Invitation for Nominations for 1964 American Institute of Nutrition Awards

Nominations are requested for the 1964 annual awards administered by the American Institute of Nutrition to be presented at the next annual meeting. Nominations may be made by anyone, including members of the Nominating Committees and non-members of the Institute.

The following information must be submitted: (1) Name of the award for which the candidate is proposed. (2) A brief convincing statement setting forth the basis for the nomination. A bibliography and supporting letters are not to be submitted. (3) Five copies of the nominating letter must be sent to the chairman of the appropriate nominating committee *before October 1, 1963*, to be considered for the 1964 awards.

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

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

1964 Borden Award in Nutrition

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

Former recipients of this award are:

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

NOMINATING COMMITTEE:

ROBERT E. SHANK, *Chairman*
BOYD L. O'DELL
L. D. WRIGHT

Send nominations to:

DR. ROBERT E. SHANK
Department of Preventive Medicine and Public Health
School of Medicine
Washington University
St. Louis 10, Missouri

1964 Osborne and Mendel Award

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

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

Former recipients of this award are:

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

NOMINATING COMMITTEE:

MAX K. HORWITT, *Chairman*
F. W. HILL
NEVIN SCRIMSHAW

Send nominations to:

DR. M. K. HORWITT
L. B. Mendel Research Laboratory
Elgin State Hospital
Elgin, Illinois

1964 Mead Johnson Award for Research in Nutrition

The Mead Johnson Award for Research in Nutrition has been re-established by the Mead Johnson Company. This award of $1000 and a certificate will be presented each year to an investigator who has not reached his 46th birthday during the calendar year in which the award is given and will be based on a single outstanding piece of research in nutrition published in the year preceding the annual meeting, or on a series of papers on the same subject published within not more than the three years preceding the annual meeting.

Former recipients of this award are:

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

NOMINATING COMMITTEE:

ROBERT OLSON, *Chairman*
CARL BAUMANN
B. CONNOR JOHNSON

Send nominations to:

DR. ROBERT OLSON
Department of Biochemistry and Nutrition
Graduate School of Public Health
University of Pittsburgh
Pittsburgh 13, Pennsylvania

Invitation for Nominations for 1964 American Institute of Nutrition Fellows

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

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

Nominations (in 5 copies) are due by October 1. A supporting statement giving the reason for the nomination is desirable but not necessary.

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

Fellows Committee:

Icie Macy Hoobler, *Chairman*
H. E. Robinson
James Waddell
Charlotte M. Young
Paul E. Howe

Send nominations to:

Dr. Icie Macy Hoobler
502 Burson Place
Ann Arbor, Michigan

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

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

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

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

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

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

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

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

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

Committee on Honorary Memberships:

C. GLEN KING, *Chairman*
HELEN BURCH
W. D. SALMON

Send nominations to:

DR. C. GLEN KING
Nutrition Foundation
99 Park Avenue
New York 16, N. Y.

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

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

In vivo Interactions of Cadmium with Copper, Zinc and Iron[1,2]

C. H. HILL, G. MATRONE, W. L. PAYNE AND C. W. BARBER
North Carolina State College, Raleigh, North Carolina

ABSTRACT The chemical similarities between copper, zinc, and cadmium led to the speculation that there existed a copper component of cadmium toxicity in addition to the previously shown zinc component. Cadmium was found to be toxic to chicks at dietary levels of 25 to 400 ppm in a copper- and iron-deficient diet. The toxicity resulted in a reduced growth rate, mortality, microcytic hypochromic anemia, and atony and elongation of the gizzard. The growth depression and gizzard abnormality were corrected by increased dietary zinc. The mortality was reversed by added copper indicating that the speculation which prompted the study was valid. Increased dietary iron partially corrected both the mortality and the growth depression, indicating a previously unsuspected iron component of cadmium toxicity.

The interrelationship which exists between dietary zinc and copper has been well documented (1–5). This relationship is revealed by the reversal of some of the symptoms of zinc toxicity by added copper. Zinc has also been found to have a biological relationship to cadmium (6–8). In this instance added zinc prevents some of the symptoms of cadmium toxicity.

Cadmium and zinc have several common chemical parameters. The valence shell of the ions of these elements are isoelectronic, for instance. Both elements usually have a coordination number of 4, with tetrahedral configuration.

These considerations suggested the possibility of a mutual effect between cadmium and copper analogous to that found between zinc and copper. The studies presented in this report were designed to investigate this thesis.

EXPERIMENTAL

The chicks used in these studies were White Plymouth Rocks obtained from a commercial hatchery. They were housed in a conventional battery brooder with raised wire floors.

The basal diet used was based on dried skim milk, glucose, vitamins, minerals, and amino acids as described previously (9). It was found by analysis to contain 25 ppm zinc, 10 ppm iron, and 1 ppm copper. The watering and feeding troughs used were made of stainless steel. The drinking water was demineralized by passing it through an ion exchange column. Mineral supplements to the basal diet were made by the use of reagent grade $FeSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, ZnO, and $CdSO_4 \cdot H_2O$.

Erythrocyte numbers and packed cell volumes were determined by standard laboratory techniques. Hemoglobin was determined as the oxyhemoglobin.

Cytochrome oxidase was determined on heart homogenates by the method of Cooperstein and Lazarow (10). The protein content of the homogenates was evaluated by the method of Lowery et al. (11).

In the histological studies of the aorta, the tissues were stained with Mollier's quadruple stain (Gridley) in order to distinguish the elastic tissue.

The results were evaluated for statistical significance by analysis of variance.

RESULTS AND DISCUSSION

The results of the first 4 experiments are presented in table 1. The objective of this series of experiments was to determine the effect of the addition of copper and iron to the basal diet on the toxicity of cadmium fed at various levels up to 400 ppm.

The first experiment revealed that in the absence of copper or iron 25 ppm cad-

Received for publication January 21, 1963.

[1] The studies presented in this report were supported in part by grant A-5651 from the National Institutes of Health and grants from the Herman Frasch Foundation and the Moorman Manufacturing Company, Quincy, Illinois.

[2] Published with the approval of the Director of the Experiment Station as Paper no. 1552 of the journal series.

TABLE 1

Effect of copper and iron on the alleviation of cadmium toxicity

Supplement		Exp. no.	Days observed	Cadmium (ppm)					
Cu	Fe			0	25	50	100	200	400
ppm	*ppm*								
			Mortality (whole numbers)						
0	0	1[1]	17	5	13	10	16	19	20
10	100	1[1]	17	1	1	2	2	5	20
0	100	2[2]	15	3	1	4	18	16	24
40	0	2[2]	15	3	1	3	3	12	24
10	100	2[2]	15	1	0	1	7	8	24
10	100	3[1]	14	0	2	0	2	16	20
10	500	3[1]	14	0	1	0	1	8	19
50	100	3[1]	14	0	1	0	1	15	20
0	50	4[1]	21	4			19	15	
0	200	4[1]	21	5			17	20	
0	500	4[1]	21	2			15	20	
10	50	4[1]	21	1			7	17	
10	200	4[1]	21	1			7	16	
10	500	4[1]	21	0			1	14	
			Body weight in grams						
0	0	1	14	93	77	75	78	85	
10	100	1	14	152	136	131	97	76	
0	100	2	15	129	99	82	58	65	
40	0	2	15	125	99	82	76	63	
10	100	2	15	153	134	114	82	66	
10	100	3	14	120	124	93	68	53	
10	500	3	14	115	130	115	87	55	59
50	100	3	14	126	119	101	85	85	
0	50	4	21	171			75	75	
0	200	4	21	186			73		
0	500	4	21	179			81		
10	50	4	21	192			105	85	
10	200	4	21	199			100	63	
10	500	4	21	176			100	71	
			Hemoglobin g/100 ml (mean of 5 chickens)						
0	100	2	15	7.2	4.5	3.6	5.3	7.1	
40	0	2	15	3.6	3.6	4.5	4.0	7.0	
10	100	2	15	7.4	6.1	5.4	4.9	5.7	
0	50	4	21	7.3			4.1	4.5	
0	200	4	21	7.3			4.9		
0	500	4	21	8.2			3.9		
10	50	4	21	7.1			4.9	5.7	
10	200	4	21	8.4			4.8	5.4	
10	500	4	21	7.0			6.3	6.3	

[1] Started with 20 chicks/treatment combination.
[2] 24 chicks/treatment.

mium increased mortality, but in the presence of these 2 elements 200 ppm cadmium were required before a significant increase in mortality occurred. All the animals died when the diet contained 400 ppm cadmium regardless of the presence of copper and iron. Subsequent to the seventeenth day, all the chicks fed the basal diet died from iron and copper deficiencies. These mortality results indicate that relatively low levels of cadmium hastened the death of chicks fed a diet deficient in copper and iron.

Although the chicks receiving the copper- and iron-supplemented diet survived the effects of the lower levels of cadmium, their weights were progressively depressed as the cadmium level was increased. This

aspect of cadmium toxicity, therefore, was not reversed by the addition of copper and iron to the diet.

The results of this study could be interpreted to indicate that there was a direct interaction between copper plus iron and cadmium. However, the possibility also had to be considered that this apparent interaction was nonspecific; that is, chicks under the physiological stress of any dietary dificiency might be more susceptible to the attrition of cadmium. To assess this possibility and at the same time to determine which of the 2 elements, copper or iron, was responsible for the apparent interaction with cadmium, the second experiment was undertaken. In this experiment the basal diet was supplemented with either copper or iron, or both.

The chicks receiving the copper-supplemented diets were much more resistant to the toxic effect of cadmium as expressed by mortality than those groups receiving only the iron-supplemented diets. This is especially true when the groups receiving the intermediate level of 100 ppm cadmium are compared. Although the groups receiving only the copper-supplemented diets were more resistant to the effect of cadmium, they were anemic and therefore must be considered deficient in iron. This observation indicates that a nutritional deficiency, per se, does not reduce a chick's ability to withstand the deleterious effects of cadmium and lends strong support to the hypothesis that cadmium interferes with copper metabolism.

A comparison of the data from experiments 1 and 2 also shows that the mortality of the doubly deficient chicks in the first experiment was greater than those fed iron alone in the second experiment. The explanation offered for these results is that copper depletion of chicks receiving neither copper nor iron is faster than for chicks receiving iron but not copper (9).

Despite the sparing effect of copper on mortality in this experiment, there was again evidence from both the weight data and the hemoglobin levels that cadmium at 25 ppm was inhibitory even in the presence of copper. It was possible that the toxicity still manifested in the presence of copper in the diet was due to the quantitative inadequacy of the supplement. Accordingly, a third experiment was conducted in which higher levels of copper or iron were tested.

Cadmium toxicity as evidenced by growth retardation was evident at 50 ppm in this experiment except in that group receiving the higher level of iron, in which case 100 ppm cadmium depressed growth. The higher level of iron also decreased the mortality in those groups receiving 200 ppm cadmium. By these 2 criteria, prevention of growth depression and survivorship, this study indicates that there is an iron component of cadmium toxicity as well as the previously shown copper component.

For additional verification of this observation, the fourth experiment was conducted to test the consequences of adding higher levels of iron in both the presence and absence of added copper.

Again in this experiment, as in the third, the highest level of iron reduced the mortality caused by an intermediate level of cadmium, in this case 100 ppm. In the presence of copper, the highest level of iron also increased the hemoglobin concentration at this level of cadmium. These observations, then, confirm those of the previous experiment and indicate that there is an iron component of cadmium toxicity. The data suggest that this component is considerably less critical than that of the copper antagonism as measured by mortality differences. In view of this, in the subsequent experiments to be reported iron was added at a level of 100 ppm in all the diets.

Although copper was an important component in the toxicity of cadmium, increasing the copper level from 10 to 50 ppm in the presence of 100 ppm iron had no effect on the reduction in growth or mortality caused by cadmium (experiment 3). This indicates that there is a component of cadmium toxicity that is neither copper nor iron dependent. Attention was therefore directed toward the known antagonism between cadmium and zinc. The basal diet contained 25 ppm zinc, a level which would not, under ordinary conditions be considered especially deficient in this element.

To study the role of zinc in the toxicity of cadmium, zinc, copper, and cadmium

were added to the basal diet containing 100 ppm iron, in a factorially designed experiment. Two lots of 20 chicks each were fed each diet. The results of this experiment are presented in table 2. Copper significantly increased the weights of the chicks and cadmium significantly decreased them. Zinc had no overall effect alone, but there was a significant zinc-cadmium interaction. This interaction is indicative of the fact that in the presence of zinc, cadmium is much less effective in reducing the growth of the chicks. There was no copper-cadmium interaction indicating that the effect of cadmium on the growth of chicks was independent of the level of copper fed, thus confirming the previous experiments.

The mortality was significantly less in those lots receiving copper and significantly greater in those receiving cadmium. In this instance there was a significant interaction between cadmium and copper. This indicates that in the absence of copper, cadmium increased mortality but not in its presence. There was no zinc-cadmium interaction as far as mortality was concerned.

These data, then, make it possible to distinguish between 2 effects of dietary cadmium. The depression in growth is responsive to the addition of zinc, and is, therefore, presumably due to the antagonism of cadmium for zinc. The mortality is responsive to copper additions and thereby reflects the antagonism of cadmium for copper.

The observation that the cadmium-copper interaction is reflected in mortality indicates some fundamental biological derangement, possibly enzymological, in which copper plays a key role. One possible site at which this could occur was heart cytochrome oxidase, a copper sensitive enzyme. To determine the effect of these treatments on cytochrome oxidase activity, 4 chicks fed each diet were killed at 20 days and the activity determined.

Copper significantly increased the activity of the enzyme, but the other treatments, singly, had no effect. There was, however, a significant zinc-cadmium interaction. This interaction is a reflection of the fact that when either cadmium or zinc was fed alone the cytochrome oxidase activity was depressed, but when fed together the activity was increased.

These observations do not support the hypothesis that the effect of cadmium on mortality and its reversal by copper is a reflection of the influence of these 2 elements on heart cytochrome oxidase. In the absence of copper and zinc, cadmium greatly increased mortality, but had no effect on cytochrome oxidase activity. In the presence of copper, cadmium had no effect on mortality, but greatly affected the enzymatic activity. The interaction of

TABLE 2

Biological effects of copper, zinc and cadmium

Levels of copper	Experimental period	Zinc and cadmium levels (ppm)				Overall significant effects
		Zn 0 Cd 0	0 100	200 0	200 100	
ppm	*days*					
		Body weight in grams[1]				
0	14	214	131	174	149	Cu**, Cd** and
10		246	163	222	187	Zn × Cd**
		Mortality[2]				
0		4	17	13	19	Cu**, Cd**
10		1	1	0	1	Cd × Cu**
		Heart cytochrome oxidase activity[3] (OD change/min/mg protein)				
0		6.03	5.83	4.19	6.46	Cu** and
10		11.36	8.64	6.96	14.91	Zn × Cd**

[1] Average of 20 chicks at 2 weeks of age.
[2] Number dead at 19 days.
[3] Average of 4 chicks.
** Indicates highly significant ($P \leq 0.01$).

cadmium and zinc observed in this study is an example of the interrelationship of these 2 elements at a fundamental level of biochemical activity which reinforces the conclusions reached on the basis of growth data and is reassuring that the growth effect is not due to some trivial influence.

Another recently noted effect of copper deficiency which has been advanced as the cause of death of copper-deficient animals is the abnormal development of the elastic tissue of the aorta.[3] To assess the influence of these 8 diets on this tissue 4 chicks from each group were killed and the aortas removed and histological observations were made. The results of that study are presented in table 3. All of the copper-deficient chicks had some degeneration of the elastic tissue of the aorta. The presence of cadmium plus zinc in those lots not receiving copper intensified the degeneration somewhat. The addition of copper resulted in normal aortas. The addition of zinc to those groups receiving copper had no effect, whereas the addition of cadmium resulted in a slight degeneration in all the aortas. The addition of cadmium and zinc to the copper-containing diet resulted in normal aortas in 3 of the 5 examined. The other 2 showed only slight degeneration. An example of marked degeneration of the aortic elastic tissue is shown in figure 1 along with a normal aorta.

The degenerative changes in this tissue do not parallel the increased mortality of chicks fed cadmium or zinc, or both, in the absence of copper. Although both cadmium and zinc greatly increased mortality in these groups, the aortic scores were almost identical. This may be an indication that the increase in mortality is due to the interference of these 2 elements with some vital copper-dependent system other than that concerned in maintaining the integrity of the aortic elastic tissue. It is also possible that histological techniques used have not revealed subtle differences in the degree of degenerative changes of this tissue which might be meaningful for survival.

Another gross abnormality was observed only in those lots receiving cadmium in the diet. There was marked atony and elongation of the gizzard with dilation of the posterior portion of the proventriculus. The relative degrees of this condition are presented in table 4. Copper addition alone had no effect on this condition, but zinc addition partially corrected the abnormality. Copper plus zinc addition prevented the condition. The genesis of this syndrome, whether nervous or muscular, is unknown; but these results leave little doubt that it is precipitated by the presence of dietary cadmium, and probably reflects the antagonism of cadmium for zinc.

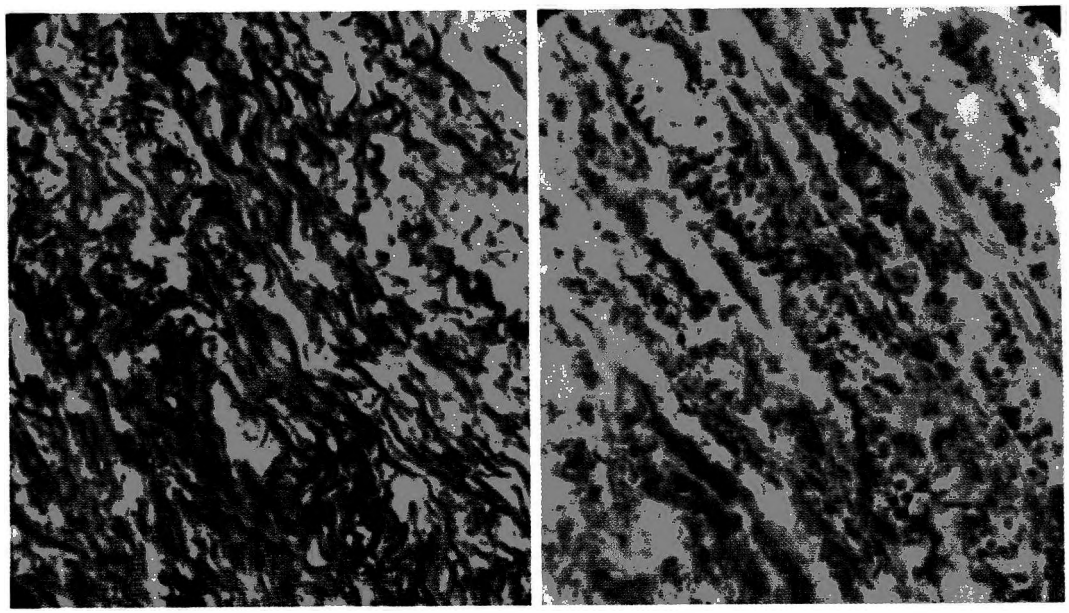
To summarize the results of this experiment, there was a significant zinc cadmium interaction on growth and heart cytochrome oxidase activity and a significant cadmium-copper interaction on mortality. While supplementation with cadmium plus zinc resulted in consistent marked degenerative changes in the elastic tissue of the aorta, this could be completely prevented by the addition of 10 ppm copper to the diet. Cadmium supple-

[3] J. E. Savage, D. A. Rosa, G. Reynolds and B. L. O'Dell 1962 Copper deficiency and beta-amino propionitrile toxicity in turkeys. Federation Proc., 21: 311 (abstract).

TABLE 3

Effect of zinc, copper, and cadmium on elastic tissue degeneration of the aorta

Treatment	Degrees of degenerative changes			
	None	Slight	Moderate	Marked
Basal	0	1	1	2
Zn, 200 ppm	0	1	1	2
Cd, 100 ppm	0	2	0	2
Cd, 100 ppm + Zn, 200 ppm	0	0	0	4
Cu, 10 ppm	4	0	0	0
Cu, 10 ppm + Zn, 200 ppm	3	0	0	0
Cu, 10 ppm + Cd, 100 ppm	0	4	0	0
Cu, 10 ppm + Cd, 100 ppm + Zn, 200 ppm	3	2	0	0



(a) Normal aorta (b) Copper-deficient aorta

Fig. 1 Degeneration of aortic elastic tissue in copper deficiency; (a) normal aorta, (b) copper-deficient aorta — note absence of black elastic fibers. Stained with Mollier's quadruple stain (Gridley). × 430.

TABLE 4

Effect of cadmium, copper, and zinc on the atony and elongation of the gizzard

Supplement	Degree of pathological change[1]
Cadmium, 100 ppm	++++
Cadmium, 100 ppm + copper, 10 ppm	++++
Cadmium, 100 ppm + zinc, 200 ppm	++
Cadmium, 100 ppm + zinc, 200 ppm + copper, 10 ppm	—

[1] Number of +'s reflect increasing severity of the condition.

mentation resulted in marked atony and elongation of the gizzard which could be partially prevented by zinc additions and completely prevented by zinc and copper supplementation.

In this experiment the addition of zinc greatly reduced the effect of cadmium on body weights, but it did not completely overcome it. There were 3 possible explanations for this. It was possible that the copper supplementation was inadequate to allow the zinc addition to fully express its effect on the reversal of cadmium- induced weight reduction, or the zinc additions were inadequate to completely overcome the cadmium toxicity, or cadmium interfered with some system other than the copper- and zinc-dependent ones. To assess the adequacy of the copper and zinc supplements, 2 further experiments were conducted. In the first the effects of copper, zinc, and cadmium were examined at 3 levels of each element and in all combinations. The results are presented in table 5.

In the absence of cadmium, zinc retarded growth at the 2 lower levels of copper supplementation. At the highest level, 50 ppm, it did not. Cadmium at 100 ppm retarded growth. The previously observed interaction between cadmium and zinc was again evident in that in the presence of 100 ppm cadmium the additions of zinc increased growth instead of further decreasing it. Again, however, growth was not restored to that of the control animals not fed zinc or cadmium.

Both cadmium and copper had a highly significant effect on the hemoglobin levels,

TABLE 5

Effect of cadmium, copper and zinc on body weights and blood values

Cadmium	Levels of copper and zinc (ppm)									Overall significant effects
	Cu	0		10			50			
	Zn	100	200	0	100	200	0	100	200	
				Body weights in grams[1]						
0	119	88	91	144	122	84	137	127	128	Cu**, Cd**
100	66	74	79	87	111	115	76	99	97	CuZn**, CuCd**
200	61	70	77	72	90	77	86	86	90	CdZn**, CuZnCd**
				Hemoglobin concentrations (g/100 ml)[2]						
0	7.6	6.1	5.5	8.3	8.5	8.2	8.4	8.6	8.5	Cu**, Cd**, Zn*
100	7.3	6.2	4.6	5.4	7.1	6.3	6.4	6.0	6.7	Cu × Zn**
200	6.6	5.5	5.4	6.8	6.4	6.7	6.2	5.7	6.1	Cu × Cd**
				Erythrocyte concentration (10^6/mm^3)[2]						
0	2.40	2.42	1.86	2.55	2.75	2.57	2.89	2.73	2.50	Cu**, Cd*
100	2.16	2.32	2.04	2.15	2.46	2.36	2.53	2.34	2.38	Cu × Zn*
200	2.19	2.41	2.04	2.67	2.50	2.46	2.58	2.29	2.54	Cu × Zn × Cd**
				Mean cell hemoglobin ($\mu\mu$g/cell)[2]						
0	31.6	26.0	30.0	32.9	31.1	32.0	29.1	31.5	35.2	Cd**
100	34.4	26.6	22.6	25.6	29.0	26.5	25.6	25.5	28.5	Cu × Zn**
200	30.4	22.9	26.5	26.0	25.8	26.9	24.2	25.1	24.7	

[1] Two weeks weight, 20 chicks/mean.
[2] Eight chicks/treatment.
* Indicates significant ($P \leq 0.05$).
** Indicates highly significant ($P \leq 0.01$).

cadmium reducing it and copper increasing it. The effect of zinc in reducing hemoglobin concentrations was significant at the 5% level of probability. There was a significant interaction between copper and zinc in that zinc had no effect on hemoglobin in the presence of copper. There was also a significant copper-cadmium interaction since the effect of copper in increasing the hemoglobin level was much reduced in the presence of cadmium.

The hemoglobin concentration is in reality made up of 2 components, the concentration of erythrocytes and the amount of hemoglobin in each erythrocyte. It was possible that these 3 elements were affecting the hemoglobin concentration by different mechanisms which would be revealed by an analysis of the erythrocyte concentration and mean cell hemoglobin. Accordingly, the number of erythrocytes per cubic millimeter was determined by standard procedures. The analysis of the data indicated that only the addition of copper had a highly significant effect on the number of erythrocytes. The effect of cadmium in reducing the number was significant at the 5% level of probability. There was a significant copper-zinc interaction which indicated that zinc reduced the number of erythrocytes only in the absence of copper.

With the hemoglobin concentration and erythrocyte numbers determined, the mean cell hemoglobin values were calculated and the results subjected to analysis of variance. The only significant effect of any single treatment was that of cadmium in decreasing this value. There was a significant copper-zinc interaction which is a reflection of the observation that zinc decreased this value in the absence of copper, had no effect when copper was fed at 10 ppm, and increased the value when the level of copper in the diet was 50 ppm.

The packed cell volume was also determined in this experiment and the mean cell volume calculated. The analysis of the results indicated that the only single treatment that had a significant effect on this value was cadmium which reduced it.

The results of these findings indicate that the lowering of the concentration of hemoglobin caused by the presence of cadmium is partly due to a decrease in the number of erythrocytes, but mainly due

to the decreased hemoglobin content of each erythrocyte. Since the cells are smaller, the anemia caused by the presence of cadmium in the diet would be classified as microcytic, hypochromic.

Since the addition of zinc did not completely prevent the cadmium-induced growth retardation in the presence of as much as 50 ppm copper, the final experiment was conducted in which the levels of zinc were increased to 400 ppm. The results of that experiment are presented in table 6.

Both cadmium and zinc depressed growth in the absence of copper. In the presence of copper, however, only cadmium reduced growth. This reduction was completely overcome by the addition of 400 ppm zinc to the diet.

The effects of these levels of the elements on the hemoglobin concentration and erythrocyte numbers were also determined in this experiment. Copper had a highly significant effect in increasing the hemoglobin concentration and cadmium in reducing it. There was a significant copper - cadmium interaction indicating that cadmium was much more effective in reducing the hemoglobin levels in the presence of copper. Copper was effective in increasing the number of erythrocytes and cadmium in reducing them. There were no significant interactions between treatments.

Cadmium and zinc significantly reduced the mean cell hemoglobin. The interaction between copper and cadmium, which was highly significant, indicated that cadmium was more effective in this respect in the presence of copper than in its absence.

Cadmium, alone, had a significant effect in reducing the size of the erythrocytes. The overall effect of dietary cadmium was again, then, to produce a microcytic, hypochromic anemia.

The results of this series of experiments indicate that cadmium toxicity can be the result of at least 3 components. These components are characterized by being iron sensitive, zinc sensitive, or copper sensitive. The addition of iron can partially correct both the weight depression and mortality caused by cadmium, as in experiment 3. The addition of zinc can reverse the cadmium-induced growth depression, whereas the addition of copper can reduce the mortality caused by cadmium. The thesis that prompted this series of investigations is, thus, affirmed.

TABLE 6

Effect of cadmium, copper and zinc on body weights and blood values

Level of cadmium	Levels of copper and zinc (ppm)						Overall significant effects
	Cu	0			25		
	Zn	0	200	400	0	200	400	
				Body weight in grams[1]			
0	112	101	88	135	120	124	Cu**, Cd**
100	86	73	80	83	99	125	Cu × Zn**
							Zn × Cd**
							Zn × Cd × Cu**
				Hemoglobin concentrations (g/100 ml)[2]			Cu**
0	5.9	5.6	5.1	8.6	7.3	7.5	Cd**
100	5.4	4.4	5.0	5.4	5.6	6.2	Cu × Cd**
				Erythrocyte concentrations (10^6/mm^3)[2]			
0	2.16	1.94	2.14	2.54	2.51	2.45	Cu**
100	1.85	1.75	2.06	2.21	2.32	2.39	Cd**
				Mean cell hemoglobin ($\mu\mu$g/cell)[2]			
0	27.7	29.1	23.9	34.2	28.8	25.9	Cd**, Zn*
100	29.2	25.3	24.0	24.9	23.9	25.9	Cu × Cd**

[1] Two weeks body weight, 20 chicks/mean.
[2] Eight chicks/treatment.
* Indicates significant ($P \leq 0.05$).
** Indicates highly significant ($P \leq 0.01$).

The chemical similarities that exist between cadmium, zinc, and copper suggest an explanation of these results. Cadmium may replace both copper and zinc at active metabolic sites, probably enzymatic, thereby rendering them inactive. (Druyan and Vallee[4] have shown that cadmium can replace zinc in liver alcohol dehydrogenase and therewith inactivate it.) In the presence of cadmium, therefore, addition of both copper and zinc to the diet is required to restore the nutritional status of the animal to that of the controls not receiving cadmium. This presumably takes place because the additional copper and zinc displaces the interfering cadmium and restores normal metabolism.

Although there is an iron component of cadmium toxicity, there may be no direct metabolic interaction between these two elements. In the presence of cadmium the animals are, in effect, deficient in both copper and zinc. It has been shown (9) that an inverse relationship exists between the requirements for iron and copper. In the presence of iron less copper is required by the chick. In the present circumstances, then, the addition of iron would make more copper metabolically available to overcome the cadmium toxicity. This would account for the increased survivorship of the cadmium-fed chicks when iron was added to the diet.

There are no data on the relationship between the dietary requirements of zinc and iron comparable to that of copper and iron. If the same relationship were present, then additional iron could increase the metabolic pool of zinc available for counteracting the cadmium toxicity and thereby account for the partial correction of the weight depression due to cadmium.

ACKNOWLEDGMENTS

The authors are deeply indebted to J. Carter, W. Britton, Mrs. B. Baker, and Mrs. C. Bunn for their excellent technical assistance throughout these studies.

