weighting data unduly with rats from large litters. Similar numbers of both sexes were included in samples of newborn rats. Since litter size was not affected by diet, any effect of number of fetuses on development should not influence interpretation of the data presented. After weaning, female offspring were used for other experiments. Males were fed a diet containing 15% protein and were killed at 3, 6 or 24 weeks of age, or permitted to die naturally. Before removal of

[4] Composed of three parts Hawk-Oser formulation and one part $CaHPO_4$ with sulfates of manganese, zinc and copper. This mixture provided: (in milligrams per kilogram diet) Ca, 6200; P, 4250; Na, 920; K, 4900; Mg, 500; Mn, 50; Fe, 76; I, 0.9; F, 7.0; Cu, 5.0; and Zn, 0.04.

[5] The cornstarch-based mix provided: (in milligrams per kilogram diet) thiamine·HCl, 1.88; riboflavin, 3.75; pyridoxine·HCl, 1.80; niacin, 22.5; Ca pantothenate, 12.0; choline chloride, 1125; vitamin B_{12}, 0.0075; folic acid, 1.5; biotin, 0.30; ascorbic acid, 75; *p*-aminobenzoic acid, 30.0; menadione, 0.15; *dl-a*-tocopheryl acetate powder (250 IU/g), 360; retinol palmitate (water-dispersible beadlets, 0.41 IU/μg), 43.3. Vitamins A and E were added immediately before incorporating the mix into the diet.

organs for weighing, rats were anesthetized with sodium pentobarbital and exsanguinated. Data for weanling and older animals are for individual males from litters of six or more at birth. In each study all the adult rats surviving at the time, despite their different ages, were immunized in a single day. One milliliter 2% sheep red cells suspended in 0.85% sodium chloride was injected intravenously. Tail blood was taken 6 days later for titration of antibodies, as described previously (1).

In study C, four or five females fed each of the two diets were killed each day from day 16 through day 21 of gestation. An additional 5 control and 10 depleted rats were allowed to deliver one litter; all were fed the control diet after delivery. Some pups were killed from these litters at birth and on each of the next 3 days. Pregnant or newborn animals were decapitated, and the time recorded to the nearest hour. The intact uterus was placed on ice, and fetuses removed and dissected one at a time, to a maximum of eight per litter. Fetal age was estimated by assuming that pregnancy began at midnight before day zero.

Means and regression coefficients were compared by t tests or by F tests in analysis of variance or covariance (11). Differences or treatment effects were considered significant only when P was less than 0.05.

RESULTS AND DISCUSSION

Maternal protein deficiency from the day after conception to parturition decreased the absolute weights of spleen and thymus in newborn rats (table 1). In contrast to a previous report (7), the thymus was decreased in proportion to body weight in the young of females fed 5% protein throughout pregnancy. The spleen-to-body weight ratio also tended to be small, but the apparent difference was not significant. When the protein intake had been increased from 5 to 15% on day 18, body and thymus weights of young equaled those of control litters at birth, but spleens were small. Thus, when adequate protein was fed after day 18, adverse effects of protein restriction on thymic development were prevented or corrected before birth. Spleen weight by the end of pregnancy, however, had not reached that of neonatal controls.

In study A, repletion of females for 3 or 4 days before parturition resulted in pups similar in many respect to those of controls and helped to prevent the neonatal deaths so common in litters of females depleted throughout pregnancy. Study C provided information about the late fetal and early postnatal periods in young of females fed 5 or 15% protein throughout gestation and 15% after delivery. The weight of the fetal spleen after day 18 increased logarithmically at about twice the rate of the thymus, which paralleled gain in body weight (table 2). Growth rates (or slopes of regressions (table 2) for organs in depleted fetuses were not detectably altered by the dietary restriction. Mean thymus weight and spleen weight in relation to body weight, however, were significantly less than in controls at an average gestational age of 20 days. The difference in response of thymus and of spleen to repletion after day 18 (table 1) may be related to the differences in their characteristic rates of growth in the fetus.

Growth of both organs was slower after birth than in the fetus. Nevertheless, the spleen grew continuously in controls during the first 3 days after birth, increasing

TABLE 1

Effects of protein deficiency during gestation on weights of spleen and thymus in the young at birth

Treatment	No.	Spleen wt		Thymus wt		Body wt
		mg	*mg/g body wt*	*mg*	*mg/g body wt*	*g*
Control [1]	61	13.5 ± 0.6 [2]	2.26	11.0 ± 0.6	1.84	5.9
Depleted to delivery [1]	24	10.0 ± 1.4 [3]	1.98	8.1 ± 0.8 [3]	1.64 [3]	4.7
Control [4]	14	16.4 ± 0.8	2.61	11.6 ± 1.0	1.87	6.2
Depleted to day 18 [4]	8	13.2 ± 1.0 [3]	2.16 [3]	10.8 ± 0.7	1.82	6.0

[1] Litters 1 and 2 of studies A, B and C.
[2] Mean ± SE.
[3] Different from controls, from t tests ($P < 0.05$).
[4] Litter 3, study A.

TABLE 2

Effects of maternal protein deficiency in pregnancy on spleen and thymus weights in young from day 18 of gestation through day 3 after birth (study C)

	Control (15% protein)				Depleted (5% protein)			
	No.	Mean age	Spleen wt	Thymus wt	No.	Mean age	Spleen wt	Thymus wt
		days	*mg*	*mg*		*days*	*mg*	*mg*
Fetal day 18	3	18.8	0.7	2.8	1	18.8	0.6	1.6
Fetal day 19	4	19.5	1.5	3.4	5	19.6	1.3	3.2
Fetal day 20	4	20.6	4.6	7.0	4	20.5	3.2	4.6
Fetal day 21	4	21.3	6.2	7.0	5	21.6	7.1	8.0
Covariance analysis:	15	20.3			15	20.3		
Log wt, mg			0.44 [1] (0.384) [2]	0.70 (0.187)			0.38 (0.337)	0.62 [3] (0.206)
Wt per body wt, mg/g			0.96 (0.364)	1.58			0.89 [3] (0.354)	1.43
Birth	5	22.3	11.9	10.1	10	22.3	10.3	7.5
Birth + 1 day	5	23.4	16.1	10.0	6	22.8	7.8	7.3
Birth + 2 days	5	24.3	21.3	10.6	5	23.8	8.2	7.3
Birth + 3 days	5	25.3	26.7	13.4	2	24.9	12.1	8.8
Covariance analysis:	20	Birth + 1.2 days			23	Birth + 1.2 days		
Log wt, mg			1.20 (0.101)	1.02			0.94 [3]	0.87 [3]
Wt per body wt, mg/g			1.73 (0.548)	1.70			1.91 [3]	1.58

[1] Adjusted means.
[2] Slope of regression on age, where greater than zero ($P < 0.05$).
[3] Different from control ($P < 0.05$).

in proportion to body size, whereas thymus weight and thymus-to-body weight ratios did not change (table 2). In depleted pups, no significant gain occurred in either organ; organs of controls were approximately twice the size of those in depleted young on day 3 after birth. Apparent increases in organ weights on day 3 suggested that the lag period in their development had ended. During this lag, which was most pronounced in depleted pups, 8 of the 10 depleted litters were lost. Pups from all five control litters survived after the 3 days of observation. Poor development and high mortality of depleted young have been attributed to weakness, poor suckling of the pups and limited milk supply (7). When young rats in study C were randomly selected for killing from among surviving littermates, stomachs of depleted pups contained about half as much milk as those of controls. Whatever the primary cause, differences at birth between control and deficient pups often increased in the next 3 days, even though the depleted dams were fed the control diet after parturition.

Male progeny from studies A and B were killed at various ages to determine whether effects of maternal protein deficiency seen earlier had persisted. After weaning, these animals were fed an adequate diet containing 15% protein. The spleen and thymus grew similarly between birth (table 1) and weaning (table 3), increasing 15- to 20-fold in controls in 3 weeks, while body weight increased about 7-fold. In weanling controls both spleen and thymus were heavier in study B than in study A (table 3), perhaps because rats had been slightly larger at birth in study B than in the earlier study. Spleen weight continued to increase through week 24; thymus weight at 6 weeks was about 0.7 g in controls, exceeding the weight at 24 weeks.

Maternal depletion for the first 18 days of gestation (group 2) produced no detrimental effects on thymus weight at birth or later; weight of the thymus at 3 weeks actually exceeded that of controls (group 1, table 3). In contrast, either prenatal deficiency extending to parturition, with (group 5) or without foster-mothers (group

TABLE 3

Weights of thymus and spleen in progeny following maternal protein deficiency: studies A (groups 1 and 2) and B (groups 3 through 7)

Group	Treatment [1] Gestation	Treatment [1] Lactation	No. of rats	Body wt	Spleen wt		Thymus wt	
3 weeks of age				*g*	*mg*	*mg/g body wt*	*mg*	*mg/g body wt*
A1	—	—	21	41	180 ± 11 [2]	4.77 ± 0.21	185 ± 11	4.91 ± 0.20
A2	5% protein to day 18	—	4	39	166 ± 15	4.04 ± 0.29	252 ± 37 [3]	6.04 ± 0.52 [3]
B3	—	—	10	44	221 ± 19 [4]	4.99 ± 0.27 [4]	214 ± 18	4.84 ± 0.30
B4	—	depleted foster-mother	6	48	286 ± 17 [3]	5.98 ± 0.36 [3]	240 ± 11	5.02 ± 0.31
B5	5% protein to birth	control foster-mother	6	37	234 ± 22	6.50 ± 0.99	174 ± 26 [3]	4.52 ± 0.51
B6	5% protein to birth	—	5	40	182 ± 19 [3]	4.66 ± 0.64	177 ± 25 [3]	4.36 ± 0.28
B7	—	5% protein to weaning	13	21	62 ± 9 [3]	2.91 ± 0.29 [3]	54 ± 6 [3]	2.56 ± 0.16 [3]
24 weeks of age					*g*		*g*	
A1	—	—	12	498	0.73 ± 0.05	1.49 ± 0.01	0.59 ± 0.06	1.19 ± 0.11
A2	5% protein to day 18	—	5	470	0.67 ± 0.06	1.43 ± 0.11	0.53 ± 0.11	1.14 ± 0.11
B3	—	—	8	479	0.78 ± 0.55	1.62 ± 0.10	0.42 ± 0.02	0.87 ± 0.04
B4	—	depleted foster-mother	5	454	0.77 ± 0.08	1.74 ± 0.26	0.43 ± 0.04	0.93 ± 0.06
B5	5% protein to birth	control foster-mother	2	466	0.70 ± 0.08	1.51 ± 0.16	0.38 ± 0.01	0.82 ± 0.03
B6	5% protein to birth	—	3	367	0.56 ± 0.05	1.56 ± 0.20	0.32 ± 0.03	0.91 ± 0.16
B7	—	5% protein to weaning	10	371	0.58 ± 0.04 [3]	1.58 ± 0.14	0.36 ± 0.03	0.96 ± 0.05

[1] Except as otherwise indicated, females were fed 15% protein and pups left with the natural mother during lactation.
[2] Mean ± SE.
[3] Different from a group of the same age, based on comparison of A2 with A1; B4, B5, B7 with B3; B6 with B4 ($P < 0.05$).
[4] Interaction ($P < 0.05$) between diet in gestation (B3 and 4 versus B5 and 6) and condition of dam at the start of lactation (B3 and 5 versus B4 and 6).

6), or depletion during lactation (group 7) led to low thymus weights at 3 weeks of age. By 24 weeks, weights for these groups were not significantly less than those for controls (group 3).

Spleen weights at 3 weeks of age in progeny of rats depleted for 18 days (group 2), or for the entire gestation period (groups 5 and 6), were comparable to those of controls left with the natural mother (groups 1 and 3). Control pups given to a depleted foster-mother (group 4) had spleens larger than littermates left with the natural mother (group 3), and larger than those of depleted pups nursed together with them by a depleted female (group 6). This could have resulted from competition between depleted and normal pups for what may have been a limited supply of milk, although body weights of young rats in groups 4 and 6 at weaning were not significantly different. When females were depleted during lactation, spleens of offspring remained small even at 24 weeks of age. The apparently permanent stunting of the spleen following postnatal deficiency was associated with a reduction in adult body size. Consequently, spleen-to-body weight ratio was normal in this as in all other groups at that age.

To determine if interference with normal growth of spleen and thymus in young rats had impaired ability to respond to antigenic stimulus, offspring were immunized between 3 and 8 months of age. In controls of study A, which included animals ranging in age from 12 to 30 weeks, antibody titers increased with the age of the animal at the time of immunization ($P < 0.05$). When means were adjusted for the age difference between the groups, titers of both hemolysin and agglutinin were significantly elevated in offspring of rats depleted 18 days in pregnancy (table 4). This enhancement of immune response may have been related to thymic development, since the rapid preweaning growth of the thymus was one of the few unusual characteristics of that group. Because numbers of animals in groups 5 and 6 were quite small, failure to demonstrate a significant effect of maternal restriction throughout gestation does not exclude the possibility that immune response might be altered by such treatment. It is unlikely, however, that a detrimental effect of depletion in lactation would have gone undetected, since groups 7 and 3 each contained eight animals. Further, the groups most severely depleted according to some measures (groups 6 and 7) had the highest mean antibody titers in the study. In primary immune response described by Sinclair (5), thymectomized mice not only produced less antibody but exhibited a longer lag period before its appearance in the circulation than did sham-operated animals. Since the peak hemolysin titer normally occurs in the rat on about day 6 after exposure to antigen, any qualitative resemblance of nutritional retardation of thymic growth to thymectomy should have been detected by the measurements reported here. There was no evidence, however, of a decrease or delay in antibody production in offspring after maternal protein restriction during pregnancy or lactation.

TABLE 4

Immune response of progeny following maternal protein deficiency during pregnancy or lactation

Group	Treatment [1]		No. of rats	Avg age at immunization	Hemolysin	Agglutinin
	Gestation	Lactation				
				weeks	*log titer* [2]	*log titer* [2]
A1	—	—	23	22	2.70	2.29
A2	5% protein to day 18	—	12	17	2.94 [3]	2.51 [3]
B3	—	—	8	17	3.28	1.81
B4	—	depleted foster-mother	4	18	3.07	1.80
B5	5% protein to birth	control foster-mother	2	24	3.14	1.50
B6	5% protein to birth	—	3	20	3.57	2.06
B7	—	5% protein to weaning	8	19	3.32	1.84

[1] Except where otherwise indicated, females were fed 15% protein and pups left with the natural mother during lactation.
[2] Means adjusted to 20 weeks (study A), or 19 weeks (study B), after analysis of covariance.
[3] Different from control group A1 ($P < 0.05$).

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Urinary Simple Phenols in Rats Fed Purified and Nonpurified Diets [1]

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ABSTRACT The simple phenol fraction of rat urine after hydrolysis in 3 N H_2SO_4 and with β-glucuronidase + sulfatase has been analyzed in several thin-layer and gas chromatographic systems. Only phenol and *p*-cresol, together with minute quantities of 4-ethylphenol, were found when a purified casein-sucrose diet was fed. Rats fed diets containing plant material excreted considerable amounts of 4-ethylphenol, catechol and resorcinol and smaller quantities of several other simple phenols in addition to those excreted when fed a purified diet. Some of these compounds (guaiacol, 4-methylguaiacol, 4-methylcatechol and 4-vinylphenol) have not previously been shown to be normal urinary constituents. The possible naturally occurring precursors of the urinary simple phenols are discussed.

The toxic effects of some simple phenols have long been recognized (1, 2) and the formation and excretion of such compounds in mammals have received considerable attention in biochemical and medical research (3–7). Williams (2) and Bray and Thorpe (8) have reviewed the species variations in the urinary excretion of simple phenols and pointed out that the nature and quantity of these compounds also depend on the diet. The relative importance of these factors, however, has not been established.

The metabolism of some plant phenolics (9–13) and other cyclic compounds occurring in plant material (14) yield simple phenols. Also, *p*-cresol and phenol have been identified as products of the bacterial degradation of tyrosine in vitro (3). The metabolism of tyrosine by intestinal microorganisms has been suggested as the origin of 4-ethylphenol (2, 3) which is excreted in large amounts in the urine of some species (15).

Studies on urinary simple phenols have hitherto been hampered by relatively nonspecific or insensitive analytical methods. Recently, sensitive and specific gas chromatographic methods have been developed which are superior to the earlier methods, especially for the analysis of monohydric phenols (16).

The present report describes the use of thin-layer and gas chromatographic analysis of the simple phenol fraction of enzyme- or acid-hydrolyzed rat urine. Evidence is provided for the excretion of some previously undetected simple phenols in rats fed plant-containing diets. Also, some conclusions are reached about the relative importance of nonprotein plant constituents and dietary protein as precursors of the quantitatively more important simple phenols. A major aim of the present study was to provide basic information essential for further investigations on the formation and possible pathophysiological significance of simple phenols.

MATERIALS AND METHODS

Animals. Male albino rats aged 5 to 6 months and weighing 300 to 350 g were used. The animals were caged individually and fed diet 1 and water ad libitum before the study.

Diets. The following diets were used: 1 (commercial pellet diet [2]), 2 (purified

Received for publication December 27, 1968.
[1] Supported by a grant from Det Videnskapelige Forskningsfond av 1919, Oslo, Norway.
[2] Diet 1 was obtained from Felleskjøpet, Oslo, Norway and contained: (in percent) herring meal, about 10; soya meal, 12; wheat meal, 12; barley meal, 30; oat meal, 15; dried milk, 8; brewer's yeast, 2; grass meal, 5; seaweed meal, 0.2; molasses, 5; chalk, 0.3; salt, 0.3; and a commercial vitamin A, D and E mixture (Stjernegranulat, Martens and Company, Bergen, Norway), 0.2.

diet[3]), 3 (vegetable diet[4]) and 4 (purified except for the crude main protein source of diet 1[5]).

Experiments. During the diet treatment the rats were kept in individual metabolic cages and allowed free access to food and water. Urine collection was started 3 days after changing to the experimental diets except with diet 1 which was given continuously. The metabolic cages were equipped with separators which disposed of the feces. The urines were collected during two successive 24-hour periods in 1 N HCl (5 ml) which was sufficient to maintain the samples below pH 2 during the collection period. All urines were stored at − 20° until analyzed. Immediately before analysis, the frozen urines were thawed and filtered and the urine volumes were recorded. The two 24-hour urine portions of each animal were combined and diluted to 60 ml. The preliminary gas chromatographic analysis needed to choose an appropriate internal standard, and also thin-layer chromatography was carried out on 25-ml portions of pooled urine containing equal volumes of the above dilutions from the animals in each diet group. Ten milliliters of the dilutions from each animal were used for quantitative gas chromatographic analysis.

Conjugate hydrolysis. Acid hydrolysis was the method employed routinely for quantitative and qualitative analysis. In addition, pooled and some individual urines were analyzed after enzyme hydrolysis. The use of both methods was necessary because it had previously been shown that neither method alone permitted adequate recoveries of all simple phenols, and that treatment with hot acids may decarboxylate phenolic acids to yield simple phenols (17).

For acid hydrolysis the diluted urines (10 ml) were refluxed for 60 minutes in Kjeldahl flasks after the addition of 1 ml water and 1 ml concentrated H_2SO_4. For enzyme hydrolysis the diluted urines (10 ml) were adjusted to approximately pH 5 with $NaHCO_3$, and 0.2 M acetic acid–sodium acetate buffer, pH 5.0 (2 ml), aqueous neomycin sulfate 1.5 mg/ml (0.5 ml) and 12,500 Fishman Units of β-glucuronidase (type H1)[6] with sulfatase were added. These additions were increased correspondingly when pooled urines (25 ml) were used. The mixtures were shaken well and incubated for 20 hours at 37°.

Extraction. For gas chromatography the simple phenols were extracted after addition of *p*-methoxyphenol (0.3 mg in 1 ml of water) as internal standard. Preliminary analysis showed this compound to be absent from the pooled urines. The acid-hydrolyzed urines and the enzyme-hydrolyzed urines adjusted to pH 1 with 4 N HCl were extracted three times with 20 ml of redistilled, peroxide-free diethyl ether. The combined ether phases were extracted three times with 5% sodium bicarbonate solution (50 ml) prepared from freshly boiled water. After drying the ether phases over anhydrous sodium sulfate (10 g) for 15 minutes, the ether was decanted; then the sodium sulfate was washed with small volumes of ether and the combined ether solution was evaporated to 0.5 ml on a steam bath.