LITERATURE CITED

1. Smith, S. E., and E. J. Larson 1946 Zinc toxicity in rats and antagonistic effects of copper and liver. J. Biol., Chem., *163:* 294.
2. Van Reen, R. 1953 Effects of excessive dietary zinc in the rat and the interrelationship with copper. Arch. Biochem. Biophys., *46:* 337.
3. Duncan, G. D., L. F. Gray and L. J. Daniels 1953 Effect of zinc on cytochrome oxidase activity. Proc. Soc. Exp. Biol. Med., *83:* 625.
4. Magee, A. C., and G. Matrone 1960 Studies on growth, copper metabolism and iron metabolism of rats fed high levels of zinc. J. Nutrition, *72:* 233.
5. Hill, C. H., and G. Matrone 1962 A study of copper and zinc interrelationships. Proc. Twelfth World's Poultry Congress, p. 219.
6. Parizek, J. 1957 The destructive effect of cadmium ion on testicular tissue and its prevention by zinc. J. Endocrinol., *15:* 56.
7. Gunn, S. A., T. C. Gould and W. A. Anderson 1961 Zinc protection against cadmium injury to rat testis. Arch. Path., *71:* 274.
8. Supplee, W. C. 1961 Production of zinc deficiency in turkey poults by dietary cadmium. Poultry Sci., *40:* 827.
9. Hill, C. H., and G. Matrone 1961 Studies on copper and iron deficiencies in growing chickens. J. Nutrition, *73:* 425.
10. Cooperstein, S. J., and A. Lazarow 1951 A microspectrophotometric method for the determination of cytochrome oxidase. J. Biol. Chem., *189:* 665.
11. Lowery, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall 1951 Protein measurement with the Folin phenol reagent. Ibid., *193:* 265.
12. O'Dell, B. L., B. C. Harwick, G. Reynolds and J. E. Savage 1961 Connective tissue defect in the chick resulting from copper deficiency. Proc. Soc. Exp. Biol. Med., *108:* 402.
13 Shields, G. S., W. F. Coulson, D. A. Kimball, W. H. Carnes, G. E. Cartwright and M. M. Wintrobe 1962 Studies on copper metabolism. XXXII. Cardiovascular lesions in copper deficient swine. Am. J. Path., *41:* 603.

[4] R. Druyan and B. I. Vallee 1962 Exchangeability of the zinc atoms in liver alcohol dehydrogenase. Federation Proc., *21:* 247 (abstract).

Intestinal Synthesis of Folic Acid in Monoflora Chicks[1]

HERMAN T. MILLER[2] AND T. D. LUCKEY
Biochemistry Department, University of Missouri Medical Center, Columbia, Missouri

ABSTRACT A new germfree chicken cage which has been described in the literature, was used to rear germfree and monoflora chicks for studies on the intestinal synthesis of folic acid. Seven-day-old chicks shown previously to be germfree and fed 100 μg of added folic acid/kg of diet were monoinoculated with *Escherichia coli*. These chicks were allowed to grow for 14 days. The stimulatory effects of the bacteria were detected in the growth rate, hematocrits and hemoglobin values. Analysis of the water at the termination of the experiments indicated that the chicks could not have drunk enough water to obtain the necessary amount of folic acid. When modified cages were used, in which the water was changed periodically, analysis showed conclusively that there was no significant amount of folic acid synthesis taking place in the water trough. The contributions due to coprophagy, attempts to monoinoculate with other strains of bacteria and possible growth stimulation using a complete diet were considered.

The use of germfree animals as the control for monoflora animals has been reviewed by Luckey (1) under the title of gnotophoric animals. Gnotophoric animals were defined as animals that are in intimate contact with only known species. They are expected to be more valuable than germfree animals for research on intestinal synthesis, the in vivo metabolism of microorganisms and the illucidation of immunologic phenomena.

Intestinal synthesis as applied herein means the synthesis of a material by intestinal microbes with subsequent absorption and utilization by the host without coprophagy. Mickelsen (2) has reviewed data supporting the theory of intestinal synthesis in classic (equivalent to, but preferred over, the term conventional) animals. Evidence against this type of data has been suggested by Luckey (3), and data from germfree animals which may be interpreted for and against this theory has been reviewed by Luckey (1) and Mickelsen (4). In the nonruminant, there is no unequivocal proof that the vitamins which are synthesized are subsequently absorbed by the host. The evidence from experiments with radiothiamin by Wostmann and Knight (5), indicates that thiamine was synthesized but was not absorbed.

In this investigation, chickens were reared germfree for short periods of time before the experimental group was monoinoculated with a pure culture of bacteria that was known to synthesize folic acid. The sterilized diet was low in folic acid. Hematocrit, hemoglobin and growth were taken as indicators of the extent of folic acid deficiency. These criteria were chosen because of the role of folic acid in methyl group biosynthesis and indirectly in cell nucleus formation. Differences between the performance of germfree and monoflora chicks were attributed directly or indirectly to the activities of the microorganisms.

MATERIALS AND METHODS

Eggs for this investigation were obtained from the Poultry Department of the University of Missouri. The procedures for washing the eggs, sterilizing the shell surface, and determination of the germfree status of the chicks have been reported by Reyniers et al. (6).

The cage, a stainless steel box with a glass top for viewing, and a "Bactytector" for the determination of the microbiological status, was sterilized in an autoclave. Previously sterilized diet was added to the cage by using a large autoclave as a sterile hood. The steam was turned on, the glass

Received for publication February 27, 1963.

[1] Portion of a dissertation presented as a partial fulfillment of the requirement for the Ph.D. degree: This investigation was supported by National Science Foundation Grant no. G-4503.

[2] Present address: Protein Chemistry Laboratory, Department of Food Science and Technology, University of California, Davis, California.

top was pulled to the back position, and the sterile diet was poured into the feed bin. The steam from the autoclave was used as positive pressure to keep out air-borne microbes. The cage with two 5-day-old chicks is shown in figure 1. Characteristics of this cage, the operating procedure and the hatching of chicks have been presented by Miller and Luckey (7).

The composition of the complete syntype (synthetic-type) diet as mixed is shown in table 1. The folic acid was deleted or varied for this investigation. The diet was double-wrapped in heavy paper and autoclaved 15 minutes at 120°C.

The hematocrits were run according to the procedure of Wintrobe (8). Several samples of blood were obtained by touching heparinized capillary tubes to a formed blood droplet made by cutting the wing vein of the chicken. The tubes were centrifuged and read on a standard microhematocrit reader. The method of hemoglobin analysis made use of the cyanomethemoglobin formed in the presence of small amounts of cyanide. This method was outlined by Drabkin (9). For the analysis, a hemoglobin standard[3] was used to construct the standard curve. The samples were evaluated by comparison with this standard curve. Blood samples were

[3] Acuglobin, the hemoglobin standard, was obtained from Ortho Pharmaceutical Corporation, Rariton, New Jersey.

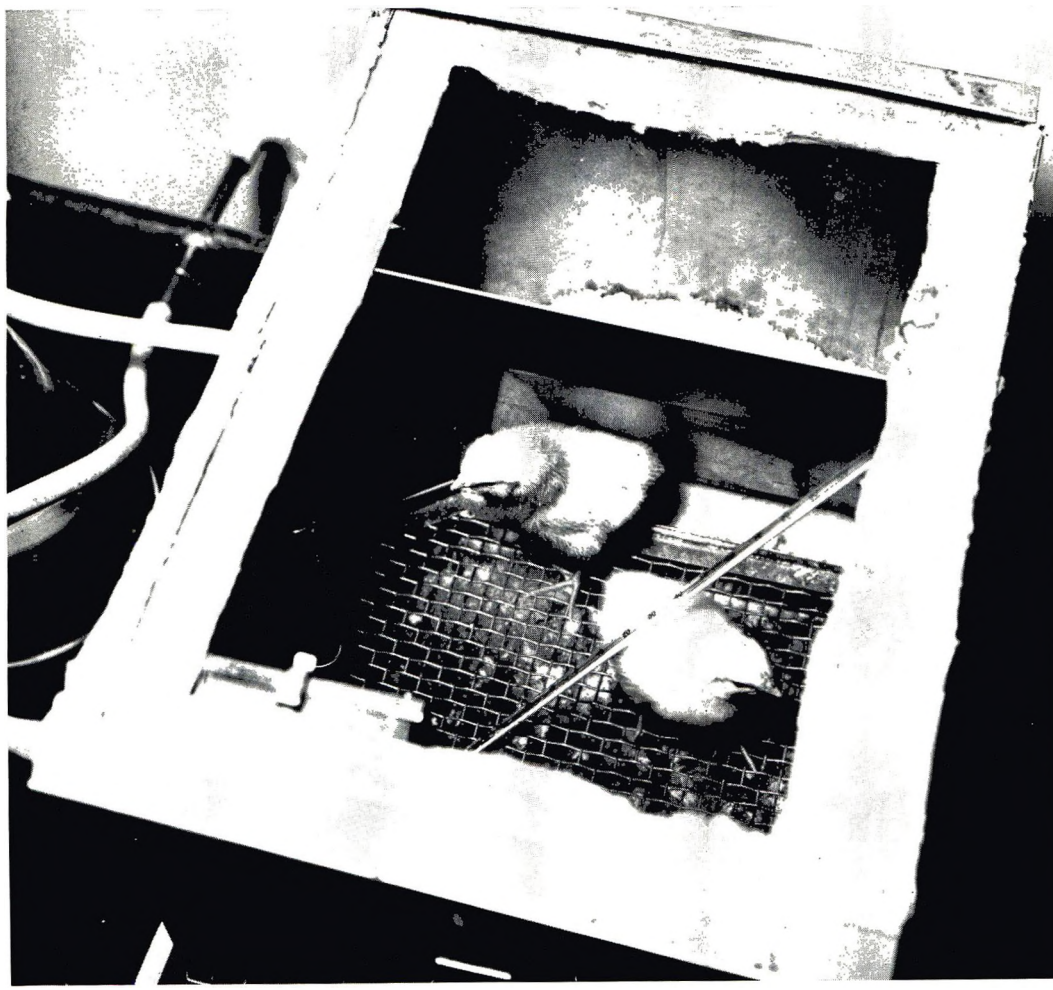


Fig. 1 A top view into the new germfree chicken cage.

TABLE 1

Composition of syntype diet

Ingredients	g/kg
Cellulose[1]	60
Starch	425
Corn oil	80
Casein, vitamin-free	330
Glycine	9
L-Cystine	4
L-Arginine·HCl	10
Choline Cl	2.4
Salts	80
p-Aminobenzoic acid	0.02
Thiamine	0.24
Riboflavin	0.09
Niacin	0.30
Pantothenic acid	0.60
Pyridoxine·HCl	0.30
Biotin	0.0015
Folic acid	0.06
Vitamin B_{12}	0.00006
Inositol	2.00
Ascorbic acid	2.00
	amount/kg
Vitamin A	20,000 IU
Vitamin D_3	2,000 IU
Vitamin E	0.30 g
Vitamin K	0.02 g

Salt mix	g/kg
$CaCO_3$	18.0
$Ca_3(PO_4)_2$	3.3
K_2HPO_4	13.5
NA_2HPO_4	12.0
NaCl	3.0
KI	0.045
$MgSO_4$	2.15
$MnSO_4$	0.53
$Fe(C_6H_5O_7)$	4.5
$CuSO_4$	0.23
$CoCl_2$	0.03
$ZnSO_4$	0.06
$Na_2B_4O_7 \cdot 10\ H_2O$	0.03
$KAl(SO_4) \cdot 12\ H_2O$	0.045
$Na_2MoO_4 \cdot 2\ H_2O$	0.30

[1] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

taken at the termination of the experiment, immediately after the chicks were removed from the cage.

EXPERIMENTAL

To show that the diet was adequate, classic White Leghorn chicks were fed the sterilized syntype diet (table 1) for 3 to 5 weeks. Classic chicks fed a nonsterile commercial ration in a conventional brooder were used as controls. Some germfree chicks were fed the complete syntype diet; another group of control chicks, reared in the germfree state, were fed a steam sterilized commercial diet.

To determine a proper level of dietary folic acid for subsequent studies, preliminary experiments were run with groups of germfree chicks fed diets containing zero, 100, 200, 500 and 1250 μg of folic acid/kg of diet.

Gnotobiotic techniques. The culture of *Escherichia coli* used in this investigation was isolated from the conventional chicken cages using the standard methods of bacteriology. The organism was shown definitely to be *E. coli* by morphologic and physiologic characteristics. The organism from agar slants was inoculated into an appropriate medium[4] for 18 hours prior to the time of use. The suspended cells were centrifuged and the medium was decanted. The cells were resuspended in saline, centrifuged, and the supernatant decanted 3 times.

Seven-day-old chicks, shown to be germfree, were monoinoculated after the drinking water had been withheld for 12 to 15 hours. Five milliliters of a medium heavy suspension of the bacteria from a sterile syringe and needle were added to the cage by flaming the rubber tubing of the water inlet until it melted slightly and thrusting the needle into the tube and into the water trough. The resulting needle hole was closed by re-heating the tube and applying glue. The inoculum was immediately made available to the chicks by lifting the water container and administering water as usual. Since the chicks were very thirsty, they drank vigorously and an adequate intake of the pure culture was assured. The chicks were allowed to eat and drink ad libitum until they were 21 days old. At 3 weeks, the cage and chicks were examined for contaminating microbes other than *E. coli*, blood samples were taken and the chicks were weighed and killed. Chicks that were monoinoculated, as well as those for the germfree experiments, were examined microbiologically as outlined by Wagner (10).

To be able to determine whether the bacteria were making the vitamin available to the chicks by synthesizing folic acid in the water trough, the water was

[4] Micro Inoculum Broth from Difco, Dertoit, Michigan.

analyzed microbiologically for folic acid (11). The analyses were performed at the termination of the experiments when the concentration of folic acid should have been maximum. The amount found was negligible. Further, the cage was modified by attaching a copper pipe to a hole drilled in the bottom of the water trough. This pipe in turn was connected to a sterile rubber tube containing a pinch clamp, and its open end was held beneath a germicidal solution. Each morning and early afternoon, the water trough was emptied by this modified outlet and fresh water was added. This frequent change of water did not allow long periods of time for the bacteria to synthesize folic acid. This prevented the observed effects from being attributed to microbial synthesis in the water trough.

RESULTS

Germfree chicks reached an average weight of 210 g in 3 weeks when fed either a sterilized commercial ration or the complete syntype diet. This rate of growth was comparable to that of classic chicks fed these diets. This average weight was obtained from about 20 germfree birds in each group reared before the start of the experiments in which the levels of folic acid in the diet were varied. The results from many separate cages (fig. 2) indicate the folic acid requirement for these germfree chicks fed this diet may be slightly under 0.5 mg/kg diet. There was no significant difference between the weights of germfree chicks fed 0.5 mg of folic acid/kg of diet and those fed 1.25 or 60.0 mg of folic acid/kg of diet. When diet with no added folic acid was fed, the germfree chicks usually died of folic acid deficiency before 21 days. Those that survived had an average weight of 95 g. The chemical and morphologic characteristics of folic acid deficient germfree birds have been reviewed (1).

The next level of folic acid fed to germfree chicks was 100 μg/kg of diet. Results of such feeding experiments at 3 weeks are shown in table 2. Both hematocrit and hemoglobin values are less than 50% of the values found when more was fed. These birds had blood that was only faintly red in color as compared with the dark red color of normal chicken blood. These chicks did routinely live for 21 days under germfree conditions.

When chicks were reared germfree for 21 days with a diet containing 200 μg of added folic acid/kg of diet, they appeared to be nearly normal. However, their average weight was 158 g. Although these chicks did not grow at the maximal rate,

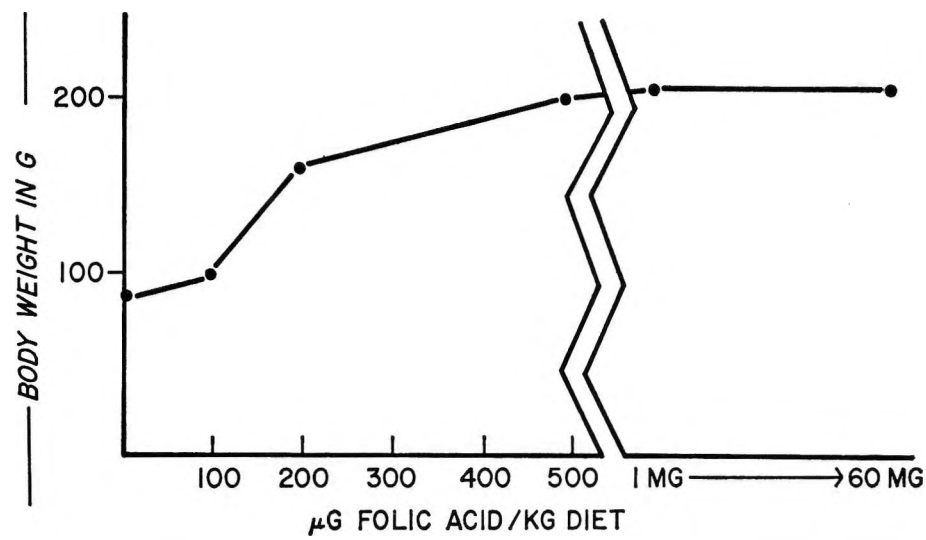


Fig. 2 Weight of germfree chicks at 3 weeks when the level of folic acid was varied in the syntype diet.

TABLE 2

Comparison of germfree and monognotophoric (Escherichia coli) chicks

	Germfree				P	Gnotophoric, *E. coli*				Gnotophoric, *E. coli*[1]		
	No.	Mean	Range	σ		No.	Mean	Range	σ	No.	Mean	Range
100 μg Folic acid/kg diet												
Body weight, g	9	106	89–117	9.26	< 0.001	15	145	131–160	9.17	4	149	135–167
Hemoglobin, %	8	4.45	4.0–5.0	0.384	< 0.001	14	7.90	5.9–9.6	1.13	4	8.3	7.5–8.9
Hematocrit, %	8	12.9	10.5–16.5	2.21	< 0.001	15	30.1	23.0–37.0	4.01	3	29.8	27.5–31.7
Liver folate, μg/g	8	0.750	0.68–0.82	0.0444	< 0.001	7	1.32	1.23–1.39	0.0674	–	—	—
Muscle folate, μg/g	9	0.0456	0.04–0.07	0.100	< 0.001	9	0.104	0.09–0.14	0.017	–	—	—
Brain folate, μg/g	5	0.050	0.04–0.06	0.0020	< 0.001	6	0.257	0.20–0.29	0.0104	–	—	—
200 μg Folic acid/kg diet												
Body weight, g	6	158	144–170	—	—	—	—	—	—	–	—	—

[1] Water trough cleaned twice daily.

their blood development was much improved. Since germfree chicks grew much better when fed 200 μg of folic acid/kg of diet than when they were fed 100 μg of folic acid/kg of diet, the 100-μg level was chosen as the diet to use in the monoflora studies with *E. coli*.

Davis showed (12) that *E. coli* can synthesize the benzene ring; however in this investigation, *p*-aminobenzoic acid was included in all of these syntype diets in order to give optimal opportunity for folic acid biosynthesis. Growth of chicks that were fed 100 μg of added folic acid/kg of diet and inoculated with a single strain of *E. coli* on the seventh day is shown in table 2. These birds were shown to be infected with only *E. coli* at the termination of the experiment. The average weight of these chicks was 145 g and the hematocrit and hemoglobin values increased almost twofold above the values obtained when germfree chicks were fed the same diet. Four inoculated chicks were reared without *p*-aminobenzoic acid in the diet and there was no apparent difference in the results. When monoflora chicks were reared in the modified cage where the water in the cages was changed periodically, and fed 100 μg of folic acid/kg of diet, the results (table 2) showed no significant difference between these chicks and those reared in the monoflora state without frequent water changes.

In the gnotophoric cages where the water was not changed, the average value for folic acid was 6.0 mμg/ml. The water trough had a capacity of 250 ml or a total content of folic acid at any one day of 1.50 μg. Analysis of the basal diet with no added folic acid yielded 200 μg of folic acid/kg of diet. Consistent analysis of the basal diet plus 200 μg of added folic acid (all after autoclaving) yielded only 325 μg of folic acid/kg of diet. This analysis showed that from one-quarter to one-third of the added folic acid had been destroyed by autoclaving. Most of the experiments used 3 chicks for 21 days and 1200 g of diet were added to each cage. The amount of diet and the calculations from the analysis of the diet showed that each chick received about 6 μg of folic acid/day from the diet with 200 μg added folic acid. The monoflora chicks reached

almost the same weight in 3 weeks as had the germfree chicks that received 200 μg of folic acid/kg of diet. In order for the monoflora chicks to obtain enough folic acid from the water trough, they would have had to drink about 500 ml/day instead of the expected 20 ml/day. The water analysis from the modified cage yielded only 1.1 mμg/ml. The chicks that were reared in the modified cage as monoflora chicks would have had to drink about 2700 ml of water/day to receive the needed amount of folic acid. Assuming no coprophagy, analysis and calculations provide strong evidence that the bacteria were synthesizing folic acid in the chicken intestine.

DISCUSSION

A comparison of the data from germfree and monoflora chicks reared with diets containing 100 μg of folic acid/kg of diet showed that there was a difference in body weight, hematocrit and hemoglobin values at a level of significance of < 0.001 (table 2). Analysis of the water at the termination of the experiments showed that the chicks would have had to drink impossible volumes of water to obtain the necessary amount of folic acid from this source. It was further observed that the growth and hematopoiesis of the monoflora chicks was not affected by the amount of *p*-aminobenzoic acid in the diet.

Since the chicks were reared on wire floors, it was assumed that they did not practice coprophagy to a great extent. Therefore, folic acid in the feces was probably not recycled into the absorptive areas of the intestine. It was therefore assumed that the amount of folic acid gained by this practice was minimal.

The strain of *E. coli* used in this investigation was isolated from conventional chicken brooders since it had been observed that other strains of *E. coli* would not establish themselves in the chicken intestine. This same phenomena was noted in studies with *Streptococcus faecalis*, an organism used in the studies of host-bacterium competition for folic acid by Miller.[5] Some strains would not grow well enough to yield more than 10 to 15 cells per microscope field when the feces slides were observed.

From the growth rates indicated (fig. 2), it appears that about 500 μg of folic acid/kg of diet is close to the minimal requirement for germfree White Leghorn chicks fed this diet. This is similar to that reported by Luckey et al. (13) for classic White Leghorn chicks fed a high protein, starch diet. Such comparison suggests that the net effect of the microorganisms may be zero. Any positive effect from one species (as *E. coli*) may be counteracted by other species of microorganisms.

Growth stimulation of the monoflora chicks is strong indirect evidence for the intestinal synthesis theory since the diet was deficient in folic acid. It is possible that a pure culture of *E. coli* might stimulate growth of chicks fed a complete synthetic type diet by some unknown mechanism. For this reason, such a control group would have been desirable.

A comparison of the tissues (table 2) showed that the gnotophoric chicks fed diets containing 100 μg of folic acid/kg of diet had about twice as much folic acid per gram of tissue as did the germfree birds fed the same diet. These data have been reported in detail by Miller.[5]

LITERATURE CITED

1. Luckey, T. D. 1963 Germfree Life and Gnotobiology. Academic Press, Inc., New York.
2. Mickelsen, O. 1956 Intestinal synthesis of vitamins in the non-ruminant. Vitamins and Hormones, *14:* 1.
3. Luckey, T. D., J. R. Pleasants and J. A. Reyniers 1955 Germfree chicken nutrition. II. Vitamin interrelationships. J. Nutrition, *55:* 105.
4. Mickelsen, O. 1962 Nutrition-germfree animal research. Ann. Rev. Biochem., *31:* 515.
5. Wostmann, B. S., and P. L. Knight 1961 Synthesis of thiamine in the digestive tract and the gut. J. Nutrition, *74:* 103.
6. Reyniers, J. A., P. C. Trexler, R. F. Erwin, M. Wagner, T. D. Luckey and H. A. Gordon 1949 Rearing Germfree Chickens. LOBUND Rept. 2:1.
7. Miller, H. T., and T. D. Luckey 1962 A new germfree chicken cage — characteristics and use. Appl. Microbiol., *10:* 52.
8. Wintrobe, M. M. 1956 Clinical Hematology, ed. 4. Lea and Febiger. Philadelphia, p. 1184.
9. Drabkin, D. L., and J. A. Austin 1935 Spectrophotometric studies: V. Techniques

[5] Miller, Herman T., unpublished Ph.D. thesis. University of Missouri, Columbia, Missouri, 1962.

for the analysis of undiluted blood and concentrated hemoglobin solutions. J. Biol. Chem., *112:* 105.

10. Wagner, M. 1959 Determination of germ-free status. Ann. New York Acad. Sci., *78:* 95.
11. Flynn, L. N., V. P. Williams, B. L. O'Dell and A. G. Hogan 1951 Medium for assay of vitamins with lactic acid bacteria. Anal. Chem., 23: 180.
12. Davis, B. D. 1951 Aromatic synthesis. I. The role of shikimic acid. J. Biol. Chem., *191:* 315.
13. Luckey, T. D., P. R. Moore, C. A. Elvehjem and E. B. Hart 1946 Effect of diet on the response of chicks to folic acid. Proc. Soc. Exp. Biol. Med., 62: 307.

A Comparison of Several Amino Acid and Casein Diets for the Growing Rat[1]

L. H. BREUER, JR., W. G. POND, R. G. WARNER AND J. K. LOOSLI
Department of Animal Husbandry, Cornell University, Ithaca, New York

ABSTRACT Weight gains and feed consumption of weanling male rats were measured when fed purified diets containing either casein or one of six different amino acid mixtures, and accompanied by variations in the level of fat as well as source of carbohydrate and salts.

The casein diets (14 and 20%) were superior to any of the amino acid diets tested. The amino acid diet of Rechcigl et al. (7) containing 15.68% essential amino acids and no nonessential amino acids supported significantly better growth than the diets containing from 5.03 to 7.06% essential amino acids and nonessential amino acids to provide a total of from 12.00 to 19.45% total amino acids. Diets containing the amino acid mixture of Rama Rao et al. (8) with 12% fat and the mixture of Warner (9) supplying the lowest level of total amino acids, were inferior to the other amino acid diets tested. Similar performance was noted with diets containing the amino acid mixtures of Rechcigl et al. (6), Rama Rao et al. (8) with 2% fat, Warner (9) with a higher level of nonessential amino acids and with a diet containing a mixture of essential and nonessential amino acids intended to simulate casein. The significance of these results is discussed.

There appeared to be no difference between sucrose and a dextrin-glucose mixture as the carbohydrate source or the salt mixtures of Warner (9) and Jones and Foster (16) when used in diets containing either casein or the amino acid mixture of Rechcigl et al. (6).

To study properly the essential and nonessential amino acid requirements, as well as many aspects of their metabolism, it is necessary to have a completely purified amino acid basal diet which will support performance equal to that which may be obtained with intact proteins. In earlier studies, performance with amino acid diets has been inferior to that with protein. Peptides and growth factors have been suggested as accounting for these observed differences (1, 2). However, more recent studies (3, 4, 5) have indicated that proper levels and proportions of essential and nonessential amino acids in protein-free diets will promote growth equal to that obtained with protein.

In the studies reported here, the 4 basal amino acid mixtures of Rechcigl et al. (6, 7), Rama Rao et al. (8) and Warner (9) were studied under similar experimental conditions to evaluate their relative effectiveness in supporting rat growth. In addition, observations were made on the effects of variations in nonspecific nitrogen levels, fat levels and kinds of minerals and carbohydrates.

EXPERIMENTAL

Methods. Male weanling Holtzman rats (Sprague-Dawley strain) were assigned to diets in weight outcome groups after feeding for 2 or 3 days a commercial stock diet. The rats were placed in a constant temperature room (23 ± 1°C) in wire-mesh cages which had been sterilized prior to the trial. Diets and water were supplied ad libitum. Diets were prepared prior to the experiments and stored in a refrigerator (4°C) prior to and during each experiment. Individual feed consumption was recorded daily and body weights at 2-day intervals throughout the experiments.

The compositions of the amino acid mixtures used are shown in table 1. The essential amino acids were supplied as the L-isomers[2] in all purified diets. Different approaches were used by the workers who formulated the basal amino acid mixtures used. The essential amino acid patterns of the Rechcigl et al. (6, 7), (hereafter re-

Received for publication January 17, 1963.

[1] Supported in part by a financial grant from Esso Research and Engineering Company, Linden, New Jersey.

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

TABLE 1

Composition of amino acid mixtures, experiments 1, 2 and 3[1]

	B series[2]	C[3]	D series[4]	E series[5]	F[6]
	% of diet	*% of diet*	*% of diet*	*% of diet*	*% of diet*
Essential amino acids					
L-Arginine·HCl	0.21	2.12	0.20	0.93	0.53
L-Histidine·HCl·H_2O	0.25	0.79	0.30	0.38	0.45
L-Lysine·HCl	0.90	2.84	0.90	1.25	1.10
L-Tryptophan	0.15	0.23	0.15	0.10	0.13
L-Isoleucine (allo-free)	0.55	2.08	0.50	0.46	0.65
L-Valine	0.55	1.63	0.70	0.72	0.77
L-Leucine	0.70	1.93	0.80	0.85	0.98
L-Threonine	0.50	1.16	0.50	0.51	0.52
L-Methionine	0.16	0.50	0.60	0.22	0.54
L-Cystine	0.34	0.45	—	0.20	0.32
L-Phenylalanine	0.42	1.09	0.90	0.48	0.53
L-Tyrosine	0.30	0.86	—	0.38	0.67
Total	5.03	15.68	5.55	7.06	7.01
Nonessential amino acids					
L-Glutamic acid	3.26	—	2.68	12.39	2.38
L-Aspartic acid	0.99	—	0.82	—	0.76
DL-Serine	0.96	—	0.79	—	0.67
DL-Alanine	0.46	—	0.38	—	—
L-Alanine	—	—	—	—	0.32
L-Proline	1.86	—	1.53	—	1.20
Glycine	0.30	—	0.25	—	0.29
Total	7.83	—	6.45	12.39	5.62

[1] The casein diets are designated "A series." Modifications in diets containing these basal amino acid mixtures are indicated by A-1, A-2, B-1, B-2, etc. (see table 2).
[2] Rama Rao et al. (8).
[3] Rechcigl et al. (7), (mixture 2-L). L-Isoleucine allowance twice that reported since source used in the above diet was allo-free.
[4] Warner (9). Diet D-2 modified in nonessential amino acid content to contain 4.48% L-glutamic acid, 1.37% L-aspartic acid, 1.32% DL-serine, 0.64% DL-alanine, 2.55% L-proline and 0.41% glycine.
[5] Rechcigl et al. (6). Diet E-1 used in trial 2 contained HCl-free forms of L-arginine and L-histidine at levels of 0.77% and 0.28%, respectively.
[6] Patterned after the amino acid content of casein (11) with added methionine.

ferred to as Rechcigl-57 and -60) mixtures were based on the results of the carcass composition studies of Williams et al. (10). The Rama Rao pattern was determined through studies of amino acid-supplemented casein diets since it appeared that unknown factors associated with intact protein were necessary for the optimal performance of rats. The Warner formulation was an untested mixture derived from a review of the literature through 1959 on the amino acid requirements of rats. Nonspecific nitrogen was supplied as glutamic acid in the Rechcigl-57 mixture to provide a diet with a protein equivalent of 13.2% (% N × 6.25), whereas the Rechcigl-60 formulation provided the same level of nitrogen through an increase in the essential amino acid levels, maintaining the same pattern as in the original mixture. The Rama Rao mixture supplied nonspecific nitrogen to a protein equivalent of 10% as an array of nonessential amino acids, whereas the same source was used in the Warner mixture to provide protein levels of 9.3 and 12.4%. An additional amino acid mixture patterned after the amino acids supplied by casein (11) when included at the 14% level with added methionine was tested in experiment 3.

The complete composition of each of the diets used in experiments 1, 2 and 3 is shown in table 2. Experiments 1 and 2 included comparisons using the diets as published by the original workers (6–9) and several modifications of the diets to reduce the number of dietary variables other than the amino acids.

Experiment 3 was designed to test the effect of several carbohydrates and salt mixtures on performance obtained with purified diets. Several reports in the literature (3, 13, 12) indicate that the type of

TABLE 2

Composition of experimental diets, experiments 1, 2 and 3

Ingredient[1]	A-1	A-2	A-3	A-4	A-5	A-6	B-1	B-2	B-3	C-1	D-1	D-2	E-1	E-2	E-3	E-4	F-1
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
Sucrose	75.62	69.60	65.62		66.62			12.80	12.80		77.80	73.48		61.35		60.35	66.99
Dextrin				43.61		43.61	64.14	64.14	54.14	42.61			43.61		43.61		
Glucose[2]				22.01		23.01				15.00			16.74		17.74		
Cellulose							2.00	2.00	2.00	2.00							
Lard	5.00	5.00									5.00	5.00					
Corn oil[3]							2.00	2.00	2.00								
Hydrogenated vegetable oil[4]			14.00	14.00	14.00	14.00			10.00	14.00			14.00	14.00	14.00	14.00	14.00
Casein[5]	14.00	20.00	14.00	14.00	14.00	14.00											
Amino acids[6]							12.86	12.86	12.86	15.68	12.00	16.32	18.87	18.87	18.87	18.87	12.81
DL-Methionine	0.18	0.18	0.18	0.18	0.18	0.18											
Vitamin supplement[7]	2.20	2.20	2.20	2.20	2.20	2.20	15.00	2.20	2.20	2.20	2.20	2.20	2.20	2.20	2.20	2.20	2.20
Mineral supplement	3.00 (W)[8]	3.00 (W)	4.00 (J&F)[9]	4.00 (J&F)	3.00 (W)	3.00 (W)	4.00 (M&J)[10]	4.00 (J&F)	4.00 (J&F)	4.00 (J&F)	3.00 (W)	3.00 (W)	4.00 (J&F)	3.00 (W)	3.00 (W)	4.00 (J&F)	4.00 (J&F)
$NaHCO_3$										2.51			0.58	0.58	0.58	0.58	

[1] 0.0125% ethoxyquin (Santoquin, Monsanto Chemical Company, St. Louis, Mo.), added to all diets.
[2] Cereiose, Corn Products Company, Argo, Illinois.
[3] Mazola, Corn Products Company, Argo, Illinois.
[4] Crisco, Procter and Gamble, Cincinnati.
[5] "Vitamin-free" casein, Nutritional Biochemicals Corporation, Cleveland, Ohio.
[6] See table 1.
[7] Vitamin Diet Fortification Mixture in Dextrose, Nutritional Biochemicals Corporation, Cleveland, Ohio, except diet B-1 which contained the vitamin mixture of Rama Rao et al. (3).
[8] Warner (9), see table 3.
[9] Jones and Foster (16), see table 3.
[10] Mameesh and Johnson, see table 3.

carbohydrate in the diet will influence performance on amino acid diets. Harper et al. (14) observed a greater effect of carbohydrate with 9% casein as compared with 18%, and more recently Chang (15) suggested that the utilization of protein is most markedly affected by the nature of the carbohydrate when the protein quality is poor or the quantity is inadequate. In the present work, sucrose, which had been used as the carbohydrate source in the casein diets, was compared with a dextrin-glucose mixture which had been used in some of the previous amino acid diets. The salt mixtures compared were those of Jones and Foster (16) and a mixture formulated to supply the mineral levels suggested by Warner (9). The Warner mixture was designed to meet the minimal mineral requirements when fed at the 3% level. The Warner mixture includes a source of selenium not present in the Jones and Foster mixture and exhibits several other differences (see table 3).

Probabilities of differences between treatments cited in the text were determined by the method of Duncan (17).

RESULTS

The results of experiments 1 and 2 are summarized in table 4. Daily gain was significantly ($P < 0.01$) greater with casein (diet A-1) or either stock diet than with any of the amino acid diets (B-1, B-2, C-1 or D-1). There was no significant difference in daily gain between the Warner amino acid (D-1) and the Rama Rao diet (B-1) or the Rama Rao diet (B-2) which was modified to observe any possible effects from differences in vitamin and salt content. The Rechcigl-60 amino acid diet (C-1), providing all nitrogen in the form of essential amino acids, promoted significantly ($P < 0.01$) faster rate of gain, higher daily feed intake and more gain per gram of feed than any of the other amino acid diets, although significantly lower ($P < 0.01$) than the protein diets in the first

TABLE 3

Composition of salt mixtures used

	Jones and Foster (16)	Mameesh and Johnson[1]	Warner (9)
	g/100 g of mixture	*g/100 g of mixture*	*g/100 g of mixture*
NaCl	13.93	10.81	2.84
Na_2HPO_4			1.87
KH_2PO_4	38.90		
K_2HPO_4		7.73	
$KHCO_3$			15.91
$K_3C_6H_5O_7 \cdot H_2O$		23.69	
$Ca_3(PO_4)_2$			37.79
$Ca(H_2PO_4)_2 \cdot H_2O$			37.79
$CaHPO_4 \cdot 2H_2O$		35.56	
$CaCO_3$	38.14	16.36	
$MgCO_3$		4.09	2.39
$MgSO_4$	5.73		
$FeC_6H_5O_7 \cdot 3H_2O$		1.60	
$FeSO_4 \cdot 7H_2O$	2.70		0.43
$CuSO_4 \cdot 5H_2O$	0.0005	0.0178	0.07
$MnSO_4$		0.124	
$MnSO_4 \cdot 2H_2O$	0.0045		
$MnSO_4 \cdot H_2O$			0.53
KI	0.008	0.0044	
KIO_3			0.00087
$K_2Al_2(SO_4)_4 \cdot 24H_2O$		0.0089	
$CoCl_2 \cdot 6H_2O$	0.00002	0.0089	
$ZnCO_3$		0.0044	0.16
$ZnCl_2$	0.0003		
NaF		0.0001	
Na_2SeO_3 (anhydrous)			0.00033

[1] Mameesh, M. S., and B. C. Johnson, J. Nutrition, 65: 162, 1958.

TABLE 4

Comparison of various amino acid diets with intact protein diets

Diet	Description	Avg daily wt gain	Avg daily feed intake	Feed efficiency
		g	*g*	*gain/feed*
Experiment 1[1]				
Stock 2	Dog pellets[2]	6.69 ± 0.24	15.23 ± 0.51	0.439 ± 0.004
Stock 1	Laboratory food[3]	6.47 ± 0.14	15.51 ± 0.31	0.418 ± 0.006
A-1	Casein, 14%	5.79 ± 0.17	13.40 ± 0.34	0.435 ± 0.007
C-1	Rechcigl-60 amino acid (7), 2-L mixture	4.74 ± 0.23	10.43 ± 0.42	0.453 ± 0.009
D-1	Warner amino acid mixture (9)	2.31 ± 0.17	7.83 ± 0.43	0.293 ± 0.013
B-2	Rama Rao amino acid diet (8) modified in vitamin and salts	2.11 ± 0.21	8.24 ± 0.46	0.250 ± 0.015
B-1	Rama Rao amino acid diet (8)	1.90 ± 0.17	7.83 ± 0.43	0.238 ± 0.012
Experiment 2[4]				
A-2	Casein, 20%	6.27 ± 0.11	12.57 ± 0.28	0.500 ± 0.012
A-1	Casein, 14%	5.82 ± 0.24	12.76 ± 0.40	0.455 ± 0.007
D-2	Warner amino acid mixture (9) with nonessential amino acids increased by 1.6 ×	3.57 ± 0.19	9.35 ± 0.36	0.373 ± 0.010
B-2	Rama Rao amino acid mixture (8), 2% fat	3.57 ± 0.20	10.86 ± 0.37	0.327 ± 0.011
E-1	Rechcigl-57 amino acid mixture (6)	3.47 ± 0.17	11.20 ± 0.35	0.309 ± 0.009
D-1	Warner amino acid mixture (9)	3.12 ± 0.17	9.32 ± 0.34	0.333 ± 0.009
B-3	Rama Rao amino acid mixture (8), 12% fat	2.55 ± 0.14	8.30 ± 0.48	0.302 ± 0.007

[1] Ten rats/treatment except diet stock 2 with 9; average initial weight 59 g; 21-day experiment.
[2] Big Red Dog Food Pellets, Grange League Federation (GLF) Feed Store, Ithaca, New York.
[3] Big Red Laboratory Animal Food, GLF Feed Store, Ithaca, New York.
[4] Ten rats/treatment except diets A-2 and B-3 with 6; average initial weight, 68 g; 19-day experiment.

2 criteria. Consumption of the other amino acid diets (B-1, B-2 or D-1) was approximately 60% of that of the protein diets, with corresponding low feed efficiencies.

Results of experiment 2 again indicated that the casein diets were superior to the amino acid diets in all criteria, with 20% casein promoting a slightly higher (not statistically significant) weight gain and feed efficiency than 14% casein. Better performance was obtained from the amino acid diets of Rama Rao (B-2) and Warner (D-1) in this experiment than in experiment 1. Increasing the nonessential amino acid fraction of the Warner diet (D-1) to increase the total dietary N content from 1.49 to 1.99% (diet D-2) tended to improve performance by all criteria.

Increasing the fat content of the Rama Rao diet (B-2) from 2 to 12% (diet B-3) inhibited general performance with this amino acid mixture. The diet (E-1) of Rechcigl-57 using glutamic acid as a source of nonspecific nitrogen supported performance similar to that of the modified Rama Rao diet (B-2) and the Warner diet (D-2) containing the higher level of nonspecific nitrogen.

Performance was significantly better by all criteria ($P < 0.01$) with the casein diets than with the amino acid diets in experiment 3 (table 5). The performance with the amino acid diet patterned after casein was comparable to that which had been obtained with the other amino acid mixtures. No evidence was noted of any

TABLE 5

Effect of carbohydrate and salt source in purified diets (experiment 3)[1]

Diet	Description	Avg gain in wt	Avg daily feed intake	Feed efficiency
		g	*g*	*gain/feed*
A-4	14% casein, dextrin-glucose, Jones and Foster (16) salts	5.89 ± 0.14	14.32 ± 0.31	0.412 ± 0.006
A-3	14% casein, sucrose, Jones and Foster (16) salts	5.88 ± 0.21	13.77 ± 0.49	0.427 ± 0.004
A-5	14% casein, sucrose, Warner (9) salts	5.64 ± 0.19	13.50 ± 0.37	0.423 ± 0.006
A-6	14% casein, dextrin-glucose, Warner (9) salts	5.23 ± 0.19	13.30 ± 0.42	0.392 ± 0.005
F-1	Amino acid diet patterned after 14% casein	3.45 ± 0.38	10.64 ± 0.70	0.317 ± 0.019
E-4	Rechcigl-57 amino acid mixture (6), sucrose, Jones and Foster (16) salts	3.26 ± 0.18	11.19 ± 0.51	0.291 ± 0.004
E-1	Rechcigl-57 amino acid mixture (6), dextrin-glucose, Jones and Foster (16) salts	2.99 ± 0.26	11.36 ± 0.82	0.253 ± 0.026
E-3	Rechcigl-57 amino acid mixture (6), dextrin-glucose, Warner (9) salts	2.79 ± 0.18	11.64 ± 0.45	0.238 ± 0.007
E-2	Rechcigl-57 amino acid mixture (6), sucrose, Warner (9) salts	2.75 ± 0.35	9.88 ± 0.65	0.271 ± 0.020

[1] Eight rats/treatment except diet E-2 with 7; average initial weight, 53 g.

effect from carbohydrate source in either amino acid or casein diets. Weight gains tended to be lower with both types of diets containing Warner salts, although these differences were not statistically significant.

DISCUSSION

Table 6 shows rat performance and actual intakes of amino acids and fat for several of the diets used in the experiments (treatments are arranged in order of weight gain supported). Four diets, F-1, E-2, B-2 and D-2, although furnishing different amounts of individual amino acids, total essential amino acids, total nitrogen and fat, supported similar weight gain of approximately 3.5 g/day. Since this performance cannot be considered optimal, it might be concluded that the demonstration of any superiority that any particular amino acid mixture had over another was limited by some deficiency of that diet. Lowering the level of nonessential amino acid-nitrogen supplied by diet D-2 reduced weight gains slightly (diet D-1) indicating that a higher level of nonessential nitrogen than that present in diet D-1 (protein equivalent of 9.11%) is required for a higher growth rate.

Weight gains were significantly higher with diet C-1 in which the essential amino acids in the relative proportions supplied by diet E-1 were increased to supply all of the amino acid nitrogen. This indicates that levels of at least certain of the essential amino acids in the previously discussed diets may not be adequate for maximal growth. Research in this laboratory using several modifications of the basal amino acid mixture of Sauberlich (4) has indicated that the levels of isoleucine and threonine in diets E-1, B-2 and D-2 are not adequate.[3] The consumption of the casein diets was higher than that of the amino acid diets including the simulated casein diet (F-1). It has been postulated that amino acids are less palatable to rats than protein, or affect feed intake for other reasons and thereby limit performance of rats (18). This problem is under study in this laboratory.

Increasing the fat level in the Rama Rao diet (B-3) significantly reduced feed consumption and weight gain. A similar

[3] Unpublished results.

TABLE 6

Average daily weight gains and intakes of feed, amino acids and fat in the experiments

Diet description	B-3 Rama Rao (8) 12% fat	D-1 Warner (9) 5% fat	F-1 Simulated casein, 14% fat	E-1 Rechcigl-57 (6) 14% fat	B-2 Rama Rao (8) 2% fat	D-2 Warner (9) 5% fat	C-1 Rechcigl-60 (7) 14% fat	A-1 14% casein 5% fat	A-2 20% casein 5% fat
Experiment no.	2	2	3	2	2	2	1	2	2
Avg wt gain, g	2.55	3.12	3.45	3.47	3.57	3.57	4.74	5.82	6.27
Avg feed intake, g	8.30	9.32	10.64	11.20	10.86	9.35	10.43	12.76	12.57
Avg amino acid intakes, mg									
Arginine	17.4	18.6	—	86.2	22.8	18.7	221.1	61.8	87.6
Arginine·HCl	—	—	56.4	—	—	—	—	—	—
Histidine	20.8	28.0	—	31.4	27.2	28.1	82.4	46.6	66.2
Histidine·HCl·H_2O	—	—	47.9	—	—	—	—	—	—
Lysine	—	—	—	—	—	—	—	124.7	175.3
Lysine·HCl	74.7	83.9	117.0	140.0	97.7	84.2	296.2	—	—
Tryptophan	12.5	14.0	13.8	11.2	16.3	14.0	24.0	18.4	25.7
Isoleucine	45.7	46.6	69.2	51.5	59.7	46.8	216.9	92.2	130.4
Valine	45.7	65.2	81.9	80.6	59.7	65.5	170.0	109.6	153.8
Leucine	58.1	74.6	104.3	95.2	76.0	74.8	201.3	139.9	196.6
Threonine	41.5	46.6	55.3	57.1	54.3	46.8	121.0	74.8	104.7
Methionine	13.3	55.9	57.5	24.6	17.4	56.1	52.2	52.1	72.7
DL-Methionine	—	—	—	—	—	—	—	23.0	22.6
Cystine	28.2	—	34.0	22.4	36.9	—	46.9	4.3	6.4
Phenylalanine	34.9	83.9	56.4	53.8	45.6	84.2	113.7	75.9	106.8
Tyrosine	24.9	—	71.3	42.6	32.6	—	89.7	95.5	134.6
Total essential amino acid	417.5	517.3	745.9	761.6	546.3	518.9	1635.4	895.8	1260.8
Total essential amino acid nitrogen	55.26	66.94	98.89	104.02	72.27	67.18	248.29	126.97	178.01
Total nonessential amino acid	649.9	601.1	598.0	1387.7	850.3	1007.0	—	801.6	1128.3
Total nonessential amino acid nitrogen	74.51	68.89	68.76	132.11	97.43	115.37	—	92.11	129.76
Total amino acid nitrogen	129.77	135.83	167.65	236.13	169.70	182.55	248.29	219.08	307.77
Avg fat intake, mg	996.0	466.0	1489.6	1568.0	217.2	467.5	1460.2	638.0	628.5

effect of high-fat levels in this diet has been reported by Rama Rao et al. (3). Since 2 diets containing 14% fat (F-1 and E-1) supported gain equal to that obtained with the Rama Rao diet containing 2% fat (B-2), a deficiency in the amino acid content of the Rama Rao diet appears to have been caused by the higher level of fat. At the same level of feed intake, these 2 high-fat diets supply higher levels of lysine, valine, leucine, methionine, phenylalanine and tyrosine, but in the case of diet F-1, similar levels of total nitrogen. Of these amino acids, methionine is probably the most closely associated with fat metabolism in its role as a methyl-group donor. Menon and Lucas (19) showed that the addition of methionine to a low-methionine diet containing 12% fat increased the net synthesis of lecithin and that this effect was independent of the level of dietary choline. These results indicate that the level of fat in the diet will have to be considered when determining the amino acid requirements of the rat.

LITERATURE CITED

1. Woolley, D. W. 1946 Some correlations of growth-promoting powers of proteins with their strepogenin content. J. Biol. Chem., *162:* 383.
2. Womack, M., and W. C. Rose 1946 Evidence for the existence of an unidentified growth stimulant in proteins. Ibid., *162:* 735.
3. Rama Rao, P. B., V. C. Metta and B. C. Johnson 1960 The amino acid composition and the nutritive value of proteins. II. Amino acid mixtures as a dietary source of nitrogen for growth. J. Nutrition, *71:* 327.
4. Sauberlich, H. E. 1961 Growth of rats fed protein-free diets supplemented with purified amino acid mixtures. Ibid., *74:* 298.
5. Bosshardt, D. K., E. Brand and R. H. Barnes 1961 Nutritional equivalence of β-lactoglobulin and its corresponding amino acids. Proc. Soc. Exp. Biol. Med., *107:* 979.
6. Rechcigl, M., Jr., J. K. Loosli and H. H. Williams 1957 The net utilization of non-specific nitrogen sources for the synthesis of non-essential amino acids. I. Growth and nitrogen utilization. J. Nutrition, 63: 177.
7. ——— 1960 D-Amino acids as source of non-specific nitrogen for growth of rats. Proc. Soc. Exp. Med., *104:* 448.
8. Rama Rao, P. B., H. W. Norton and B. C. Johnson 1961 The amino acid composition and the nutritive value of proteins. IV. Phenylalanine, tyrosine, methionine and cystine requirements of the growing rat. J. Nutrition, 73: 38.
9. Warner, R. G. 1963 The Laboratory Rat. National Research Council Committee on Animal Nutrition, pub. 990. National Academy of Sciences—National Research Council, Washington, D. C.
10. Williams, H. H., L. V. Curtin, J. Abraham, J. K. Loosli and L. A. Maynard 1954 Estimation of growth requirements for amino acids by assay of the carcass. J. Biol. Chem., 208: 277.
11. Hawk, P. B., B. L. Oser and W. H. Summerson 1954 Practical Physiological Chemistry, ed. 13. McGraw-Hill Book Company, New York.
12. Marshall, M. W., and M. Womack 1954 Influence of carbohydrate, nitrogen source and prior state of nutrition on nitrogen balance and liver composition in the adult rat. J. Nutrition, 52: 51.
13. Harper, A. E., and M. C. Katayama 1953 The influence of various carbohydrates on the utilization of low protein rations by the white rat. I. Comparison of sucrose and cornstarch in 9% casein rations. Ibid., 49: 261.
14. Harper, A. E., W. J. Monson, D. A. Arata, D. A. Benton and C. A. Elvehjem 1953 Influence of various carbohydrates on the utilization of low protein rations by the white rat. II. Comparison of several proteins and carbohydrates. Growth and liver fat. Ibid., 51: 523.
15. Chang, V. 1962 Effect of carbohydrates on utilization of protein and lysine by rats. Ibid., 78: 21.
16. Jones, J. H., and C. Foster 1942 A salt mixture for use with basal diets either low or high in phosphorus. Ibid., 24: 245.
17. Duncan, D. B. 1955 Multiple range and multiple F tests. Biometrics, *11:* 1.
18. Chen, M. L., Q. R. Rogers and A. E. Harper 1962 Observations on protein digestion in vivo. IV. Further observations on the gastrointestinal contents of rats fed different dietary proteins. J. Nutrition, 76: 235.
19. Menon, K. K. G., and C. C. Lucas 1961 Effects of dietary methionine and vitamin B_{12} on the net synthesis of choline in rats. Canad. J. Biochem. Physiol., 39: 683.

Relationship of 2,5-Diamino-4,6-diketopyrimidine, 2,4-Diaminobutyric Acid and a Crude Preparation of β-Cyano-L-Alanine to the Toxicity of Common and Hairy Vetch Seed Fed to Chicks[1]

G. H. ARSCOTT AND J. A. HARPER
Department of Poultry Science, Oregon State University, Corvallis, Oregon

ABSTRACT Two experiments involving the feeding of 2,5-diamino-4,6-diketopyrimidine (DDP), 2,4-diaminobutyric acid (DBA), a crude 30% ethanol-soluble preparation representing β-cyano-L-alanine (BCA) prepared from common vetch seed (CVS), a similarly prepared fraction of hairy vetch seed (HVS) and 30% of CVS and HVS per se were conducted with chicks up to 4 weeks of age. Complete mortality occurred with 30% of CVS and a crude fraction (of BCA) equivalent to 90 % of CVS. DBA (1.0%) resulted in considerable incidence of crooked toes as well as some symptoms of odoratism and blindness coupled with some mortality. Levels of DDP (0.15 and 1.0%), DBA (0.3%), 30% of HVS and a fraction equivalent to 90% of HVS had no effect on mortality. Body weights were significantly ($P < 0.05$) depressed by 30% of CVS (at 13 days), 1.0% of DBA, 30% of HVS and a fraction thereof. The relationship between these various effects is discussed.

In the course of investigations on the toxic effects of common vetch seed, *Vicia sativa*, L. var. *Willamette*, in contrast to hairy vetch seed, *V. villosa*, var. *glabrescens* Koch,[2] it has been reported that a number of compounds are responsible for the development of neurolathyrism (1). Anderson et al. (2) noted neurotoxic effects in young guinea pigs following subcutaneous injection of divicine or 2,5-diamino-4,6-diketopyrimidine (DDP) which appeared similar to toxic symptoms noted from feeding common vetch, *V. s.*, L. var. *angustifolia*. Ressler et al. (3) reported on the isolation of 2,4-diaminobutyric acid (DBA) from the perennial sweet pea, *Lathyrus latifolius*, and the flat pea, *L. sylvestris Wagneri*, to which neurotoxic symptoms in the hind legs of male rats supplied an aqueous extract were ascribed. More recently Ressler (4) reported on the isolation and identification of β-cyano-L-alanine (BCA) from common vetch seed, *V. s.*, L., and narrow leaf vetch, *V. angustifolia* L., as a possible causative agent of neurolathyrism in rats.

It is the purpose of this paper to report on experiments involving the use of these various drugs per se or, in the absence of the pure drug, a crude fraction prepared from common vetch seed (CVS) and a similarly prepared fraction of hairy vetch seed (HVS) when included in the diet. Comparisons involving CVS and HVS additions to the diet were also made.

EXPERIMENTAL

Duplicate lots of day-old White Vantress × Nichols 108 chicks, equalized for sex, were raised to 4 weeks of age in electrically heated batteries equipped with raised wire floors, subfloor heaters and continuous flow waterers. The chicks were housed in a battery room equipped with forced-draft ventilation, supplemental heaters and 24-hour lights. The number of chicks used is indicated in tables 1 and 2. Where indicated, length of experiment and number of chicks were limited by the amount of drug available.

The composition of the diet used is shown in table 3. Ground vetch seed replaced an equal amount of corn by weight. The diet was provided ad libitum. Mortality was recorded daily and body weights

Received for publication February 20, 1963.

[1] Technical paper no. 1645, Oregon Agricultural Experiment Station.

[2] Also known as smooth vetch but sold under the name hairy vetch (6).

TABLE 1

Effect of common vetch seed (CVS) and levels of 2,5-diamino-4,6-diketopyrimidine (DDP), 2,4-diaminobutyric acid (DBA) on growth, mortality and survival time of chicks

Treatment	Average data				
	Body weights		Mortality		Survival
	13 Days	28 Days	13 Days	28 Days	
	g	*g*	%	%	*days*
None	163	514(24)[1]	0	0	28.0
30% of CVS	133	— (24)	80.0	100	12.9
0.15% of DDP	148	492(8)	0	0	28.0
1.0% of DDP	149	457(8)	0	0	28.0
0.3% of DBA[2]	162	535(8)	0	0	28.0
1.0% of DBA[2]	101	394(8)[3,4]	12.5	25	24.6
L.S.D. $P < 0.05$	28	73			

[1] Figures in this column represent number of chicks started.
[2] Treatment discontinued after 13 days because of limited drug supply. Control diet used thereafter.
[3] Three of six birds appeared blind at 13 and 28 days.
[4] Two birds showed symptoms typical of β-aminoproprionitrile toxicity prior to death. At 13 days 6 out of 7 birds showed evidence of crooked toes.

TABLE 2

Effect of common (CVS) and hairy vetch seed (HVS) and crude fractions thereof on growth, mortality and survival time of chicks

Treatment	Average 28-day data		
	Body weights	Mortality	Survival
	g	%	*days*
None	506(24)[1]	0	28.0
30% of CVS	— (24)	100	13.0
30% of HVS	398(24)	0	28.0
30% Ethanol-soluble fraction of CVS ≅ 90% of CVS[2]	— (24)	100	11.7
30% Ethanol-soluble fraction of HVS ≅ 90% of HVS	414(24)	0	28.0
L.S.D. $P < 0.05$	73		

[1] Figures in this column represent number of chicks started.
[2] Contains β-cyano-L-alanine activity.

were determined at the end of the experiment.

The experimental outlines are shown in tables 1 and 2. The level of DDP[3] used was estimated from reports (2,5) showing CVS to contain 0.3% vicine (equivalent to 0.14% DDP) with the lower level representing an amount equivalent to about 100% of CVS in the diet. With DBA,[4] the level used was estimated from the report (4) that CVS contains about 0.1% BCA and that this drug may serve as a possible intermediate for DBA (3). For this experiment, a value of 3 times the reported amount was used for the lower level. The crude fraction involving CVS[5] representing BCA was obtained using the procedure (4) wherein an amount of ground CVS equivalent to 90% of vetch seed in the diet was extracted with 2 liters of 30% of ethanol per 454 g of vetch seed. The material was stirred continuously overnight in a cooler (2°C) and then condensed under vacuum and over a steam bath to a usable volume. A crude fraction obtained from HVS[6] using the procedure described above was also used.

RESULTS AND DISCUSSION

The experiment involving CVS and levels of DDP and DBA is shown in table 1

[3] Obtained through purchase from Nutritional Biochemicals Corporation, Cleveland, Ohio.
[4] See footnote 3.
[5] Obtained through purchase from Corvallis Feed and Seed Company, Corvallis, Oregon.
[6] See footnote 5.

TABLE 3
Composition of basal diet

	%
Corn, yellow, ground	69.25
Animal fat[1]	0.6
Soybean meal, dehulled (50% protein)	15.55
Fish meal (70% protein)	4.9
Meat and bone meal (50% protein)	6.2
Alfalfa meal, dehydrated (20% protein)	2.0
Limestone flour	0.8
Salt, iodized	0.3
Methionine hydroxy analogue (90%)	0.15
Vitamin and trace mineral premix[2]	0.25
Coccidiostat[3]	+
	100.00

[1] Calogen (Swift and Company, Inc., Portland, Oregon), stabilized with Tenox R which is composed of 20% citric acid (anhydrous), 20% butylated hydoxyanisole and 60% propylene glycol.
[2] Nopcosol M-5 (Nopco Chemical Company, Richmond, California), supplied/454 g of mixture: vitamin A, 600,000 USP units; vitamin D_3, 200,000 ICU; vitamin E, 200 IU; vitamin K, 100 mg; riboflavin, 600 mg; D-pantothenic acid, 1 g; niacin, 4 g; choline, 40 g; vitamin B_{12}, 1 mg; Zn bacitracin, 800 mg; butylated hydroxy toluene, 22.68 g; Mn, 10.8 g; Fe, 3.6 g; Cu, 363 mg; I, 218 mg; Zn, 5 g.
[3] Zoamix (Dow Chemical Company, Midland, Michigan), to supply 227 mg/454 g of diet.

Complete mortality was evident for all chicks fed 30% of CVS with an average survival time of 12.9 days. A significant ($P < 0.05$) decrease in growth was obtained at 13 days for the surviving chicks fed 30% of CVS. Levels of 0.15 and 1.0% of DDP in the diet had no effect on mortality and survival time although some decrease in growth was evident at 28 days with 1.0% of DDP that was not present at 13 days. No adverse effects on growth at 13 or 28 days, mortality or survival time were noted from using 0.3% of DBA. In the presence of 1.0% of DBA growth was significantly ($P < 0.05$) depressed at 13 days with 3 of the 7 birds surviving showing symptoms of blindness. These birds had apparently learned to eat and drink and when fed the control ration were able to survive, although blind, through 28 days of age but with growth significantly ($P < 0.05$) reduced. The 2 birds that died at 13 and 14 days of age which account for the mortality noted showed symptoms of odoratism typical of β-aminoproprionitrile (BAPN) toxicity[7] prior to death. Also at 13 days of age 6 out of 7 birds showed evidence of crooked toes that persisted for the remainder of the trial.

The effects of CVS versus HVS including fractions prepared from these seeds are shown in table 2. Excessive mortality to 30% of CVS is again evident in contrast to that obtained with 30% of HVS. A 30% ethanol-soluble fraction of CVS in the diet also proved highly toxic with survival time approximating that noted with 30% of CVS in the diet. The high degree of excitability, nervous symptoms and convulsions prior to death exhibited by these chicks were similar to those exhibited with CVS. The 30% ethanol-soluble fraction of HVS did not affect mortality as the CVS preparation did. Body weights were significantly ($P < 0.05$) depressed in the presence of 30% of HVS or the HVS fraction. Whether the effects noted between CVS and HVS reflect differences in concentration of toxicity or different toxic properties cannot be ascertained from these results.