For thin-layer chromatography the acid hydrolysates and the acidified incubation mixtures were extracted three times with equal volumes of ether. The combined ether phases were dried over anhydrous sodium sulfate for 15 minutes and evaporated to dryness. The residues were dissolved in 1 ml of acetone.

Gas chromatography. An F and M model 402 gas chromatograph equipped with a flame ionization detector was used. By means of a Hamilton microsyringe, 5 μl of the final ether concentrates were injected into three gas chromatographic columns with different eluting characteristics: column C (1% Carbowax 20 M[7]), column S (15% silicone rubber UC–W 98) and column T (2% tricresylphosphate).

[3] Diet 2 contained: (in percent) casein (light white soluble, BDH Chemicals Ltd., Poole, England), 20; sucrose, 68; soya oil, 6; USP XIV salt mixture, 4; and vitamin mixture, 2. The vitamin mixture contained thiamine·HCl, 25 mg; riboflavin, 22 mg; pyridoxine·HCl, 22 mg; calcium pantothenate, 33 mg; nicotinamide, 220 mg; ascorbic acid, 350 mg; vitamin A, 5000 IU; vitamin D_2, 600 IU; and tocopherol acetate, 51 mg, in sufficient glucose to make 20 g.

[4] Diet 3 contained: (in percent) carrot, 10; potato, 20; apple, 10; banana, 10; cabbage, 10; wheat meal, 30; soya oil, 6; USP XIV salt mixture, 2; and vitamin mixture (as in diet 2), 2. The vegetables and fruits (wet weight) were ground and mixed well with the salt and vitamin mixtures.

[5] Diet 4 contained: (in percent) herring meal, 40; cornstarch, 54; USP XIV salt mixture, 4; and vitamin mixture (as in diet 2), 2.

[6] Sigma Chemical Company, St. Louis, Mo.

[7] The solid support and the stationary phases were supplied by Hewlett-Packard Company, F and M Division, Avondale, Penna.

The qualitative analysis of the simple phenols was carried out by estimating the peak areas of these compounds, and the internal standard by planimetry and by the use of calibration curves. The 24-hour excretion of each of the compounds (Q) was calculated using the following formula:

$$Q = \frac{\text{Simple phenol in injected sample}}{\text{Internal standard in injected sample}} \times 0.9 \text{ mg}$$

Further details concerning the reference compounds and the gas chromatographic analysis are given elsewhere (16).

The data were analyzed statistically using Student's t test (18).

Thin-layer chromatography. Together with appropriate standards, the extracts (in 1 ml of acetone) of the pooled urines were examined by thin-layer chromatography on 0.5 mm-thick layers of cellulose. Sigmacell Type 19 [8] was used with solvent 1 (benzene–glacial acetic acid–H_2O, 6:7:3, upper layer) and MN 300 [9] was used with solvent 2 (20% aqueous potassium chloride – glacial acetic acid, 100:1). The phenols were detected after spraying with 0.5% aqueous solution of fast blue B salt followed by saturated sodium bicarbonate solution. The R_F values, color reactions and observations are shown in table 1.

Identification criteria. To determine the identities of the substances eluting from the columns, known quantities of standard compounds not present in the urines were added to the extracts. Chromatography was then repeated on the three columns, the detector response of the standards was compared with the unknown peaks and their relative retention times were recorded. The presence on the chromatograms from the three columns of peaks with comparable relative sizes and with relative retention times identical to those of known reference compounds were the criteria used for the identification of the monohydric phenols. Also, no double contours should appear in the tracings from any of the columns when the analyses were repeated after the addition of the respective reference compounds. For trace amounts of 4-ethylphenol and 4-vinylphenol, retention data were available only with columns C and S because of the rather high detection limits for these compounds using column T.

Owing to the slow elution of the dihydric phenols from column T, thin-layer chromatography in two solvent systems without the prior treatment of the extracts with sodium bicarbonate solution was used as an appropriate substitute for column T for the identification of these compounds. Otherwise the identification criteria were the same as for the monohydric phenols.

RESULTS

The simple phenols listed in table 2 were detected in the extracts from the enzyme-hydrolyzed urines. Figures 1, 2 and 3 show chromatograms of a representative urine extract from the group fed diet 1, on columns C, S and T, respectively. The compounds listed in table 2 account for all peaks observed on the chromatograms with column T. Except for minute deflections (relative retention 0.50 and 1.44) in the chromatograms from diet group 1, all peaks are accounted for on column S.

[8] See footnote 6.
[9] Macherey, Nagel and Company, Düren, Germany.

TABLE 1

Thin-layer chromatography of urine extracts and reference compounds

Compounds	R_F solvent 1	R_F solvent 2	Color with fast blue B salt	Observations		
				Diet 1	Diets 2 and 4	Diet 3
Catechol	0.30	0.62	Pink-gray	tr [1]	—	tr
Resorcinol	0.10	0.56	Red-purple	+	—	+
Hydroquinone	0.09	0.62	Gray-brown	—	—	—
4-Methylcatechol	0.40	0.52	Gray-violet	tr	—	tr
Pyrogallol	0.04	0.55	Pink-brown	—	—	—

[1] Tr = trace; + = prominent; — = not detected.

TABLE 2
Simple phenols in extracts of enzyme-hydrolyzed urines

Diet fed	Simple phenols identified
1	Phenol p-Cresol 4-Ethylphenol Catechol 4-Methylcatechol Resorcinol (absent in acid-hydrolyzed urines) 4-Vinylphenol (trace, absent in acid-hydrolyzed urines) Guaiacol (trace) 4-Methylguaiacol (trace, reduced in acid-hydrolyzed urines)
2	Phenol *p*-Cresol 4-Ethylphenol (trace)
3	Same as diet 1
4	Same as diet 2

Analysis of the urine extracts from diet groups 1 and 3 revealed the presence of several unknown substances with long retention times on column C. These substances were not detected on columns T and S and are probably highly polar and high boiling compounds. These unknown peaks represent none of the simple phenols and derivatives which are analyzed by the present gas chromatographic methods (16). The extraction method employed is not specific for simple phenols. Therefore, complex plant phenols and other substances occurring in plants as well as metabolites of these compounds may contribute to the multitude of peaks observed on the latter part of chromatograms from column C with urine extracts from rats fed the plant-containing diets 1 and 3.

Of the simple phenols identified in the urine extracts from diet groups 1 and 3, resorcinol and 4-vinylphenol were detected only after enzyme hydrolysis. The trace of 4-methylguaiacol detected in these extracts nearly disappeared after acid hydrolysis. These findings are consistent with observations reported previously (17) and in a companion paper (19).

In the acid-hydrolyzed urines no simple phenols were identified which were not also found after enzyme hydrolysis. The results of the quantitative analysis on column C of the acid-hydrolyzed urines from diet groups 1, 2 and 3 are presented in table 3. Diet 4 was fed to two rats only to determine whether urinary 4-ethylphenol could be attributed to herring meal which was the main crude protein source of diet 1. As shown in table 2 only traces of this compound were found in the urine when diet 4 was fed.

DISCUSSION

It has long been recognized that the urine of many mammals contains phenol, *p*-cresol and 4-ethylphenol in the free state, and as their sulfate and glucuronic acid conjugates (2–4, 15, 20). The occurrence of an additional monohydric simple phenol, o-cresol, has also been claimed (4). The separate determination of the *meta* and *para* isomers of cresol and ethylphenol was not possible with the methods used in the analysis of urinary phenols in earlier investigations. Analysis on column T in the present study showed that rats excreted these substances only as their *para* isomers.

Whether tyrosine from protein (3, 4) or phenolic compounds occurring in plants (10, 12) are the major dietary precursors of the phenol, *p*-cresol and 4-ethylphenol found in urine has not been settled (2, 8, 10). The results presented in table 3 show a significantly larger excretion ($P < 0.001$) of *p*-cresol and a significantly smaller excretion ($P < 0.001$) of 4-ethylphenol in rats fed diet 2 compared with those fed the commercial ration (diet 1). The difference in phenol excretion between these two diet groups was not significant ($P > 0.2$). Whether statistically significant or not, however, quantitative differences in the excretion of simple phenols between the diet groups must be interpreted cautiously as the intestinal microflora which is concerned with the formation of simple phenols (5, 12) is extensively influenced by diet (21). Nevertheless, the large excretion of phenol and *p*-cresol in rats fed diet 2 confirms that dietary protein is a major precursor of these compounds. The amounts of phenol and *p*-cresol derived from nonprotein plant material cannot be estimated from the results presented in table 3.

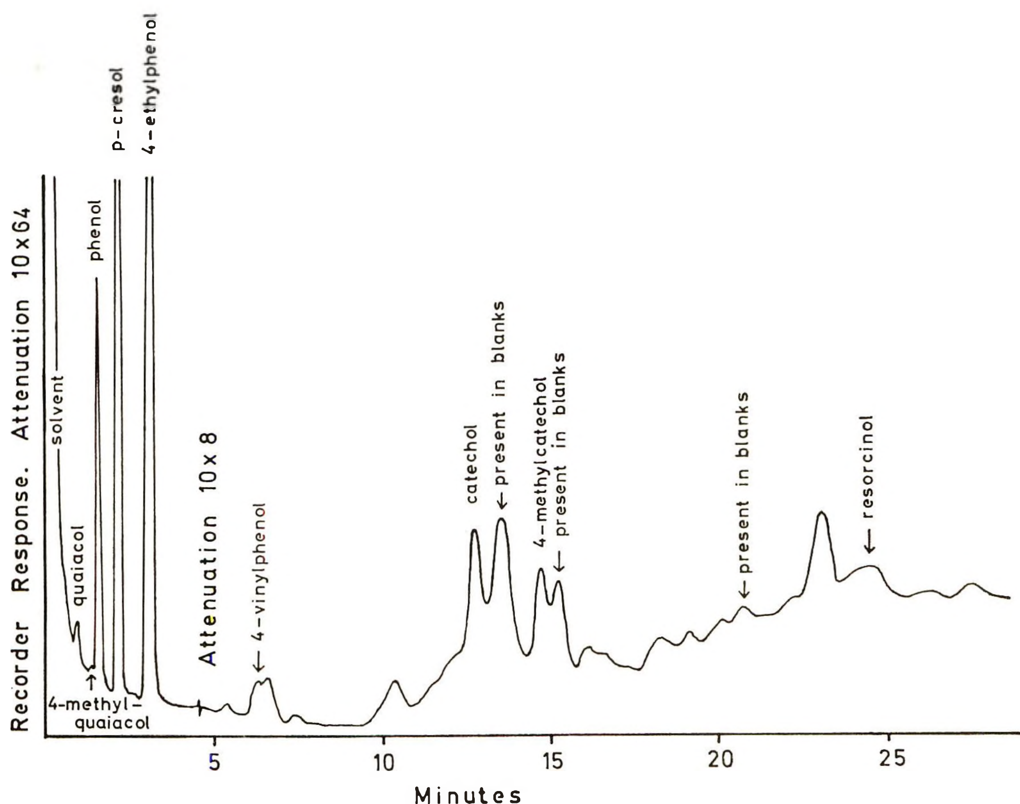

Fig. 1 Chromatogram showing temperature-programmed analysis on column C of the extract of an enzyme-hydrolyzed urine from diet group 1. Blanks were prepared by submitting 10 ml of distilled water to the hydrolysis and extraction procedure.

Baumann (3) and Williams (2) have suggested that 4-ethylphenol may be derived from dietary tyrosine. The present study demonstrated that most of the 4-ethylphenol was not derived from protein as only minute amounts were found in the urines when diet 2 was fed. It is not known which substances in plants give rise to the large quantities of this compound which were excreted by rats fed diet 1. It has been shown that *p*-coumaric acid (4-hydroxycinnamic acid), however, which is widely distributed in plants mainly as part of various esters (22), is decarboxylated and reduced to 4-ethylphenol by rat cecal contents (12).

The presence of 4-vinylphenol in the enzyme-hydrolyzed urines from rats fed diets containing plant material is a new observation. *p*-Coumaric acid is again a possible precursor because 4-vinylphenol has been identified as a metabolite of this phenolic acid after incubation with rat cecal microorganisms (12).

The trihydric and dihydric phenols which have been claimed by earlier investigators to be excreted in the urine of various species are pyrogallol (1,2,3,-trihydroxybenzene) (13), catechol (1,2-dihydroxybenzene) (23–25), resorcinol (1,3-dihydroxybenzene (26) and hydroquinone (1,4-dihydroxybenzene) (24, 25). No earlier data are available on the excretion of these compounds in rats. The extraction method used for gas chromatography in this study included treatment with sodium bicarbonate solution which resulted in considerable losses of dihydric phenols (17). However, the omission of this step in the extraction procedure employed prior to thin-layer chromatography did not reveal the presence of any simple phenols

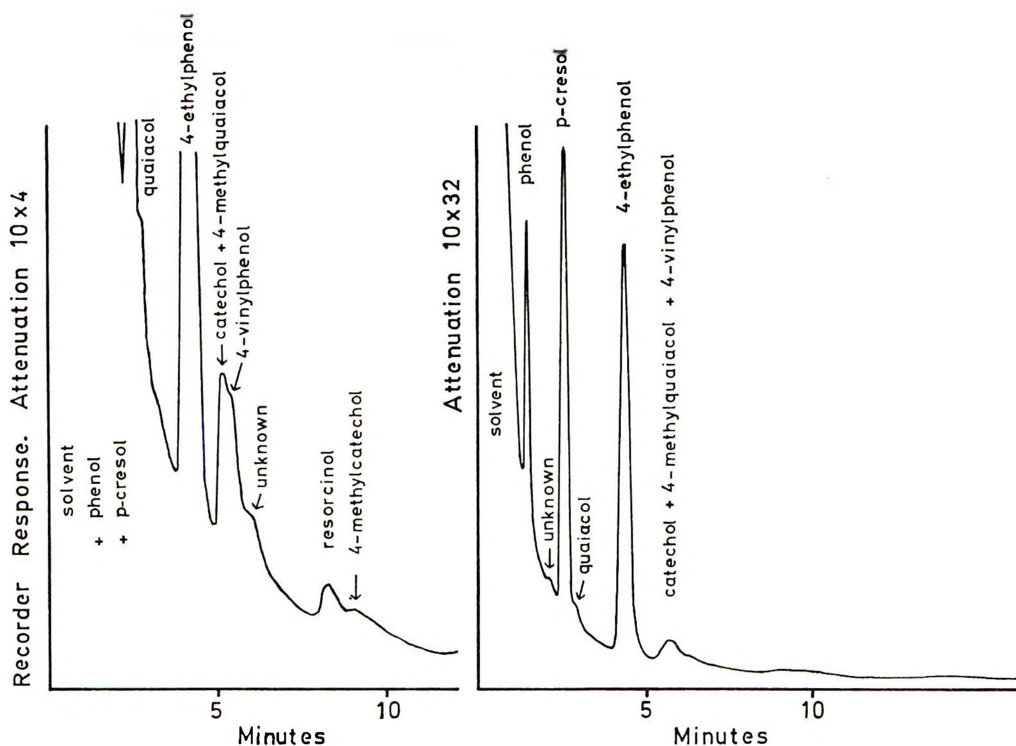

Fig. 2 Chromatograms on column S of the same urine extract as in figures 1 and 3. Tracings with two different attenuations are shown.

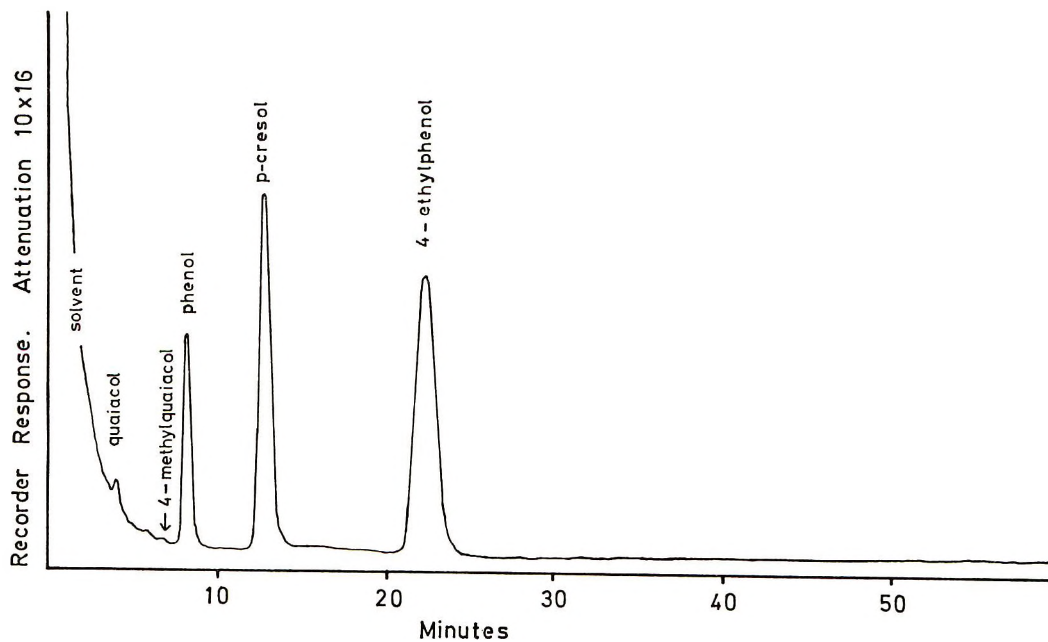

Fig. 3 Chromatogram on column T of the same urine extract as in figures 1 and 2.

TABLE 3

Quantitative determination of urinary simple phenols after acid hydrolysis

Diet fed	No. of rats	Phenol	*p*-Cresol	4-Ethylphenol	Catechol	4-Methyl-catechol
		mg/24 hours	*mg/24 hours*	*mg/24 hours*	*mg/24 hours*	*mg/24 hours*
1	11	0.57 ± 0.09 [a] [1]	0.42 ± 0.06 [a]	0.41 ± 0.03 [a]	0.20 ± 0.03 [a]	0.06 ± 0.01 [a]
2	10	0.45 ± 0.04 [a]	1.13 ± 0.07 [b]	0.012 ± 0.002 [b]	— [b] [2]	— [b]
3	5	0.27 ± 0.03 [a]	0.69 ± 0.12 [ab]	0.053 ± 0.012 [c]	0.03 ± 0.003 [c]	0.03 ± 0.004 [a]

[1] Mean ± SE of mean. Values within a column not designated with the same letter are statistically different ($P < 0.01$).
[2] Not detected.

which were not also found by gas chromatography (table 2).

Booth et al. (14) have postulated that urinary catechol is derived from dietary quinic acid (1,3,4,5-tetrahydroxycyclohexane carboxylic acid) and shikimic acid (3, 4,5 - trihydroxy-1-cyclohexene-1-carboxylic acid) because these compounds occur in plants and are metabolized to catechol in rats. This conversion has also been shown to take place when quinic and shikimic acids were incubated with rat intestinal microorganisms in vitro (27, 28). Under similar conditions protocatechuic acid (3, 4-dihydroxybenzoic acid) and vanillic acid (4-hydroxy-3-methoxybenzoic acid) yield catechol (10, 27).

Curzon and Pratt (26) suggested that resorcinol sulfate, which was excreted in the urine of some human subjects, arose from the action of intestinal microorganisms on dietary tea polyphenols. Scheline (9) has demonstrated that gallic acid (3, 4,5-trihydroxybenzoic acid), which occurs in plant material including tea leaves, is decarboxylated and dehydroxylated to resorcinol by intestinal microorganisms.

Urinary excretion of 4-methylcatechol in animals fed natural diets has not been reported previously. This compound was detected only when the plant-containing diets (1 and 3) were fed (tables 2 and 3). Bray et al. (29) showed that traces of conjugated 4-methylcatechol appeared in the urine when large doses of *p*-cresol were given to rabbits. Similarly, catechol and hydroquinone are metabolites of phenol (2). This oxidative pathway, however, is apparently not of any quantitative importance under normal conditions because no dihydric phenols were detected in the group fed diet 2. 4-Methylcatechol is a metabolite of homoprotocatechuic acid (3, 4-dihydroxyphenylacetic acid) (11) which is a metabolite of quercetin (30), a flavonoid occurring in plants. Homovanillic acid (4-hydroxy-3-methoxyphenylacetic acid) also yields 4-methylcatechol when incubated with intestinal microorganisms (12). However, the quantitative importance of these precursors of 4-methylcatechol in animals fed natural diets is unknown.

Guaiacol (2-methoxyphenol) and 4-methylguaiacol (4-hydroxy-3-methoxytoluene) are also simple phenols which have not previously been found in urine. In the case of guaiacol, Scheline (10), who found this compound after the incubation of vanillic acid with intestinal microorganisms, suggested that guaiacol might be expected in urine. The possibility that O-methylation of catechol may account for some of the guaiacol present in urine cannot be disregarded.

The natural precursors of the traces of 4-methylguaiacol found in the urine of rats fed plant-containing diets are not known. This simple phenol was not detected by thin-layer chromatography after the incubation of homovanillic acid with rat cecal contents (12). Rats fed a purified diet containing 10% tyrosine, however, excrete 4-methylcatechol and 4-methylguaiacol (19). This suggests that the latter compound may arise by O-methylation of 4-methylcatechol.

ACKNOWLEDGMENTS

The author is indebted to Dr. R. R. Scheline for his advice in the preparation of the manuscript, and to Miss E. Lund and Mr. E. Larsen for skilled technical assistance.

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Urinary Simple Phenols in Rats Fed Diets Containing Different Amounts of Casein and 10% Tyrosine [1]

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ABSTRACT Gas chromatographic analysis of extracts of hydrolyzed urines demonstrated a lower excretion of *p*-cresol ($P < 0.01$) and phenol ($P < 0.05$) in rats fed a "protein-free" diet compared with those fed 10, 20, 40 and 64% casein. The quantities of these compounds in the urine were not significantly different when rats were fed high casein diets (40 and 64%) or a diet containing 20% casein. The simple phenols excreted by rats fed a 10% tyrosine diet were analyzed by thin-layer and gas chromatography. The amounts recovered from the urines far exceed the values for urinary "volatile phenols" reported by earlier investigators. *p*-Cresol accounted for the bulk of the simple phenols found in these urines, but smaller amounts of phenol, hydroquinone, 4-methylcatechol and 4-methylguaiacol were also present. It is concluded that the formation of simple phenols is probably of considerable significance in regard to the adverse effects observed during long-term feeding of high tyrosine diets.

A previous report (1) confirmed the view of many authors (2–4) that dietary protein is a major precursor of phenol and *p*-cresol found in urine. Folin and Denis (4) and Levine et al. (5) have shown that there exists a quantitative relationship in human subjects between protein intake or nitrogen excretion and the amount of some classes of hydroxyphenyl compounds in urine. Volterra (6), however, who also determined the "volatile phenols," found that the quantities of these substances did not fluctuate significantly after high and low protein meals.

High levels of dietary tyrosine induce pathological lesions and growth inhibition (7, 8). Rats fed toxic levels of tyrosine show greatly elevated levels of urinary "total phenols" (9, 10). Bernhart and Zilliken (9) identified *p*-hydroxypyruvic acid, tyramine and possibly homogentisic acid in the urine of rats fed a 10% tyrosine diet. These investigators also found a significant elevation of volatile phenols in the urine of these animals. These earlier investigations, however, were carried out using methods of low specificity and the compounds which accounted for the bulk of the urinary total phenols and those present in the volatile phenol fraction were not identified.

The present gas chromatographic study was undertaken to obtain precise quantitative and qualitative information about the effect of protein intake and the feeding of a high tyrosine diet on the production and excretion of simple phenols.

METHODS

Animals. Male albino rats aged 5 to 6 months and weighing 300 to 360 g were used. The animals were fed a commercial pellet diet [2] and water ad libitum before the experiments.

Diet experiments. Diets containing 10, 20, 40 and 64% casein were prepared by adjusting the amounts of sucrose and casein in the purified diet described in a companion paper (1). The casein-free diet contained: (in percent) sucrose, 52; cornstarch, 32; soya oil, 10; and vitamin and salt mixtures as in the other diets. The high tyrosine diet was prepared by adding 10% *l*-tyrosine [3] to the 20% casein diet at the expense of an equivalent amount of sucrose.

The animals were placed in individual metabolic cages and allowed free access to

Received for publication December 27, 1968.
[1] Supported by a grant from Det Videnskapelige Forskningsfond av 1919, Oslo, Norway.
[2] Felleskjøpet, Oslo, Norway.
[3] Sigma Chemical Company, St. Louis, Mo.

TABLE 1

Casein intakes, urine volumes and urinary simple phenols in rats fed diets with varying casein content

Dietary casein	No. of rats	Casein intakes	Urine volumes	Phenol	p-Cresol	4-Ethylphenol	Total simple phenols
%		*g/24 hours*	*ml/24 hours*	*mg/24 hours*	*mg/24 hours*	*mg/24 hours*	*mg/24 hours*
64	5	7.2 ± 0.8 [a 1]	13.5 ± 1.2	0.44 ± 0.10 [ab 2] (0.30)	0.99 ± 0.13 [ab] (0.71)	0.013 ± 0.002 [a] (0.011)	1.44 ± 0.16 [ab] (1.02)
40	5	5.6 ± 0.4 [a]	14.1 ± 1.0	0.55 ± 0.10 [a]	0.96 ± 0.05 [a]	0.011 ± 0.001 [a]	1.52 ± 0.13 [a]
20	10	3.1 ± 0.1 [b]	9.4 ± 0.6	0.45 ± 0.04 [a] (0.31)	1.13 ± 0.07 [a] (0.89)	0.012 ± 0.002 [a] (0.012)	1.59 ± 0.07 [a] (1.21)
10	5	2.1 ± 0.1 [c]	6.1 ± 0.7	0.29 ± 0.05 [ab] (0.28)	0.76 ± 0.03 [b] (0.64)	0.011 ± 0.001 [a] (0.009)	1.06 ± 0.04 [b] (0.93)
0	5	0	5.8 ± 0.6	0.20 ± 0.03 [b] (0.14)	0.50 ± 0.06 [c] (0.35)	0.011 ± 0.002 [a] (0.009)	0.71 ± 0.07 [c] (0.50)

[1] Mean ± SE of mean. Values within a column with no superscript letter in common are statistically different ($P < 0.01$).
[2] Hydrolysis in 3 N H_2SO_4. Values in parentheses are controls after hydrolysis of pooled urine samples in 6 N H_2SO_4.

the experimental diets and to water. Urine was collected in 1 N HCl (5 ml) during two successive 24-hour periods beginning 3 days after the introduction of the experimental diets. The diet consumption and the urine volumes were recorded; the mean 24-hour casein intakes and urine volumes corrected for evaporation (0.4 ml) were calculated for each animal (table 1). The two urine portions from each rat were combined and diluted to 60 ml. Because of the differences in the mean urine volumes between the diet groups, the recovery of water pipetted into the metabolic cages was tested. For 1, 2 and 5 ml of water the recoveries were 93, 95 and 98%, respectively.

Analysis. Ten-milliliter samples of the urine dilutions from the rats fed various dietary levels of casein were hydrolyzed in boiling 3 N H_2SO_4 for 60 minutes. Ten-milliliter samples of the pooled urine dilutions from each of these diet groups were also refluxed in 6 N H_2SO_4.

Because of the large excretion of some simple phenol conjugates in the rats fed the 10% tyrosine diet, 1-ml samples were employed for the analyses of total (free + conjugated) simple phenols in these urines. These samples were diluted with water to 10 ml before hydrolysis in 3 N H_2SO_4 or with β-glucuronidase + sulfatase. For thin-layer chromatography of acid-hydrolyzed urines, and for gas chromatography of free simple phenols in nonhydrolyzed urines (table 2), 10-ml samples of the diluted urines were used.

The methods of hydrolysis and extraction and the analysis in two thin-layer chromatographic systems and on three gas chromatographic columns are described in other reports (1, 11, 12).

RESULTS

Table 1 shows the casein intakes, the urine volumes and the quantities of urinary phenol, *p*-cresol, 4-ethylphenol and their sums (total simple phenols) in the rats fed diets with different casein contents. The amounts of total simple phenols and *p*-cresol were significantly lower ($P < 0.01$) in rats fed diets devoid of casein compared with those fed the casein-containing diets. The rats fed diets devoid of casein excreted significantly lower quanti-

TABLE 2

Urinary simple phenols in rats fed a 10% tyrosine diet

Rat no.	Methods of analysis	Phenol	*p*-Cresol	4-Methyl-guaiacol	Hydro-quinone	4-Methyl-catechol
		mg/24 hours	*mg/24 hours*	*mg/24 hours*	*mg/24 hours*	*mg/24 hours*
1	F+GLC [1]	0.02	2.4	tr	—	—
	E+GLC	0.7	175	2.1	—	6.3
	A+GLC	0.6	139	tr	—	3.9
	A+TLC				—	+
2	F+GLC	0.29	1.6	—	—	—
	E+GLC	11.0	127	0.5	1.4	2.9
	A+GLC	12.1	85	—	1.7	1.9
	A+TLC				+	+
3	F+GLC	0.02	3.6	tr	—	tr
	E+GLC	0.4	184	0.9	—	3.2
	A+GLC	0.4	97	tr	—	1.3
	A+TLC				—	+
4	F+GLC	0.34	2.9	tr	—	—
	E+GLC	5.2	198	2.0	—	3.2
	A+GLC	5.1	121	tr	—	1.4
	A+TLC				+	+
5	F+GLC	0.85	3.7	—	—	—
	E+GLC	4.9	59	tr	—	1.0
	A+GLC	9.1	74	—	—	1.1
	A+TLC				+	tr

[1] F = free phenols (urine not hydrolyzed); E = enzyme-hydrolyzed urine (β-glucuronidase + sulfatase); A = acid-hydrolyzed urine (3 N H_2SO_4); GLC = gas chromatography; TLC = thin-layer chromatography; tr = trace; + = prominent; and — = not detected.

ties ($P < 0.05$) of phenol than those fed 20, 40 and 64%. The differences between the amounts of simple phenols excreted by the rats fed 10% casein and those fed the other diets are of less consistent statistical significance. 4-Ethylphenol excretion did not decrease significantly with casein intake. Urinary simple phenols failed to increase when the casein intake was doubled by increasing dietary casein from 20 to 40 or 64%.

In accordance with earlier observations (12) the absolute quantities of phenol and *p*-cresol found after hydrolysis in 6 N H_2SO_4 (pooled urines) were reduced compared with the mean values after boiling in 3 N H_2SO_4. The values obtained, however, indicate a similar relationship between the amount of simple phenols in urine and the casein intake, regardless of the acid concentration used.

The mean intake of extra tyrosine in five rats during the urine collection period was 1.6 g/24 hours (range: 1.4 to 1.8 g/24 hours). The results of the thin-layer and gas chromatographic analysis of the simple phenols in the urine of these animals are shown in table 2. There were considerable discrepancies between the amounts of *p*-cresol found with acid and with enzyme hydrolysis. This, together with the failure to detect hydroquinone by gas chromatography in rats 4 and 5, is explained by earlier observations using these methods of hydrolysis and extraction (12). Hydroquinone was detected by thin-layer chromatography only in rats 2, 4 and 5 which also showed elevated phenol excretion. Large amounts of *p*-cresol occurred together with some 4-methylcatechol in rats 1, 2, 3 and 4 whereas the smallest quantities of both compounds were found in rat 5. A similar relationship existed between the amounts of 4-methylcatechol and 4-methylguaiacol found after enzyme hydrolysis.

Traces of 4-ethylphenol were likely to have been present in the urines in addition to the simple phenols listed in table 2. However, large adjacent *p*-cresol peaks interfered with its detection on the most sensitive of the gas chromatographic columns.

DISCUSSION

Earlier investigators (4, 5) have shown that the urinary excretion of "phenolic compounds" as determined by the Folin-Ciocalteu reaction increases with increasing protein intake or nitrogen excretion. Only a few of these compounds, mainly the three simple phenols identified in the present study are volatile and these have been determined collectively by Volterra (6) using a steam-distillation method. Volterra found no diurnal variations in the excretion of volatile phenols in patients fed low protein meals at breakfast, animal protein at lunch and only vegetable proteins at dinner. The processes involved in their formation and excretion, however, may be too lengthy for these compounds to appear in the urine within a few hours after ingestion of protein.

Cornish and Ryan (13) showed that fasted rats, presumably due to a lack of dietary sulfate, excreted phenolic benzene metabolites mainly as acid-resistant glucuronides, whereas the easily hydrolyzable sulfate conjugates decreased in the urine. As shown by hydrolysis in 6 N H_2SO_4, such factors did not influence the results of the present casein study. The metabolic cages permitted nearly complete collection of the urine. It is, therefore, not likely that the differences in urine volumes (table 1) have significantly affected the recovery of simple phenols.

Bernhart and Zilliken (9) reported recoveries of 98 to 100% with phenol and *p*-cresol using acid hydrolysis, steam distillation and the Folin-Ciocalteu phenol reagent for the analysis of volatile phenols in the urine of rats fed a 10% tyrosine diet. The urinary excretion of volatile phenols did not exceed 12 mg/day in any of these rats which weighed about 150 g. This value is less than 10% of the amount of *p*-cresol alone which was found in the present study. However, these authors used a low acid concentration (0.5 N HCl) for conjugate hydrolysis. Also, their rats were fed high tyrosine diets for several weeks, whereas in this study the urine collection started only 3 days after the introduction of the experimental diet. The influence of different experimental procedures on the intestinal microflora which is involved in the degradation of tyrosine may contribute to the discrepancies between the results of the above authors and those presented here.

Baumann (3) and Williams (2) have pointed out that like phenol and *p*-cresol, urinary 4-ethylphenol is possibly derived from tyrosine through the action of intestinal microorganisms. The failure to detect 4-ethylphenol in the present study strongly suggests that the considerable amounts of this compound found in the urine of rats fed natural diets (1) are of nonprotein origin.

Large amounts of *p*-cresol were found in the urine of all of the rats in the present study. However, only rats 2, 4 and 5 excreted larger quantities of phenol than did rats fed the purified diet without tyrosine. Individual variations in the intestinal microflora may explain this observation. The dihydric simple phenols hydroquinone and 4-methylcatechol arise by oxidation of phenol (2) and *p*-cresol (14), respectively. *O*-Methylation of phenolic hydroxyl groups occurs with several compounds (2). It is probable that this metabolic reaction accounts for the 4-methylguaiacol recovered from the urines.

Deichmann and Witherup (15) investigated the acute toxicity of phenol and *p*-cresol in rats. Administered orally the LD_{50} of phenol was 0.34 to 0.54 g/kg body weight depending on the concentration of the aqueous solution or emulsion given. They also examined the relative toxicity of phenol and *p*-cresol administered orally as a 10% solution in olive oil and found that both compounds were about equally toxic. Schmidt et al. (16), finding that the conjugated volatile phenol fraction was elevated in uremia, determined the acute toxicity of *p*-tolylsulfate by intraperitoneal injection into mice. For this compound which is ordinarily the main conjugate of *p*-cresol (2, 12), the LD_{50} was 1.6 g/kg body weight. Hydroquinone is somewhat more toxic than phenol (2). Information is lacking about the toxicity of 4-methylcatechol and 4-methylguaiacol.

The quantities of free and conjugated simple phenols which were excreted by the rats in the present study reflect a formation on the order of 0.2 to 0.6 g/kg body weight per 24 hours of these compounds. It seems highly probable that the produc-

tion of simple phenols contributes to the toxic manifestations which have been observed during long-term feeding of high tyrosine diets. The physiological significance of the small amounts of simple phenols excreted by rats fed diets without extra tyrosine is unknown.

ACKNOWLEDGMENTS

The author thanks Miss Elin Lund and Mr. Olav Fjellbirkeland for skilled technical assistance.

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Alcohol and Amino Acid Transport in the Human Small Intestine [1]

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ABSTRACT The effect of ethanol on the absorption of L-methionine was studied in the human small intestine. It was shown that 2% ethanol significantly ($P < 0.01$) inhibited the absorption of this amino acid by 55%. A 40-g dose of alcohol was given orally to human volunteers as 10 and 20% solutions. Ethanol in the intestinal fluid reached concentrations of 2.5 to 3% and was maintained above 1% for 30 to 60 minutes. The possible significance of these findings is discussed in relation to the nutritional deficiencies that occur in chronic alcoholism in humans.

Ethanol [2] has been shown to inhibit the active transport of Na^+ and K^+ and the (Na + K)-activated ATPase in several tissues and species (1–3). It has also been reported, in rats, that the intestinal active transport of amino acids, a process known to be dependent on an asymmetrical distribution of Na^+ across the cell membrane, is also inhibited by ethanol both in vitro and in vivo (4, 5). We wish to report similar results in humans using L-methionine. We have chosen this amino acid because of its lipotropic properties and also because it is known that ethanol orally administered produces fatty liver even when it is given with well-balanced diets (6, 7). The latter might conceivably result or be aggravated by an inhibition of the intestinal absorption of this amino acid.

METHODS

This study was performed on normal volunteers and on subjects admitted to the hospital with signs of hyperexcitability from alcoholism. The subjects were fasted overnight and the absorption of L-methionine was determined early in the morning. A double catheter was introduced by mouth up to the small intestine to reach 15 to 20 cm below the ligament of Treitz (upper jejunum). The position of the catheter was checked fluoroscopically. The ^{3}H-L-methionine,[3] 3 mM (2000 cpm/µmole) in saline containing 1% polyethylene glycol (PEG), was perfused at 37° at a constant rate of 5 ml/minute through the catheter and 3-minute samples were obtained by continuous aspiration 15 cm distal to the infusion point. The intestinal absorption was calculated from the difference between the radioactivity of the perfusion fluid and that of the liquid collected distally. The radioactivity was measured in a liquid scintillation counter [4] and was corrected for quenching by internal standardization. The amino acid absorption was further corrected for water absorption using PEG as described by Schedl et al. (8). PEG in the aspirates was determined turbidimetrically by a modification of the method of Hyden (9). To determine the concentration of alcohol in the upper jejunum, intestinal fluid was aspirated through a thin catheter at different times after a standard dose of alcohol. Ethanol in the aspirates was determined spectrophotometrically using alcohol dehydrogenase (4).

RESULTS

Figure 1 shows the effect of 2% ethanol on the absorption of L-methionine in the small intestine. In a group of 10 subjects (4 normal and 6 alcoholics) methionine was perfused for an initial 36-minute control period. At this time ethanol was added to the perfusion fluid to a final concentration of 2% and the sample collection was

Received for publication January 21, 1969.

[1] Supported by grants from the National Institutes of Health(USA); the Foundation's Fund for Research in Psychiatry (USA); the Alcoholism and Drug Addiction Research Foundation of Ontario, Canada; and the Foundation for Overseas Research Grants and Education (USA).

[2] The words alcohol and ethanol are used interchangeably.

[3] The Radiochemical Centre, Amersham, Buckinghamshire, England.

[4] Nuclear-Chicago, Des Plaines, Ill.

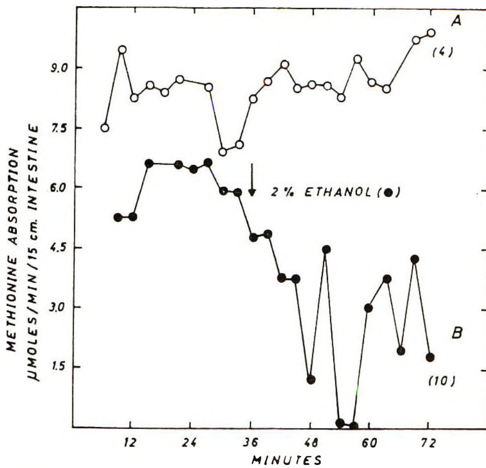

Fig. 1 Methionine absorption in the human small intestine. The points represent the average for 4 subjects in curve A and 10 in curve B. The arrow shows the time at which alcohol was added to the perfusion fluid. No alcohol was added throughout the experiment to the subjects in curve A.

continued for another 36-minute period. The mean methionine absorption (curve B of fig. 1) during this control period was 6.10 ± 0.27 (SEM) μmoles/minute per 15 cm intestine. Alcohol significantly reduced the absorption to 2.73 ± 0.5 ($P < 0.01$ by the t test. The same degree of significance was obtained when paired data were used). In this group the results for six alcoholics and for four normal subjects are presented. Because no significant difference was found between controls and alcoholics with respect to the methionine absorption or to the inhibition produced by alcohol, the values were pooled. In a separate group of four subjects, two normal and two alcoholic, the absorption of methionine was measured for 72 minutes without the addition of alcohol. Although considerable individual variation was found, the absorption remained constant for the same subject during the study period. The mean methionine absorption in the initial 36-minute period (group A of fig. 1) was 7.75 ± 0.54 μmoles/ minute per 15 cm intestine, and 8.67 ± 0.31 in period 2 ($P = 0.7$).