These results confirm our earlier observations (1) regarding the toxic effect of CVS in relation to HVS with respect to mortality. In that report the toxic fraction also was noted to be water soluble. In view of the report of Ressler (4) regarding toxicity from BCA in feeding experiments with rats, it appears that the toxic entity in CVS, as reflected in the 30% ethanol-soluble extract of CVS, in our experiments is probably BCA. More recently, Ressler[8] has noted toxic symptoms with chicks fed BCA. Neither DDP or DBA fed at levels used in this study appeared to elicit toxic symptoms similar to those noted with CVS although a neurotoxic symptom (blindness) to DBA was noted. These observations do not agree with the report of Anderson et al. (2) where toxic symptoms ascribed to DDP were reported following subcutaneous injection of this drug into guinea pigs, but in that study the drugs were administered through different routes. Also the neurotoxic symptoms between DBA and BCA through CVS are not similar for chicks. Ressler et al. (3) have proposed a biosynthetic pathway in which BCA may serve as a precursor for either DBA or BAPN dependent on species of plant involved but as they indicate evidence for this is only circumstantial. In the experiments reported some similarity of symptoms between DBA, ex-

[7] Arscott, G. H., and J. A. Harper 1962 Unpublished data.
[8] Ressler, C. 1962 Personal communication.

cluding blindness, and that reported for BAPN toxicity was noted as evidenced by the incidence of crooked toes and certain leg deformities, thus pointing to a possible relationship between DBA and BAPN. In this respect, McDonald et al.[9] failed to show toxic effects attributable to DBA that characterized BAPN toxicity in turkeys. However, the highest level used by these workers was 0.02% of the ration. Only with the 1.0% level of DBA were the leg deformities and blindness noted in the experiments reported here.

LITERATURE CITED

1. Harper, J. A., and G. H. Arscott 1962 Toxicity of common and hairy vetch seed for poults and chicks. Poultry Sci., *42:* 1968.
2. Anderson, L. A. P., A. Howard and J. L. Simonsen 1925 Studies on lathyrism. I. Ind. J. Med. Res., *12:* 613.
3. Ressler, C., P. A. Redstone and R. H. Erenberg 1961 Isolation and identification of a neuroactive factor from *Lathyrus latifolius.* Science, *134:* 188.
4. Ressler, C. 1962 Isolation and identification from common vetch of the neurotoxin β-cyano-L-alanine, a possible factor in neurolathyrism. J. Biol. Chem., 237: 733.
5. Horvath, A. A. 1945 Toxicity of vetch seed for chickens. Poultry Sci., *24:* 291.
6. Hanson, P. R., and H. A. Schoth 1959 Vetch culture and uses. U. S. Department of Agriculture Farmers' Bull. no. 1740, Washington, D. C., p. 1–24.

[9] McDonald, B. E., H. R. Bird and F. M. Strong 1962 Effects of amino acids and related compounds on aortic aneurysms in turkeys. Poultry Sci., *41:* 1665 (abstract).

Nutrition and Longevity in the Rat[1]

V. WEANING WEIGHT, ADULT SIZE, AND ONSET OF DISEASE

BENJAMIN N. BERG, HENRY S. SIMMS AND ARTHUR V. EVERITT
Department of Pathology, College of Physicians and Surgeons, Columbia University, New York, New York

ABSTRACT A study of weaning weight in relation to adult body size and to the onset of disease was made on 1051 male Sprague-Dawley rats and 451 females from 500 to 1200 days old. The animals were free from lung or ear infection, and were raised under standardized conditions of temperature, humidity and light. The diet consisted of commercial vitamin D-free pellets supplied ad libitum from time of weaning. Littermates were divided at weaning into 4 groups according to weight which ranged from 40 to 89 g. Body weight curves (based on data obtained from rats over 900 days old) remained separate according to weaning weights. The higher the weaning weight, the heavier the animal. Peak body weight, body length, and tibia length were significantly greater in adult rats starting with highest weaning weights than in animals starting with lowest weaning weights. Observations at various ages on incidence of lesions of 4 major diseases, and on incidence of 4 benign tumors showed no significant differences according to weaning weights. The results demonstrate that adult body size is related to weaning weight. However, no relationship was observed between weaning weights and the age of onset of lesions.

In previous papers (1, 2) body weights and skeletal measurements of rats at various ages were reported. It was also shown (3, 4) that onset of lesions was delayed and life expectancy was extended in rats receiving a food intake restricted to levels that had little retarding effect on skeletal growth or sexual maturity. Dietary restriction caused a reduction in body weight, and no storage of excess body fat developed as in the unrestricted animals.

In our earlier work adult body weight and skeletal measurements were expressed as mean values irrespective of weaning weight. The question arises whether adult body size and age of onset of lesions are related to weaning weight. The present study deals with this problem. Rats of both sexes were divided into 4 groups according to weaning weight, and were compared with respect to adult body weight, body length and tibia length, and incidence of disease.

METHODS AND MATERIALS

Rat selection and colony conditions. Data were obtained from 1051 male rats and 451 females from 500 to 1200 days old. Animals were allowed to complete their life span and were examined when they were moribund or had died. Before 500 days, in males, and 700 days, in females, the number of spontaneous deaths was too small to give significant results. Distribution according to weaning weight, age and sex is shown in table 1. The rats were bred from a Sprague-Dawley strain started 18 years ago in our laboratory. They were free from lung or ear infection and were raised in air conditioned quarters with uniform temperature, humidity and lighting. Each cage held 3 or 4 animals. None of the experimental rats were mated.

From the time of weaning, when the rats were 28 days old, they were fed a diet consisting of vitamin "D-free" pellets[2] containing all the nutritional elements necessary for growth, maintenance and fertility. The pellets were composed of natural foods containing 24.3% of crude protein, 4.0% of fat, and 54.2% of carbohydrate. Caloric values per 100 g of diet were: protein, 78; fat, 36; carbohydrate, 217. Food was supplied ad libitum and drinking water was always available. At weaning, littermates were divided into 4

Received for publication December 27, 1962.
[1] This work is part of the Program for Research on Aging, supported by grant HE-00945 from the National Heart Institute, National Institutes of Health, U. S. Public Health Service.
[2] Rockland "D-free" Pellets, A. E. Staley Manufacturing Company, Decatur, Illinois.

TABLE 1

Number of rats with different weaning weights examined at various ages (all rats were moribund or had died at the ages indicated)

Weaning wt range	Age range (days)					
	500–599	600–699	700–799	800–899	900+	Total no. rats
g			Males (no.)			
40–49	—[1]	26	45	66	56	193
50–59	16	67	82	99	99	363
60–69	16	69	81	89	81	336
70–89	17	33	36	37	36	159
			Females (no.)			
40–49	—	—	24	17	42	83
50–59	—	—	44	63	102	209
60–69	—	—	36	41	66	143
70–89	—	—	—	—	16[2]	16

[1] Number of rats too small to be significant.
[2] Used for weight curve (figure 2) and body measurements (table 2).

groups according to weaning weight which ranged from 40 g to 89 g.

Body measurements. Body weights were recorded at intervals of 35 days. Peak body weight in female rats was the highest recorded value before the appearance of palpable or visible tumors. Final weight was the value at death after removing tumors. The tibia was removed at autopsy and cleaned of muscle tissue. Tibia length was the calipered distance from the spines of the head to the medial malleolus. Body length was measured after death and was the calipered distance from the tip of the nose to the anus. This measurement was not as accurate as tibia length because it could not take into account variations in the curvature of the spine. However, previous observations (1) showed that the relation between tibia length and body length (nose to anus measurement) was proportional. Data on body weight curves and on final tibia length as well as body length of rats starting with different weaning weights were obtained from 272 male rats and 226 females over 900 days old.

Autopsies and microscopic examinations. Moribund animals were killed with pentobarbital sodium[3] injected intraperitoneally, and complete autopsies were performed immediately after death. Examination of rats found dead in their cages was made only when there was no gross evidence of autolysis. Specimens were taken from all tissues and, after fixation in Zenker's fluid, they were imbedded in paraffin. Sections were prepared for microscopic study by staining with hematoxylin and eosin.

Diseases studied. Four major diseases, namely, chronic glomerulonephritis, periarteritis, myocardial degeneration, and skeletal muscle degeneration developed in our rat strain. The pathology of these lesions was described previously (3, 5). A fifth condition, myelin degeneration, was described recently (6, 7), and is not included in the present study.

Many types of spontaneous tumors developed in our rat strain. The neoplasms were mostly benign, the commonest being adenoma of the thyroid, chromophobe adenoma of the pituitary, and pheochromocytoma of the adrenal. These tumors occurred in both sexes and incidence at different ages is expressed as total number (in per cent) of rats having one or more of the 3 types. Another common neoplasm, mammary fibroadenoma, developed only in females and its incidence is considered separately.

RESULTS

Body weight and measurement. A comparison of body weight, body length and tibia length of rats over 900 days old shows statistically significant differences in both sexes between animals starting with lowest and highest weaning weights (table 2). In the 2 intermediate categories, values for body weight and measurement

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

are between the lowest-highest weaning weight groups.

Figures 1 and 2 show that body weight curves of the 4 groups of rats over 900 days old remained separate according to weaning weights; the greater the weaning weight, the heavier the adult animal. Peak weights were reached at about 700 days in the males and at about 800 days in females. At these ages the difference between rats starting with lowest and highest weaning weights (table 2) amounted to 67 g for males ($P < 0.001$) and 38 g for females ($P < 0.001$). Subsequently,

TABLE 2

Measurements and body weights of 900- to 1200-day-old rats with different weaning weights

Weaning wt		Mean peak body wt	Mean body length	Mean tibia length
Range	Mean			
g	*g*	*g*	*cm*	*cm*
		Males		
40–49	44	(56)[1]430 ± 34[2]	(51) 23.5 ± 0.8	(8) 4.50 ± 0.03
50–59	54	(99) 463 ± 36	(94) 23.5 ± 0.9	(17) 4.55 ± 0.10
60–69	64	(81) 483 ± 37	(77) 24.0 ± 0.7	(25) 4.60 ± 0.09
70–89	77	(36) 497 ± 37	(34) 24.0 ± 0.8	(11) 4.61 ± 0.09
		P[3] < 0.001	$P < 0.01$	$P < 0.005$
		Females		
40–49	44	(42) 272 ± 23	(35) 20.4 ± 0.6	(14) 3.95 ± 0.06
50–59	54	(102) 285 ± 29	(90) 20.8 ± 0.9	(45) 4.01 ± 0.08
60–69	63	(66) 283 ± 27	(59) 20.9 ± 0.8	(43) 4.02 ± 0.07
70–89	75	(16) 310 ± 29	(13) 21.0 ± 0.6	(11) 4.03 ± 0.08
		$P < 0.001$	$P < 0.005$	$P < 0.02$

[1] Figures in parentheses are the number of rats used.
[2] SD.
[3] The P values are computed for the differences between the lightest (40–49 g) and the heaviest (70–89 g) groups.

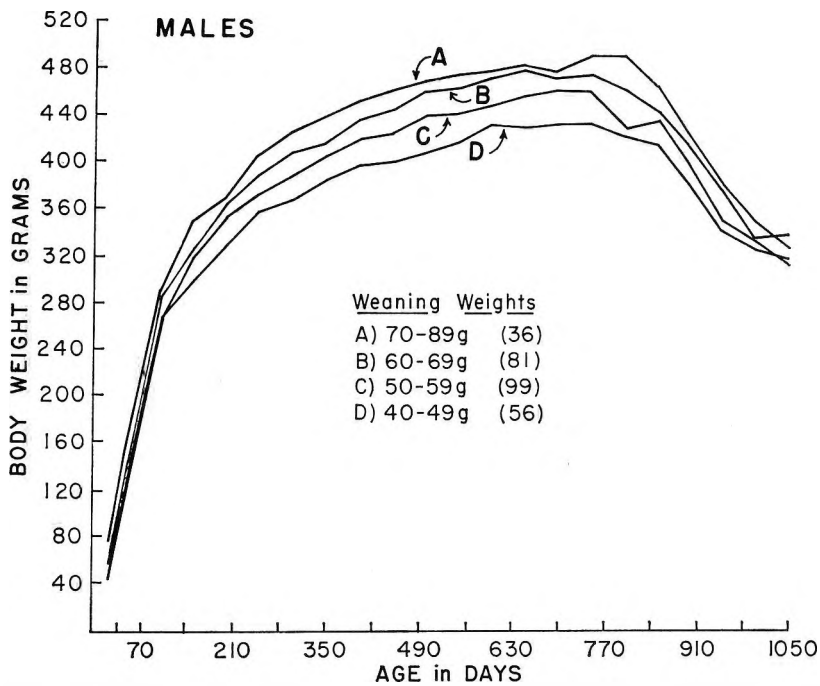


Fig. 1 Body weight curves of male rats over 900 days old starting with different weaning weights. Figures in parentheses represent number of rats in each category.

the course of the curves followed a plateau and then a decline. Because of the high percentage of mammary tumors in females, late loss of body weight appeared to be less than in males. Weight curves according to weaning weights were reported previously (2). However, this observation did not extend beyond 800 days.

Tibia length of male rats over 900 days old starting with lowest weaning weights

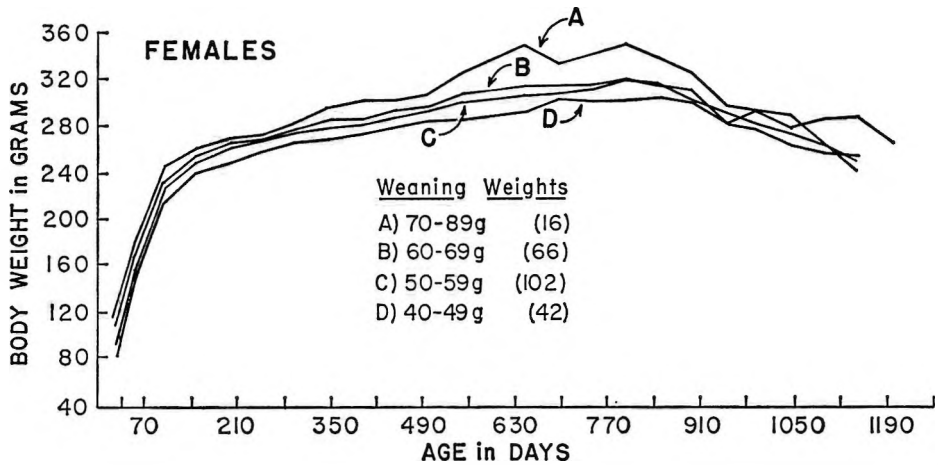


Fig. 2 Body weight curves of female rats over 900 days old starting with different weaning weights.

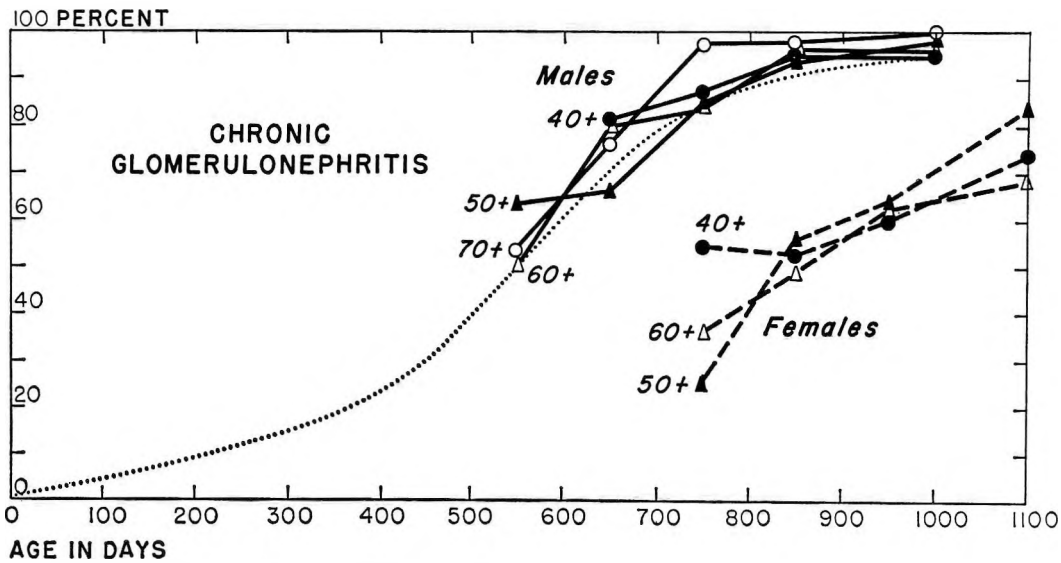


Fig. 3 Incidence of microscopically observed lesions of chronic glomerulonephritis, expressed as percentage of animals having lesions at various ages, in 1051 male rats and 451 females, starting with different weaning weights. The latter are represented as follows: "40+" means 40 to 49 g at weaning; "50+" means 50 to 59 g; "60+" means 60 to 69 g; "70+" means 70 to 89 g. The number of rats in each category at different ages is given in table 1. There is no curve for the "70+" female group because the number of rats is too small to be significant. Note the later onset of lesions in females than in males. The smooth, dotted sigmoid curve represents total incidence of lesions in 1410 male rats including the 1051 reported in this paper, irrespective of weaning weight.

was 4.50 cm as compared with 4.61 cm for those with highest initial weights ($P < 0.005$); in females, the corresponding values were 3.95 cm and 4.03 cm ($P < 0.02$). Body length differences were also significant. Measurements for males in the lowest-highest weaning weight categories were 23.5 cm and 24.0 cm ($P < 0.01$); in females the values were 20.4 cm and 21.0 cm ($P < 0.005$).

Incidence of major diseases and tumors. Curves representing incidence of lesions of 4 major diseases in both sexes at various ages are shown in figures 3 to 6. Pathological changes were mostly moderate or severe. There was no constant relation-

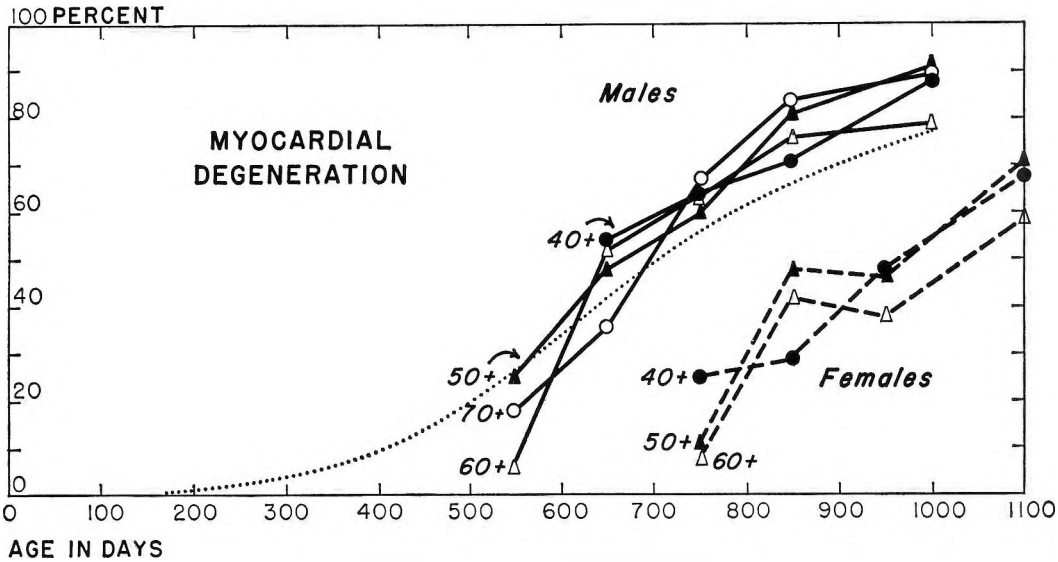


Fig. 4 Myocardial degeneration.

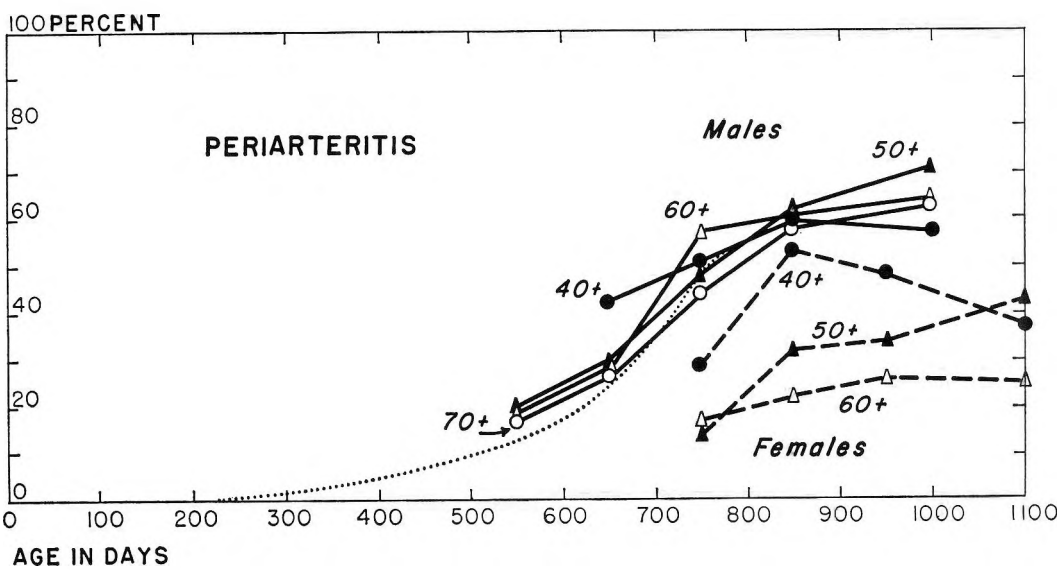


Fig. 5 Periarteritis.

ship between percentage of lesions at different ages and weaning weights. The curves were similar in shape and slope, and often overlapped. The curves for periarteritis remained separate in females, but we do not consider that the present data indicate a significant correlation. Skeletal muscle degeneration developed later than other lesions (5) and increase in frequency was more rapid.

In accord with previous observations (3, 4, 8, 9), incidence of lesions increased

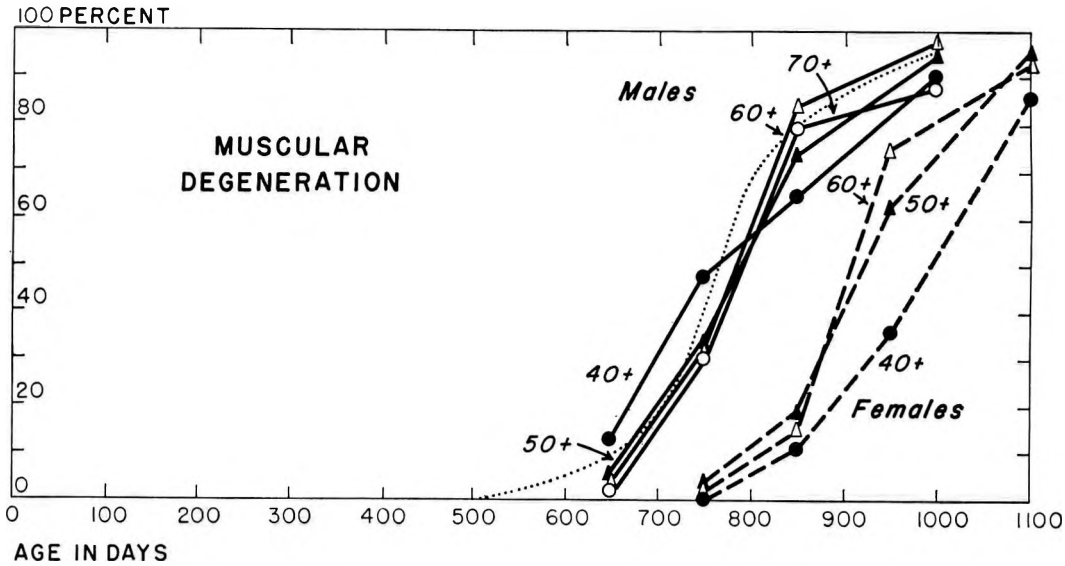


Fig. 6 Skeletal muscle degeneration. Note the late onset of lesions and rapid increase in frequency as compared with other diseases.

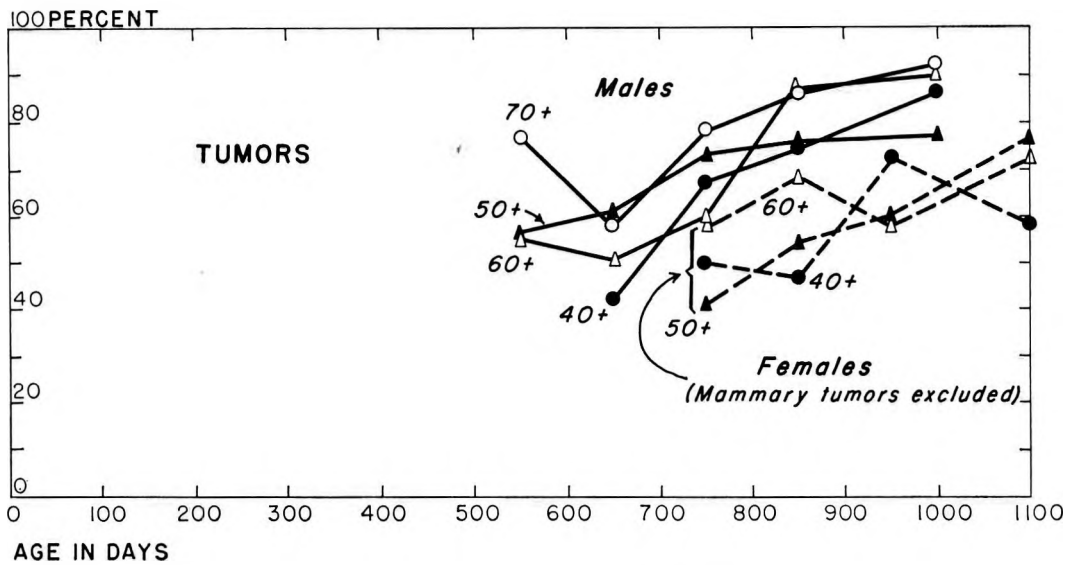


Fig. 7 Incidence of tumors expressed as percentage of rats with one or more of the following: thyroid adenoma, pituitary chromophobe adenoma, and adrenal pheochromocytoma.

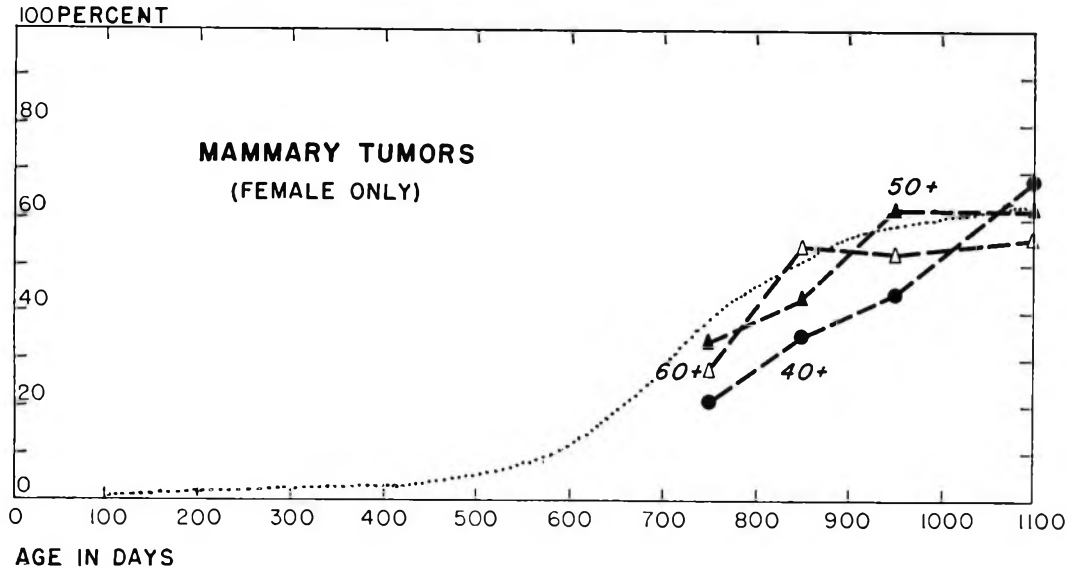


Fig. 8 Incidence of mammary fibroadenoma in female rats. This tumor did not develop in males. The smooth, dotted sigmoid curve represents total incidence of mammary tumors at different ages in 581 females including the 451 reported in this paper, irrespective of weaning weight.

with age, and onset was earlier in males than in females. McCay et al. (10) observed that the survival rate of male rats was shorter than that of females. Berg and Simms (3, 4) showed that the shorter life expectancy of males was related to earlier onset of lesions.

Total tumor incidence (thyroid adenoma, pituitary adenoma, and adrenal pheochromocytoma) at various ages for rats starting with different weaning weights is shown in figure 7. Frequency of mammary fibroadenoma in females is shown in figure 8. The graphs reveal no evidence of separation according to weaning weights.

DISCUSSION

The data presented here demonstrate that onset of lesions with age is unrelated to weaning weight. A 100% difference in weight at weaning had no observed effect on the time of development of disease in the adult rat. Incidence of lesions at various ages was similar to that observed previously in ad libitum-fed animals regardless of initial weight. Curves representing percentage of lesions in male rats starting with different weaning weights (figures 3, 4, 5 and 6) agree with the latter part of the sigmoid-shaped curves (dotted lines in the same figures based on data obtained from 1410 male rats including the 1051 reported in the present paper) irrespective of weaning weights (8). From these sigmoid curves it was shown that probability of onset of new lesions was related to the age of the animal by the same equation that applies to mortality of the rat and humans. In figure 4 (myocardial degeneration) the curves do not coincide accurately owing to the difference in number of rats represented by the graphs. The sigmoid curve for mammary tumors (fig. 8) is based on data obtained from 581 female rats including the 451 reported here, regardless of weaning weights,[4] and coincides with the curves representing breast tumor incidence in females with different weaning weights.

The difference in peak body weights (lightest-heaviest weights) amounting to 16% for males and 14% for females, may have consisted partly of body fat but not entirely. The significance of increased body fat in aging rats is discussed by Zucker et al. (11) and by Berg (2). The greater body length of the heavier animals,

[4] Unpublished experiments.

while not large, does indicate a greater body size. The difference in body length corresponds to a 6.5% difference in body volume for the male rats, and 8.8% for the females.