It was of interest to determine whether the concentrations of alcohol that inhibit the absorption of methionine can be found in the human small intestine in moderate drinking. Figure 2 shows that these intestinal concentrations do indeed occur and that they are maintained for rather long periods of time. When 40 g of ethanol were administered to two normal subjects in a final volume of 200 and 400 ml (20 and 10% solutions) the concentration of alcohol in the jejunum reached maximum values of 2.5 to 3% and was maintained above 1% for 30 and 60 minutes, respectively (fig. 2).

DISCUSSION

Our results demonstrate that 2% ethanol, a concentration that can be found in the human intestine in moderate drinking, inhibits by 55% the intestinal absorption of L-methionine. In our previous work with rats in vivo (4), ethanol given orally as a 25% solution to a final dose of 250 mg/100 g body weight, was found to inhibit the intestinal absorption of L-phenylalanine by about 50%. In that study it could be seen that alcohol prevented the amino acid absorption rather than delaying it. When 40 g of ethanol were given as 10 and 20% solutions to control subjects, alcohol in the intestine was found to reach values above 2% and was maintained over 1% for up to 1 hour when it was given in the more diluted form. It should be considered that some alcoholics consume amounts as high as 500 g eth-

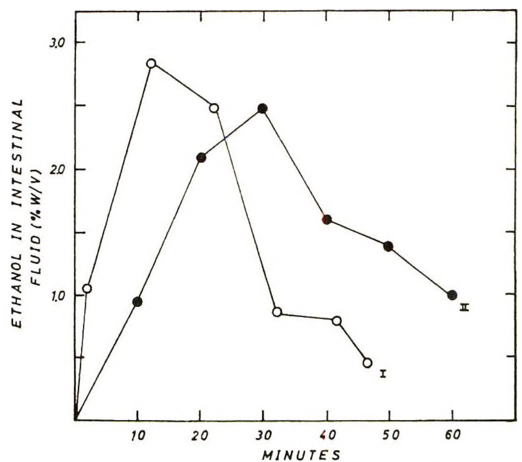

Fig. 2 Ethanol concentration in the intestinal fluid after the oral administration of 40 g of alcohol. (I) Alcohol was given as a 20% solution. (II) Ethanol was given as a 10% solution.

anol/day, divided in small doses of relatively low concentrations. Thus, it is not unlikely that these subjects could maintain constant high concentrations of alcohol not only in the proximal but also in the distal segments of the small intestine. Recent work by Schedl et al. (8) has shown that the maximum rate for the absorption of methionine in the distal segments is only 1/5 to 1/6 that in the proximal regions.

It is conceivable, therefore, that the syndromes of protein deficiency in humans might be aggravated, or even produced, by the inhibitory effect of alcohol on the intestinal amino acid absorption that we are describing. To what extent is this effect responsible for the production of liver steatosis, known to occur in alcoholism, should be a matter of further studies.

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Effect of α-Aminoisobutyric Acid on Arginine Metabolism in Chicks [1,2]

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ABSTRACT The addition of 0.5% α-aminoisobutyric acid (AIB), a presumably nonmetabolizable amino acid, to a casein basal diet supplemented with graded levels of L-arginine·HCl caused an accumulation of free arginine and a depression of free ornithine in both plasma and muscle of chicks, and also altered the levels of some other amino acids. The differential effect on free amino acids was more pronounced in muscle than in plasma. Injection of AIB into the breast muscle of chicks fed the casein diet supplemented with 3% L-arginine·HCl gave an effect on arginine, ornithine and other amino acids similar to that given by oral feeding of AIB. Further observations of significance were that the weight gain and feed efficiency of chicks fed the casein basal diet were improvd by supplementation with AIB, and that kidney arginase activity was greatly depressed when AIB was fed. Arginine metabolism was also affected by AIB in chicks fed a basal diet based on wheat gluten supplemented with arginine, but the growth response to AIB supplementation found with the casein diet was not observed. Possible mechanisms of the influence of AIB on arginine metabolism are discussed in relation to the experimental findings.

The behavior of α-aminoisobutyric acid (AIB) in living cells and mammalian tissues has been extensively studied (1–4). Owing to its nonmetabolizable characteristics in tissue, this amino acid has been used to study problems of amino acid transport or absorption, and Smith (5) suggested that AIB might serve as an internal indicator in chick plasma amino acid studies.

In some of our experiments designed to study the distribution of free amino acids between plasma and muscle of chicks, AIB was incorporated into purified diets as a reference amino acid. The chicks receiving AIB, however, revealed some unusual amino acid patterns in the plasma and muscle tissue. The experiments reported here were conducted to study these effects.

EXPERIMENTAL

Commercially obtained White Plymouth Rock pullets were fed a practical starter ration from 1 day to 7 days of age. At day 7, chicks within a narrow range of the average body weight were fed preliminary diets for an adaptation period of 4 days. At the end of this period, the chicks were further selected on the basis of body weight and distributed into groups (10 or 15 chicks) of the same average weight. The composition of the basal diets is given in table 1. The experimental diets were formulated from these basal diets by supplementation with varying levels of L-arginine·HCl at the expense of glucose. Treatment diets containing AIB were prepared from the corresponding control diets by adding 0.5% AIB [3] without compensation. The preliminary diets for the adaptation period mentioned were the experimental diets with presumably adequate supplementation of L-arginine·HCl; that is, the casein diet supplemented with 1% L-arginine·HCl, or the wheat gluten diet supplemented with 0.52% L-arginine·HCl. The experimental diets were fed to duplicate groups for 7 days. Some alterations of procedure are noted in the description of particular experiments. Throughout the experiments, all the chicks were housed in elec-

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Received for publication December 23, 1968.

[1] Supported in part by a grant from the National Research Council of Canada and from the Ontario Department of Agriculture and Food. The senior author was a recipient of a scholarship from the National Research Council of Canada.

[2] From a thesis submitted by the senior author in partial fulfillment of the requirements for the degree of Doctor of Philosophy, University of Guelph, Guelph, Ontario, Canada.

[3] α-Aminoisobutyric acid, purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio, was homogeneous by paper chromatography (manufacturer's analysis), and gave a single peak using ion exchange and gas-liquid chromatography of the butyl ester derivative.

TABLE 1
Basal diets

	Casein diet	Wheat gluten diet
	%	%
Casein [1]	20.0	
Wheat gluten [1]	—	22.64 [2]
Glucose monohydrate	63.66	60.43
Cellulose [3]	3.0	3.0
Corn oil	4.0	4.0
Salts [4]	6.0	6.0
Vitamin mixture [5]	2.0	2.0
Choline chloride [6]	0.44	0.44
Glycine	0.6	—
L-Cystine	0.3	—
L-Methionine	—	0.10
DL-Tryptophan	—	0.04
DL-Threonine	—	0.42
L-Lysine·HCl	—	0.93
	100.00	100.00

[1] Vitamin-free casein, wheat gluten, purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio.
[2] The quantity was equivalent to 20% casein on N basis.
[3] Alphafloc, purchased from Lee Chemicals, Ltd., Toronto, Ontario, Canada.
[4] Fox-Briggs N mineral mixture (16), purchased from General Biochemicals Inc., Chagrin Falls, Ohio. A supplement of 5 mg of $Na_2MoO_4 \cdot 2H_2O$/kg diet was also included.
[5] Contributed in milligrams per kilogram diet: vitamin E conc (44,000 IU/kg), 816.0; vitamin A conc (10,000 IU/g), 1060; vitamin D_3 conc (20,000 ICU/g), 48; riboflavin, 11.5; Ca pantothenate, 40.6; niacin, 105.8; biotin, 0.352; folic acid, 2.2; menadione, 20.2; thiamine·HCl, 8.0; pyridoxine·HCl, 14.0; vitamin B_{12} (0.1% triturate), 40.6; p-aminobenzoic acid, 20.0; and inositol, 1102.0.
[6] A mixture of equal parts of pure choline chloride and a 25% choline chloride concentrate.

trically heated batteries with feed and water supplied ad libitum.

At the end of the experimental period, chicks were weighed and decapitated; about 2 ml of blood were collected individually from the carotid artery into vials containing a droplet of heparin solution (10 mg/ml in 0.9% NaCl solution). The blood samples were then pooled in a stoppered centrifuge tube and stored in an ice bath until centrifuged. After centrifuging, the supernatant plasma and precipitated red blood cells were separated and 5 ml of the plasma was mixed with 2 volumes of 4.5% sulfosalicylic acid (w/v) and the precipitated protein was removed by centrifugation and filtration. The deproteinized samples were kept in tightly closed bottles and stored at − 20° until analyzed.

Muscle extracts were prepared by the following procedure: A piece of muscle was cut from the biceps fraction of the right leg immediately after the blood was drained. The tissue was immediately frozen and stored in dry ice for a few days until extractions were made. Muscle samples from each pen were minced and mixed, and 2 g of the minced muscle was homogenized with 10 ml of 4.5% sulfosalicylic acid in an ice bath with a mixer.[4] The homogenate was centrifuged and the supernatant was filtered. The muscle extracts were kept in tightly closed bottles at − 20°.

The plasma and muscle samples were analyzed for amino acids within 3 weeks by ion exchange chromatography using an amino acid analyzer (130-cm column).[5]

In the experiment to study arginase activity, both kidneys were excised and frozen immediately. Each pair of kidneys was sealed in a plastic bag and stored in dry ice. Kidney arginase activities were determined by the method of Smith and Lewis (6), except that the homogenate concentration in the reaction mixture was increased to 33.3 mg/ml (or 100 mg/ml homogenate) in an attempt to compare the high and low arginase activity of the samples from each treatment under the comparable condition of homogenate concentration. The relationship between homogenate concentration and urea formation was tested with the kidneys of chicks fed a 3% L-arginine·HCl–casein basal diet. Three concentrations: 100, 50, and 25 mg/ml homogenates were prepared with 0.001 M $MnSO_4$–maleate buffer at pH 7.0 and incubated at 37° for 70 minutes. The rate of urea formation at the concentration of 100 mg/ml homogenate was found linearly related to that at 50 mg/ml. When the concentration was diluted to 25 mg/ml the specific activity of the enzyme was slightly decreased. Urea was determined colorimetrically by using 1-phenyl-1,2-propanedione-2-oxime as reagent according to the method of Van Slyke and Archibald (7).

Statistical significance between pairs of means for planned comparisons was made

[4] Omni-Mixer Homogenizer, Ivan Sorvall Inc., Norwalk, Conn.
[5] Technicon Corporation, Ardsley, N. Y.
[6] Supplementation with arginine was found previously to cause depressions in certain amino acids. Unpublished data.

by the "least significant difference" test (8).

RESULTS AND DISCUSSION

The effect of supplementation with 0.5% AIB on the response of plasma and muscle free amino acids to three levels of arginine supplementation of the casein diet was studied (table 2, figs. 1 and 2). Plasma arginine in the AIB-treated groups was markedly elevated as compared with that in the corresponding control groups. This elevation of plasma arginine was accompanied by a depression of plasma ornithine (fig. 1). The free arginine and ornithine in the muscle were affected in a similar way by the AIB but the differential effect was more pronounced (fig. 2).

Certain other plasma amino acids showed characteristic changes when the diet was supplemented with arginine.[6] In particular, plasma lysine, threonine, glycine and valine were depressed (9, 10). The addition of AIB depressed lysine and valine still further when supplemental arginine was in the diet (table 2). However, the effect of AIB on plasma threonine and glycine was less marked. A similar depressing effect of AIB was also found for leucine when the diet was supplemented with arginine. There were apparent effects of AIB on other amino acids in plasma in the group fed the casein basal diet; however, their significance is not clear.

The depressing effect of AIB on lysine, valine and leucine was similar in muscle and plasma, but its effect on threonine and glycine was only observed in muscle. In addition, histidine was also decreased in muscle when AIB was added. Other muscle amino acids, isoleucine, glutamic acid, alanine and a dipeptide carnosine, were also affected.

It is suggested that the observed effects of AIB on arginine, ornithine and lysine might result from an alteration in the operation of one or both of two mechanisms: 1) changes occurred in intestinal absorption or renal reabsorption of lysine, arginine or ornithine by a simple competitive action of AIB or, 2) metabolic changes involving a direct effect of AIB on enzyme activities occurred. If the effects were caused chiefly by the former, the amino acid patterns in blood or muscle of chicks fed AIB for either a long period, or a short period, should show little difference. An experiment was carried out to test this assumption. Chicks were raised in a similar manner as in the previous experiment, i.e., fed a starter ration for 7 days, and fed the preliminary diet for 4 days. The long-period feeding groups were changed to the 3% L-arginine·HCl-casein diet for an additional 7 days. The short-period groups were continued on the 1% L-arginine·HCl preliminary diet for 7 days until the last 2.5 hours. At this time, feed was removed for 30 minutes and then 3% L-arginine·HCl–casein diet was fed for the final 2 hours. At the end of 2 hours the chicks in both treatment groups were killed and samples taken at the same time.

The effect of AIB on the plasma free arginine and ornithine of chicks fed the 3% L-arginine·HCl–casein diet for 7 days was similar to that observed in the previous experiment (table 3). In the 2-hour group the plasma arginine level responded to dietary arginine and was elevated to a level higher than that of the control group fed 3% L-arginine·HCl for 7 days. The conversion of excess arginine into ornithine is the likely explanation of the marked increase of plasma free ornithine. Treated chicks in the 2-hour group, however, showed no sign of the effect of AIB, that is, no accumulation of arginine accompanied by a depression of ornithine. The evidence, therefore, favors the possibility that AIB exerted its effect through a direct metabolic change involving enzyme activities.

Further evidence that the effect of AIB was not mitigated through an influence on intestinal absorption came from an experiment in which AIB was injected into chicks receiving a casein diet supplemented with 3% L-arginine·HCl (table 4). The AIB, dissolved in 0.9% NaCl, was injected daily into the breast muscle over a period of 7 days in increasing amounts, with the total intake for the 7 days being approximately equal to the AIB consumed in previous experiments over the same period of time, namely, 890 mg AIB/chick per 7 days. Plasma and muscle arginine were elevated and ornithine depressed in essentially the same manner as they were when

TABLE 2

Effect of dietary AIB on the free amino acids in the plasma and muscle of the chick fed a casein basal diet supplemented with graded levels of arginine

Amino acid	Plasma [1]						Muscle [1]					
	L-Arginine·HCl added						L-Arginine·HCl added					
	0%		1.0%		3.0%		0%		1.0%		3.0%	
	Control	+ AIB [2]	Control	+ AIB [2]	Control	+ AIB [2]	Control	+ AIB [2]	Control	+ AIB [2]	Control	+ AIB [2]
	µmoles/100 ml plasma						*µmoles/100 g tissue*					
AIB	0	54 ± 1	0	57 ± 3	0	47 ± 5	0	140 ± 1	0	167 ± 3	0	167 ± 34
Arginine	7 ± 0	11 ± 1	28 ± 0	61 ± 0 [a]	58 ± 5	183 ± 5 [a]	21 ± 3	26 ± 3	134 ± 8	472 ± 2 [a]	346 ± 5	1640 ± 29 [a]
Ornithine	4 ± 0	3 ± 1 [b]	13 ± 0	4 ± 0 [a]	73 ± 0	9 ± 1 [a]	14 ± 0	11 ± 0	46 ± 1	20 ± 4 [a]	272 ± 3	13 ± 0 [a]
Lysine	189 ± 2	180 ± 1	147 ± 3	99 ± 4 [a]	135 ± 5	76 ± 3 [a]	882 ± 7	1024 ± 23	1059 ± 79	793 ± 47 [b]	790 ± 88	552 ± 2 [b]
Threonine	257 ± 20	272 ± 8	111 ± 2	87 ± 5	68 ± 2	86 ± 1	427 ± 49	435 ± 15	311 ± 34	179 ± 13 [b]	174 ± 4	140 ± 12
Glycine	81 ± 1	93 ± 5 [b]	70 ± 0	65 ± 3	63 ± 2	60 ± 2	447 ± 19	437 ± 3	380 ± 17	243 ± 7 [a]	333 ± 9	225 ± 19 [a]
Valine	57 ± 1	57 ± 2	48 ± 2	39 ± 3 [b]	49 ± 0	38 ± 1 [a]	42 ± 2	42 ± 0	40 ± 2	33 ± 1 [b]	47 ± 3	25 ± 1 [b]
Methionine	12 ± 0	14 ± 1 [a]	13 ± 1	12 ± 0	10 ± 0	12 ± 0 [b]	23 ± 5	26 ± 0	23 ± 0	18 ± 1	22 ± 1	21 ± 1
Isoleucine	21 ± 1	22 ± 1	17 ± 1	15 ± 2	17 ± 1	14 ± 1	16 ± 1	16 ± 0	14 ± 0	9 ± 0 [a]	13 ± 2	8 ± 0 [a]
Leucine	32 ± 1	36 ± 1	32 ± 2	27 ± 1 [b]	31 ± 1	26 ± 1 [b]	26 ± 2	28 ± 2	29 ± 0	23 ± 1 [b]	30 ± 2	19 ± 2 [b]
Phenylalanine	16 ± 1	18 ± 1	18 ± 1	17 ± 1	16 ± 1	17 ± 0	19 ± 1	20 ± 1	27 ± 2	26 ± 0	24 ± 4	20 ± 0
Histidine	24 ± 1	29 ± 2 [b]	26 ± 2	24 ± 0	24 ± 1	23 ± 1	170 ± 2	131 ± 11 [a]	133 ± 6	95 ± 6 [a]	120 ± 6	66 ± 6 [a]
Aspartic acid	3 ± 0	3 ± 0	3 ± 0	3 ± 0	4 ± 0	3 ± 1	49 ± 6	44 ± 1	38 ± 3	32 ± 3	36 ± 0	29 ± 2
Serine and glutamine	178 ± 7	225 ± 10 [a]	157 ± 2	161 ± 10	138 ± 9	138 ± 1	821 ± 43	809 ± 19	804 ± 3	549 ± 7 [a]	653 ± 7	358 ± 27 [a]
Glutamic acid	15 ± 1	22 ± 2 [b]	16 ± 0	19 ± 0	15 ± 2	15 ± 1	153 ± 2	173 ± 0 [a]	168 ± 6	132 ± 4 [a]	163 ± 0	122 ± 3 [a]
Proline	—	175 ± 15	—	131 ± 6	—	124 ± 9	—	301 ± 0	—	296 ± 30	—	398 ± 36
Alanine	64 ± 4	93 ± 3 [a]	81 ± 0	90 ± 4	69 ± 0	75 ± 5	277 ± 1	314 ± 1	352 ± 14	295 ± 26 [b]	312 ± 6	174 ± 7 [a]
Cystine (1/2)	11 ± 0	13 ± 1	8 ± 0	8 ± 0	7 ± 1	8 ± 1	—	—	—	—	—	—
Tyrosine	33 ± 1	43 ± 0 [a]	31 ± 2	27 ± 1	22 ± 3	26 ± 1	36 ± 3	47 ± 2 [b]	41 ± 4	29 ± 2 [b]	26 ± 1	27 ± 1
Carnosine	—	—	—	—	—	—	156 ± 14	216 ± 10 [a]	295 ± 1	287 ± 11	252 ± 7	101 ± 8 [a]

[1] Average ± range/2 of two determinations, each on a pooled sample from replicate pens of 15 chicks. Range is the difference between the results for the two determinations.

[2] Added at a level of 0.5%.

[a] Value is significantly different from control value ($P < 0.01$).

[b] Value is significantly different from control value ($P < 0.05$).

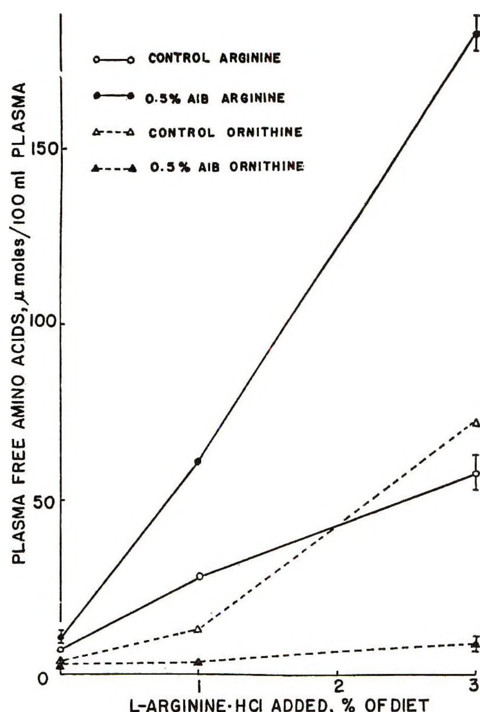

Fig. 1 Effect of dietary AIB on plasma arginine and ornithine in chicks fed a casein diet supplemented with graded levels of arginine (data from table 2).