From our autopsy findings we conclude that the decline in body weight of older rats was caused by disease. We have observed no loss in body weight as long as the animals remained free from disease (1). The time required for an early lesion to develop into a severe one is about 225 days (8), and it is during this interval that weight loss occurs. Decline in body weight appeared earlier in males than in females and corresponded to earlier onset of lesions. Everitt (12), on the other hand, attributed the senescent loss of body weight of rats to an aging process. However, in his rats unlike ours, there was a high incidence of lung abscesses. Complete gross or microscopic findings at postmortem examination of his rats was not reported, except for a few grossly observed tumors. Lindop (13) also believed that the weight loss of older mice was due to aging, although a high percentage of her animals had lung infection as well as renal, hepatic and intestinal diseases.

Difference in adult body size of rats starting with lowest and highest weaning weights appears to be related to level of food intake and growth rate in early life. Unpublished data obtained from rats used in previous experiments (2) showed that food intake, from time of weaning to 70 days of age, was 24% less for male rats in the lightest weaning weight category as compared with males in the heaviest category; in females the difference amounted to 12%. After 70 days of age in females and after 160 days in males, there was no observable difference in food consumption although body weight curves remained separate according to weaning weights.

LITERATURE CITED

1. Berg, B. N., and C. R. Harmison 1957 Growth, disease and aging in the rat. J. Gerontol., *12:* 370.
2. Berg, B. N. 1960 Nutrition and longevity in the rat. I. Food intake in relation to size, health and fertility. J. Nutrition, *71:* 242.
3. Berg, B. N., and H. S. Simms 1960 Nutrition and longevity in the rat. II. Longevity and onset of disease with different levels of food intake. J. Nutrition, *71:* 255.
4. ——— 1961 Nutrition and longevity in the rat. III. Food restriction beyond 800 days. Ibid., *74:* 23.
5. Berg, B. N. 1956 Muscular dystrophy in aging rats. J. Gerontol., *11:* 134.
6. Berg, B. N., A. Wolf and H. S. Simms 1962 Degenerative lesions of spinal roots and peripheral nerves in aging rats. Gerontologia, *6:* 72.
7. ——— 1962 Nutrition and longevity in the rat. IV. Food restriction and the radiculoneuropathy of aging rats. J. Nutrition, *77:* 439.
8. Simms, H. S., and B. N. Berg 1957 Longevity and the onset of lesions in male rats. J. Gerontol., *12:* 244.
9. ——— 1962 Longevity in relation to lesion onset. Geriatrics, *17:* 235.
10. McCay, C. M., G. Sperling and L. L. Barnes 1943 Growth, ageing, chronic diseases and life span in rats. Arch. Biochem., *2:* 469.
11. Zucker, L., L. Hall, M. Young and T. F. Zucker 1941 Quantitative formulation of rat growth. Growth, *5:* 415.
12. Everitt, A. V. 1957 The senescent loss of body weight in male rats. J. Gerontol., *12:* 382.
13. Lindop, P. J. 1961 Growth rate, lifespan and causes of death in SAS/4 mice. Gerontologia, *5:* 193.

Response of Thyroid Hormone-deficient Rats to a Growth Factor in Duodenal Tissue[1]

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

ABSTRACT A procedure for preparing a water-soluble fraction of duodenal tissue is described. This fraction was administered in the drinking water to growth-arrested thyroidectomized and hypophysectomized rats, and its effect was compared with 3,5,3′-triiodo-L-thyronine. Both substances evoked comparable growth responses in these rats, but exerted different effects on certain body tissues. In addition, the duodenal fraction did not prevent goiter in the usual goiter prevention assay. When administered intraperitoneally to thyroidectomized, hypophysectomized or thiouracil-fed rats, it had no effect on growth, but it reinstated growth of sulfaguanidine-fed rats whether it was administered orally or parenterally. When the duodenal fraction was digested with pancreatin and then administered to growth-arrested thiouracil-fed rats, a growth response was observed. The data from these experiments suggest that an unknown substance is present in duodenal tissue which is capable of eliciting a growth response in thyroid hormone-deficient rats.

Previous reports have demonstrated that duodenal powder and certain other tissues are capable of maintaining growth or of reinstating growth of goitrogen-fed rats (1,2). Growth arrest which occurs after 28 days of goitrogen feeding (2), is a symptom of a thyroid hormone deficiency and the growth response which results when duodenal powder is added to the goitrogen-containing diet implies that this tissue contains thyroid hormones. Therefore, in a continuation of studies to characterize the effect of duodenal tissue, it became desirable to determine the effect of duodenal powder on rats in which a thyroid hormone deficiency was produced by thyroidectomy or hypophysectomy and to compare the effect with that produced by thyroxine or 3,5,3′-triiodothyronine. For such studies it would be desirable to administer duodenal powder or fractions derived therefrom parenterally. Preliminary experiments[2] have been reported in which fractions obtained after acid hydrolysis of duodenal powder were effective in reinstating growth of thyroidectomized and hypophysectomized rats when the fractions were added to the diet. These fractions were unsuitable for parenteral administration. For the studies reported here, enzymatic hydrolysis of duodenal powder was used to prepare a water-soluble fraction which was suitable for parenteral administration, and the effect of this fraction was compared with triiodothyronine in thyroidectomized and hypophysectomized rats.

MATERIALS AND METHODS

Sodium-L-thyroxine-5H_2O[3] (T_4) and 3,5,3′-triiodo-L-thyronine[3] (T_3) were dried over H_2SO_4, and used without further purification.

Hypophysectomized and parathyroid-thyroidectomized rats were obtained from Charles River Breeding Laboratory, Brookline, Massachusetts.

Total iodine and plasma protein bound iodine (PBI) were determined by the method of Grossman and Grossman (3). Hemoglobin was determined by the acid hematin method with 0.1 ml of blood drawn from the tail vein. The tibias were split, stained with silver nitrate, and the width of the uncalcified portion of the proximal epiphyseal cartilage was measured under a microscope with an ocular micrometer according to the procedure of

Received for publication January 14, 1963.
[1] Supported in part by a grant from the Williams-Waterman Fund of the Research Corporation, New York.
[2] Ackerman, C. J., and V. Tsou 1960 Effect of certain tissue extracts on the growth of goitrogen-fed and hypophysectomized rats. Abstracts, Fifth International Congress on Nutrition, Washington, D. C., p. 27.
[3] Mann Research Laboratories, New York.

Evans et al. (4). The measurements are reported as micrometer divisions or units.

Growth assays (5) were conducted as follows: Weanling rats were fed a complete diet which contained 1% sulfaguanidine. When growth arrest was established, the samples to be tested were added to the diet and the body weight gain during the next 2 weeks was a qualitative measure of the growth-promoting activity of the sample. One or two rats may be used per assay since the weight gain prior to the assay is negligible and the weight gain of control rats is also negligible (2).

A fraction which reinstates growth of growth-arrested, sulfaguanidine-fed rats was prepared from duodenal powder[4] as follows: 250 g of duodenal powder were extracted successively with 600 ml of boiling acetone, benzene, methanol, and petroleum ether. The insoluble residue was suspended in 500 ml of 0.2 N acetate buffer at pH 5.0 containing 1.0 g of papain. After 3 days at 39°, the entire mixture was acidified to pH 1 with HCl and then extracted with an equal volume of *n*-butanol and then 5 times with one-half volume of *n*-butanol. The butanol-insoluble fraction was air dried and saved. The butanol extract was evaporated at reduced pressure to a syrup and then air dried at 60°. The brown residue was dissolved in 25 ml of 0.2 N NaOH, centrifuged, and the supernatant then adjusted to pH 7.0 with HCl. The precipitate which formed was separated by centrifugation and discarded. The pH 7 supernatant was adjusted to pH 6 with HCl, cooled overnight, and then centrifuged. The pH 6 precipitate and the butanol-insoluble residue contained all of the growth-promoting activity of the original duodenal powder. The pH 6 precipitate dissolved readily in water and a stock solution for these experiments was prepared by dissolving the precipitate in 50.0 ml of water. This preparation contained 49.9 mg of dry residue/ml and will be referred to as the growth factor (GF). It contained 3.1 μg of iodine/ml. The butanol-insoluble residue and GF were assayed for growth activity in growth-arrested, sulfaguanidine-fed rats. The growth response to 0.50 ml of GF or to 3% of the butanol-insoluble residue was comparable to 10 μg of sodium-L-thyroxine (table 1, exp. A).

Thyroxine was tested at only one level in this experiment, but if it is assumed that all the iodine in 0.5 ml of the GF solution (1.5 μg) was thyroxine iodine it would represent 2.3 μg of thyroxine (or 2.6 μg of 3,5,3′-triiodo-L-thyronine). It appeared unlikely that thyroxine was responsible for the growth activity of GF. However, when triiodothyronine was assayed for its ability to reinstate growth of growth-arrested rats, it was observed that 3 μg/100 g diet of this hormone effected a gain of 39 g in 2 weeks (table 1). Thus, the growth-promoting activity of GF could be due to triidothyronine. Therefore, GF was compared with triiodothyronine in the experiments with thyroidectomized and hypophysectomized rats.

[4] Duodenal powder was purchased from Cudahy Laboratories, Omaha, Nebraska, and was a partially defatted dry powder.

TABLE 1

Growth response of growth-arrested rats to fractions of duodenal powder and to thyroxine

Exp.	Addition to diet		No. male rats	Results
				2-week gain
		mg/100 g diet		*g ± range*
A	Growth factor	24.9[1]	2	34 ± 3
	Butanol-insoluble residue	2000	2	22 ± 4
	Butanol-insoluble residue	3000	3	33 ± 4
	Na-L-thyroxine	0.010	4	38 ± 5
	None	—	4	2 ± 2
B	3,5,3′-triiodo-L-thyronine	0.003	6	39 ± 3[2]
	3,5,3′-triiodo-L-thyronine	0.006	6	44 ± 3
	3,5,3′-triiodo-L-thyronine	0.012	6	51 ± 4
	None	—	6	1 ± 1

[1] This represents 0.5 ml of the GF solution.
[2] Average ± SD.

TABLE 2

Response of thyroidectomized male rats to the growth factor (GF) and to triiodothyronine (T_3)

Treatment, dose/100 g body wt/day	Initial wt	2-week gain	Plasma protein bound iodine	Tissue weights			
				Thymus	Heart	Adrenal	Testes
	g	*g*	*μg/100 ml*	*mg/100 g body wt*	*mg/100 g body wt*	*mg/100 g body wt*	*mg/100 g body wt*
None (6)[1]	136	−2 ± 2[2]	1.68 ± 0.45	175 ± 91	243 ± 17	17.1 ± 3.6	1456 ± 349
GF oral (6) 0.05 ml	144	30 ± 7	1.48 ± 0.70	178 ± 52	280 ± 55	15.9 ± 0.2	1612 ± 231
GF injected (5) 0.05 ml	145	5 ± 3	1.42 ± 0.16	174 ± 36	242 ± 23	16.1 ± 1.9	1741 ± 67
T_3 oral (5) 0.3 μg	151	33 ± 12	0.91 ± 0.35	199 ± 33	291 ± 18	15.5 ± 0.1	1490 ± 92
GF + T_3 oral (6) 0.05 ml + 0.3 μg	149	39 ± 8	1.39 ± 0.26	207 ± 38	289 ± 39	16.9 ± 2.1	1510 ± 193

[1] Number of rats indicated in parentheses.
[2] S.D.

EXPERIMENTAL AND RESULTS

Thyroidectomized rats. Forty male (100 to 120 g) thyroidectomized rats were maintained for 3 weeks with a low iodine diet[5] with 1% of calcium gluconate in the drinking water. Thirty rats which had gained less than 6 g during the third week were selected and divided into 5 groups of 6 rats each. The GF (0.05 ml; 0.15 μg of iodine), and T_3 (0.3 μg; 0.17 μg of iodine) were administered daily per 100 g of body weight. When administered orally, the daily dose was diluted to 2 ml with 1% calcium gluconate solution and pipetted into 3-ml beakers which had been strapped inside of each cage. The usual drinking cups were removed from the cage until the daily dose had been consumed (2 to 4 hours). For intraperitoneal administration, GF and T_3 were diluted to 0.2 ml with sterile saline. Controls received an equal volume of sterile saline. After 14 days, the animals were anesthetized with sodium 5-allyl-5-(1-methylbutyl)-2-thiobarbiturate.[6] Blood was drawn by cardiac puncture into heparinized syringes. After the animals were killed, certain organs were removed and weighed to the nearest 0.2 mg on a Roller Smith torsion balance.

The results of this experiment are summarized in table 2. One rat in the third group had died during the experiment and one rat in the fourth group was not included in the results because it had exhibited an abnormal growth response (61 g gain in 2 weeks) that suggested thyroidectomy was not complete.

The GF effected a body weight gain of 30 g when it was given in the drinking water, but its effect was negligible when it was given intraperitoneally; GF had no appreciable effect of plasma PBI, thymus, heart, adrenal or testes. When administered in the drinking water, T_3 effected a growth response comparable to that of GF. It tended to decrease PBI, but it had no effect on the relative weights of the tissues.

Evans et al. (6) have shown that as little as 0.25 μg of thyroxine/day given intraperitoneally maintained nearly nor-

[5] Nutritional Biochemicals Corporation, Cleveland, Ohio.
[6] Sodium Surital, Eli Lilly Company, Indianapolis, Indiana.

TABLE 3

Response of male hypophysectomized rats to the growth factor (GF) and to triiodothyronine (T_3)

Treatment, dose/100 g body wt[1]	Initial wt	21-day gain	Width epiphyseal cartilage	Hemoglobin	Tissue weights				
					Thymus	Heart	Kidney	Adrenal	Testes
	g	*g*	*micrometer units*	*g/100 ml*	*mg/100 g body wt*	*mg/100 g body wt*	*mg/100 g body wt*	*mg/100 g body wt*	*mg/100 g body wt*
None (6)[2]	80	2 ± 2.1[3]	54.9 ± 6.5	12.3 ± 2.4	181 ± 83	293 ± 72	636 ± 159	12.4 ± 3.1	233 ± 46
GF oral (7) 0.07 ml	80	12 ± 2.5	75.8 ± 3.6	13.3 ± 1.1	227 ± 62	206 ± 27	653 ± 53	13.2 ± 3.2	313 ± 20
GF injected (5) 0.07 ml	80	2 ± 5.1	63.5 ± 3.5	—	177 ± 33	293 ± 68	634 ± 85	18.8 ± 6.9	727 ± 58
T_3 oral (4) 1.4 μg	79	15 ± 2.2	80.7 ± 6.3	13.6 ± 0.93	307 ± 47	384 ± 71	738 ± 69	11.9 ± 1.7	182 ± 33
GF + T_3 oral (6) 0.07 + 1.4 μg	81	17 ± 3.3	100.2 ± 6.3	13.2 ± 0.23	409 ± 50	277 ± 37	744 ± 86	12.7 ± 1.8	322 ± 72
GF + residue[4] (6)	79	14 ± 3.1	79.1 ± 6.3	—	223 ± 62	317 ± 22	642 ± 114	14.5 ± 1.6	236 ± 86

[1] The daily dose for the first 13 days was 0.05 ml of GF, and 1.0 μg of T_3. On day 14, the dose was increased to those shown.
[2] Number of rats in parentheses.
[3] SD.
[4] The butanol-insoluble residue that remains after extraction of the growth factor from hydrolysates of duodenal powder.

mal endocrine organ weights. By doubling the dose of thyroxine, these workers reported that growth was normal, but with the exception of the uterus, no change was observed in the relative weights of the endocrine organs. Therefore, if GF contained thyroid hormones and it was administered simultaneously with T_3, an augmentation of the growth response and possibly organ weights may be expected. To test this, T_3 and GF were administered simultaneously to the last group of rats shown in table 2. The results show that GF did not augment the effect of T_3 since growth and organ weights were no different than when T_3 alone had been administered.

Hypophysectomized rats. Forty-two male (75 to 90 g) hypophysectomized rats were maintained with a complete diet (5) with 5% dextrose in the drinking water until growth arrest had been established (less than 4 g gain during the third week). They were then divided into 6 groups of 7 rats each. The GF and T_3 were administered daily in proportion to body weight either in 2 ml of the drinking water or by intraperitoneal injection. For the first 13 days, the daily dose of T_3 was 1.0 μg/100 g of body weight,[7] and 0.05 ml of GF/100 g of body weight. On day 14, the dose was increased to 1.4 μg of T_3 and 0.07 ml of GF. On the twentieth day, 0.1 ml of blood was drawn from the tail vein for hemoglobin determinations. On the twenty-first day, the animals were killed, and certain tissues were removed, blotted and weighed. The tibia from the left leg of each rat was removed and frozen until measurements of the tibial epiphyseal cartilage were made.

The results are summarized in table 3. Two rats had died during this experiment and those rats that had exhibited growth responses out of proportion to others in their respective groups, or whose pituitary target organs were near normal in size were not included in the data on the assumption that hypophysectomy was not

[7] When T_3 was administered to hypophysectomized rats at a level of 0.3 μg/100 g body weight the effect on growth was erratic, ranging from zero to 25 g in 3 weeks. Although other factors may be responsible for this effect, it was assumed that free T_3 was inefficiently utilized by hypophysectomized rats and the dose was increased to 1.0 μg/day/100 g body weight for this experiment in order to establish an effect of T_3 which could be compared with that of GF.

complete in these rats. Therefore, the data, particularly that pertaining to tissue weights, is weakened by the low number of observations and the results are discussed in terms of the trends effected by either GF or T_3.

When GF was given in the drinking water, an increase of 12 g in body weight was accompanied by an increase in the width of the epiphyseal cartilage. This agrees with the results reported previously.[8] It had no effect on hemoglobin levels or on the relative weights of the kidney or the adrenals. The thymus increased in weight and the heart decreased in weight. When GF was administered intraperitoneally, it had no effect on growth although the epiphyseal cartilage increased from 54.9 to 63.5 micrometer units. Also, the adrenal glands and testes increased in weight.

The T_3 effected a growth response and an increase in the width of the epiphyseal cartilage comparable to that of GF. However, the effect of T_3 contrasts with that of GF in that the relative weights of the heart and kidney increased and that of the testes decreased.

When T_3 and GF were administered simultaneously in the drinking water, the growth response was 17 g, but the increase in width of the epiphyseal cartilage was the sum of the increases observed when either substance was administered alone. An additive effect of these 2 substances was also observed on the thymus, heart, kidneys, and the adrenals in spite of the variation in values within each group. For example, the negative effect of GF on the heart ($-$ 87 g, group 2) apparently counteracted the positive effect of T_3 ($+$ 91 g, group 4), since the observed result (277 mg/100 g body weight) was comparable to the control group. The effect on the thymus was approximately the sum of the increases due to GF and T_3, whereas the negative effect of T_3 on the adrenal glands was apparently offset by the positive effect of GF. In this respect, only the testes exhibited an anomolous response since T_3 had a negative effect and GF a positive effect, but the two together produced an effect comparable to GF alone.

Since the butanol-insoluble residue retains a fraction of the total growth activity of the original duodenal powder (table 1), it was used in one group of rats in this experiment to increase the total amount of growth activity that was ingested, hoping thereby to clearly delineate the effect of the growth factor. The last group of rats in table 3 were given 0.07 ml/day of GF in the drinking water and 4% of the butanol-insoluble residue was added to the diet. This would be approximately 3 times the growth activity necessary to produce a gain of 34 g in growth-arrested rats (table 1). In spite of this excess, the effect on growth and on the tibia epiphyseal cartilage was no greater than when GF was given alone. The heart tended to increase in weight, whereas the testes decreased in weight, but the thymus, kidney and the adrenal glands were no different than when GF was given alone. These results indicate that the maximal effect of the growth factor was achieved in the second group of rats which received 0.07 ml of the GF/day. However, if the butanol-insoluble residue or GF contained thyroid hormones, the result should have been comparable to that observed when T_3 and GF were administered simultaneously (group 5). The rats in groups 5 and 6 differed markedly in the response of the epiphyseal cartilage and the thymus gland. These results suggested that the growth response to these fractions of duodenal tissue was not due to the presence of thyroid hormones and further investigation was warranted.

Goiter prevention assays. The goiter prevention assay of Dempsey and Astwood (7) provides another method for determining whether thyroid hormones are present in GF. A purified diet containing 11 μg of KI/g (5) was fed to female Sprague-Dawley rats until they weighed 140 to 160 g. Thiouracil (0.1%) or sulfaguanidine (1.5%) was then added to the diet. The next day, graded levels of sodium-L-thyroxine or GF in 0.2 ml of sterile saline were administered by intraperitoneal injection. Controls received an equal volume of sterile saline. On the day after the last injection, the rats were

[8] See footnote 2.

killed and the thyroid glands removed and weighed.

The results are shown in table 4. The GF at a level of 0.12 ml/day had a slight, if any, ability to prevent goiter and it was concluded that 0.12 ml of the GF solution contained 0.34 μg or less of free thyroxine. Thus, 0.5 ml of the GF in 100 g of diet (table 1) could add no more than 1.4 μg of L-thyroxine to the diet.

From the data in table 4 it was calculated that the amount of L-thyroxine (as the anhydrous, free acid) necessary to maintain normal thyroid weight was 1.4 μg/100 g body weight/day for thiouracil-fed rats and 1.1 μg/100 g body weight/day for sulfaguanidine-fed rats.

Oral and parenteral administration of GF. The unexpected observation that GF was effective in reinstating growth when administered in the drinking water, but not when administered intraperitoneally, suggested that a comparison be made of the 2 modes of administration in goitrogen-fed rats. Table 5, experiment A, shows the results of an experiment in which 0.05 ml of GF/day, administered intraperitoneally, was compared with the effect of 0.05 ml GF/day given in the drinking water to growth-arrested thiouracil- and sulfaguanidine-fed rats. The GF was effective in reinstating growth of sulfaguanidine-fed rats by either mode of administration, but it was effective in thiouracil-fed rats only when given in the drinking water.

These results demonstrated an interesting difference between sulfaguanidine- and thiouracil-fed rats, but they suggested that further hydrolysis of GF was necessary for it to become effective in thiouracil-fed rats. Accordingly, 8.0 ml of GF was incubated with 8 mg of pancreatin and 8 ml of Krebs Ringer phosphate buffer, pH 8, at 37° for 24 hours. An amount equivalent to 0.05 ml of the original GF solution was administered daily by intraperitoneal injection or in the drinking water to growth-arrested, thiouracil-fed rats. The 2-week growth response is shown in table 5, experiment B, and demonstrates that GF reinstated growth of thiouracil-fed rats after pancreatin hydrolysis.

DISCUSSION

These experiments demonstrate that a water-soluble fraction prepared from duodenal tissue is capable of reinstating growth of rats in which a thyroid deficiency had been produced by thyroidectomy, hypophysectomy or goitrogen feeding. The data suggest that the ability of this fraction to reinstate growth is not due to thyroid hormones. However, more finite evidence is necessary to prove that thyroid hormones are absent in the preparation used for these studies.

TABLE 4

Ability of the growth factor (GF) to prevent goiter in thiouracil- or sulfaguanidine-fed rats

Treatment L-thyroxine[1]	Results, thyroid wt	Treatment GF	Results, thyroid wt
μg/100 g/day	*mg/100 g*	*ml/100 g/day*	*mg/100 g*
A 1.5% Sulfaguanidine, 14 days			
0.0	13.8 ±1.3[2]		
0.37	9.0 ±0.48	0.01	12.6±2.0
0.67	7.6 ±0.25	0.03	11.8±1.7
1.12	5.7 ±0.27	0.05	12.9±1.0
0.00 (normal control)	5.8 ±0.6		
B 0.1% Thiouracil, 10 days			
0.0	14.3 ±2.6		
0.345	12.4 ±2.4	0.04	12.6±2.4
0.690	10.0 ±2.4	0.08	14.0±1.4
1.035	8.68±0.84	0.12	12.3±1.2
0.00 (normal control)	6.14±0.40		

[1] Anhydrous; free acid.
[2] SD; 6 female rats/group.

TABLE 5

Response of goitrogen-fed rats to the oral and parenteral administration of the growth factor (GF)

Experiment[1]	Treatment	Two-week gain of rats fed	
		0.1% Thiouracil	1% Sulfaguanidine
		g	*g*
A[2]	None	2 ± 3[3]	2 ± 2
	GF injected	−1 ± 2	33 ± 5
	GF oral	27 ± 3	37 ± 4
B[4]	GF hydrolyzed with pancreatin 24 hours		
	None	3 ± 1	
	GF injected	28 ± 3	
	GF oral	22 ± 4	

[1] In both experiments GF was administered at a level of 0.05 ml/100 g body wt/day.
[2] Four female and 2 male rats/group.
[3] SD.
[4] Two male and 2 female rats/group.

The inability of thiouracil-fed rats to respond to intraperitoneal administration of the growth factor preparation contrasts with that of sulfaguanidine-fed rats and suggests a striking difference in the mode of action of these 2 goitrogens. No adequate explanation is available at the present time, but it appears that the active principle in the growth factor preparation is bound in an unavailable form since the growth factor is effective in thiouracil-fed rats when it is administered orally or intraperitoneally after pancreatin digestion. The inability of thiouracil-fed rats to respond to the growth factor administered intraperitoneally is not a manifestation of a nonspecific thiouracil toxicity since thyroidectomized and hypophysectomized rats also failed to respond to the growth factor when it was administered intraperitoneally.

Because the growth factor preparation was not effective when administered intraperitoneally to thiouracil-fed rats, the results of the goiter prevention assay with thiouracil-fed rats (table 4) are questionable. However, the data serve to demonstrate a lack of thyroid hormone activity in this preparation. This is supported by the data from sulfaguanidine-fed rats which are capable of responding to intraperitoneal administration of the growth factor, but GF failed to prevent goiter in these rats (table 4).

ACKNOWLEDGMENT

The authors are indebted to Mr. N. O. Price for the iodine analysis of the duodenal fraction.

LITERATURE CITED

1. Ackerman, C. J., and V. Tsou 1961 Effect of various tissues on the growth of sulfaguanidine-fed rats. J. Nutrition, 74: 84.
2. Ackerman, C. J. 1963 The effect of potassium iodide and duodenal powder on the growth and organ weights of goitrogen-fed rats. J. Nutrition, 79: 140.
3. Grossman, A., and G. F. Grossman 1955 Protein bound iodine by alkaline incineration and a method for producing a stable cerate color. J. Clin. Endocrinol. Metab., 15: 354.
4. Evans, H. M., M. E. Simpson, W. Marx and E. Kibrick 1943 Bio-assay of the pituitary growth hormone. Width of the proximal epiphyseal cartilage of the tibia in hypophysectomized rats. Endocrinology, 32: 13.
5. Ackerman, C. J. 1959 Growth promoting properties of meat meal and certain tissues in goitrogen-fed rats and chicks. J. Nutrition, 67: 589.
6. Evans, E. S., L. L. Rosenberg and M. E. Simpson 1960 Relative sensitivity of different biological responses to thyroxine. Endocrinology, 66: 433.
7. Dempsey, E. W., and E. B. Astwood 1943 Determination of the rate of thyroid hormone secretion at various environmental temperatures. Ibid., 32: 509.

Observations on Protein Digestion in vivo

V. FREE AMINO ACIDS IN BLOOD PLASMA OF RATS FORCE-FED ZEIN, CASEIN, OR THEIR RESPECTIVE HYDROLYZATES[1]

CARL PERAINO[2] AND ALFRED E. HARPER[3]
Department of Biochemistry, University of Wisconsin, Madison, Wisconsin

ABSTRACT Concentrations of free amino acids in the portal and systemic blood plasma of rats force-fed zein, casein, or their respective hydrolyzates were determined in an effort to show a relationship between the change in plasma amino acid pattern and the relative digestibility of the proteins. Force-feeding zein had little effect on the concentrations of free amino acids in the portal and systemic plasma but force-feeding casein caused a substantial increase in the concentrations of free amino acids in the portal plasma. When hydrolyzates of these proteins were force-fed, much larger increases in the plasma free amino acid concentrations were observed with the largest responses occurring after ingestion of casein hydrolyzate.

In an earlier part of this study (1) the rates of disappearance of different proteins from the gastrointestinal tracts of rats fed a single meal were compared. Casein and zein emptied from the stomach at slightly different rates but much more nitrogen accumulated in the small intestines of rats fed zein than could be accounted for by the difference between the rates at which the 2 proteins emptied from the stomach. Further investigation (2) revealed that the residual nitrogen recovered after feeding zein was mainly in an insoluble fraction and probably consisted of undigested protein.

The objective of the present study was to determine whether differences in the digestibility of these proteins would be reflected in the concentrations of free amino acids in the blood plasma. Comparisons were made between the concentrations of free amino acids in the plasma of rats force-fed a given amount of protein (zein or casein) and of others force-fed the same amount of nitrogen from the corresponding protein hydrolyzates.

METHODS

The proteins were commercial products. Hydrolyzates were prepared by autoclaving (15 pounds pressure) 100 g of protein in 2 liters of 4 N sulfuric acid for 24 hours; neutralizing the resulting solution with solid calcium hydroxide; filtering off the precipitate of calcium sulfate; decolorizing the filtrate with Norite;[4] and removing water by evaporation.

Male white rats (200 g) which had previously been fed a stock diet and had been starved for 24 hours were force-fed an amount of test protein or hydrolyzate which contained 0.16 g of nitrogen (i.e., an amount equivalent to 1 g of pure protein). The hydrolyzates were not supplemented with amino acids. The test nitrogen sources were administered as aqueous suspensions or solutions (10% w/v). Blood samples were taken from the portal vein and heart of individual rats at successive time intervals after feeding. The procedure has been described (3). Equal quantities of heparinized portal blood taken from 2 rats at each interval were combined; systemic blood samples (obtained by heart puncture) were similarly combined and the cells were removed from each pooled sample by centrifugation.

Free amino acids in the resulting plasma samples were then determined quantitatively by a paper chromatographic

Received for publication December 5, 1962.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the National Live Stock and Meat Board, Chicago, Illinois.

[2] Present address: McArdle Institute for Cancer Research, University of Wisconsin, Madison, Wisconsin.

[3] Present address: Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts.

[4] Pfanstiehl Laboratories, Inc., Waukegan, Illinois.

procedure (4). Results are presented for the following amino acids: glutamic acid, glutamine, serine, threonine, proline, alanine, glycine, valine, leucine + isoleucine (not separated on the chromatogram), and lysine.

RESULTS

The quantity of the particular amino acid in the protein or hydrolyzate that the rats received is shown in the legend for each figure. The concentrations of the designated amino acids in both the portal and systemic plasma at each half-hour interval after force-feeding the test protein or its corresponding hydrolyzate are indicated by the points on the curves. The points on the vertical axes of the figures directly above the letter F indicate the concentration of the particular amino acid in both the portal and systemic plasma of animals kept without food for 24 hours (fasting concentration).