AIB was given orally. The pattern of other amino acids, lysine, threonine, glycine, valine and histidine as affected by arginine and AIB (0.5%), was also similar to that previously observed (table 2).

If the effect of AIB on plasma arginine and ornithine is due to a metabolic change in the body, the enzyme arginase or transamidinase might be directly involved. Because arginase in the chick kidney is very active (6) and sensitive to dietary arginine (11, 12), a study was made of kidney arginase activity and plasma urea level in chicks fed diets supplemented with 3% L-arginine·HCl and 0.5% AIB. The results are given in table 5.

The individual variation in the kidney arginase activity of the chick was very large in the present study. This has been found by other workers (11–13). However, it is clear that AIB in the diet greatly depressed kidney arginase activity of chicks. Plasma urea was decreased in the AIB-treated chicks, although the depression was not of the same degree as that observed for arginase. This lesser effect might be explained by some renal reabsorption of urea.

In the experiments which yielded the amino acid data given in tables 2 and 3, weight gain and feed efficiency of the chicks were recorded (table 6). Of considerable interest is the apparent improvement in weight gain and feed efficiency when AIB was added to the arginine-deficient basal diet. A simple explanation of this effect could be a sparing effect of AIB on arginine, through a depression of arginase activity. The casein basal diet is excessively high in lysine content, however, and, in view of the known antagonism of lysine to arginine (14, 15), the possibility exists that an interrelationship of lysine and AIB might be concerned. To test this possibility an experiment was conducted in which casein was replaced by wheat gluten (see table 1), and the lysine

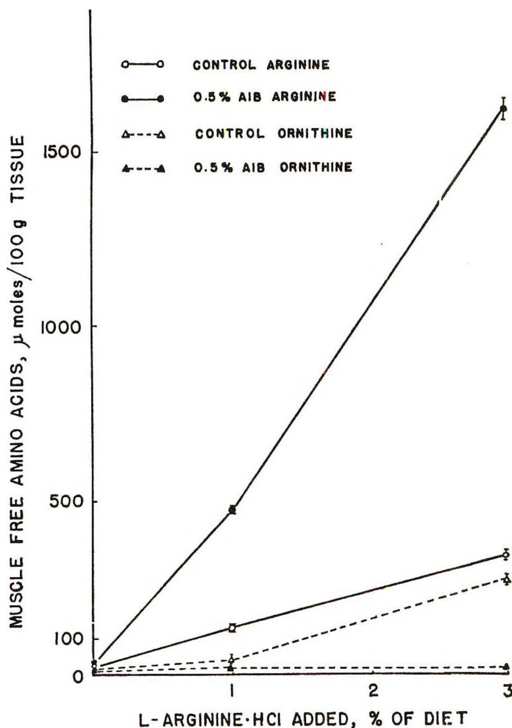

Fig. 2 Effect of dietary AIB on muscle arginine and ornithine in chicks fed a casein diet supplemented with graded levels of arginine (data from table 2).

TABLE 3

Effect of feeding period with 0.5% AIB on plasma amino acid pattern of chicks fed a casein diet supplemented with 3% L-arginine·HCl

Amino acid	7 day[1]		2 hour[1]	
	Control	AIB	Control	AIB
	μmoles/100 ml plasma		*μmoles/100 ml plasma*	
AIB	0	60±6	0	nm[2]
Arginine	48±1	170±5[a]	65±4	72±3
Ornithine	51±8	11±5[a]	36±1	43±3
Lysine	154±4	86±4[a]	155±8	148±3

[1] Average ± range/2 of two determinations, each on a pooled sample from replicate pens of 15 chicks. Range is the difference between the results for the two determinations.
[2] Small peak, not measurable.
[a] Value is significantly different from control value ($P < 0.01$).

TABLE 4

Effect of AIB injection on plasma and muscle amino acids of chicks fed a casein diet supplemented with 3% L-arginine·HCl

Amino acid	Plasma[1]		Muscle[1]	
	Saline	AIB	Saline	AIB
	μmoles/100 ml plasma		*μmoles/100 g tissue*	
AIB	0	34±2	0	151±4
Arginine	49±2	167±15[b]	287±21	1461±6[a]
Ornithine	56±2	10±1[a]	179±12	22±1[a]
Lysine	103±1	63±1[a]	640±6	495±18[b]
Threonine	62±1	70±2	149±3	124±3[b]
Glycine	53±5	55±5	327±32	184±3[b]
Valine	49±0	38±2[b]	37±1	24±0[a]
Isoleucine	17±1	13±0	10±3	7±1
Leucine	32±2	26±0[b]	27±2	18±1[b]
Phenylalanine	18±1	16±1	16±2	14±0
Histidine	19±1	19±1	153±4	79±1[a]
Aspartic acid	4±1	3±0	46±0	28±1
Serine and glutamine	128±3	118±0	580±22	326±17
Glutamic acid	15±0	14±0	146±1	119±2
Alanine	71±4	67±4	352±16	183±8[b]
Tyrosine	23±1	23±2	36±0	30±3
Carnosine	—	—	298±35	155±14

[1] Average ± range/2 of two determinations, each on a pooled sample from replicate pens of 15 chicks. Range is the difference between the results for the two determinations.
[a] Value is significantly different from control value ($P < 0.01$).
[b] Value is significantly different from control value ($P < 0.05$).

level was adjusted to avoid an excess of lysine and give approximately the National Research Council (NRC) requirement.

The results of this experiment are given in table 7. The growth data show that the wheat gluten diet was deficient in arginine, since supplementation of the basal diet with 0.52% of L-arginine·HCl improved the growth. However, 0.5% AIB in the basal diet did not improve the growth as it did when added to the casein basal diet. The plasma and muscle amino acid data also showed that AIB did not affect plasma and muscle free arginine and ornithine of the chick fed the basal diet without arginine supplementation. When supplemental arginine was added, however, the effect of AIB was evident, particularly, in the case of chicks receiving the diet supplemented with 3% L-arginine·HCl.

The foregoing data clearly show that supplementation with AIB at the 0.5% level caused an alteration of plasma and muscle free arginine and ornithine of chicks. Furthermore, the addition of 0.5%

TABLE 5

Effect of dietary AIB on kidney arginase and plasma urea of chicks fed a casein diet with and without supplemental arginine

		Arginase activity [1]	Urea [2,3]
		μmoles urea/ hour per kidney	*μg urea/ ml plasma*
Basal diet (table 1)	Control	9970 ± 1970	37 ± 14
	0.5% AIB	780 ± 250 [a]	22 ± 3
Basal diet + 0.3% L-arginine·HCl	Control	20,250 ± 1920	129 ± 18
	0.5% AIB	680 ± 240 [a]	54 ± 16 [b]

[1] Mean ± SE is based on individual determinations on 10 chicks housed in two pens, 5 chicks in each.
[2] Average ± range/2 of determinations on two samples of plasma, each a pooled sample for a pen of five chicks.
[3] Determined by method of Archibald (17) with sulfosalicylic acid in the urea standard solution.
[a] Value is significantly different from control value ($P < 0.01$).
[b] Value is significantly different from control value ($P < 0.05$).

TABLE 6

Effect of dietary AIB on weight gain and feed efficiency of chicks fed a casein basal diet supplemented with graded levels of L-arginine·HCl

	Exp. 1		Exp. 2	
	Wt gain [1]	Feed/gain	Wt gain [1]	Feed/gain
	g/chick per week		*g/chick per week*	
(1) Basal diet	35 ± 1	3.62 ± 0.04	39 ± 4	3.72 ± 0.29
(2) (1) + 0.5% AIB	63 ± 8 [b]	2.41 ± 0.17	59 ± 2 [b]	2.47 ± 0.01
(3) (1) + 0.5% L-arginine·HCl	—	—	99 ± 4	1.92 ± 0.05
(4) (3) + 0.5% AIB	—	—	106 ± 5	1.76 ± 0.01
(5) (1) + 1% L-arginine·HCl	110 ± 1	1.65 ± 0.01	109 ± 6	1.71 ± 0.10
(6) (5) + 0.5% AIB	115 ± 1	1.56 ± 0.02	108 ± 7	1.72 ± 0.06
(7) (1) + 3% L-arginine·HCl	113 ± 1	1.58 ± 0.01	109 ± 1	1.65 ± 0
(8) (7) + 0.5% AIB	108 ± 6	1.54 ± 0.04	104 ± 3	1.73 ± 0.02

[1] Average ± range/2 of two pens of 15 chicks each. Range is the difference between the average for two pens.
[b] Value is significantly different from corresponding control value ($P < 0.05$).

AIB to the casein basal diet or this basal diet supplemented with 3% L-arginine·HCl depressed kidney arginase activity and also reduced the level of urea in plasma (table 5). If it is accepted that excess arginine is converted into ornithine and urea by arginase in the kidney of chicks, the observed depression of kidney arginase activity could be considered responsible for the reduced plasma urea, the accumulation of arginine and the depression of ornithine in plasma and muscle.

The growth-promoting effect of AIB for the chick fed the casein basal diet is of considerable interest. Casein is known to be a protein containing an excess of lysine for the chick (14). Nesheim (13) reported that there were high arginine-requirement and low arginine-requirement strains of chicks. In both strains, dietary excess of lysine caused a marked increase in kidney arginase activity, but the strain with the high arginine requirement was particularly sensitive. It is not known whether the chicks used in the present experiments were of a high or low arginine requirement strain. However, a comparison of kidney arginase of the chicks fed the casein basal diet (table 5) with arginase activity as reported by Nesheim (13) suggests that most of chicks used in the present study were of a high arginine requirement strain. Jones et al. (11) reported that excess lysine in a casein-gelatin diet or a crystalline amino acid diet caused a reduction in growth rate and an increase in kidney arginase of chicks. O'Dell et al. (12) found that there was an inverse correlation of the growth rate and the kidney arginase activity in the chicks fed a casein basal diet supplemented with a suboptimal level of arginine.

TABLE 7

Effect of dietary AIB on the free amino acids in the plasma and muscle of the chick fed a wheat gluten diet supplemented with graded levels of arginine

Amino acid	Plasma [1]						Muscle [1]					
	L-Arginine·HCl added						L-Arginine·HCl added					
	0%		0.52%		3.0%		0%		0.52%		3.0%	
	Control	+ AIB [2]	Control	+ AIB [2]	Control	+ AIB [2]	Control	+ AIB [2]	Control	+ AIB [2]	Control	+ AIB [2]
	μmoles/100 ml plasma						*μmoles/100 g tissue*					
AIB	0	50 ± 3	0	44 ± 2	0	41 ± 2	0	127 ± 15	0	149 ± 18	0	103 ± 7
Arginine	8 ± 1	5 ± 0	39 ± 2	44 ± 2	60 ± 1	185 ± 13 [a]	13 ± 2	10 ± 1	154 ± 16	190 ± 17	341 ± 29	1388 ± 11 [a]
Ornithine	1 ± 0	2 ± 0	4 ± 0	3 ± 0	109 ± 4	9 ± 2 [a]	7 ± 1	5 ± 0	16 ± 1	13 ± 1	453 ± 24	19 ± 6 [a]
Lysine	78 ± 5	75 ± 2	37 ± 0	37 ± 0	52 ± 2	31 ± 8 [b]	335 ± 19	341 ± 16	196 ± 1	200 ± 11	263 ± 41	129 ± 31 [a]
Threonine	204 ± 4	218 ± 7	97 ± 1	115 ± 8 [b]	85 ± 1	88 ± 1	376 ± 28	348 ± 26	233 ± 6	199 ± 2	182 ± 5	125 ± 2 [b]
Glycine	67 ± 2	70 ± 4	43 ± 1	44 ± 1	40 ± 1	41 ± 0	417 ± 19	439 ± 15	149 ± 1	142 ± 12	160 ± 10	116 ± 3 [b]
Valine	33 ± 2	32 ± 4	16 ± 0	17 ± 1	14 ± 0	16 ± 2	22 ± 0	22 ± 1	8 ± 0	9 ± 0	5 ± 1	6 ± 1
Methionine	9 ± 1	9 ± 1	9 ± 1	8 ± 0	6 ± 0	8 ± 1	11 ± 1	10 ± 2	10 ± 1	10 ± 0	5 ± 0	8 ± 0
Isoleucine	16 ± 1	18 ± 0	12 ± 0	12 ± 1	12 ± 1	11 ± 1	14 ± 1	13 ± 0	9 ± 0	9 ± 0	8 ± 0	6 ± 1
Leucine	26 ± 3	26 ± 1	21 ± 0	22 ± 2	22 ± 1	19 ± 1	24 ± 1	23 ± 1	20 ± 1	18 ± 1	19 ± 0	13 ± 1 [a]
Phenylalanine	16 ± 2	17 ± 1	15 ± 0	15 ± 0	15 ± 0	14 ± 1	16 ± 2	21 ± 4	20 ± 2	19 ± 2	16 ± 2	11 ± 1
Histidine	26 ± 2	27 ± 0	16 ± 0	13 ± 1	14 ± 1	15 ± 2	235 ± 8	160 ± 2 [a]	201 ± 8	145 ± 14 [a]	162 ± 6	100 ± 3 [a]
Aspartic acid	4 ± 0	4 ± 0	3 ± 1	3 ± 0	3 ± 0	3 ± 1	45 ± 1	35 ± 7	39 ± 1	31 ± 5	39 ± 2	23 ± 4 [b]
Serine and glutamine	183 ± 16	166 ± 13	137 ± 2	135 ± 5	114 ± 3	111 ± 1	683 ± 38	582 ± 67	551 ± 21	454 ± 36	476 ± 34	259 ± 22 [a]
Glutamic acid	22 ± 2	30 ± 1 [b]	19 ± 2	19 ± 2	14 ± 1	14 ± 1	144 ± 5	138 ± 17	141 ± 11	112 ± 2	130 ± 12	82 ± 1 [b]
Proline	—	252 ± 4	—	204 ± 11	179 [3]	164 [3]	—	439 ± 73	—	512 ± 17	—	542 ± 8
Alanine	80 ± 3	81 ± 2	59 ± 0	62 ± 2	53 ± 1	54 ± 0	304 ± 5	248 ± 20 [a]	241 ± 9	211 ± 9	241 ± 7	141 ± 6 [a]
Cystine (1/2)	12 ± 1	14 ± 1	9 ± 0	9 ± 1	7 ± 1	8 ± 0	—	—	—	—	—	—
Tyrosine	28 ± 2	26 ± 1	18 ± 1	18 ± 1	15 ± 0	17 ± 1	35 ± 1	37 ± 5	37 ± 1	35 ± 0	20 ± 0	15 ± 1
Carnosine	—	—	—	—	—	—	318 ± 48	316 ± 53	459 ± 21	379 ± 23	276 ± 34	150 ± 7 [b]
Wt gain, g/chick per week	81 ± 9	77 ± 7	123 ± 2	121 ± 8	106 ± 1	102 ± 4						
Feed/gain	2.30	2.29	1.71	1.70	1.73	1.83						

[1] Average ± range/2 of two determinations, each on a pooled sample from replicate pens of 10 chicks. Range is the difference between the results for the two determinations.
[2] Added at a level of 0.5%.
[3] Single value.
[a] Value is significantly different from control value ($P < 0.01$).
[b] Value is significantly different from control value ($P < 0.05$).

The possible arginine-sparing effect, caused by the inhibition of kidney arginase in the chick fed the casein basal diet and 0.5% AIB supplement (table 5), might be one of the main factors responsible for the increased growth rate and feed efficiency. In a preliminary experiment an in vitro inhibition of arginase activity by AIB using kidney homogenate was observed.[7] Further work is needed, however, to determine whether the inhibition is competitive or noncompetitive.

Christensen and Cullen (4) reported that high levels of AIB produced aminoaciduria in the rat, including excretion of the cationic as well as the neutral amino acids, with the early effect on lysine excretion being very strong. Jones (14) attributed the relatively poor growth of chicks fed a casein diet to the high lysine content of the diet. Since the depression of plasma and muscle free lysine by arginine was increased in the presence of AIB (tables 2 and 7), an interaction among AIB, lysine and arginine in the renal reabsorption process may also be a factor in the growth-promoting effect in the present experiments. It was difficult to distinguish an effect of AIB on the plasma lysine of chicks fed a diet first limiting in arginine, since plasma lysine was usually elevated on diets with this deficiency. The growth data obtained when the chicks were fed a wheat gluten diet and 0.5% AIB supplement (table 7) did not favor the interpretation that competition at the site of renal reabsorption was primarily the cause of the growth-promoting effect observed with the casein diet. If competition with lysine was involved, the growth rate and feed efficiency of the chicks fed the wheat gluten diet might be expected to be reduced when the diet was supplemented with AIB, since the lysine content in this diet was supplemented to be marginal on the basis of the NRC requirement. Consequently, if AIB were increasing renal excretion of lysine as it did in the rat, growth depression resulting from a lysine deficiency could be anticipated.

Generally, the mechanism by which AIB improved the growth rate of chicks fed the casein basal diet needs to be further investigated. Whether AIB behaves as a nonmetabolizable amino acid to avian species as it does to mammals is not yet clear. It will be of great interest if AIB can alleviate the growth depression and promote feed efficiency through the suppression of kidney arginase, as this would suggest a possibility of employing a synthetic nontoxic but metabolically inert chemical as an enzyme inhibitor to alleviate an amino acid imbalance.

An explanation of the effect of AIB on other amino acids in the plasma and muscle is not available. It may relate to a competition of AIB with other amino acids during the transport process across the muscle membrane. It appeared that the response of plasma amino acid patterns to AIB was different in the chick and the rat. In a preliminary experiment, a 20% casein basal diet, similar to the chick diet with and without additional 3% L-arginine·HCl was fed to rats with graded levels of AIB from 0 to 2.0%. The AIB did not affect rat plasma arginine and ornithine as it did in the chick, except that in the 3% L-arginine·HCl-supplemented group, AIB at 1 and 2% decreased the plasma lysine of the rat. It is obvious that if AIB is used as an internal indicator in the chick for amino acid studies, much care is needed in the interpretation of the data.

ACKNOWLEDGMENTS

The authors thank Dr. I. Motzok for his encouragement and advice during the experimental work and the preparation of the manuscript, and Mr. T. L. Ashby for his technical assistance for the latter part of the amino acid analysis.

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Effect of Food Restriction on Metabolic Alterations in "Control Animals" Used in Studies on Biotin-deficient Rats [1,2]

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ABSTRACT Different types of "control animals" used in investigations on biotin-deficient rats have been studied. The effect of restricted food intake (10 g), offered as one, two, or four equal meals a day, on metabolic patterns of control animals was investigated. Although the rate of glycogen synthesis in livers of control animals was not altered, it was considerably greater in diaphragms of animals given a restricted amount of food than in animals fed ad libitum. This increase was even more significant in animals fed multiple meals. The synthesis in vitro of fatty acids from labeled glucose or acetate by adipose tissues from animals given a restricted amount of food as a single meal was greatly increased compared with animals fed ad libitum. This increase was less marked in animals given multiple meals. The present study shows that metabolic alterations are induced in control animals as a consequence of food restriction. In view of these findings the noncritical use of "pair-fed," "pair-weighed" and "trained-fed" animals as "controls" in nutritional studies could lead to erroneous conclusions.

It is known that biotin deficiency results in a marked reduction in food intake of animals. To restrict food intake of control animals, pair-feeding (1, 2), pair-weighing (3, 4) and trained-feeding (4) techniques have been employed. Recently, we (5) observed systematic oscillations in body weight, liver weight, the level of hepatic glycogen and spontaneous activity of control animals given a restricted amount of food (10 g) as a single meal or as multiple meals a day. In view of these systematic oscillations the effect of food restriction on the synthesis in vivo of glycogen, and the synthesis in vitro of fatty acids in various tissues of control rats, has been studied.