Zein and zein hydrolyzate. Force-feeding zein did not cause the concentration of glutamic acid in either the portal or systemic plasma to increase much above the fasting value, but after administration of the hydrolyzate a substantial increase was observed in the portal plasma and a smaller increase in the systemic plasma (fig. 1).

The concentration of alanine in both portal and systemic plasma (fig. 1) increased above the fasting value after force-feeding zein, but larger increases occurred in both after force-feeding the hydrolyzate, with the portal concentration showing the greater response. Although the amount of hydrolyzate given contained less alanine

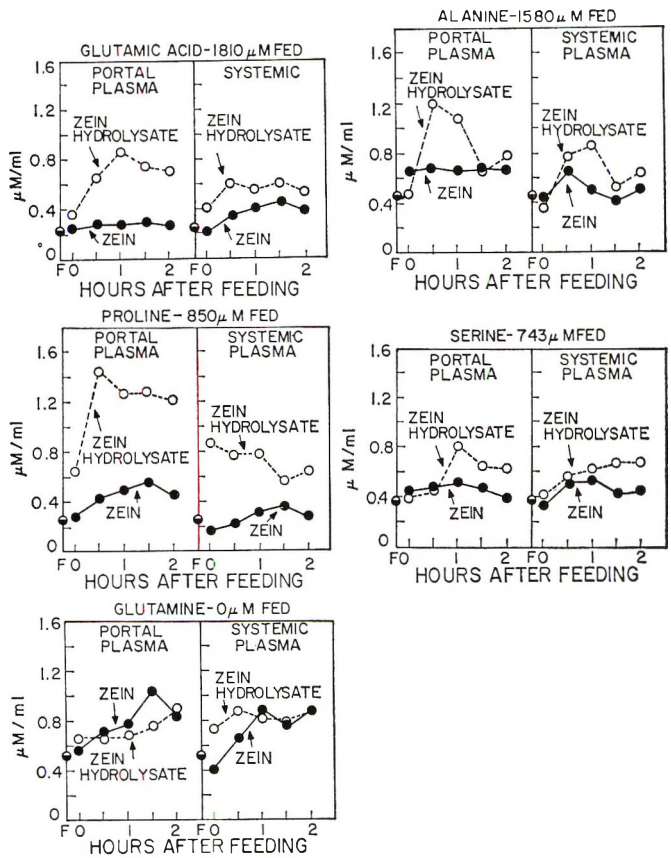


Fig. 1 Concentrations of amino acids in portal and systemic plasma at successive intervals after force-feeding zein or zein hydrolyzate.

(1580 μmoles) than glutamic acid (1810 μmoles), alanine concentration rose to a maximum of 1.2 μmoles/ml, whereas glutamic acid concentration rose to only 0.85 μmoles/ml.

The concentration of proline (fig. 1) increased only slightly in the portal and systemic plasma after force-feeding zein, but increased much more in both after force-feeding the hydrolyzate. Proline increased less in the systemic than in the portal plasma. The amount of hydrolyzate administered contained only 850 μmoles of proline compared with 1580 μmoles of alanine and 1810 μmoles of glutamic acid, but the concentration of proline in the portal plasma increased more than the concentrations of either alanine or glutamic acid.

The effect of prior hydrolysis of zein on plasma serine concentration (fig. 1) was much less than its effect on the plasma concentrations of glutamic acid, alanine and proline. Also, differences between the plasma serine concentrations of groups fed zein and zein hydrolyzate did not occur until one hour after feeding.

The concentration of glutamine (fig. 1) gradually increased in both portal and systemic plasma of rats force-fed zein or zein hydrolyzate, although this amino acid is not present in zein. The plasma concentration of glycine (fig. 2), another amino acid which is not found in zein, did not change appreciably from the fasting value in any of the trials.

When zein was force-fed the concentration of valine did not increase above the

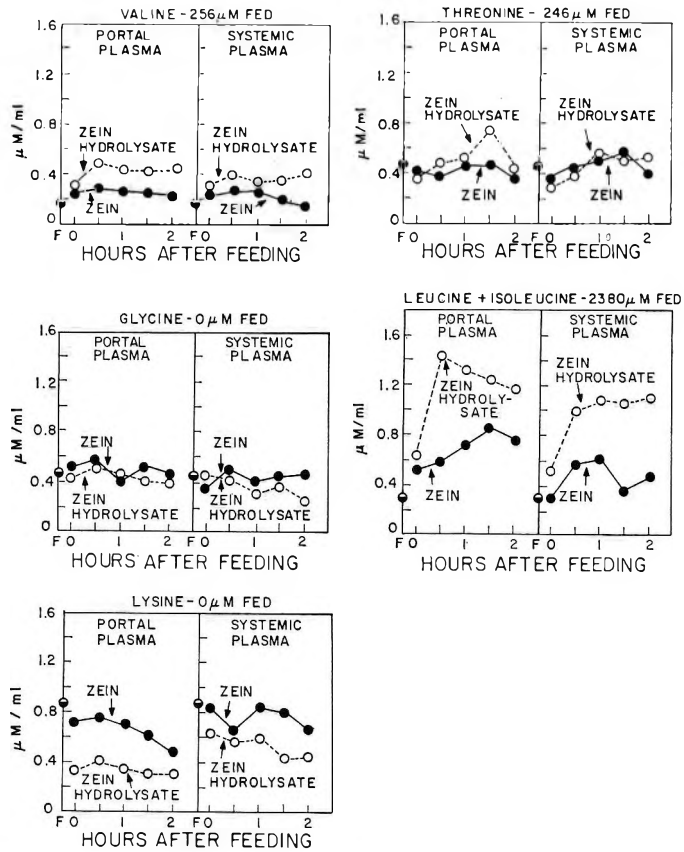


Fig. 2 Concentrations of amino acids in portal and systemic plasma at successive intervals after force-feeding zein or zein hydrolyzate.

fasting value in either the portal or systemic plasma (fig. 2). A small increase in valine concentration occurred in both portal and systemic plasma of the rats force-fed the zein hydrolyzate. Similar results were obtained for threonine (fig. 2). Both valine and threonine are present in relatively low concentrations in zein (both 3%).

The concentration of leucine plus isoleucine in the plasma after feeding zein or zein hydrolyzate is shown in figure 2. The value for the amount fed represents the sum of the concentrations of the 2 amino acids in the meal. The concentration of leucine plus isoleucine increased in both the portal and systemic plasma after force-feeding zein. However, the increase was much greater in both after administration of zein hydrolyzate. The curve for leucine plus isoleucine in the portal plasma after feeding zein hydrolyzate appears to be similar to the corresponding curve for proline (fig. 1) although the hydrolyzate contained almost 3 times as much leucine plus isoleucine (2380 μmoles) as proline (850 μmoles). The systemic plasma curves for these amino acids differ markedly, with the leucine plus isoleucine values being much higher than those for proline after the first hour.

The effect of force-feeding zein or zein hydrolyzate on the plasma concentration of lysine, an indispensable amino acid not present in zein, is also shown in figure 2. In contrast with the results shown for the dispensable amino acids, glutamine and glycine, the plasma concentration of lysine decreased after the administration of zein or zein hydrolyzate, the decrease being greater with the latter.

Casein and casein hydrolyzate. Glutamic acid is the amino acid present in highest concentration (23%) in casein. Although a relatively large quantity (1568 μmoles) of this amino acid was force-fed in the casein or casein hydrolyzate, its concentration did not increase appreciably in either case (fig. 3). Also, plasma glutamic acid concentration increased to the same extent whether the meal contained casein or casein hydrolyzate and the concentration of glutamic acid in the portal plasma increased only slightly above that in the systemic plasma. Comparison of the portal plasma glutamic acid concentrations of rats that received intact zein, or casein (figs. 1 and 3) shows that the value for those receiving casein increased more.

Although the casein meal contained less alanine (453 μmoles alanine ingested) than the zein (1580 μmoles alanine ingested) the concentration of alanine in both portal and systemic plasma increased more after force-feeding casein (fig. 3) than zein (fig. 1). When intact casein was given, the concentration of alanine in the portal plasma increased as much as when the hydrolyzed protein was fed, but the maximal concentration was not reached quite as early. The alanine concentration in the systemic plasma did not increase after force-feeding casein hydrolyzate.

The concentration of proline in the portal plasma increased substantially after force-feeding casein and a somewhat smaller increase occurred in the systemic plasma (fig. 3). Comparable increases were not observed when intact zein (fig. 1) was given. A greater increase occurred in the concentration of proline in the portal plasma after force-feeding the casein hydrolyzate (fig. 3). This increase was comparable to that observed after force-feeding zein hydrolyzate, although the amount of proline ingested in the casein hydrolyzate was somewhat greater (1071 μmoles for casein hydrolyzate as opposed to 850 μmoles for zein hydrolyzate). The increase in the concentration of proline in the systemic plasma after the ingestion of hydrolyzates of zein or casein was always much less than the increase in the proline concentration in the portal plasma.

The concentration of serine increased to the same extent in both portal and systemic plasma when casein was force-fed (fig. 3). This increase was somewhat greater than that observed when zein was force-fed (fig. 1), although in the latter case more serine was ingested (743 μmoles as opposed to 656 μmoles ingested when casein was fed). When casein hydrolyzate was given, the increase in plasma serine was similar to, but slightly less than, that observed after giving intact casein.

Force-feeding casein caused a marked increase in the concentration of glutamine

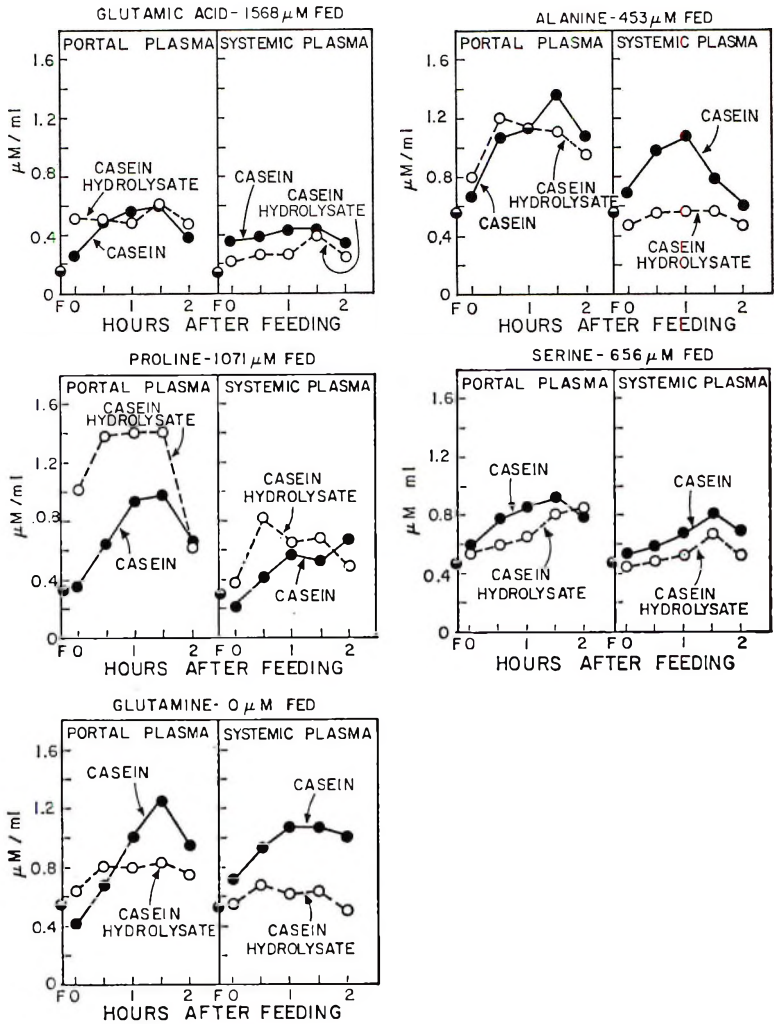


Fig. 3 Concentrations of amino acids in portal and systemic plasma at successive intervals after force-feeding casein or casein hydrolyzate.

in both portal and systemic plasma; yet force-feeding the hydrolyzate had much less effect (fig. 3).

Force-feeding casein resulted in an increase in the concentration of valine in both portal and systemic plasma, the increase in the systemic plasma being somewhat smaller (fig. 4). When casein hydrolyzate was force-fed, much larger increases in valine concentration occurred in both portal and systemic plasma, the concentration again being lower in the systemic plasma.

The concentration of threonine in the portal plasma increased well above the fasting value when casein was force-fed (fig. 4), but the systemic plasma concentration did not increase appreciably. The portal plasma concentration increased more rapidly during the first hour after force-feeding the hydrolyzate, but the maximum was not quite as high as that observed when intact casein was given.

The concentration of glycine did not increase appreciably in either portal or systemic plasma after force-feeding casein

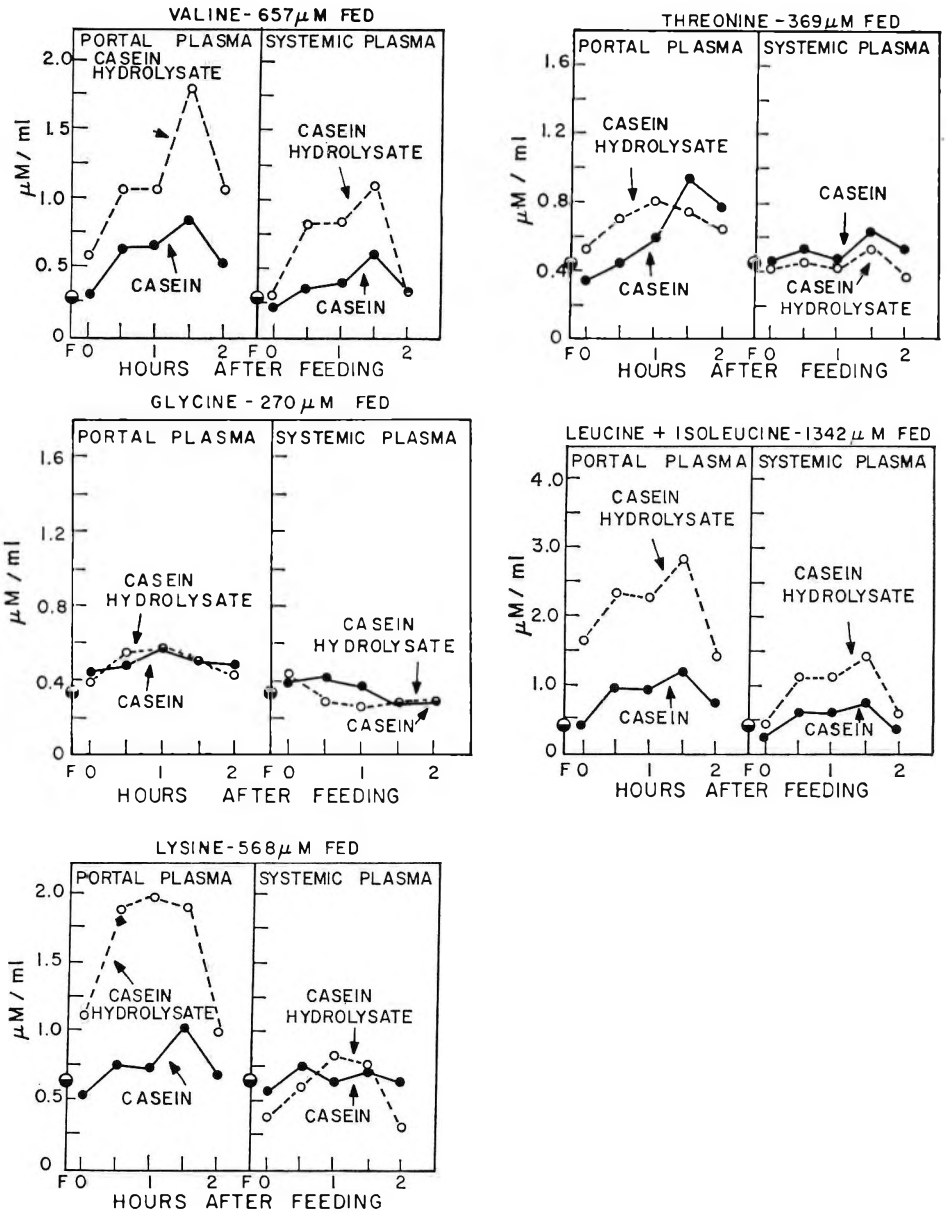


Fig. 4 Concentrations of amino acids in portal and systemic plasma at successive intervals after force-feeding casein or casein hydrolyzate.

or casein hydrolyzate (fig. 4). The curves closely resemble those for glycine after the feeding of zein which contains no glycine (fig. 2). Casein contains only 2% of glycine.

The concentration of leucine plus isoleucine increased moderately in the portal plasma and only slightly in the systemic plasma after casein was force-fed (fig. 4). The concentration of leucine plus isoleucine increased much more in the portal plasma after administration of casein hydrolyzate; however, the general shape of the curve closely resembled that obtained

with intact casein. The concentration of leucine plus isoleucine in the systemic plasma after force-feeding the hydrolyzate was greater than that observed after intact casein but was still well below the maximal leucine plus isoleucine concentration observed in portal plasma. When casein hydrolyzate was force-fed, the concentration of leucine plus isoleucine in the plasma increased more in proportion to the amount ingested (1342 μmoles) than when zein hydrolyzate (containing 2380 μmoles) was fed (fig. 2).

The concentration of lysine (fig. 4) increased moderately in the portal plasma, but changed very little from the fasting value in the systemic plasma after casein was force-fed. Force-feeding casein hydrolyzate caused a rapid increase in the concentration of lysine in the portal plasma, but the concentration in the systemic plasma was not appreciably affected.

DISCUSSION

The changes which occur in the concentrations of free amino acids in the plasma after the ingestion of dietary protein depend upon the amino acid composition of the dietary protein; the rate at which the protein empties from the stomach; the rate of release of free amino acids from the protein during digestion; the rates of absorption of the amino acids; the extent to which the amino acids are metabolized by the intestinal tissues during absorption; and the rates of removal of the absorbed amino acids from the blood. Guggenheim et al. (5, 6) have suggested that, although the concentration of amino acids in the portal blood may serve as a guide to amino acid availability, the relationship between portal blood concentrations and availability is neither direct nor simple. Denton and Elvehjem (7) and Morrison et al. (8) have shown that differences in the availability of amino acids in dietary protein may be reflected in blood amino acid concentrations.

An overall comparison of the amino acid patterns in the portal plasma after the test proteins or their hydrolyzates were fed shows that the amino acids of zein and about one-half of those of casein were absorbed into the portal blood more rapidly when hydrolyzates were fed. This indicates that digestion of the protein was for the most part a more limiting factor than amino acid absorption in determining the rate of entry of the dietary amino acids into the portal blood in these experiments.

The relatively low concentrations of amino acids in the portal plasma after feeding intact zein indicates that the amino acids of zein were less available for absorption than those of casein. This might be expected owing to the high concentration of nitrogen, apparently from undigested protein, found in the intestinal contents of animals fed zein (12), and is in agreement with the observation of Goldberg and Guggenheim (6).

In these experiments even when the diet contained hydrolyzed protein some amino acids were not absorbed into the portal blood in direct proportion to their concentrations in the test meal. In this connection Orten et al.[5] and Pinsky and Geiger (9) found that the rate of absorption of individual amino acids by the intestine was affected by the presence of other amino acids, and Orten[6] found that the overall rate of absorption of an amino acid mixture, as well as the pattern of the mixture that is absorbed, are affected by the qualitative composition of the mixture present in the intestine.

The frequent lack of correlation between the rates of absorption of dietary amino acids (as manifested by increases in their concentrations in the portal plasma) and the quantities of these amino acids ingested may thus be due in part to the effects of the particular amino acid mixture present in the intestine on the absorption process. The presence of relatively large quantities of certain amino acids may depress the absorption of other amino acids. For example, when zein hydrolyzate is fed, the proportion of leucine ingested is very large. The presence of a relatively large concentration of leucine in the intestinal contents could conceivably depress the rates of absorption of other amino acids.

Also, the rates of absorption of individual amino acids differ markedly (10–12). These differences could have considerable

[5] Orten, A. H., K. Korzumi and D. J. France 1951 Federation Proc., *10*: 390 (abstract).
[6] Orten, A. H. 1961 Federation Proc., *20*: 2d.

effect on the response of the plasma amino acid pattern to the feeding of protein or protein hydrolyzate.

The observation that the ingestion of a large quantity of casein or its hydroyzate caused only a relatively small increase in the concentration of glutamic acid in the portal plasma is in agreement with results obtained by Dent and Schilling (12) with dogs fed casein. In other experiments the administration of glutamic acid alone resulted in a relatively small increase in the concentration of this amino acid in the portal plasma (3). As was previously suggested (3) the relatively small increase in portal plasma glutamic acid may be the result of a relatively low rate of absorption of glutamic acid from the intestinal lumen (11) coupled with a high rate of metabolism of this amino acid by the intestinal cells during absorption (13).

The decrease in the concentration of plasma lysine observed after feeding zein, and the still larger decrease observed after feeding the hydrolyzate, suggests that the free lysine in the plasma was drawn into the cells during stimulation of protein synthesis caused by the influx of amino acids from the intestine. The more pronounced lowering effect of the hydrolyzate would then be expected as a result of more rapid absorption of the free amino acids. Wu (14) also observed a decrease in the concentration of lysine in the plasma of rats fed a zein diet for 12 days; and Hill et al. (15) in experiments on chicks found that the addition of 15% of zein to a diet containing 9.5% of soybean protein resulted in a marked decrease in the concentration of lysine in the plasma.

The lack of change in the concentration of glycine after the feeding of zein or zein hydrolyzate indicates either that the influx of the dietary amino acids had no effect on the metabolism of glycine; or that the utilization of glycine by the tissue increased but *de novo* synthesis of glycine compensated for the increased utilization so that no net change in the concentration of glycine in the plasma was observed.

The increase in plasma glutamine concentration after the feeding of zein or zein hydrolyzate indicates synthesis of this amino acid in response to the production of ammonia during the metabolism of the ingested amino acids (16). However, the larger increase in plasma glutamine observed after feeding casein as opposed to its hydrolyzate cannot be adequately explained on this basis.

When considered as a whole, the results of this investigation indicate that the response of the plasma amino acid pattern to the ingestion of a protein or its hydrolyzate is complex, and depends on many factors in addition to the digestibility of the dietary nitrogen source. However, comparison of the concentrations of free amino acids in the portal plasma after feeding the unhydrolyzed proteins does show that the amino acids in casein are more available for absorption than those of zein and indicates that such comparisons may provide useful information about the relative digestibility of dietary proteins.

LITERATURE CITED

1. Rogers, Q. R., M-L. Chen, C. Peraino and A. E. Harper 1961 Observations on protein digestion *in vivo*. III. Recovery of nitrogen from the small intestine at intervals after feeding diets containing different proteins. J. Nutrition, 72: 331.
2. Chen, M-L., Q. R. Rogers and A. E. Harper 1962 Observations on protein digestion *in vivo*. IV. Further observations on the gastrointestinal contents of rats fed different dietary proteins. Ibid., 76: 235.
3. Peraino, C., and A. E. Harper 1962 Concentration of free amino acids in blood plasma of rats force-fed L-glutamic acid, L-glutamine or L-alanine. Arch. Biochem. Biophys., 97: 442.
4. ——— 1961 Quantitative paper chromatography of plasma amino acids. Anal. Chem., 33: 1863.
5. Guggenheim, K., S. Halevy and N. Friedman 1960 Levels of lysine and methionine in portal blood of rats following protein feeding. Arch. Biochem. Biophys., 91: 6.
6. Goldberg, A., and K. Guggenheim 1962 The digestive release of amino acids and their concentrations in the portal plasma of rats after protein feeding. Biochem. J., 83: 129.
7. Denton, A. E., and C. A. Elvehjem 1954 Availability of amino acids *in vivo*. J. Biol. Chem., 206: 449.
8. Morrison, A. B., J. M. McLaughlin, F. J. Noel and J. A. Campbell 1961 Blood amino acid studies. III. Effects of amount and quality of dietary protein and length of test period on plasma free lysine levels in the rat. Canad. J. Biochem. Physiol., 39: 1681.

9. Pinsky, J., and E. Geiger 1952 Intestinal absorption of histidine as influenced by tryptophane in the rat. Proc. Soc. Exp. Biol. Med., *81:* 55.
10. Wilson, R. H., and H. B. Lewis 1929 The rate of absorption of amino acids from the gastrointestinal tract of the white rat. J. Biol. Chem., *84:* 511.
11. Wiseman, G. 1956 Active transport of amino acids by sacs of everted small intestine of the golden hamster. J. Physiol., *133:* 626.
12. Dent, C. E., and J. A. Schilling 1949 Studies on the absorption of proteins: the amino-acid pattern in the portal blood. Biochem. J., *44:* 318.
13. Spencer, R. P., and W. E. Knox 1960 Comparative enzyme apparatus of the gut mucosa. Federation Proc., *19:* 886.
14. Wu, C. 1954 Metabolism of free amino acids in fasted and zein-fed rats. J. Biol. Chem., *297:* 775.
15. Hill, D. C., E. M. McIndoo and E. M. Olsen 1961 Influence of dietary zein on the concentration of amino acids in the plasma of chicks. J. Nutrition, *74:* 16.
16. Meister, A. 1956 Metabolism of glutamine. Physiol. Rev., *36:* 103.

Influence of Various Carbohydrates on the Utilization of Low Protein Rations by the White Rat

V. RELATIONSHIPS AMONG PROTEIN INTAKE, CALORIE INTAKE, GROWTH AND LIVER FAT CONTENT[1]

RUTA P. WIENER,[2] MINORU YOSHIDA[3] AND A. E. HARPER[4]
Department of Biochemistry, University of Wisconsin, Madison, Wisconsin

ABSTRACT Growth, nitrogen balance, carcass analysis and liver fat studies were made on rats fed low protein, low fat diets with sucrose or dextrin as the dietary carbohydrate. Substitution of dextrin for sucrose in isonitrogenous diets stimulated growth by stimulating food, and hence protein, intake. Growth was proportional to protein intake irrespective of the type of dietary carbohydrate; therefore, although protein or amino acid requirements expressed as *percentage of the diet* differed with the type of dietary carbohydrate, expressed as *protein required per unit of weight gained* they did not. Nitrogen balance experiments indicated that the type of dietary carbohydrate affected neither protein digestibility nor nitrogen retention; however, metabolic fecal nitrogen increased and endogenous urinary nitrogen decreased when dextrin was substituted for sucrose in a low protein diet containing fibrin. Calorie intake per unit of body weight increased when dextrin was substituted for sucrose, particularly in diets containing low levels of high quality proteins, and this was accompanied by elevation of carcass fat content. Liver fat content was elevated when sucrose was substituted for dextrin in low protein diets containing methionine-supplemented casein; however, when liver fat content was expressed per unit of protein consumed, the difference due to the change in carbohydrate was small.

Growth rate and food intake of the young rat fed a low protein diet ad libitum increase and liver fat content decreases when dextrin[5] or cornstarch is substituted for sucrose or glucose as the dietary carbohydrate (1–3). Originally the more rapid growth, the lower fat content of the liver and the greater food intake of rats fed low protein diets containing the more complex carbohydrates were attributed to improved protein utilization. However, when Spivey et al. (4) observed that improvement in efficiency of protein utilization as a result of substituting dextrin for sucrose was too small to account for these effects, and that an inverse relationship existed between the food intake of rats fed a low protein diet and the capacity of the dietary carbohydrate to exert osmotic pressure, a direct effect of the type of dietary carbohydrate on food intake appeared to be the most plausible explanation for the various observations. Also, since all of these effects could be eliminated by increasing the protein content of diets containing sucrose, it seemed probable that most of the differences due to the type of dietary carbohydrate could be accounted for if growth and liver fat content were expressed as a function of protein intake. That such is the case for rats fed several different proteins is shown in this paper.

EXPERIMENTAL

The experimental diets contained: corn oil,[6] 5%; salts,[7] 5%; water-soluble vitamin mixture,[8] 0.25%; choline chloride, 0.15%; protein, type and level as indicated in the

Received for publication December 28, 1962.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station and supported in part by grants from the National Live Stock and Meat Board, Chicago, Illinois, and from the Research Committee of the Graduate School from funds provided by the Wisconsin Alumni Research Foundation.

[2] Present address: Linden, New Jersey.

[3] Present address: National Institute of Agricultural Sciences, Chiba, Japan.

[4] Present address: Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge 39, Massachusetts.

[5] The term "dextrin" refers to the product obtained when moist cornstarch is heated in an autoclave at 120°C for 3 hours, then dried and ground.

[6] Fat-soluble vitamins A, D and E were included in the corn oil, to provide the following concentrations per 100 g of diet: A, 400 IU; D, 200 IU; and E, 10 mg.

[7] Harper, A. E. 1959 J. Nutrition, 68: 405.

[8] See footnote 7.

tables and figures; and dextrin or sucrose to make 100%. Lysine was included in one series of wheat gluten diets at a level of 4.7% of the wheat gluten content. Methionine was included at a level of 0.3% of the diet in the casein diets used for growth and carcass composition studies and 3.3% of the casein content in the liver fat study. The proteins (wheat gluten, egg albumen, soya bean protein[9] and fibrin) were commercial products. The casein was extracted with hot ethanol.

Male Holtzman rats weighing 45 to 55 g were used throughout. Before being offered the experimental diets, they were fed a diet containing the lowest level of the appropriate protein and a 1:1 mixture of sucrose and dextrin for 2 to 4 days. Each group contained 5 rats selected so that the average weights and weight ranges were the same for all groups. They were housed in individual cages and fed ad libitum. Food consumption was recorded daily and weight gain weekly during the 2-week experimental periods. Calorie intakes were calculated.

There were 6 rats/group in the nitrogen balance experiment. Urinary and fecal collections were made for a 7-day period. This was preceded by a 5- to 7-day adjustment period. During both the experimental and the adjustment periods the animals were housed in individual metabolism cages. After the first 7-day collection period, the diets of groups 1 and 3, and groups 2 and 4, were exchanged and the experiment was repeated.

Nitrogen was determined by the Kjeldahl method using mercuric oxide as a catalyst on the total 7-day fecal collections and on aliquots of the 7-day urine collections. Dilute sulfuric acid was used to preserve the urine specimens. Apparent and true digestibilities were calculated according to the following formulae:

$$\text{Apparent digestibility} = \frac{\text{N intake} - \text{fecal N}}{\text{N intake}} \times 100.$$

$$\text{True digestibility} = \frac{\text{N intake} - (\text{fecal N} - \text{metabolic fecal N})}{\text{N intake}} \times 100.$$

Metabolic nitrogen excretion was determined experimentally on rats fed a 4% egg albumen diet with either sucrose or dextrin as the carbohydrate. These rats were of the same age and approximately the same weight as those used for the determination of total nitrogen excretion. The amount of metabolic fecal nitrogen was expressed as milligrams of N per gram of food consumed and the amount of endogenous urinary N as milligrams of N per square centimeter of body surface using the formula of Carman and Mitchell (5) to relate body surface area to body weight. The average of the initial and final body weights was used in the calculation. Percentage of nitrogen retention was calculated using the following equations:

$$\text{Apparent retention} = \frac{\text{N intake} - (\text{fecal N} + \text{urinary N})}{\text{N intake} - \text{fecal N}} \times 100.$$

$$\text{True retention} = \frac{\text{N absorbed} - (\text{urinary N} - \text{endogenous urinary N})}{\text{N absorbed}} \times 100.$$

The technique used for determining endogenous nitrogen excretion was a slight modification of the method of Nehring and Haesler (6), Columbus (7) and Njaa (8). Njaa showed that the value for endogenous nitrogen excretion obtained using littermates does not differ significantly from the value obtained when the animal is used as its own control as in the classical method of Mitchell and Carman (9). The procedure used in the present study differs from Njaa's in that weanling rats of the same strain, sex and age were used instead of littermates.

The rats used for the carcass composition study were killed by ether anesthesia. Livers and gastrointestinal tracts were removed. The carcasses were frozen, then ground 4 to 7 times until they appeared homogeneous. A 10-g aliquot was used for each analysis. Moisture content was determined by drying the sample at 110°C for 24 hours or until the weight remained constant. Fat was determined by ethyl ether extraction of the dried and ground residue in a Goldfisch continuous extractor for 24 hours and nitrogen by the semimicro-Kjeldahl method.

The rats used for the liver fat study were decapitated at the end of the 2-week period. Livers were removed and homogenized. The homogenates were dried at

[9] ADM C-1 Assay Protein, Archer-Daniel-Midland, Minneapolis.

60°C for 12 hours and then at 110°C for 4 hours. The moisture-free residue was ground and fat was determined as for the carcass analysis.

Student's *t* test was used to determine the significance of differences.

RESULTS

Growth. Weight gains of rats fed the diets containing different levels of the various proteins with either dextrin or sucrose as the carbohydrate were plotted as functions of the protein content of the diet and the average protein intake of the group (fig. 1).

When weight gains were plotted as a function of protein content of the diet, the growth-response curves for groups receiving dextrin were above those for groups receiving sucrose with casein (fig. 1-A), methionine-supplemented casein (fig. 1-C), soya bean protein (Drackett) (fig. 1-E), egg albumen (fig. 1-G), fibrin (fig. 1-I), and lysine-supplemented wheat gluten (fig. 1-K) as the dietary proteins. The growth-stimulating effect of dextrin was most pronounced when the diets contained the lower levels of protein. With diets containing 15% or more of proteins other than wheat gluten the differences between the weight gains of the groups receiving the 2 different carbohydrates were seldom statistically significant. This can be seen from the curves for groups receiving methionine-supplemented casein and fibrin (fig. 1-C and 1-I), which were studied over the widest range of levels.

With diets containing 16, 23, or 30% of unsupplemented wheat gluten, groups receiving dextrin grew more rapidly than those receiving sucrose, but with 44 or 51% of wheat gluten in the diet, groups receiving sucrose grew more rapidly (fig. 1-M).

When weight gain was plotted as a function of protein intake, differences between the growth-response curves of the corresponding sucrose and dextrin series were much smaller, particularly when the protein intake was low (fig. 1-B, -D, -F, -H, -J, -L, -N). However, groups receiving dextrin tended to gain a little more rapidly per unit of protein consumed than the comparable groups receiving sucrose. Student's *t* test was used to determine the significance of differences between the protein intakes of sucrose and dextrin groups that received the same protein and gained about the same amount of weight. Comparisons of this type are not valid at the upper parts of the curves where protein is no longer limiting but over the ranges where protein is limiting, except for 3 comparisons in the unsupplemented casein and 3 in the fibrin series, the differences were not statistically significant ($P > 0.05$).

The observations that dextrin supported slightly but significantly more rapid growth per unit of protein consumed than did sucrose when the dietary protein was either fibrin or unsupplemented casein, and that a similar, but not statistically significant, trend was observed in several comparisons suggested that some factor in addition to increased protein intake contributed to the better growth of dextrin groups. The effect is much smaller than that due to the stimulation of food intake, but has been consistent in several experiments.

In an effort to explain this observation, an experiment was carried out in which the difference of 5% between the caloric values of dextrin and sucrose was compensated for by increasing the corn oil content of the sucrose diets. This adjustment did not affect growth as a function of either protein level of the diet or protein intake.

Nitrogen balance. The possibility that a small difference between the percentages of nitrogen retained by the sucrose and the dextrin groups might account for this effect was also considered. Spivey et al. (4) observed that substituting dextrin for sucrose resulted in a slight improvement in the apparent nitrogen retention of weanling rats fed low casein diets supplemented with methionine. A more complete study was made, therefore, to determine whether further evidence of improved nitrogen retention could be obtained. The results are presented in table 1.

The growth rates of the sucrose group receiving 8% of fibrin and of the dextrin group receiving 6% of fibrin were similar in each collection period, but the average food intake of the dextrin group was

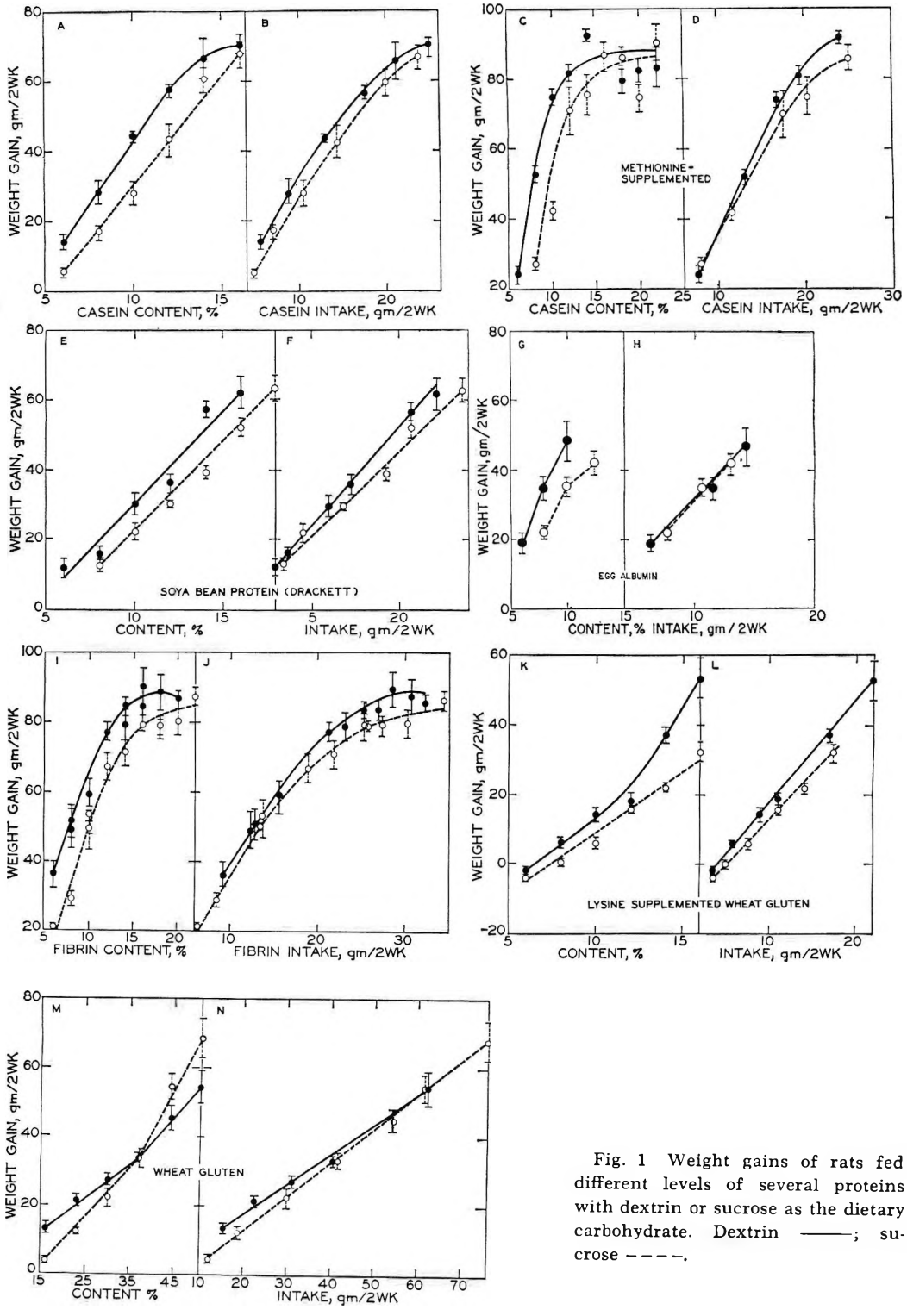


Fig. 1 Weight gains of rats fed different levels of several proteins with dextrin or sucrose as the dietary carbohydrate. Dextrin ——; sucrose – – – –.

TABLE 1

Effect of type of dietary carbohydrate on digestibility of fibrin and on nitrogen retention

	Trial 1		Trial 2	
	6% Fibrin Dextrin	8% Fibrin Sucrose	6% Fibrin Dextrin	8% Fibrin Sucrose
Initial weight (g)	68	63	81	72
Weight gain (g/week)	33	29	31	26
Food intake (g/week)	81	68	89	65
N intake (mg/week)	860	898	935	813
Fecal N (mg/week)				
Total	132	59	143	50
Metabolic	136	44	163	51
Digestibility (%)				
Apparent	85	94	85	94
True	100	98	100	100
N absorbed (mg)	860	884	935	813
Urinary N (mg/week)				
Total	89	117	88	127
Endogenous	68	105	71	138
N retained (%)				
Apparent	90	87	91	84
True	98	99	98	100

greater than that of the comparable sucrose group.

Significantly more nitrogen ($P < 0.05$) was excreted in feces by rats receiving dextrin than by those receiving sucrose. The estimated amounts of metabolic nitrogen excreted per gram of food consumed by the dextrin and sucrose groups were, respectively, 1.63 mg and 0.65 mg in the first collection period and 1.85 mg and 0.78 mg in the second. The metabolic fecal nitrogen thus accounted for nearly all of the fecal nitrogen excreted.

In contrast, rats fed sucrose excreted significantly more nitrogen ($P < 0.05$) in the urine than those fed dextrin. The average values for milligrams of endogenous nitrogen per square centimeter of body surface for the dextrin and the sucrose groups were: 0.31 and 0.51 during the first collection period and 0.30 and 0.63 during the second collection period. Endogenous nitrogen excreted by rats receiving low fibrin diets containing sucrose accounted, within the limits of error, for all of the urinary nitrogen they excreted.

Replacing sucrose with dextrin resulted in a lowered apparent digestibility of fibrin. The difference was statistically significant ($P < 0.05$). The true digestibility, on the other hand, was not affected by the type of dietary carbohydrate. The ingested fibrin was completely digested in all cases. The apparent nitrogen retention was slightly but not significantly ($P > 0.05$) higher for the dextrin-fed rats but true nitrogen retention was nearly 100%, irrespective of the type of dietary carbohydrate.

Food and calorie intake. When groups receiving the same dietary level of protein are compared, regardless of the type of protein, those receiving dextrin nearly always have greater food, protein and calorie intakes than the corresponding groups receiving sucrose (table 2). Only with unsupplemented wheat gluten as the dietary protein were there exceptions in which the groups fed the higher levels of this protein with sucrose consumed more food, protein and calories than the corresponding groups receiving dextrin.

The average daily calorie intake, expressed as calories consumed per 100 g of body weight, was plotted against protein level in the diet in an effort to detect relationships among the various factors studied. Three types of graphs which appear to depend upon the nutritive quality of the dietary proteins were obtained. The first type, obtained with high quality proteins (fibrin, methionine-supplemented casein and egg albumen) (fig. 2-A, -B, -C), indicate that: 1) the daily calorie intakes of groups receiving sucrose remained fairly constant per unit of body weight, regardless of the protein content of the diet; 2) groups receiving dextrin had greater daily calorie intakes per gram of body weight than those receiving sucrose, the differences being particularly large and highly signficant ($P > 0.01$) when dietary protein level was low; and 3) at protein levels above 10 to 12% groups receiving dextrin showed fairly constant

TABLE 2

Food intake of rats fed various diets with "dextrin" or sucrose as carbohydrate

Protein level	Casein		Casein + methionine		Drackett protein		Egg albumen		Fibrin		Wheat gluten + lysine		Protein level	Wheat gluten	
	Dextrose	Sucrose	Dextrose	Sucrose	Dextrose	Sucrose	Dextrose	Sucrose	Dextrose	Sucrose	Dextrose	Sucrose		Dextrose	Sucrose
%	*g/2 weeks*		*g/2 weeks*		*g/2 weeks*		*g/2 weeks*		*g/2 weeks*		*g/2 weeks*		%	*g/2 weeks*	
6	92	80	121		96		105		154	104	59	60	16	98	75
8	110	87	157	94	90	85	139	95	160	104	72	63	23	98	81
10	130	105	160	113	119	90	142	103	155	136	86	76	30	104	100
12	147	119	161	142	121	113		109	176	157	93	92	37	110	112
14	152	142	172	141	154	132			180	155	122	101	44	123	140
16	157	149		153	152	134			178	160	138	109	51	122	149
18						151			170	152					

daily calorie intakes per unit of body weight.

Graphs of the second type, obtained for groups fed the less nutritionally adequate proteins (unsupplemented casein, soya bean protein and wheat gluten supplemented with lysine) (fig. 2-D, -E, -F), indicate that: 1) calorie intakes of groups receiving sucrose were fairly constant over the entire range of protein levels tested with casein, but only over the range of 12 to 16% with lysine-supplemented wheat gluten and 10 to 16% with soya bean protein, and that a gradual increase in the daily calorie intake occurred when the levels of the last 2 proteins were increased from 6% to 10 or 12%; and 2) the average daily calorie intakes of groups receiving dextrin were higher than those of groups receiving sucrose and increased over the lower portion of the ranges tested with lysine-supplemented wheat gluten and soya bean protein.

The third type of graph, obtained when unsupplemented wheat gluten was the source of protein, indciates that: 1) the average daily calorie intake per 100 g of body weight was fairly constant for the animals receiving dextrin; 2) it increased gradually as the wheat gluten content of the diet was increased from 16 to 44% for the rats receiving sucrose; and 3) the daily calorie intake per unit of body weight was greater for rats receiving dextrin at protein levels of 16 to 30%.

Carcass composition. Since average daily calorie intake per unit of body weight was higher when dextrin was substituted for sucrose as the sole source of carbohydrate, particularly in groups receiving low levels of methionine-supplemented casein or fibrin, an effort was made to determine whether the higher daily calorie intake resulted in greater fat deposition in the carcass.

Values for carcass composition for fibrin and methionine-supplemented casein groups are shown in table 3. Carcass moisture content of the rats receiving sucrose was in each comparison significantly greater ($P < 0.05$) than that of rats receiving dextrin. The carcass fat content showed the reverse pattern and was significantly higher ($P < 0.05$) for rats fed

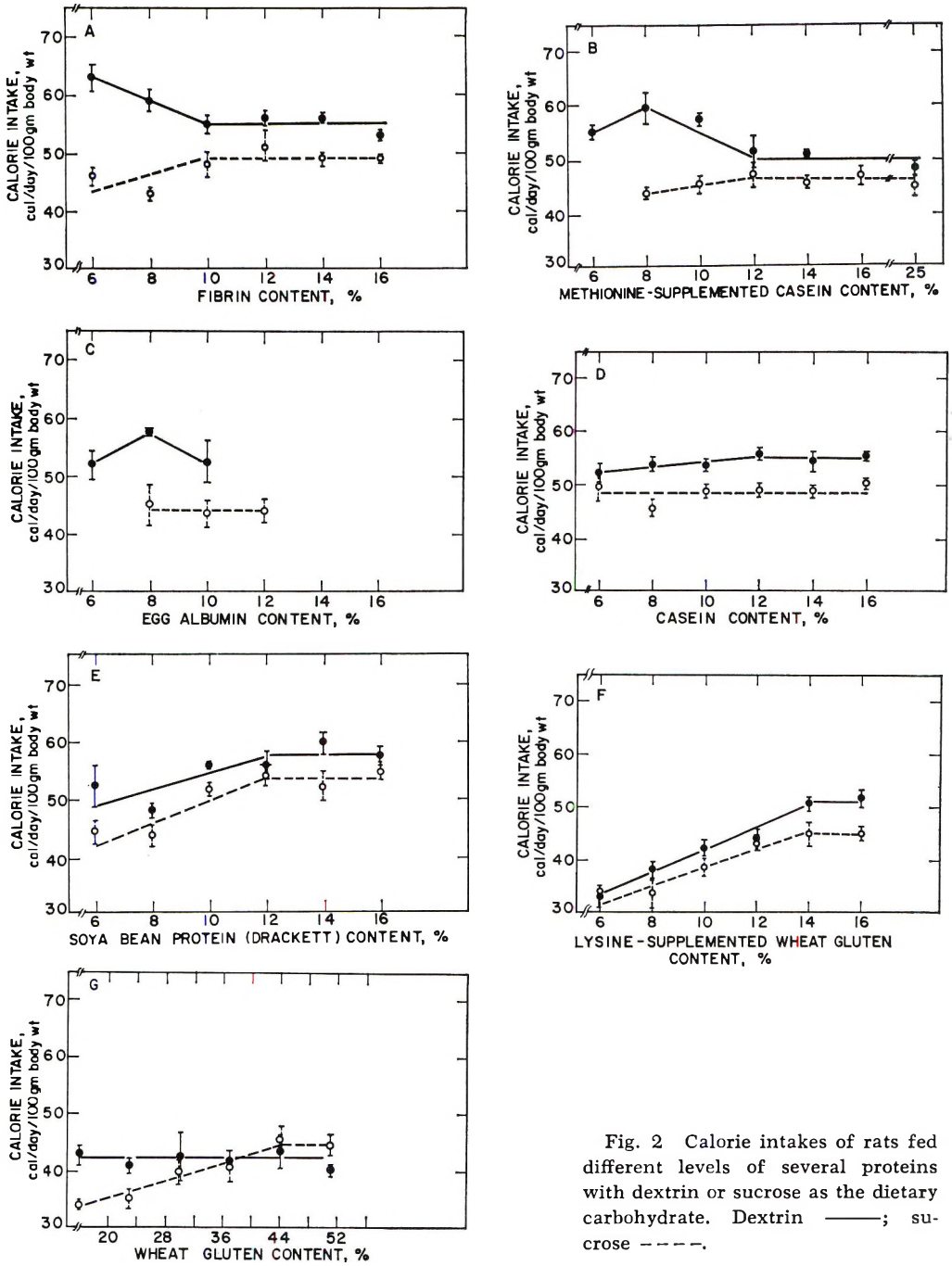


Fig. 2 Calorie intakes of rats fed different levels of several proteins with dextrin or sucrose as the dietary carbohydrate. Dextrin ——; sucrose – – – –.

TABLE 3

Carcass composition of rats fed low protein diets containing "dextrin" or sucrose

Group	Protein	Carbohydrate	Weight gain	Carcass			Calorie intake/day	Calorie intake/day/100 g body wt[1]
				Moisture	Fat	Protein		
			g/2 weeks	%	%	%		
1	6% Fibrin	dextrin	32 ± 1.1[2]	63.6 ± 0.4[2]	15.4 ± 0.4[2]	16.8 ± 0.2[2]	40.3	56.7
2	8% Fibrin	sucrose	35 ± 2.4	66.8 ± 0.7	11.4 ± 0.9	17.0 ± 0.2	34.2	47.1
3	8% Fibrin	dextrin	49 ± 5.2	62.7 ± 0.8	15.6 ± 1.0	17.1 ± 0.3	45.9	57.6
4	10% Fibrin	sucrose	48 ± 2.7	66.4 ± 0.5	10.5 ± 0.5	17.2 ± 0.5	38.6	48.5
5	8% Casein + methionine	dextrin	48 ± 3.4	62.1 ± 0.8	15.5 ± 0.5	16.7 ± 0.4	42.6	56.8
6	8% Casein + methionine	sucrose	28 ± 2.5	65.9 ± 0.8	12.1 ± 0.5	16.0 ± 0.4	30.9	48.3

[1] Calculated for one-half maximal weight gain.
[2] S.E.M.

dextrin than for those fed sucrose. The differences between the values for fat content of the dextrin and sucrose groups were approximately equal to the differences in moisture content. The values for carcass protein content were similar for all groups.

Liver fat. When the diets contained the same percentage of protein more fat accumulated in the livers of rats receiving sucrose than in the livers of those receiving dextrin (fig. 3-A). Approximately equal liver fat concentrations were observed for groups receiving the different carbohydrates when the protein content of the sucrose diet was 2% higher than that of the dextrin diet. The differences between the sucrose and dextrin groups were much smaller when liver fat was plotted against protein intake (fig. 3-B). Variations in liver fat content were large within both expermiental groups, a common observation in this type of study.

DISCUSSION

Growth in relation to protein level and protein intake. In agreement with previous observations (1, 2, 10–15), the growth rate of rats fed low protein, high carbohydrate diets increased when dextrin was substituted for sucrose as the dietary carbohydrate. When growth rate was plotted as a function of dietary protein level, only the curves for groups fed unsupplemented wheat gluten deviated from the general pattern and then only when the protein content was high. In diets in which protein is limiting the beneficial effect of dextrin diminishes as its concentration in the diet decreases (2), but in no instance previously has sucrose proved superior to dextrin.

Because of its hydrophilic properties, wheat gluten ingested in large amounts may form a mass in the stomach that disperses with difficulty. This could lower the rate of stomach-emptying and hence decrease food consumption. Harper and Spivey (14) and Peraino et al. (16) noted that stomach contents were more fluid when sucrose was substituted for dextrin. Sucrose may therefore result in more rapid emptying of total solids from the stomach and hence in greater food intake when the diet is high in wheat gluten.

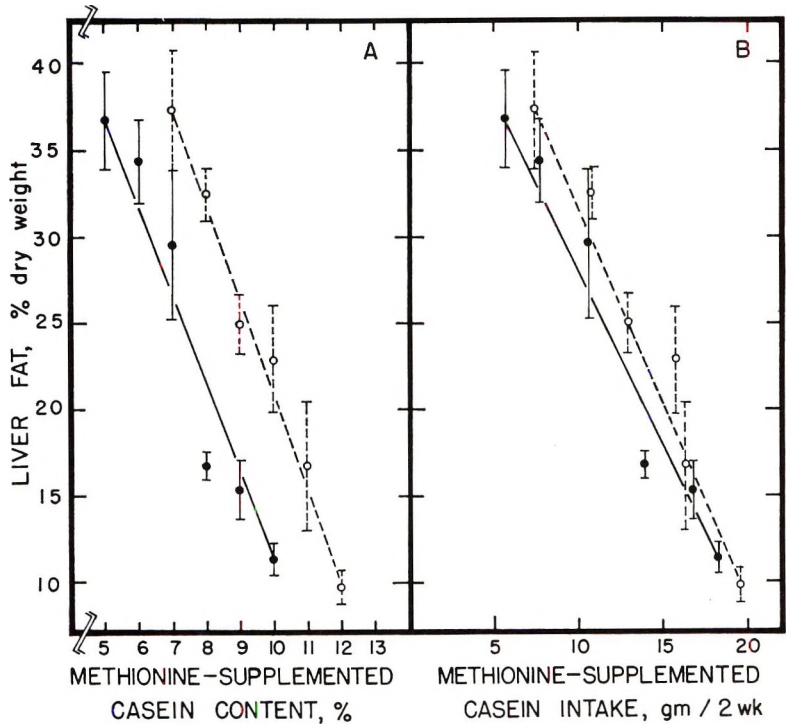


Fig. 3 Fat content of liver from rats fed various levels of methionine-supplemented casein with dextrin or sucrose as the dietary carbohydrate. Dextrin ———; sucrose – – – –.

The observation that only a very small growth-stimulating effect of dextrin can be demonstrated when growth rate is plotted as a function of protein intake, instead of as a function of dietary protein content, indicates that protein is not used appreciably more efficiently in dextrin than in sucrose diets. This is in accord with the hypothesis that the difference between the osmotic pressures exerted by sucrose and dextrin results in a difference in stomach distension and, hence, in food consumption which is, in turn, responsible for the difference in growth rate (14). Middleton et al. (17) and Derse (18) have observed that the protein efficiency ratios (gain in weight/g protein consumed) of 3 different proteins are not affected by the type of dietary carbohydrate.

From this it is clear that the type of dietary carbohydrate has little effect on the *absolute* amino acid requirements of an animal (15). However, it should be emphasized that amino acid requirements of the rat expressed as a percentage of the diet do vary with the type of dietary carbohydrate. Gupta et al. (19) observed that the requirement for lysine, expressed as a percentage of the diet, was lower with dextrin than with sucrose as the dietary carbohydrate, but when the lysine requirement was expressed as total lysine required per 2 weeks for maximal growth, the type of carbohydrate was without effect.

Nitrogen retention. The substitution of dextrin for sucrose resulted in greater metabolic nitrogen excretion in feces and, hence, in a significantly lower apparent digestibility of fibrin.

Booher et al. (20) noted that the apparent digestibility of protein is directly related to the digestibility of dietary starches. Yoshida and Harper[10] observed that the percentage of ingested dry matter excreted in feces was 6.0 and 0.8, respectively, for groups receiving dextrin and sucrose in low casein diets supplemented

[10] Unpublished data.

with methionine and threonine. The greater excretion of dry matter by groups fed dextrin probably accounts for their greater excretion of fecal metabolic nitrogen.

Marshall and Womack (21) and Spivey et al. (4) observed that the apparent nitrogen retention of rats fed a low protein diet containing dextrin also was greater than that of comparable animals receiving sucrose. In the present study most of the difference was accounted for by a difference between the values for endogenous urinary nitrogen excretion for the 2 groups so that true nitrogen retention values were essentially the same. This further emphasizes that low protein diets containing dextrin are not more efficiently ultilized than low protein diets containing sucrose.

Effect of protein level on calorie intake. The daily calorie intakes per 100 g of body weight of young, small laboratory animals are fairly constant (22–29). Hegsted and Haffenreffer (30) suggested that food intake is controlled by some means at a relatively constant level above the basal caloric requirement and that animals eat primarily to satisfy their need for calories. However, Meyer (31) has suggested that an animal fed a low protein, high calorie diet attempts to compensate for its inadequate protein intake by increasing its total food intake and that the disposal of the extra calories ingested limits the extent to which it can do so. Excess energy ingested must be deposited as body fat (25, 31, 32) or eliminated as heat (33, 34). Meyer (31) and Peterson et al. (25) observed greater gain in fat and lower gain in fat-free carcass in animals fed low protein diets and Meyer and Hargus (35) observed that food intake, weight gain and gain in lean body mass of rats fed low protein diets increased when they were forced to expend energy by a low environmental temperature or exercise.

In the present study the calorie intakes per unit of body weight of rats receiving sucrose and the better-quality proteins (fibrin, egg albumen and methionine-supplemented casein) remained fairly constant regardless of the protein content of the diet in agreement with observations of Hegsted and Haffenreffer (30). However, when dextrin was the dietary carbohydrate and the protein level was below 10 or 12%, conditions in which protein was the growth-limiting nutrient, the animals ate more per unit of body weight than when the protein level was high and much more than the comparable groups receiving sucrose. The higher food and energy intakes of rats fed diets low in fibrin or methionine-supplemented casein and containing dextrin were reflected in their carcass compositions by an increase in fat content. The failure of sucrose groups to eat as much as dextrin groups would be excepted if rats receiving sucrose were unable to increase their food and calorie intakes, not because their protein or energy needs were satisfied, but because the nature of the diets was such that the animals were physically unable to ingest additional amounts of food. Again, this could be accounted for by the high osmotic capacity of sucrose.

When dextrin was substituted for sucrose in diets containing low levels of proteins of intermediate or low nutritional quality, calorie intake per unit of body weight was not appreciably elevated. Such diets contain more calories per unit of *utilizable* protein and this might prevent the animal from increasing its food intake enough to satisfy a greater portion of its need for protein. Such a relationship could also account for the smaller response to dextrin with 6% than 8% of egg albumen and methionine-supplemented casein and also for the observation that calorie intake per 100 g of body weight increased with increasing protein level over a fairly wide range with either carbohydrate when the protein was of low quality.

Mayer and Vitale (36) reported that in the albino rat the thermochemical efficiency, defined as the ratio of calories deposited to calories ingested, stays approximately constant over the period 21 to 50 days when animals are maintained with low protein diets (10%). The extra calories in the carcasses of the 37- to 39-day-old rats receiving dextrin in the present study accounts for only approximately one-fourth of the extra calories they ingested. Therefore, dextrin appears to decrease the thermochemical efficiency with which low protein diets are used by

young rats. This would be expected if the efficiency of energy utilization for fat synthesis is low. The gross efficiency of growth, defined as the ratio of weight gain to the amount of food consumed, on the other hand, is slightly better for groups fed the low protein diets containing dextrin.

Effect of protein intake on liver fat accumulation. The concentration of liver fat in rats fed low casein diets supplemented with methionine was reduced when dextrin was substituted for sucrose as had been observed previously (2, 37). An increase in the protein content of the sucrose-type diets overcame this difference just as it did the growth difference. The difference between the values for liver fat content of dextrin and sucrose groups was largely eliminated by expressing fat content as a function of protein intake rather than as a function of the protein content of the diet, indicating that the elevated liver fat content of sucrose groups was also due to their lower food, and, hence, protein intakes.