METHODS AND MATERIALS

Animals. Nineteen-day-old weanling male rats of the Sprague-Dawley strain weighing 32 to 38 g were used. The animals were housed individually in metal cages with raised wire screens in a temperature-regulated room. Biotin deficiency was produced by feeding ad libitum a basal diet (6) containing 71% glucose, 20% spray-dried egg white, 4% corn oil and all the other nutrients; biotin was not added to the diet. The control animals were cured of biotin deficiency by injecting intramuscularly 100 µg biotin in physiological saline three times a week during the 2-week curative period. During this treatment the visible symptoms of biotin deficiency progressively disappeared. Water was available to animals at all times.

Experimental design. Sixty deficient animals, after being fed the basal diet for 5 weeks, were divided into five groups of 12 animals/group, as shown in table 1. The curative treatment and various feeding schedules were carried out for 2 weeks. Animals fed 10 g once (group 3), 5 g twice (group 4) and 2.5 g four times (group 5) a day, completely ate their individual offering in about 6 hours, 30 and 15 minutes, respectively, as reported in our earlier study (5). These animals (groups 3 to 5) consumed their total food allotment of 10 g every day during these 2 weeks. The average food intake of deficient animals fed ad libitum (group 1) and control (group 2) animals in week 2 of the treatment was about 10 and 17 g, respectively.

Received for publication January 15, 1969.
[1] Supported in part by Public Health Service Research Grant no. AM 08373 and National Science Foundation Grant no. GB-1417.
[2] Taken in part from a thesis submitted in partial fulfillment of the requirements for a Ph.D. degree at the University of Illinois, Urbana.
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TABLE 1
Experimental design [1]

Group	Biotin status	Feeding pattern
1	Deficient	Ad libitum
2	Cured-normal	Ad libitum
3	Cured-normal	Restricted food intake, 10 g once a day (10 g × 1)
4	Cured-normal	Restricted food intake, 5 g twice a day at 12-hour intervals (5 g × 2)
5	Cured-normal	Restricted food intake, 2.5 g four times a day at 6-hour intervals (2.5 g × 4)

[1] Sixty animals were fed ad libitum an egg-white diet for the first 5 weeks of the experiment. The animals were then grouped (12 in each group) and fed the same diet for another 2 weeks as indicated above. Animals in control groups were cured of biotin deficiency.

On the last day of the treatment the control animals in groups 3, 4 and 5 were given 2 g of food at the zero hour (12 noon), which was consumed in about 15 minutes. This procedure brought the animals (groups 3 to 5) to the same nutritional status. At this time food cups were removed from animals in groups 1 and 2. All animals were then fasted for 24 hours, but water was available at all times. At the end of the fast, six animals from each group were decapitated and exsanguinated; the glycogen in livers and diaphragms was determined as described below. Adipose tissues from control animals (groups 2 to 5) were used to study in vitro synthesis of fatty acids as described below. The remaining six animals from each group (groups 1 to 5) received, intragastrically, 5 mmoles glucose containing 1.5 μCi labeled glucose [4] in a 3-ml solution per animal. After 3 hours the animals were killed, and the radioactivity and amount of glycogen in livers and diaphragms were determined.

Determination of glycogen. Immediately after decapitation about 300 mg liver and 100 mg diaphragm were removed; they were quickly weighed on a torsion balance,[5] transferred to tubes containing 2 ml of hot 30% KOH and digested in a boiling-water bath for 30 minutes. Glycogen was coprecipitated with Na_2SO_4 from the KOH digest using 95% ethanol (7), and the precipitate was washed once with 65% ethanol, as suggested by Fong et al. (8). The precipitate containing the radioactivity was dissolved in water and reprecipitated with 95% ethanol. After repeating this treatment, the precipitate was dissolved in water and glycogen was estimated by the anthrone method (9). Radioactivity was determined by counting an aliquot with 15 ml of Bray's scintillation solution (10) in a scintillation counter [6] and a counting efficiency of about 65%.

In vitro synthesis of fatty acids. As mentioned above, after the 24-hour fast six animals from groups 2 to 5 (table 1) were decapitated, and the epididymal adipose tissues were rapidly removed; one fat pad was used for studying fatty acid synthesis from glucose; the other pad was used for the incorporation of acetate into fatty acids. About 100 mg of the tissue were weighed on a torsion balance and immediately transferred to a 25-ml Erlenmeyer flask containing 3 ml of calcium-free Krebs-Ringer biocarbonate buffer, pH 7.4 (11). The buffer contained per milliliter: a) 5 μmoles glucose-U-^{14}C (0.180 μCi) and 0.1 unit of insulin [7]; or b) 10 μmoles labeled acetate [8] (0.179 μCi), 5 μmoles of glucose and 0.1 unit of insulin. Glucose and insulin are required for optimal synthesis of fatty acids from labeled acetate, or pyruvate-2-^{14}C, or pyruvate-3-^{14}C by adipose tissue (12). The levels employed were the same as used in earlier studies (12, 13).

Each flask was gassed with 95% O_2–5% CO_2 and sealed with a rubber serum stopper having a hanging polyethylene well which contained a 2 cm by 2 cm piece of Whatman no. 1 filter paper. The flasks were then shaken in a reciprocating water bath (90 strokes/minute) at 38° for 3 hours. At the end of the incubation period, through the rubber stopper, 0.1 ml of 25% KOH was introduced with a syringe and needle onto the filter paper; similarly, 0.5 ml of 0.2 N H_2SO_4 was added to the incubation medium to stop the reaction and to release CO_2. To ensure complete liberation and trapping of CO_2, shaking was continued for another 30 minutes. The filter

[4] Glucose-U-^{14}C (specific activity 3.83 mCi/mmole), New England Nuclear Corporation, Boston, Mass.
[5] Roller-Smith Precision Balances, Newark, N. J.
[6] Packard Tri-Carb, Packard Instrument Company, Inc., Downers Grove, Ill.
[7] Crystalline pork insulin, Eli Lilly and Company, Indianapolis, Ind.
[8] Acetate-U-^{14}C (specific activity 28.2 mCi/mmole), Nuclear-Chicago Corporation, Des Plaines, Ill.

paper containing trapped $^{14}CO_2$ was transferred to a counting vial and allowed to dry; the paper was flattened, and 10 ml of toluene scintillation solution (13) was added and counted in a scintillation counter [9] as described by Buhler (14). The overall efficiency of the method was calculated to be 24% using the buffer containing $NaH^{14}CO_3$ (13).

After incubation the adipose tissue was treated essentially as described by Cahill et al. (15). The tissue was rinsed three times in physiological saline and transferred to a tube containing 15 ml chloroform–methanol solution (2:1, v/v), and the lipids were extracted with constant shaking for 6 hours. To remove radioactive contamination of nonlipid material, the lipid extract was washed with 4 ml of "salty wash" and then with 7.5 ml of "salty wash upper phase" (16). After adding two or three boiling chips, the solvent was evaporated to dryness in a water bath at 60°.

The lipids were saponified by refluxing in 10 ml of 3% methanolic KOH at 80 to 85° for 45 minutes. The tubes were allowed to cool and 10 ml water was added to each tube. The nonsaponifiable lipids were removed by three 5-ml extractions with petroleum ether (BP 30 to 60°). The saponified lipid fraction was acidified with concentrated HCl (tested with Congo red test paper). The fatty acids were extracted with three 5-ml portions of petroleum ether, and each portion was transferred to a scintillation vial. The combined extract was evaporated under a stream of O_2-free N_2. The fatty acids were dissolved in 10 ml of toluene scintillation solution (13), and radioactivity was determined in a scintillation counter [10] and a counting efficiency of 70%.

RESULTS

The effect of various feeding patterns on the synthesis in vivo of glycogen in livers of control animals is shown in table 2. Restricted feeding, as a single meal or multiple meals (groups 3 to 5), had no effect on the repletion of hepatic glycogen compared with ad libitum feeding (group 2). The incorporation of labeled glucose into glycogen supported this finding. A very marked reduction in the repletion of glycogen in livers of biotin-deficient rats was observed.

In contrast to the finding in the liver, a marked increase in glycogen repletion was observed in the diaphragm of animals given a restricted amount of food (table 3, groups 3 to 5) compared with animals fed ad libitum (group 2). Furthermore, this increase was most marked in animals given two or four meals a day compared with animals receiving a single meal (compare groups 4 and 5 with group 3). The radioactivity incorporated into glycogen correlated with these observations. A marked reduction in the repletion of glycogen in diaphragms of biotin-deficient animals was observed.

The effect of feeding patterns on the synthesis in vitro of fatty acids from labeled glucose and its oxidation to $^{14}CO_2$ is shown in table 4. Biotin-deficient animals were not used in these studies because under our experimental conditions they had hardly any subcutaneous or epididymal fat. Glu-

[9] See footnote 6.
[10] See footnote 6.

TABLE 2

Effect of feeding patterns on glycogen synthesis in livers of biotin-deficient and control animals [1]

Group	Biotin status	Feeding pattern	Glycogen per gram liver after hours				P
			0	3	Difference	3	
			mg	*mg*	*mg*	*dpm × 10⁻³*	
1	Deficient	Ad libitum	0.1 ± 0.02 [2]	1.9 ± 1.1	1.8	7 ± 6	< 0.001
2	Cured-normal	Ad libitum	8.4 ± 2.0	31.1 ± 3.0	22.7	87 ± 6.6	
3	Cured-normal	10 g × 1	0.4 ± 0.1	28.3 ± 4.8	27.9	89 ± 16.3	ns [3]
4	Cured-normal	5 g × 2	1.5 ± 0.3	23.3 ± 1.3	21.8	82 ± 5.2	ns
5	Cured-normal	2.5 g × 4	1.2 ± 0.2	29.8 ± 1.2	28.6	105 ± 5.2	ns

[1] The animals were fasted for 24 hours and given 5 mmoles (1.5 μCi) of labeled glucose in 3 ml by stomach tube.
[2] Each result is the mean ± SE of the mean of six animals.
[3] ns = not significant.

cose oxidation and incorporation into fatty acids was markedly greater in adipose tissues of control animals given restricted amount of food (table 4, groups 3 to 5) than in animals fed ad libitum (group 2). This increase appeared to be more in animals fed a single meal (group 3) than in animals fed multiple meals (groups 4 and 5). As seen in table 5, when labeled acetate instead of glucose was used, similar results were obtained except that this increase was even more marked in animals fed a single

TABLE 3

Effect of feeding patterns on glycogen synthesis in diaphragms of biotin-deficient and control animals [1]

Group	Biotin status	Feeding pattern	Glycogen per gram diaphragm after hours				P
			0	3	Difference	3	
			mg	*mg*	*mg*	*dpm* × 10^{-3}	
1	Deficient	Ad libitum	0.3 ± 0.1 [2]	0.8 ± 0.3	0.5	3 ± 1.7	< 0.01
2	Cured-normal	Ad libitum	1.2 ± 0.1	5.1 ± 0.5	3.9	13 ± 1.2	
3	Cured-normal	10 g × 1	0.4 ± 0.1	5.9 ± 0.3	5.5	20 ± 1.2	< 0.01
4	Cured-normal	5 g × 2	0.6 ± 0.1	7.2 ± 1.0	6.6	29 ± 4.1	< 0.01
5	Cured-normal	2.5 g × 4	0.7 ± 0.1	7.9 ± 0.4	7.2	30 ± 2.2	< 0.001
3 and 4							< 0.05
3 and 5							< 0.01
4 and 5							ns [3]

[1] The animals were fasted for 24 hours and given 5 mmoles (1.5 μCi) of labeled glucose in 3 ml by stomach tube.
[2] Each result is the mean ± SE of the mean of six animals.
[3] ns = not significant.

TABLE 4

Effect of feeding patterns on the synthesis in vitro of fatty acids from glucose-U-^{14}C *and its oxidation to* $^{14}CO_2$ *by adipose tissues of control animals*

Group	Biotin status	Feeding pattern	Glucose-U-^{14}C [1] converted to			
			$^{14}CO_2$	P	Fatty acids	P
			mμmoles of glucose converted/100 mg tissue in 3 hr			
2	Cured-normal	Ad libitum	708 ± 60 [2]		1019 ± 163	
3	Cured-normal	10 g × 1	1897 ± 309	< 0.01	3181 ± 579	< 0.01
4	Cured-normal	5 g × 2	1201 ± 151	< 0.02	2028 ± 364	< 0.05
5	Cured-normal	2.5 g × 4	1122 ± 84	< 0.01	1831 ± 260	< 0.05
3 and 4				ns [3]		ns
3 and 5				< 0.05		ns
4 and 5				ns		ns

[1] The buffer contained per milliliter: 5 μmoles (0.180 μCi) labeled glucose and 0.1 unit insulin.
[2] Each result is the mean ± SE of the mean of six animals.
[3] ns = not significant.

TABLE 5

Effect of feeding patterns on the synthesis in vitro of fatty acids from acetate-1,2-^{14}C *and its oxidation to* $^{14}CO_2$ *by adipose tissues of controls animals*

Group	Biotin status	Feeding pattern	Acetate-1,2-^{14}C [1] converted to			
			$^{14}CO_2$	P	Fatty acids	P
			mμmoles of acetate converted/100 mg tissue in 3 hr			
2	Cured-normal	Ad libitum	751 ± 78 [2]		879 ± 79	
3	Cured-normal	10 g × 1	2238 ± 297	< 0.001	4440 ± 317	< 0.001
4	Cured-normal	5 g × 2	1303 ± 115	< 0.01	3124 ± 295	< 0.001
5	Cured-normal	2.5 g × 4	1354 ± 227	< 0.05	2454 ± 227	< 0.001
3 and 4				< 0.02		< 0.02
3 and 5				< 0.05		< 0.001
4 and 5				ns [3]		ns

[1] The buffer contained per milliliter: 10 μmoles (0.179 μCi) sodium acetate, 5 μmoles glucose and 0.1 unit insulin.
[2] Each result is the mean ± SE of the mean of six animals.
[3] ns = not significant.

meal (group 3) than in animals fed multiple meals (groups 4 and 5).

DISCUSSION

To overcome differences in food intake between biotin-deficient and control animals, pair-feeding (1, 2), pair-weighing (3, 4) and trained-feeding (4) techniques have been used. Patel and Mistry (5) observed systematic oscillations in the steady-state level of hepatic glycogen of control animals given a restricted amount of food (10 g) as a single meal a day. Control animals, however, given four equal meals 6 hours apart were able to maintain the level of hepatic glycogen in the range observed in animals fed ad libitum (5). This could be a result of increased synthesis or decreased breakdown of hepatic glycogen. Since the rate of glycogen synthesis in the liver was not altered in animals fed multiple meals (table 2, groups 4 and 5), the effect observed in the earlier study (5) could have been a result of decreased breakdown of glycogen. Also, no change in glycogen synthesis in the liver was observed in animals given a single meal (table 2, group 3).

In contrast to the findings in the liver, glycogen synthesis was markedly increased in diaphragms of control animals given a restricted amount of food as a single meal compared with animals fed ad libitum (table 3, group 2 compared with group 3). A similar increase in the diaphragms of meal-fed rats compared to nibbling animals has been observed by Leveille and Chakrabarty (17). The significance of this finding in relation to energy storage has been discussed by these authors. Furthermore, this increase was even more marked in animals given multiple meals than in animals given a single meal (table 3, groups 4 and 5 compared with group 3). It is possible that the avoidance of a prolonged postabsorptive state between two successive meals, and the rapid influx of a substantial amount of dietary glucose at regular intervals result in the maintenance of a high level of glycogen synthetase activity, the rate-limiting enzyme, on the pathway of glycogen synthesis.

A similar increase in lipogenesis also was observed in animals given a restricted amount of food when compared with animals fed ad libitum (tables 4 and 5, groups 3, 4 and 5 compared with group 2). As mentioned earlier, these results are maximal responses obtained under optimal conditions of fatty acid synthesis by the adipose tissue. Whether the criterion was fatty acid synthesis or CO_2 production from labeled substrates such as glucose or acetate, the results were similar. The enhanced lipogenic capacity of adipose tissues from meal-fed rats compared with nibbling animals has been observed by many investigators (18–21). The significance of this finding in meal-fed rats in terms of energy storage has been discussed by Leveille and Hanson (20). This would also explain the increase in lipogenesis induced in the adipose tissue of control animals fed a single meal. It is of interest to note, however, that in the present study lipogenesis was greater in control animals even after feeding four meals a day than in animals fed ad libitum. The explanation offered above for the increased synthesis of glycogen in diaphragms of animals fed multiple meals would also explain this observation.

The usefulness and limitations of the pair-feeding technique have been discussed by Mitchell (22). Very little is known about metabolic alternations induced, by pair-feeding or restricting food intake, in rats used as controls in studies on biotin deficiency; however, in the field of nutrition some scattered observations have been reported. In a study on the effect of pantothenic acid, protein, and calorie intake on the respiration of duodenal mucosa of the rat, Vitale et al. (23) pointed out that the pair-feeding technique might not be an adequate or satisfactory method of separating the metabolic effects of nutritional deficiency from the associated inanition which sometimes accompanies it. Castor and Armstrong (24), in studying the effect of X-irradiation on loss of potassium from rats, cautioned that the noncritical use of the pair-feeding technique might constitute a distinct hazard in the interpretation of the data. They suggested an alternate approach, namely, to use the animal fed ad libitum as a standard for comparison, and to express the effects of the treatment as deviations from the control state. In studying the response of lipogenesis to repletion of pyridoxine in deficient rats, Radhakrishnamurty et al. (25) reported

marked differences in the incorporation of labeled acetate into fatty acids by adipose tissues from animals fed ad libitum and pair-fed animals.

In the light of marked metabolic alterations observed in animals fed a restricted amount of food given as a single meal, the noncritical use of "pair-fed," "pair-weighed" and "trained-fed" rats as "controls" in nutritional studies could lead to erroneous conclusions. In the present study control animals fed a restricted amount of food in four equal meals 6 hours apart were still not comparable to animals fed ad libitum with respect to alterations in metabolism. Whether increasing the number of meals offered to control animals would overcome the limitations of pair-feeding needs further investigation.

ACKNOWLEDGMENTS

The authors thank Mr. L. Nash for assistance in caring for the animals, and Mrs. Alma White for assistance in the preparation of the manuscript. We are indebted to Dr. Gilbert A. Leveille for critically reading the manuscript.

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Relationship Between Formula Concentration and Rate of Growth of Normal Infants [1]

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ABSTRACT The recent advent of ready-to-use infant formulas supplied in disposable units has made it feasible to record precise food intakes of normal infants living at home. Thirty-seven normal infants with birth weights of 2500 g or more were enrolled in a study designed to explore the relationship between formula concentration and growth rate during ad libitum feeding in the first 112 days of life. Thirty-two of the subjects completed the entire period of observation. The infants were divided into three groups according to diet and sex. Comparisons are made between two groups of male infants receiving formulas that were similar except for water content, one group being fed a formula that provided 133 kcal/100 ml whereas the other group received a formula providing 67 kcal/100 ml. Comparison of male and female infants receiving the same formula (67 kcal/100 ml) is also presented. In observations of male infants during the interval 8 to 42 days of age, feeding of the more concentrated formula was associated with smaller volume of food consumed but generally greater intake of calories. The greater calorie intakes were accompanied by greater rates of gain in length and weight; these differences were statistically significant. Rate of gain in weight per unit of calories consumed was not significantly different. Between 42 and 112 days of age, calorie intake per unit of body weight, and rates of gain in length and weight were nearly the same for the two groups of male infants.

Calorie intake per unit of body weight, and rates of gain in length and weight were less for female than for male infants fed the same formula during the interval 8 to 42 days of age. Differences between the two groups in rate of gain in length but not in rate of gain in weight were statistically significant. Calorie intake per unit of body weight and rates of gain in length and weight were also less for females than for males during the interval 42 to 112 days of age. The regression of gain in weight (grams per day) on calorie intake (kilocalories per kilogram per day) for all males in the period 8 to 42 days of age was statistically significant. Using data from the present study, together with those from previously published reports, speculations are presented on the composition of the weight gained and on the partition of metabolizable energy into that for growth and that for maintenance. It is tentatively concluded that the composition of weight gained by the two groups of male infants was different: lipid appeared to account for a larger percentage of weight gain by infants receiving the higher calorie intakes. It is also tentatively concluded that energy requirements for maintenance per unit of body weight are less for female than for male infants receiving the same diet.

Numerous studies of rapidly growing animals have demonstrated that rate of gain in weight is significantly correlated with calorie intake. Preferable experimental design for such studies consists of alteration in intake of carbohydrate or fat, or both, without alteration in intake of other nutrients.

Although such a design is not currently feasible in relatively long-term studies of human infants, ad libitum feeding of infants receiving formulas identical except for water content can be carried out with safety. Several investigators (1–4) have demonstrated that formula concentration is correlated with rate of gain in weight when infants of low birth weight are fed ad libitum. A single study (5) with two groups of full-size infants also demonstrated that mean rate of gain in weight was greater by the group receiving the more concentrated formula.

In all these studies and in that to be reported here, intakes of protein and of various vitamins and minerals, as well as intakes of carbohydrate, fat and calories were greater by infants fed the more con-

Received for publication October 21, 1968.

[1] Supported in part by Public Health Service Research Grant no. HD 00383.

centrated formula. Nevertheless, as will be discussed subsequently, there is reason to believe that the greater rates of growth observed in infants fed more concentrated formulas result from greater calorie intakes rather than from greater intakes of protein or of other nutrients.

The relationship between calorie intake and growth rate in the human can probably be demonstrated best with full-size infants during their period of most rapid growth, the first few weeks or months of life. Fortunately, the recent advent of ready-to-use infant formulas supplied in disposable units has made it feasible to record precise food intake of normal infants living at home.

The present study, although not providing definitive answers, presents data that may at least offer a better basis than any previously available for speculation about the following questions: When rate of gain in weight is accelerated by dietary alterations, is the composition of the weight gained the same as that of infants of the same sex who gain less rapidly? What is the partition of metabolizable energy between growth and maintenance in normal infants of various ages? Is the composition of gain the same in infant males and females fed the same diet?

SUBJECTS AND INTERVALS OF STUDY

Normal full-term infants with birth weights of 2,500 g or more were considered eligible for enrollment in the study. All were Caucasian. Nearly all were children of students or younger staff members were the University of Iowa and several were siblings of children who had served as subjects of other studies reported from the Infant Metabolic Unit in recent years. All were enrolled in the program during the first 9 days after birth.

The infants were weighed and measured between 6 and 9 days of age, within 2 days of each of the following ages: 14, 28, 42 and 56 days; and within 4 days of ages 84 and 112 days.

In describing mean size of the three groups of infants (table 1, fig. 1), recorded measurements were "corrected" by linear interpolation or extrapolation to reflect values applicable to ages 8, 14, 28, 42, 56, 84 and 112 days. For convenience, these same age designations have been employed throughout although data on food intake, change in size and serum albumin have not been so "corrected." For example, body weight gain has been calculated from the difference between the measurement made at 6 to 9 days and that made at 12 to 16 days, divided by the number of days between the times of the two measurements. In reporting data on fecal excretion of fat, the age recorded represents day 1 of a 72-hour metabolic balance study.

Lengths and weights at 8 days are recorded in table 1 for the 37 infants enrolled in the study. Thirty-two of these infants were available for the entire 112 days of observation. Data on mean birth weights and mean lengths and weights at 8 days of age, in relation to sex and feeding group, are presented in figure 1.

FEEDINGS

Experimental formulas 29B and 29C [2] were prepared from nonfat cow milk, lactose, vegetable oils (equal parts of corn and coconut oils), vitamins and minerals. As may be seen from table 2, the two formulas were similar in many respects except for the amount of water; formula 29C had approximately twice the caloric concentration of formula 29B. The amino acid composition of the protein, as determined by microbiologic assay, has been described previously (6).

The formula was supplied in 120- and 240-ml disposable units. A supply of formula sufficient for 48 hours was weighed and delivered to the family. When a new supply was delivered 2 days later, the bottles from the previous supply (including any unconsumed amounts of formula) were collected and again weighed. At 30 days of age an iron supplement [3] was introduced and provided 15 mg of elemental iron daily as ferrous sulfate.

The infants were permitted strained foods according to the following schedule: 28 days, oatmeal with bananas and applesauce; 56 days, pears; and 84 days, applesauce and bananas. Although introduction of these foods was permitted at the ages specified, no attempt was made to en-

[2] The experimental formulas were prepared by Ross Laboratories, Columbus, Ohio.

[3] Fer-in-Sol was supplied by the Mead Johnson Company, Evansville, Ind.

TABLE 1

Size of subjects at various ages

	Subject no.	Size at 8 days of age		Size at 42 days of age		Size at 112 days of age	
		Length	Weight	Length	Weight	Length	Weight
		cm	*g*	*cm*	*g*	*cm*	*g*
Males fed formula 29C (133 kcal/100 ml)	25	52.6	3955	57.6	5626	64.2	7610
	26	50.5	3455	55.7	5343	62.8	8737
	27	51.0	3540	56.0	5620	63.2	8594
	28	48.0	3225	53.8	4819	59.9	6010
	29	52.2	3693	56.8	5257	63.9	7040
	30	52.8	3788	58.1	5740	64.8	8052
	31	53.4	3965	58.7	5542	64.0	7130
	32	46.4	2475	51.6	3936	59.7	5505
	33	51.2	3706	56.5	5386	65.7	6404
	34 [1]	52.2	3590	57.5	5245	—	—
	35 [1]	51.5	3633	55.6	4979	—	—
Mean [2]		50.9	3534	56.1	5252	63.1	7231
Males fed formula 29B (67 kcal/100 ml)	1	50.2	3230	54.8	4280	61.1	6014
	2	50.8	3280	55.3	4443	62.3	6598
	3	50.9	3064	55.8	4185	63.4	6444
	4	49.1	3289	53.5	4740	61.2	7355
	5	48.8	2674	52.8	3780	59.9	6365
	6	52.4	4000	57.3	5080	63.2	7599
	7	52.3	3545	56.5	4624	62.4	6121
	8	51.2	3930	54.9	5482	60.7	7134
	9	53.8	3954	58.3	5149	65.4	6597
	10	47.5	2581	52.3	3992	59.9	5897
	11	52.7	3850	56.4	4879	62.2	6523
	12	48.8	2655	52.7	3639	59.7	5922
	23	49.2	2885	54.6	4390	61.4	6512
	24 [1]	53.2	3640	—	—	—	—
	36 [1]	51.4	3539	—	—	—	—
	37 [1]	50.7	3420	—	—	—	—
Mean [2]		50.6	3302	55.0	4513	61.8	6545
Females fed formula 29B (67 kcal/100 ml)	13	49.3	3130	54.5	4093	60.2	5959
	14	51.8	3295	56.2	4440	64.2	5977
	15	49.4	3049	52.5	3935	60.0	5397
	16	51.9	3600	55.9	4718	62.4	6529
	17	51.7	3796	55.8	5000	64.1	6950
	18	52.1	3715	56.4	5019	62.1	6030
	19	50.6	3337	53.6	4372	59.8	6156
	20	52.3	3785	56.5	5011	61.9	6496
	21	49.4	3530	53.1	4357	58.5	5336
	22	51.8	4080	55.2	4668	61.1	5858
Mean		51.0	3532	55.0	4561	61.4	6069

[1] Did not complete 112 days of study.
[2] Excluding subjects who did not complete 112 days of study.

courage the feeding of foods other than the formula. Parents of experimental subjects were advised that addition of such foods to the diet was optional and that the experimental formula was a complete food. Strained foods were purchased from one manufacturer [4] and empty (or partially empty) jars were collected and weighed. Volume of formula and of pureed foods consumed by each infant was calculated on the basis of weight of each food consumed divided by its determined density.

PROCEDURES AND METHODS

Rates of growth in length and weight were determined as described previously (6, 7a). Procedures employed in metabolic balance studies with infants have been presented in detail (7b).

[4] The Gerber Company, Fremont, Mich.

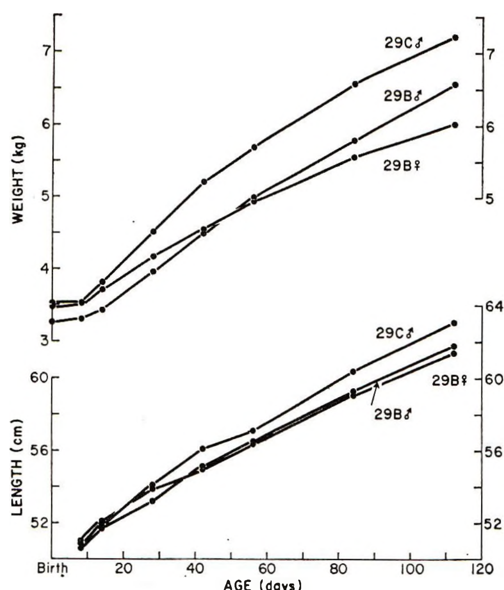

Fig. 1 Mean body weight and body length at various ages for each of the three groups of infants.

Fecal excretion of fat was determined by a minor modification of the method of Van de Kamer et al. (8). With 28 of the 32 specimens, fat excretion was also determined by the following gravimetric method: an aliquot of about 15 g of fecal homogenate was transferred to a tared alundum thimble and weighed. Extraction was carried out with 40 ml of 95% ethyl alcohol for 6 hours. The extract was set aside and the homogenate was again refluxed with 40 ml of ethyl alcohol for 6 hours. Two additional 6-hour extractions were carried out with Skellysolve-F. The four extracts were combined in a separatory funnel and shaken with 80 ml distilled water. After standing 6 hours, the Skellysolve-F fraction was separated, washed with distilled water, dried over sodium sulfate, filtered into a tared flask and evaporated to constant weight with a flash evaporator. The mean excretion of fat as determined by the method of Van de Kamer et al. amounted to 6.5% of intake while the extraction method gave a value equal to 7.2% of intake.

Blood for serum protein determinations at 112 days of age was obtained by venipuncture utilizing the external jugular vein. With few exceptions, blood was drawn between 1:00 and 1:30 PM; there was no restriction relating to the time of feeding. Concentration of total protein in serum was determined by the biuret method and was standardized with the use of control sera.[5] These sera were, in turn, standardized by determination of nitrogen using the Dumas method.

Separation of serum proteins into the various fractions was carried out by electrophoresis on cellulose acetate.[6] Frozen serum from a normal adult subject was stored in small vials and serum of one vial was thawed and used as a control with each set of unknown sera. Standardization of results with the control serum was accomplished by comparison with results of analysis of a mixture of albumin and gamma globulin in a solution of sodium chloride.

TABLE 2
Composition of formulas

	Formula 29B	Formula 29C
Concentration, kcal/100 ml	67	133
Major constituents, g/100 ml		
Protein	1.03	2.00
Fat	3.55	7.33
Carbohydrates	7.80	15.60
Mineral content per liter		
Calcium, mg	480	830
Phosphorus, mg	290	590
Sodium, mEq	10	15
Potassium, mEq	13	25
Chloride, mEq	11	20
Magnesium, mg	55	69
Iron, mg	8.2	5.7
Vitamin content per liter		
Vitamin A, IU	1800	1800
Thiamine, μg	900	1100
Riboflavin, μg	1600	2700
Niacin, μg	2900	3300
Pyridoxine, μg	230	220
Pantothenate, μg	1740	3120
Ascorbic acid, mg	92	99
Vitamin D, IU	400	400
Vitamin E, mg	3.4	6.2

[5] Lab-trol and Path-trol, Dade Reagents, Inc., Miami, Fla.

[6] Beckman Instrument Manual, RM-IM-3 (August, 1965) using the Beckman Microzone Electrophoresis Cell, Model R-101. Scanning of the cellulose acetate membranes was performed with the Beckman Analytrol, Model RB, using the Microzone Scanning Attachment, Model R-102, with speed control set at 30.

CALCULATIONS OF CALORIE INTAKE

From the weight of each food and knowledge of its composition, calorie intakes were calculated utilizing the factors 4, 9 and 4 kcal/g, respectively, for protein, fat and carbohydrate. These values may be considered to represent metabolizable energy rather than heat of combustion. Actual heats of combustion of milk are approximately 5.65, 9.25 and 3.95 kcal/g, respectively (9), but the apparent digestibility of protein, as previously described (6), and fat, as will be shown, result in a lesser value for metabolizable energy than for heat of combustion. The magnitude of the difference is such that the factors 4, 9 and 4 kcal/g for protein, fat and carbohydrate appear reasonable.

Use of a value for calorie intake that approximates metabolizable energy is of considerable convenience in discussion of partition of energy intake between growth and maintenance. In addition, it facilitates comparison with other reports of calorie intake by infants.

RESULTS

Although voluntary participation in a study of this type is in itself a factor of considerable selectivity, it appeared important to account for the performance of each of the 37 infants initially enrolled. Five of these subjects, all males, failed to complete the planned 112 days of observation. In three instances the families moved from town: subjects 24 and 37 after the visit at 28 days of age, subject 35 after the visit at 56 days of age. Subject 36 failed to appear for the visit scheduled at 28 days of age, and was therefore eliminated from the study. The parents of subject 34 elected to withdraw him from the study after the visit at 56 days of age, at the time he was admitted to the hospital for repair of an indirect inguinal hernia. Information about the size of these infants at 8 days of age is presented in table 1, and information regarding their performance to the time of withdrawal from the study is included in figure 2.

Data concerning the five infants who failed to complete 112 days of observation are not included in the summary presented in table 3, in figures other than figure 2, nor in the statistical analyses. Thirty-two infants completed 112 days of study, each making seven visits.

Intake of Total Food, Calories and Proteins

Volume of intake. Data on volume of intake presented in figures 2 and 3 and in table 3 refer to combined volume of formula and pureed foods. In general, pureed foods accounted for a rather small percentage of total food intake (see *Calorie intake*). As might be anticipated, males fed formula 29B demonstrated greater mean intake with increasing age, reaching a mean intake of 899 ml/day in the interval from 84 to 112 days of age.

Although mean intakes by females fed formula 29B were less in each age interval than those by males receiving the same formula, the pattern was also one of increasing intakes with increasing age. On the other hand, males fed formula 29C in each age interval consumed significantly less volume of food than did males fed formula 29B, and the pattern of intake was different: maximal mean intakes were reached in the interval 56 to 84 days, with a lesser mean intake in the interval 84 to 112 days.

Per unit of body weight, volume of intake was greatest in each instance in the interval between 14 and 28 days of age, thereafter decreasing in each succeeding age interval.

Noteworthy was the great variability in volume of intake by infants fed formula 29C during the age interval 56 to 84 days and 84 to 112 days.

Calorie intake. Because the infants received no pureed foods during the first 28 days of life, the ratio of calorie intake (kilocalories per day) to volume of intake (milliliters per day) was 0.67 for infants fed formula 29B (i.e., 67 kcal/100 ml), and 1.33 for infants fed formula 29C. Thereafter, pureed foods were taken in small amounts. Between 8 and 42 days of age foods other than formula accounted for less than 1% of the calorie intake of male infants fed formulas 29C and 29B, and for 2.3% of the calorie intake of females fed 29B. Between 42 and 112 days of age foods other than formula accounted for 5.0, 4.7 and 6.2% of the calorie intake, respectively, for males fed formulas

TABLE 3

Intake of food and rate of growth at various ages

Formula	Conc, kcal /100 ml	Sex	Ages, days								
			8–14	14–28	28–42	42–56	56–84	84–112	8–42	42–112	8-112
			volume of intake, ml/day								
29C	133	M	362 [1] (75) [2]	438 (83)	488 (67)	473 (69)	548 (161)	526 (193)	445 (61)	526 (149)	499 (117)
29B	67	M	523 (83)	670 (89)	761 (101)	802 (127)	829 (92)	899 (118)	675 (84)	851 (92)	792 (80)
29B	67	F	504 (70)	644 (69)	664 (104)	687 (78)	746 (72)	808 (94)	629 (64)	759 (64)	719 (57)
			volume of intake, ml/kg per day								
29C	133	M	98 (13)	104 (13)	100 (13)	86 (9)	89 (24)	76 (25)	102 (9)	83 (20)	90 (16)
29B	67	M	156 (21)	180 (19)	179 (20)	168 (27)	153 (18)	146 (21)	175 (17)	153 (17)	161 (16)
29B	67	F	140 (22)	164 (18)	153 (26)	145 (16)	141 (11)	139 (17)	155 (18)	141 (10)	146 (9)
			calorie intake, kcal/day								
29C	133	M	485 (100)	587 (111)	647 (96)	618 (91)	702 (207)	684 (266)	593 (84)	680 (201)	652 (159)
29B	67	M	349 (55)	448 (60)	510 (69)	539 (86)	556 (64)	605 (80)	451 (56)	572 (63)	530 (55)
29B	67	F	337 (47)	430 (46)	446 (68)	462 (51)	500 (49)	545 (64)	419 (42)	511 (42)	480 (34)

			calorie intake, kcal/kg per day								
29C	133	M	131 (17)	*140* (18)	133 (18)	113 (13)	114 (31)	99 (35)	*136* (12)	108 (27)	117 (22)
29B	67	M	104 (14)	120 (13)	120 (14)	113 (19)	103 (12)	98 (14)	117 (12)	103 (12)	108 (11)
29B	67	F	93 (15)	109 (12)	*103* (17)	97 (10)	95 (7)	93 (12)	*103* (12)	95 (6)	97 (6)
			gain in weight, g/day								
29C	133	M	50 (17)	50 (8)	*51* (10)	34 (13)	31 (13)	23 (13)	*51* (6)	29 (11)	36 (9)
29B	67	M	26 (13)	37 (7)	39 (9)	36 (11)	28 (7)	27 (5)	36 (7)	29 (6)	31 (4)
29B	67	F	32 (12)	33 (6)	27 (9)	28 (6)	22 (4)	*17* (8)	30 (7)	22 (5)	24 (4)
			gain in length, mm/day								
29C	133	M	1.81 (1.02)	*1.53* (0.55)	1.46 (0.21)	*0.76* (0.28)	1.16 (0.25)	0.99 (0.47)	*1.56* (0.09)	0.99 (0.16)	*1.19* (0.12)
29B	67	M	1.62 (0.61)	1.10 (0.36)	1.34 (0.33)	1.02 (0.23)	1.02 (0.16)	0.86 (0.19)	1.30 (0.15)	0.95 (0.12)	1.08 (0.11)
29B	67	F	1.59 (0.68)	1.32 (0.36)	*0.78* (0.29)	1.04 (0.31)	0.91 (0.21)	0.87 (0.25)	*1.15* (0.18)	0.92 (0.15)	1.00 (0.12)
			gain in weight per unit of calorie intake, g/100 kcal								
29C	133	M	10.6 (3.9)	8.8 (2.2)	7.9 (1.3)	5.7 (2.1)	4.5 (1.7)	3.4 (1.7)	8.6 (1.3)	4.3 (1.6)	5.6 (1.4)
29B	67	M	7.8 (4.9)	8.4 (1.5)	7.8 (1.5)	6.6 (1.5)	5.0 (1.2)	4.5 (1.1)	8.0 (1.3)	5.1 (1.0)	5.9 (0.8)
29B	67	F	9.3 (3.6)	7.6 (1.0)	5.9 (1.6)	6.1 (1.0)	4.4 (0.7)	*3.1* (1.4)	7.1 (1.0)	4.2 (0.8)	*5.1* (0.7)

[1] Numbers in italics indicate value is significantly different at the 95% level of confidence from the corresponding value for males fed formula 29B.
[2] Values in parentheses are standard deviations.

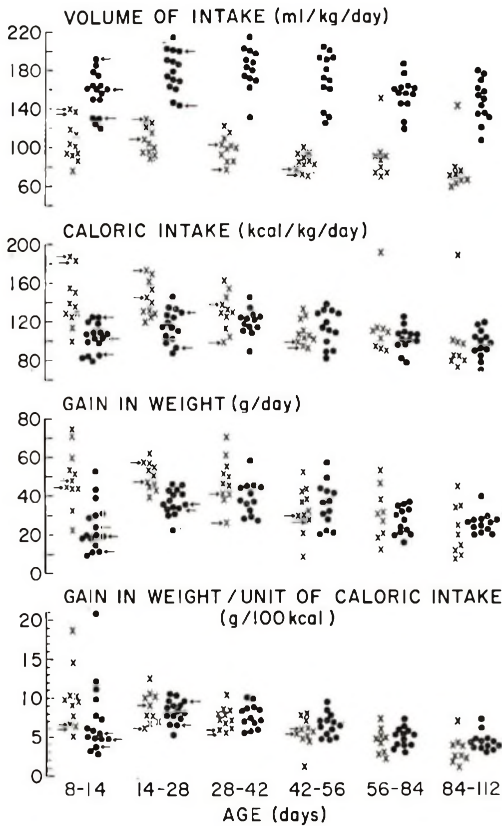

Fig. 2 Performance of the male infants. X refers in the specified age interval to the mean volume of intake, intake of calories or gain in weight of one infant fed formula 29C. ● refers similarly to the performance of one infant fed formula 29B. Performances of five infants who did not complete 112 days of study are indicated by arrows.

29C and 29B and females fed formula 29B.

Mean calorie intakes, whether expressed in absolute terms or in relation to body weight, were significantly greater by infants fed formula 29C than by those fed formula 29B. As may be seen from table 3, however, the difference was greatest between 8 and 84 days of age; in fact, between 84 and 112 days of age, calorie intakes per unit of body weight were nearly identical. Similarly, calorie intake (in absolute terms or per unit of body weight) between 8 and 112 days of age was greater by males than by females fed formula 29B.

Protein intake. Because formula 29B provided the greatest part of total food intake, and because protein content of pureed foods averaged about 1 g/100 g of food, protein intake may be calculated on the basis of protein content of the formula: 1.03 g/100 ml. Data on protein intakes are, therefore, not included in table 3. In addition, protein intakes of 22 of the 23 infants fed formula 29B have been summarized previously (6).

In the case of infants fed formula 29C, protein intake may be calculated for the age intervals 8 to 14, 14 to 28, and 28 to 42 days from volume of intake on the basis of protein content of the formula: 2.00 g/100 ml. In the age intervals 42 to 56, 56 to 84, and 84 to 112 days, the mean ratios of protein intake to volume of

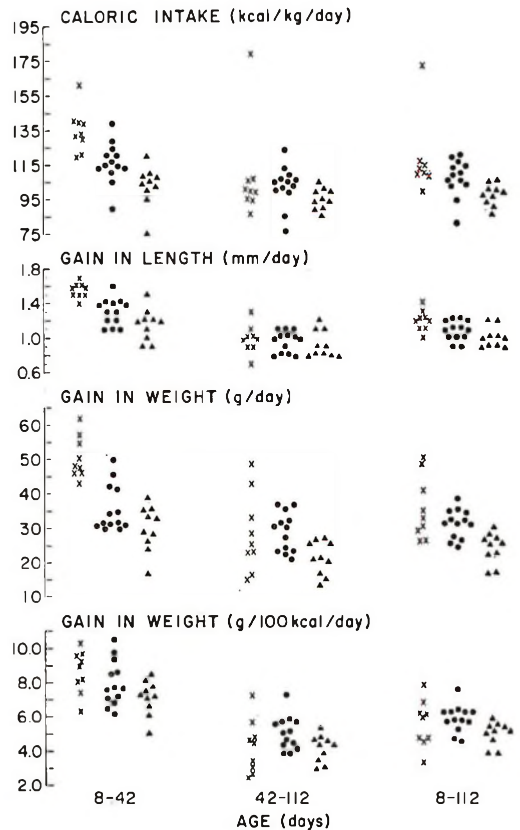

Fig. 3 Performance of infants who completed 112 days of study. Symbols for male infants are as indicated in figure 2. ▲ refers to performance of one female infant fed formula 29B.

intake were 1.97, 1.95 and 1.90 g/100 ml, respectively.

For males fed formula 29C, males fed formula 29B and females fed formula 29B, mean intakes of protein were 2.03, 1.80 and 1.61 g/kg per day, respectively, in the age interval 8 to 42 days (standard deviations 0.17, 0.18 and 0.18 g/kg per day, respectively). Corresponding mean intakes in the age interval 42 to 112 days were 1.62, 1.57 and 1.45 g/kg per day (standard deviations 0.41, 0.17 and 0.10).

Fecal Excretion of Fat

Although urinary losses are relatively unimportant in energy balance, fecal losses may account for a substantial percentage of calorie intake, especially loss of fat. Therefore, interpretation of data on calorie intake is difficult unless an estimate can be made of fecal losses of fat.

A total of 32 3-day metabolic balance studies was performed with five of the male infants fed formula 29B (subjects 3, 5, 8, 15 and 18) between 7 and 115 days of age. Age, body weight and body length on day 1 of each study, together with tabular data on nitrogen balance, have been presented previously (6).

Excretion of fat, as estimated by the method of Van de Kamer et al. (8), amounted to 11% of intake for subject 3 at age 7 days and 13% of intake for subject 5 at age 11 days. With one exception (subject 3 at age 43 days: fat excretion 24% of intake), fat excretion of infants from 12 to 115 days of age amounted to 3 to 9% of fat intake. Thus, with few exceptions, fecal loss of fat amounted to 1.5 to 4.5% of calorie intake. The results, therefore, agree quite closely with those reported previously (7c) for infants fed another formula in which the fat consisted of equal parts of corn and coconut oils.

Because other studies of normal infants (7d) have failed to demonstrate a sex-related difference in fecal excretion of fat, it may be assumed that excretion of fat expressed as a percentage of intake was similar for males and females fed formula 29B. Because males fed formula 29C ingested greater amounts of fat (although percentage of total calories from fat was the same), excretion of fat may have accounted for slightly greater percentage of intake. The magnitude of the difference, however, is likely to be small and has been ignored in the speculations presented.

Mean fecal excretion of nitrogen during 33 metabolic balance studies with five of the infants fed formula 29B was 16% of intake. The range was 8 to 22%.

Size and Gain

Size. By interpolating or extrapolating from the growth curves for the individual infants, the weight (and length) of each infant was recorded at exactly 8, 14, 28, 42, 56, 84 and 112 days. The means of the measurements at these ages were employed to construct a composite growth curve for each group of infants (fig. 1). It may be seen that the mean weights at birth and at 8 days of age were slightly less for males fed formula 29B than for infants of the other feeding groups.

Gain in weight. Since one would anticipate that greater calorie intakes would be responsible for greater gains in weight, it is not surprising that mean rate of gain was greatest for males fed formula 29C, next for males fed formula 29B and least for females fed formula 29B (fig. 3, table 3). Rates of gain in weight by infants fed formula 29B were generally similar to those reported from other studies of normal infants in the Metabolic Unit at the University of Iowa (7a). The difference in mean rates of gain in weight between infants fed formula 29C and formula 29B in the age interval 8 to 112 days is due to differences in rates of gain during the interval 8 to 42 days; mean rates of gain were identical (29 g/day) during the interval 42 to 112 days.

Considerably greater variability in rates of gain in weight was noted for male infants fed formula 29C than for those fed formula 29B during the age intervals 56 to 84 days and 84 to 112 days. Thus, although mean rates of gain in weight were identical during the interval 42 to 112 days, the standard deviation was 11 g/day for males fed formula 29C and only 6 g/day for males fed formula 29B.

Gain in length. As was true of gain in weight, data on gain in length of infants fed formula 29B were similar to those previously reported (7a) from other stud-

ies of normal infants. Males fed formula 29C gained in length more rapidly during the interval 8 to 42 days than did males fed formula 29B (table 3). During the interval 42 to 112 days, mean rates of gain in length were only slightly greater by males receiving formula 29C than by those receiving formula 29B (0.99 versus 0.95 mm/day).

Relation of gain in weight to calorie intake. For reasons to be presented (see Discussion), it was assumed that the only difference between formulas 29B and 29C that was biologically meaningful was the difference in calorie intake. From this point of view, male infants fed the two formulas may be considered a homogeneous group in which the influence of calorie intake on rate of gain in weight may be evaluated.

The regression of gain in weight on calorie intake for the 22 male infants (fig. 4) is described by the equation $y = 0.24x + 19.3$, where y is gain in weight expressed as grams per day and x is calorie intake expressed as kilocalorie per kilogram per day. The correlation coefficient is 0.426 and the slope is statistically significant at the 95% level of confidence.

As may be seen from figure 4, one infant fed formula 29C had an extremely high calorie intake without a correspondingly rapid gain in weight. Because metabolic balance studies were not performed with this infant, it was impossible to know

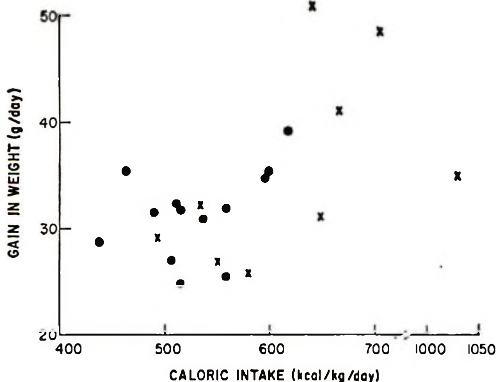

Fig. 4 Relation between gain in weight and calorie intake of male infants in the interval 8 to 112 days of age. Each X refers to this relation in one infant fed formula 29C, and each ● to the relation in one infant fed formula 29B.

whether some degree of malabsorption accounted for his unusually large food intake. Excluding data pertaining to this infant, the regression of gain in weight on calorie intake for the remaining 21 male infants is described by the equation, $y = 0.066x - 3.9$. The correlation coefficient is 0.664 and the slope is statistically significant at the 99.9% level of confidence.

In each group of infants, weight gain per unit of calorie intake generally decreased with increasing age. Such a result might be anticipated since increasing body size would demand increasing energy expenditures for maintenance.

Serum Concentration of Total Protein and Albumin

Concentrations of total protein and albumin were determined at age 112 days in sera of 8 males fed formula 29C, 12 males fed formula 29B and 10 females fed formula 29B. Mean concentrations of albumin were 4.33, 4.24 and 4.22 g/100 ml, respectively (standard deviations 0.44, 0.33, and 0.34 g/100 ml), for male infants fed formula 29C, male infants fed formula 29B and female infants fed formula 29B. Corresponding values for male and female breast-fed infants were 4.33 and 4.29 g/100 ml, respectively (standard deviations 0.40 and 0.36).

DISCUSSION

In addition to greater intakes of calories, infants fed formula 29C had greater intakes of protein and of various vitamins and minerals than did infants fed formula 29B. As already pointed out, however, infants fed formula 29B grew in length and weight at the same rate and demonstrated concentrations of serum albumin similar to those of breast-fed infants and of infants receiving formulas with higher percentages of calories from protein. For example, previous studies of normal full-size infants fed ad libitum with formulas providing 6 to 20% of calories from protein (and generally proportional intakes of minerals and most vitamins), demonstrated (7a) similar rates of gain in weight — gains almost identical to those of infants fed formula 29B in the present study. In considering the possible effects of differences

in intakes of minerals and vitamins on rates of growth in the present study, it is important to note that intakes of these nutrients by infants fed formula 29B were greater than current estimates of requirements. With these considerations in mind, it appears exceedingly unlikely that the observed effects on growth — namely, a supernormal rate of gain in length and weight by infants fed formula 29C during the period from 8 to 42 days of age — could be a result of the particular intakes of protein, vitamins and minerals.

That no investigators have previously demonstrated a statistically significant correlation between rate of gain in weight and calorie intake of individual infants is, perhaps, not surprising. Despite all attempts at classification of human infants of low birth weight, any group of these subjects represents a much more heterogeneous population than would be considered acceptable in studies of experimental animals. Full-size human infants represent a more homogeneous population, but one with a lesser rate of growth per unit of body weight than is true of the commonly employed animal models. Therefore, the proportion of calories used for growth will be relatively low and individual variability in energy requirements for maintenance may obscure the relationship between calorie intake and rate of gain in weight. Further, the procedural aspects of carrying out satisfactory experiments with human infants are obviously greater than is the case with experimental animals.

Three previously reported studies have failed to demonstrate a statistically significant relationship betweeen calorie intake and rate of gain in weight during several months of observation of normal full-size infants. Doxiadis and Paschos (5), in a residential nursery in Athens, studied 8 infants fed a formula providing 73 kcal/100 ml and 12 infants fed a formula providing 50 kcal/100 ml. Daily food intakes were measured. As might be expected, rates of growth were relatively slow in infants fed the formulas providing only 50 kcal/100 ml. Unfortunately, the data have not been presented in sufficient detail to permit the reader to accept with confidence the conclusion of the authors that rate of gain in weight was not significantly correlated with calorie intake.

Rueda-Willamson and Rose (10) studied 67 infants serially between the ages of 2 and 15 months. Food intakes were estimated twice monthly between 2 and 5 or 6 months of age and monthly thereafter. These estimates were based on diet histories and observations in the home. Failure to establish a statistically significant correlation between calorie intake and rate of gain in weight is not surprising since a relatively small percentage of total calorie expenditure is used for growth after 4 months of age (and especially after 6 months of age), and because dietary recall has serious limitations as a method for determining intakes.

A recent study by Rose and Mayer (11) concerned calorie intake (estimated by history) and rate of gain in length and weight of infants between 4 and 6 months of age. The authors concluded that rate of growth was not related to calorie intake; it is certainly true that the relatively large expenditures of calories for maintenanace at this age and the large and highly variable expenditures for activity might overshadow the relatively small expenditures for growth. In addition, several serious deficiencies in reporting (if not in procedures themselves) have been noted (12). Nevertheless, it has been pointed out (12) that the five infants with greatest calorie intakes gained an average of 1.3 kg during the period of observation while the five infants with lowest calorie intakes gained an average of 1.1 kg.

In the present report a statistically significant relationship between rate of gain in weight and calorie intake was demonstrated for the male infants (fig. 4).

SPECULATIONS

In examining the relationship between calorie intake and rate of growth in the present study, it is useful to combine available data from several sources with a number of assumptions. It will be seen that in most instances the individual assumptions may be quantitatively modified within rather wide limits without altering the conclusions.

Total calorie intake must, of course, be accounted for in terms of calorie losses

in urine and feces, calorie expenditures for growth and calorie expenditures for maintenance. In this context, maintenance is interpreted broadly as applying to all metabolic expenditures (including activity and specific dynamic action) except those for growth. As indicated previously (see Calculations of Calorie Intake), data on calorie intake in the present report are assumed to represent metabolizable energy and calorie losses in urine and feces are, therefore, ignored in the discussion that follows.

If one is willing to assume that maintenance requirements per kilogram of body weight are similar in infants of the same sex and same approximate size and body composition, it is possible to examine the hypothesis that composition of weight gained by the more rapidly growing male infants fed formula 29C was the same as that by male infants fed formula 29B. If the hypothesis is sound, the following equation will yield approximately the same result for both groups of infants:

$$\frac{\text{energy intake} - \text{rate of gain} \times K}{\text{body weight}} = \text{energy for maintenance},$$

where energy intake represents the mean value for total calorie intake expressed as kilocalories per day, rate of gain represents mean gain in weight in grams per day, body weight is mean body weight in kilograms, and energy for maintenance is expressed as kilocalories per kilogram per day. The constant, K, expressed as kilocalories per 100 g gain, indicates calorie expenditure per unit of weight gain. Thus, rate of gain $\times$ K represents total calorie expenditure for growth. It will be shown subsequently that for the purpose of testing the hypothesis, the value assigned to K need not be precise.

In an attempt to assign a reasonable value to the constant, K, the composition of weight gained by the "male reference infant" (13) between birth and 4 months of age was used in conjunction with the assumption that 4 kcal were required for synthesis of each gram of protein and 9 kcal for synthesis of each gram of lipid. Thus, 413 kcal would be required for gain of 100 g of body weight.

Employing data from table 3,[7] energy for maintenance for the interval 8 to 112 days was found to be 82 kcal/kg per day for male infants fed formula 29B. Calculations from data for the individual male infants fed formula 29B yielded results from 60 to 92 kcal/kg per day. As already mentioned, one infant fed formula 29C had an unexplainably high calorie intake (fig. 4). The calculated value for caloric maintenance for this infant was 140 kcal/kg per day. Calculated caloric maintenance for the other eight male infants fed formula 29C in the interval 8 to 112 days of age was 83 kcal/kg per day, with a range of 70 to 91 kcal/kg per day. Thus, the data presented here provide no reason to doubt the hypothesis that composition of weight gain was similar in male infants fed formula 29B and those fed formula 29C.

It was stated previously that the value assigned to the constant, K, need not be precise for the purpose of testing the hypothesis. If 300 kcal/100 g gain were assigned to K, mean caloric maintenance for the interval 8 to 112 days would be 91 kcal/kg per day for 13 male infants fed formula 29B, and 90 kcal/kg per day for the 8 male infants fed formula 29C. If a value of 500 kcal/100 g gain were assigned to K, corresponding mean caloric maintenance values would be 76 and 77 kcal/kg per day. In each case, the values for caloric maintenance are nearly identical for the two groups of infants.

Data from a variety of sources (14) indicate that during infancy female infants are fatter than males of corresponding age. Skin-fold thickness, as measured by calipers and by roentgenograms, is greater in females than in males, and total body water accounts for a greater percentage of body weight in males than in females. Since water content of fat-free body mass differs little between males and females of a specified age, one must conclude that lipid content of the body during infancy is greater in females than in males. Whether the relationship between calorie intake and rate of gain in weight demonstrates a sex-related difference is therefore of interest.

[7] Mean body weight during a specified age interval may, of course, be calculated by dividing the value for total calorie intake per day by that for total calorie intake per kilogram per day.

A female reference infant has not previously been described but data suitable for calculating body composition of such an infant are available. Results of whole-body chemical analyses of stillborn infants have been summarized by Owen et al. (14). Using data from analysis of body composition of those stillborn infants with birth weights greater than 2500 g, corrected as previously discussed (13) for the amount of water presumed lost between birth and time of analysis, it was found that water accounts for 72.8% of body weight, protein 10.6% and lipid 12.2%.

A method for estimating the lipid content of a female reference infant at 112 days of age can also be devised. Assuming, as discussed previously, that composition of weight gained by male infants fed formula 29B was the same as that of the male reference infant, the mean lipid content of the body at age 112 days would be 25.8% of body weight. Fat-free body mass would, therefore, comprise 74.2% of body weight. If water accounted for 81% of fat-free body mass of these male infants at age 112 days (a value equal to that of the male reference infant at age 4 months), total body water would account for 60.1% of body weight (table 4). This value is remarkably similar to the mean value of 59.8% reported by Owen et al. (15) from 61 studies of total body water performed with 16 normal male infants between 4 and 9 months of age.

Thirty-seven studies of total body water performed with 10 normal female infants of the same age yielded a mean value of 56.3% of body weight (15). This value may be used as an approximation of total body water of the female infant at 112 days of age.

Assuming that in female infants fed formula 29B, as in the males, water comprised 81% of fat-free body mass, the fat-free portion of the body would constitute 69.3% of body weight and lipid would constitute the remaining 30.7%. These values are summarized in table 4. Also presented in table 4 are rates of gain in weight of the two groups of infants during the interval 8 to 112 days of age, as well as the calculated lipid content of the gain. Lipid accounted for 40.8% of gain by males and 57.2% of gain by females.

If, as in the case of the male reference infant, protein accounted for 11.4% of weight gain in both groups, energy for growth could be estimated as 413 kcal/100 g gain for males ($11.4 \text{ g} \times 4 \text{ kcal/g} + 40.8 \text{ g} \times 9 \text{ kcal/g}$) and 560 kcal/100 g gain for females. Since the rate of growth is known, the daily requirement of energy for growth may be calculated. Subtracting this value from total calorie intake expressed as kilocalories per day, total calories for maintenance is obtained. The latter value, divided by mean body weight, is 81.7 kcal/kg per day for males and 69.8 kcal/kg per day for females.

In view of the greater thickness of subcutaneous adipose tissue in female infants than in males, it may be reasonable to suspect that the better insulated female would dissipate less heat to the environment, and therefore, have a lesser energy requirement for maintenance. Conceivably, activity is also somewhat less in females than in males but data on this point are not available.

TABLE 4

Speculation on sex-related differences in composition of weight gain and in energy required for maintenance by normal infants during early infancy

	8 days		112 days				8–112 days		
							Gain		
	Body wt	Lipid	Body wt	Total body water	Fat-free body mass	Lipid	Body wt	Lipid	Energy for maintenance
	g	*% body wt*	*g*	*% body wt*	*% body wt*	*% body wt*	*g/day*	*% gain*	*kcal/kg per day*
Males	3299	11.0	6545	60.1	74.2	25.8	31	40.8	81.7
Females	3532	12.2	6069	56.3	69.3	30.7	24	57.2	69.8

The hypothesis suggested by the calculations presented, that energy requirements for maintenanace are significantly greater for males than for females, should be tested. The greater mortality and morbidity of male infants during early life could be related to such a difference.

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