LITERATURE CITED

1. Harper, A. E., and M. C. Katayama 1953 The influence of various carbohydrates on the utilization of low protein rations by the white rat. I. Comparison of sucrose and cornstarch in 9% casein rations. J. Nutrition, *49:* 261.
2. Harper, A. E., W. J. Monson, D. A. Arata, D. A. Benton and C. A. Elvehjem 1953 Influence of various carbohydrates on the utilization of low protein rations by the white rat. II. Comparison of several proteins and carbohydrates. Growth and liver fat. Ibid., *51:* 523.
3. Harper, A. E., and C. A. Elvehjem 1957 A review of the effects of different carbohydrates on vitamin and amino acid requirements. Agr. Food Res., 5: 754.
4. Spivey, H. E., M. C. Katayama, M. Yoshida and A. E. Harper 1958 Significance of the "protein-sparing" effect of dextrin. Am. J. Physiol., *193:* 479.
5. Carman, G. G., and H. H. Mitchell 1926 Estimation of the surface area of the white rat. Am. J. Physiol., 76: 380.
6. Nehring, K., and E. Haesler 1954 Untersuchungen ueber die biologische Wertigkeit von Eiweissfutterstoffen. I. Die Bestimmung der biologischen Wertigkeit und der Ergaenzungswirkung von Eiweissfutterstoffen durch Versuche an wachsenden Ratten. Arch. Tierernaehr. Beihefte, *5:* 110.
7. Columbus, A. 1954 Stickstoff-Grundumsatz und Methodik der Bestimmung der biologischen Eiweisswertigkeit nach dem N-Bilanz-Verfahren bei wachsenden Ratten. Ibid., *4:* 51.
8. Njaa, L. R. 1959 A shortened version of Mitchell method for determination of the biological value of protein. Growth rate as a source of error. Brit. J. Nutrition, *13:* 137.
9. Mitchell, H. H., and G. G. Carman 1926 The biological value of the nitrogen of mixtures of patent white flour and animal foods. J. Biol. Chem., 68: 183.
10. Krehl, W. A., P. S. Sarma, L. J. Tepley and C. A. Elvehjem 1946 Factors affecting the dietary niacin and tryptophane requirement of the growing rat. J. Nutrition, *31:* 85.
11. Henderson, L. M., T. Deodhar, W. A. Krehl and C. A. Elvehjem 1947 Factors affecting the growth of rats receiving niacin-tryptophan-deficient diets. J. Biol. Chem., *170:* 261.
12. Hankes, L. V., L. M. Henderson, W. L. Brickson and C. A. Elvehjem 1948 Effect of amino acids on the growth of rats on niacin-tryptophan-deficient rations. Ibid., *174:* 873.
13. Lyman, R. L., and C. A. Elvehjem 1951 Further studies on amino acid imbalance produced by gelatin in rats on a niacin-tryptophan low ration. J. Nutrition, *45:* 101.
14. Harper, A. E., and H. E. Spivey 1958 Relationship between food intake and osmotic effect of dietary carbohydrate. Am. J. Physiol., *193:* 483.
15. Harper, A. E. 1959 Advances in our knowledge of protein and amino acid requirement. Federation Proc., *18:* 104.
16. Peraino, C., Q. R. Rogers, M. Yoshida, M.-L. Chen and A. E. Harper 1959 Observations on protein digestion *in vivo*. II. Dietary factors affecting the rate of disappearance of casein from the gastrointestinal tract. Canad. J. Biochem. Physiol., *37:* 1475.
17. Middleton, E. J., A. B. Morrison and J. A. Campbell 1960 Evaluation of protein in foods. VI. Further factors influencing the protein efficiency ratio of foods. Ibid., *38:* 865.
18. Derse, P. H. 1960 Evaluation of protein quality (biological method). J. Assoc. Offic. Agr. Chemists, *43:* 38.
19. Gupta, J. D., A. M. Dakroury, A. E. Harper and C. A. Elvehjem 1958 Biological availability of lysine. J. Nutrition, *64:* 259.
20. Booher, L. E., I. Behan and E. McMeans 1951 Biological utilization of unmodified and modified food starches. Ibid., *45:* 75.
21. Marshall, M. W., and M. Womack 1954 Influence of carbohydrate, nitrogen source and prior state of nutrition on nitrogen balance and liver composition in the adult rat. Ibid., 52: 51.
22. Hill, F. W., and L. M. Dansky 1950 Studies on the protein requirement of chicks and its relation to dietary energy level. Poultry Sci., 29: 763.
23. Hill, F. W., and L. M. Dansky 1954 Studies on the energy requirement of chickens. I. The effect of dietary energy level on growth and feed consumption. Ibid., *33:* 112.

24. Sellers, E. A., R. W. You and N. M. Moffat 1954 Regulation of food consumption by caloric value of the ration in rats exposed to cold. Am. J. Physiol., *177:* 367.
25. Peterson, D. W., C. R. Grau and N. F. Peek 1954 Growth and food consumption in relation to dietary level of protein and fibrous bulk. J. Nutrition, *52:* 241.
26. Sibbald, I. R., R. T. Berg and J. P. Bowland 1956 Digestible energy in relation to food intake and nitrogen retention in the weanling rat. Ibid., 59: 385.
27. Sibbald, I. R., J. P. Bowland, A. R. Robblee and R. T. Berg 1957 Apparent digestible energy and nitrogen in the food of the weanling rat. Influence on food consumption, nitrogen retention and carcass composition. Ibid., *61:* 71.
28. Vaughan, D. A., and L. N. Vaughan 1957 The effect of a low environmental temperature on the weight and food consumption of thiamine-deficient rats. Ibid., *63:* 417.
29. Yoshida, A., A. E. Harper and C. A. Elvehjem 1957 Effects of protein per calorie ratio and dietary level of fat on calorie and protein utilization. Ibid., 63: 555.
30. Hegsted, D. M., and V. K. Haffenreffer 1949 Caloric intake in relation to the quality and quantity of protein in the diet. Am. J. Physiol., *157:* 141.
31. Meyer, J. H. 1958 Interactions of dietary fiber and protein on food intake and body composition of growing rats. Ibid., *193:* 488.
32. Johnson, S. R., A. S. Hogan and U. S. Ashworth 1936 University of Missouri, Agricultural Experiment Station Research Bull. 249. Columbia, Missouri.
33. Forbes, E. B., R. W. Swift, L. F. Marcy and M. T. Davenport 1944 Protein intake and heat production. J. Nutrition, *28:* 189.
34. Black, A., K. H. Maddy and R. W. Swift 1950 The influence of low levels of protein on heat production. Ibid., *42:* 415.
35. Meyer, J. H., and W. A. Hargus 1959 Factors influencing food intake of rats fed low-protein rations. Am. J. Physiol., *197:* 1350.
36. Mayer, J., and J. J. Vitale 1957 Thermochemical efficiency of growth in rats. Ibid., *189:* 39.
37. Womack, M., and M. W. Marshall 1955 Starches, sugars, and related factors affecting liver fat and nitrogen balances in adult rats fed low levels of amino acids. J. Nutrition, *57:* 193.

Studies on the Utilization of Various Starches and Sugars in the Rat

G. REUSSNER, JR., J. ANDROS AND R. THIESSEN, JR.
Technical Center, General Foods Corporation,[1] Tarrytown, New York

ABSTRACT Various types of dietary carbohydrates were fed to growing rats for 28 days with either casein or wheat gluten as the source of dietary protein. Unmodified potato starch and potato amylose resulted in increases in body weight gains and enlarged ceca when casein supplied a dietary protein level of 6%. The same effect was shown when wheat gluten was the dietary protein at either a 9 or 15% protein level. These 2 sources of carbohydrate gave relatively poor weight gains and protein efficiency values when the casein level of the diet was increased to 15% protein. Unmodified corn, wheat, rice, tapioca, and amioca starches, as well as maltose, corn amylopectin, and potato amylopectin gave normal body weight gain values, protein efficiency ratios and ceca weights at both the high and low dietary protein levels. Lactose, unmodified arrowroot starch, fructose, and dextrose gave inferior body weight gains and protein efficiency values. The prevalance of diarrhea and an enlargement of the ceca gave evidence that commercial samples of corn dextrin and pregelatinized amioca starch were not as well utilized as unmodified corn starch or unmodified amioca starch.

Recently there has been an increased interest in the biological utilization of carbohydrates because of the development of new starch products for the food industry. During the past several years, amylose has been produced in large quantities from both corn starch and potato starch. Many new pregelatinized starch products have been developed, also, with special functional properties for food use.

It has been claimed that starches from various sources and modified starches produce varying growth responses in the rat. Booher et al. (1) carried out an extensive study in which it was shown that unmodified potato, arrowroot, and sago starches were not completely available to the growing rat, whereas the starches from wheat, corn, waxy maize, rice, and cassava were well-utilized for rat growth. It was also shown that commercial samples of hydrochloric acid-modified and hypochlorite-oxidized wheat starch performed as well as unmodified wheat starch. Commercially modified corn starch products were shown by Whistler and Belfort (2) to give body weight gains in rats which were similar to those obtained from feeding unmodified corn starch. However, these workers also showed that a very highly oxidized, laboratory-prepared starch gave poor body weight gains.

In vitro studies with amylase enzymes have indicated that whole starches of various types, and starch fractions are digested at different rates. Waxy maize starch was hydrolyzed more slowly than unfractionated corn starch, and much more slowly than the linear fraction from corn starch according to Caldwell and Adams (3); Sandstedt et al. (4) found that the resistance of high amylose corn starch products to digestibility by α-amylase was not directly associated with the amylose content of the starches, and Borchers (5) reported that high amylose corn starch gave digestibility values of 66 to 77% in the rat, which were much lower than the value of 95% given by normal corn starch.

Various dietary carbohydrates have been reported to affect protein metabolism. Thus, Harper et al. (6) showed that at low dietary protein levels, dextrin gives better rat growth than sucrose and fructose, and glucose gives a growth rate between that of dextrin and sucrose. When sucrose was the source of dietary carbohydrate in low protein diets, from 2 to 3% more protein was required to support rat growth equivalent to that obtained from

Received for publication January 28, 1963.

[1] Mailing address: Technical Center, General Foods Corporation, White Plains, New York.

a similar diet in which corn starch was the source of dietary carbohydrate (7). Chang (8) found that raw potato starch, cooked potato starch, dextrin, sucrose, corn starch, and glucose had different effects on the availability of lysine in wheat gluten diets.

The purpose of this study was to compare the protein efficiency values, and rat growth data obtained from feeding a series of unmodified starches, pregelatinized starches, disaccharides, and monosaccharides, as well as several amylose and amylopectin products.

EXPERIMENTAL

Groups of 10 rats each were fed semipurified diets containing various sources of dietary carbohydrate. The animals were Wistar strain males weighing from 50 to 55 g at the beginning of the assays. The animals were housed in individual raised-bottom wire cages in an air conditioned room kept at 75° ± 2°F. The diets contained 4% USP salts XIV, 10% corn oil, either casein[2] or wheat gluten[3] to supply protein, ample vitamins and 2% cellulose. No adjustments were made to equalize the moisture contents of the diets because previous experiments had shown that the addition of water to diets containing high levels of some sugars resulted in a poor diet texture. The maximal moisture content of the diets used in these studies was 12%.

The 15% protein diets contained 66% of each carbohydrate. Casein was also fed at 6% protein, and wheat gluten at 9% protein. These latter diets contained 77 and 73%, respectively, of the carbohydrates being evaluated. Reagent grade sugars were used as sources of fructose, maltose, dextrose, sucrose and galactose. The pregelatinized starches were obtained from various manufacturers, and therefore were subjected to different processing conditions. The amylose and amylopectin samples were also from commercial sources and probably contained small amounts of the respective branched-chain and linear fractions.

Three different rat assays were carried out to study the nutritional effects of dietary carbohydrate. At the termination of the first rat study, it was noted that the ceca of some of the animals were greatly enlarged and engorged with food. This condition would give erroneous body weight gain values in some of the test groups. Therefore, at the termination of the assays the ceca were removed and weighed so that corrections might be made for the large amounts of incompletely digested food that remained in this portion of the gastrointestinal tract. In the first assay, ceca were weighed from at least 6 animals in most test groups. In the other 2 assays all the ceca were weighed except where the ceca burst due to extreme enlargement. The cecal weights were not deducted from the body weight gains given in the tables since they were not available from all the animals in each assay.

The body weight gains, with and without the cecal weights, and the protein efficiency values were analyzed statistically by analysis of variance technique as described by Federer (9). The variation of animals within each diet was calculated. No differences in these variances could be detected among diets within an assay. Hence a weighted average variance was used for each assay as the measure of animal to animal variation. Such an average variance is more precise than the variance of a single diet because the average variance is based on more observations. Also, because it is based on more observations, the average variance permits more sensitive tests of differences among diets.

The 95% confidence limits in the tables show ± 2 standard deviations of the average, based on the averaged variance of each assay.

RESULTS AND DISCUSSION

First study with casein as dietary protein. A large selection of starches and sugars was included in the first study to determine the effects of dietary carbohydrate on the utilization of protein. As indicated in table 1 there was considerable variation in the protein efficiency values obtained from feeding different carbohydrates to weanling rats. Of the unmodified

[2] A.N.R.C. Casein, Sheffield Chemical Company, Norwich, New York.
[3] Pro 80 Wheat Gluten, General Mills, Minneapolis, Minnesota.

TABLE 1

Summary of data from first 28-day study with casein as the dietary protein

Carbohydrate source	6% Dietary protein level				15% Dietary protein level			
	Body wt gain[1]	Protein efficiency[1]	Dry food consumed[1]	Cecum wt/100 g body wt[2]	Body wt gain[1]	Protein efficiency[1]	Dry food consumed[1]	Cecum wt/100 g body wt[2]
	g		*g*	*g*	*g*		*g*	*g*
Unmodified starches								
Corn	46.3 ± 7.1[3]	2.43 ± 0.36[3]	290	1.3(9)	143 ± 15.0[3]	2.42 ± 0.20[3]	361	1.4(10)
Potato	64.5 ± 7.5[4]	2.54 ± 0.37	375	9.5(7)	101 ± 15.0[4]	1.65 ± 0.20[4]	362	6.4(9)
Wheat	42.9 ± 7.1	2.27 ± 0.36	287	1.3(6)	142 ± 15.0	2.47 ± 0.20	350	1.0(7)
Arrowroot	33.1 ± 7.5[4]	1.56 ± 0.37[4]	312	5.3(10)	111 ± 15.0[4]	2.09 ± 0.20[4]	315	4.3(7)
Rice	41.9 ± 7.1	2.22 ± 0.36	285	1.3(7)	132 ± 15.0	2.27 ± 0.20	352	1.0(7)
Tapioca	37.0 ± 7.1	2.21 ± 0.36	251	1.5(7)	146 ± 15.0	2.33 ± 0.20	376	0.9(7)
Pregelatinized starches								
Potato	40.8 ± 7.5	2.62 ± 0.37	249	1.3(8)	131 ± 15.0	2.30 ± 0.20	359	1.2(10)
Arrowroot	35.1 ± 7.5[4]	2.17 ± 0.37	259	1.6(9)	118 ± 15.0[4]	2.30 ± 0.20	329	1.2(8)
Tapioca	42.2 ± 7.1	2.15 ± 0.36	315	1.2(7)	126 ± 15.0	2.21 ± 0.20	363	1.1(7)
Amioca	39.4 ± 7.1	2.33 ± 0.36	259	4.4(9)	115 ± 15.0[4]	2.03 ± 0.20[4]	348	2.8(8)
Starch derivatives								
Corn dextrin	42.6 ± 7.1	2.15 ± 0.36	314	2.8(7)	126 ± 15.0	1.97 ± 0.20[4]	401	2.2(7)
Potato amylose	62.7 ± 7.1[4]	2.49 ± 0.36	377	4.8(9)	124 ± 15.0	2.04 ± 0.20[4]	367	4.3(8)
Potato amylopectin	49.4 ± 7.1	2.65 ± 0.36	282	2.1(10)	120 ± 15.0[4]	2.37 ± 0.20	308	2.8(9)
Disaccharides								
Sucrose	27.2 ± 7.1[4]	1.57 ± 0.36[4]	285	1.2(7)	131 ± 15.0	2.42 ± 0.20	353	1.1(7)
Maltose	38.8 ± 7.1	2.16 ± 0.36	294	1.1(6)	149 ± 15.0	2.57 ± 0.20	376	1.0(7)
Lactose	1.2 ± 10.1[4]	0.13 ± 0.50[4]	157	14.4(1)	48.2 ± 21.2[4]	1.10 ± 0.28[4]	287	— (0)
Lactose-dextrose (50:50)	13.3 ± 7.1[4]	0.88 ± 0.36[4]	241	6.5(6)	102 ± 15.0[4]	2.06 ± 0.20[4]	316	4.1(7)
Monosaccharides								
Fructose	24.8 ± 7.5[4]	1.16 ± 0.37[4]	352	1.4(6)	110 ± 15.0[4]	2.00 ± 0.20[4]	357	1.5(7)
Galactose-dextrose	47.8 ± 7.1	2.29 ± 0.36	335	1.6(6)	122 ± 15.0	1.93 ± 0.20[4]	405	1.3(7)
Dextrose	37.4 ± 7.1	1.62 ± 0.36[4]	368	1.8(7)	115 ± 15.0[4]	1.95 ± 0.20[4]	364	1.3(7)

[1] Mean values based on 10 animals/group where no mortality occurred. Five animals died when fed the lactose diets at both protein levels and one death occurred with the unmodified potato starch, pregelatinized potato starch, unmodified arrowroot starch and fructose, all at the 6% protein level.

[2] Mean value with number of animals in parentheses. These include both the cecum and its contents.

[3] Mean and 95% confidence limit on the mean.

[4] Means are significantly different at the 5% significance level when compared to those obtained with unmodified corn starch.

starches, potato and arrowroot gave enlarged ceca at both the 6 and 15% protein levels and poor body weight gains at the higher protein level when casein was the dietary protein. At the lower protein level, feeding of unmodified potato starch resulted in significantly better body weight gains than those produced by the other dietary carbohydrates, and a high protein efficiency value was obtained. This superiority in weight gain from the diet containing potato starch was also evident when the cecal weights were deducted from the terminal body weights. The animals that were fed the unmodified potato starch diet consumed much more food than the other groups in this study. On the other hand, arrowroot gave a very poor protein efficiency value at the 6% protein level, and unmodified potato starch gave an unusually poor protein efficiency value at the 15% protein level. The unmodified corn, wheat, rice, and tapioca starches all gave good growth and protein efficiency values at both the high and low dietary protein levels. No evidence of enlarged ceca were noted from these starches.

Throughout these studies unmodified corn starch was used for comparisons because this carbohydrate has been used frequently as a "normal" control in the literature. In the series of pregelatinized starches which were tested at both protein levels, the arrowroot product gave growth values which were significantly lower than those obtained from unmodified corn starch. Feeding of pregelatinized amioca starch in a diet containing 15% protein resulted in significantly poorer growth and protein efficiency values when compared to corn starch in the 15% protein diets. This pregelatinized starch is a commercial product made from waxy maize corn starch. The diet containing pregelatinized amioca starch gave a diarrhea throughout the test and resulted in ceca which were enlarged as compared to the cecal weights obtained from the animals fed the other pregelatinized starches. Also, the corn dextrin diets caused a slight diarrhea. This processed starch gave ceca which were about twice the weight of those obtained with unmodified corn starch. The protein efficiency value obtained from the corn dextrin diet was also significantly poorer than the value obtained from unmodified corn starch when the dietary level of protein was 15% for both diets.

A commercial sample of potato amylose gave significantly better body weight gains and protein efficiency values at the lower casein levels when compared with the responses given by either corn dextrin or corn starch. However, as shown with the unmodified potato starch, this superiority by the amylose product was not evident when the protein level was increased to 15%. The potato amylose also resulted in enlarged ceca at both levels of dietary protein. Potato amylopectin supported normal body weight gains at the low protein level and slightly poorer body weight gains than corn starch at the higher protein level. Very good protein efficiency values were evident at both protein levels with the amylopectin. The amylopectin diets gave ceca which were slightly enlarged, but smaller than those observed in the animals fed the amylose. These data indicate that the amylopectin fraction of potato starch was better utilized than the amylose fraction at both dietary protein levels.

Of the lower molecular weight sugars, lactose produced poor weight gains, even when diluted with an equal part of dextrose. When lactose was fed as the sole source of carbohydrate there was a 50% mortality at both dietary protein levels. This was to be expected since it is known that lactose is poorly utilized by the rat at high dietary levels. Maltose and sucrose gave good growth and protein efficiency values at the 15% dietary protein level, whereas the sucrose diet gave significantly poorer body weight gains and protein efficiency when compared with unmodified corn starch at the 6% dietary protein level. Fructose or dextrose as the only source of carbohydrate gave relatively poor body weight gain and protein efficiency ratings. These values for fructose at the 6% dietary protein level were the lowest of any of the carbohydrates evaluated, with the exception of lactose.

The fructose diets resulted in somewhat larger livers, on a unit body weight basis, than the other diets. The sucrose diets also produced slightly larger livers than

the other carbohydrates (table 2). The data in the same table do not indicate that the larger livers were related to a high fat content. However, at the low casein level the sucrose diet resulted in slightly greater liver fat values than most of the diets. Most of the liver fat values were in the normal range for these levels of dietary protein with the possible exceptions of the low liver fat values obtained from feeding lactose at both protein levels, and unmodified arrowroot starch at the 6% dietary protein level. The 14.1% liver fat value obtained from the maltose diet with the higher casein level was higher than expected. Usually diets producing good protein efficiency values have liver fat values in the range of 9 to 12% on a dry basis. The animals fed sucrose, fructose, dextrose or lactose had a very "unthrifty" appearance which was especially apparent in those fed dextrose at the 15% dietary protein level.

During the last 2 weeks of the 28-day study the apparent gross digestibility of some diets was determined by a quantitative collection of the feces from 6 animals at each protein level. The average digestibility value obtained from the potato amylose was 91%, and the unmodified potato starch gave a value of 82%. The potato amylopectin, pregelatinized potato starch, pregelatinized amioca starch, and unmodified potato starch gave values of 97 to 98%. This was a further indication that the amylose, and unmodified potato starch were not completely digested.

Second study with casein as dietary protein. A second rat study was carried out to obtain data on the utilization of the amylose and amylopectin fractions of corn starch. A high amylose corn starch sample containing 55% amylose[4] and a potato amylose product from a different commercial source than the one evaluated in the first study were also included.

The data in table 3 show that the second potato amylose sample also resulted in a significantly greater body weight gain and a higher protein efficiency value at the low dietary protein level when compared with the unmodified corn starch. At both protein levels the corn amylose resulted in smaller ceca than the potato

[4] Amylon, National Starch and Chemical Corporation, New York City.

TABLE 2

Summary of liver weights and liver fat contents from first casein series[1]

Dietary carbohydrate	Liver weight / 100 g body wt		Liver fat content on dry basis[2]	
	6% Protein level	15% Protein level	6% Protein level	15% Protein level
	g	*g*	%	%
Unmodified potato starch	3.7	4.2	9.4	9.8
Unmodified arrowroot starch	3.5	3.8	6.2	9.5
Unmodified tapioca starch	3.8	4.0	9.4	10.7
Unmodified wheat starch	3.8	3.6	8.2	9.4
Unmodified rice starch	3.7	3.9	8.0	9.4
Unmodified corn starch	3.8	3.9	8.2	11.1
Pregelatinized amioca starch	3.8	4.3	8.1	8.4
Pregelatinized potato starch	4.0	3.9	10.4	8.7
Pregelatinized tapioca starch	4.1	4.3	12.0	8.7
Pregelatinized arrowroot starch	4.1	3.9	9.3	8.1
Corn dextrin	4.0	3.6	9.7	7.8
Potato amylose	3.6	3.8	9.0	9.2
Potato amylopectin	3.9	4.3	11.8	10.6
Sucrose	5.0	4.7	12.3	9.5
Maltose	4.1	3.9	10.0	14.1
Lactose	3.8	4.2	6.5	4.0
Lactose-dextrose (50:50)	3.9	4.2	6.7	11.4
Fructose	5.7	5.2	10.3	7.6
Galactose-dextrose	4.3	4.5	10.0	14.1
Dextrose	3.9	3.8	10.0	11.9

[1] Average liver weights determined from all the animals surviving in each group.
[2] Average liver fat data calculated from the livers which were pooled and dried before extraction with ethyl ether.

TABLE 3

Summary of data from second 28-day rat study with casein as the dietary protein

Carbohydrate source	6% Dietary protein level				15% Dietary protein level			
	Body wt gain[1]	Protein efficiency[1]	Dry food consumed[1]	Cecum wt/100 g body wt[2]	Body wt gain[1]	Protein efficiency[1]	Dry food consumed[1]	Cecum wt/100 g body wt[2]
	g		*g*	*g*	*g*		*g*	*g*
Unmodified corn starch	51.3 ± 6.2[3]	2.95 ± 0.20[3]	260	1.7(10)	162 ± 20.2[3]	2.74 ± 0.32[3]	355	1.1(8)
Unmodified amioca starch	46.4 ± 6.2	2.58 ± 0.20	269	1.8(9)	150 ± 18.1	2.67 ± 0.28	336	1.2(10)
Pregelatinized amioca starch	42.9 ± 6.2[4]	2.90 ± 0.20	250	4.0(10)	119 ± 18.1[4]	2.60 ± 0.28	291	2.4(10)
High amylose corn starch	49.4 ± 6.2	2.55 ± 0.20	286	6.5(4)	124 ± 19.0[4]	2.38 ± 0.30[4]	350	4.5(9)
Corn amylopectin	48.6 ± 6.2	3.15 ± 0.20	258	1.6(9)	128 ± 18.1[4]	2.71 ± 0.28	301	1.1(10)
Corn amylose	52.7 ± 6.2	3.09 ± 0.20	262	4.1(10)	151 ± 18.1	2.60 ± 0.28	354	3.4(7)
Potato amylose no. 2	73.2 ± 6.2[4]	3.25 ± 0.20	332	5.6(10)	142 ± 18.1	2.46 ± 0.28	342	4.1(10)

[1] Mean values based on an average of 10 animals/group except 8 animals in the corn starch group and 9 in the high amylose corn starch group when the protein level was 15% and 8 animals in the high amylose corn starch group when the protein level was 6%.

[2] Mean value with the number of animals in parentheses. These include both the cecum and its contents.

[3] Mean and 95% confidence limit on the mean.

[4] Means are significantly different at the 5% significance level when compared to those obtained with unmodified corn starch.

amylose. The corn amylose did not result in the large weight gains and food intakes at the lower dietary protein level as shown by both the unmodified potato starch and the potato amylose in the 2 studies where casein was the protein source. This suggests that the corn amylose may have been better utilized than the potato amylose product at the low dietary protein level. The high amylose corn starch gave the largest ceca in this series of diets; however, the enlarged ceca were not accompanied by increases in body weight gains at the lower level of protein intake. When the 15% protein diets were compared, the high amylose corn starch produced significantly poorer body weight gains and protein efficiency than the unmodified corn starch.

The corn amylopectin gave relatively poorer body weight gains at the higher dietary protein level. This appeared to be due to a depression in food intake since the protein efficiency values and ceca sizes were normal at both levels of dietary protein.

In this second study the pregelatinized amioca starch gave larger ceca and significantly poorer body weight gains than either the unmodified amioca starch or the unmodified corn starch in both the high and low protein diets. This pregelatinized starch product resulted in a diarrhea which was not observed in the animals fed the unmodified amioca starch. These data indicate that the processing conditions used in manufacturing the pregelatinized amioca starch could have been responsible for the different responses obtained. Moinuddin and Lee (10) reported that the addition of cellobiose, a poorly digested carbohydrate, to a high protein casein diet also resulted in diarrhea, poor body weight gains, and enlarged ceca in the rat.

Wheat gluten as the dietary protein. A third rat assay included a series of carbohydrates with wheat gluten as the protein source at 9 and 15% dietary protein levels. The 9% protein level was fed in this series since wheat gluten is a protein of relatively poor quality and it was doubtful whether the animals would grow on a level of 6% protein.

The data in table 4 show that unmodified potato and arrowroot starches gave

TABLE 4

Summary of 28-day data with wheat gluten as the dietary protein

Carbohydrate source	9% Dietary protein level				15% Dietary protein level			
	Body wt gain[1]	Protein efficiency[1]	Dry food consumed[1]	Cecum wt/100 g body wt[2]	Body wt gain[1]	Protein efficiency[1]	Dry food consumed[1]	Cecum wt/100 g body wt[2]
	g		*g*	*g*	*g*		*g*	*g*
Unmodified starches								
Potato	15.1 ± 3.2[3,4]	0.41 ± 0.12[3]	358	7.2(9)	48.1 ± 5.1[3,4]	0.66 ± 0.11[3]	431	6.8(9)
Arrowroot	7.4 ± 3.0	0.19 ± 0.12[4]	384	4.9(10)	23.9 ± 5.1	0.38 ± 0.11[4]	383	5.3(9)
Corn	10.4 ± 3.0	0.36 ± 0.12	286	1.4(10)	29.1 ± 4.8	0.57 ± 0.10	331	0.9(10)
Modified starches and starch derivatives								
Pregelatinized potato starch	14.1 ± 3.0	0.54 ± 0.12[4]	284	1.2(10)	32.3 ± 5.1	0.70 ± 0.11	290	1.0(10)
Pregelatinized amioca starch	10.5 ± 3.0	0.40 ± 0.12	277	3.7(10)	26.9 ± 4.8	0.65 ± 0.10	260	3.1(10)
Pregelatinized arrowroot starch	11.3 ± 3.0	0.49 ± 0.12	249	1.8(10)	26.5 ± 4.8	0.51 ± 0.10	310	1.3(10)
Potato amylose	17.8 ± 3.2[4]	0.71 ± 0.12[4]	253	5.6(10)	50.4 ± 4.8[4]	0.90 ± 0.10[4]	344	5.0(10)
Potato amylopectin	13.1 ± 3.0	0.50 ± 0.12	260	1.8(10)	37.1 ± 4.8[4]	0.80 ± 0.10[4]	283	1.6(10)
Corn dextrin	10.8 ± 3.0	0.38 ± 0.12	331	2.5(10)	36.1 ± 4.8	0.71 ± 0.10	323	2.4(10)
High dextrose dextrin	11.1 ± 3.0	0.42 ± 0.12	293	1.4(10)	32.5 ± 4.8	0.72 ± 0.10	291	1.1(10)
Di- and monosaccharides								
Maltose	8.6 ± 3.2	0.39 ± 0.12	238	1.6(9)	30.0 ± 4.8	0.68 ± 0.10	298	1.0(10)
Sucrose	5.2 ± 3.0	0.26 ± 0.12	211	1.6(10)	27.5 ± 4.8	0.71 ± 0.10	256	1.1(10)
Dextrose-fructose (50:50)	9.6 ± 3.4	0.35 ± 0.13	281	1.4(10)	31.2 ± 5.1	0.59 ± 0.11	357	1.0(9)
Dextrose	13.0 ± 3.0	0.45 ± 0.12	315	1.4(10)	30.5 ± 4.8	0.57 ± 0.10	347	1.0(10)

[1] Mean values based on 10 animals/group with the exception of 9 animals in the groups fed unmodified potato starch, pregelatinized potato starch, unmodified arrowroot starch and dextrose-fructose in the 15% protein diets; also 9 animals in the group fed amylose, unmodified potato starch, and maltose and 8 animals from the diet containing dextrose-fructose in the 6% protein diets.

[2] Mean value with number of animals in parentheses. These include both the cecum and its contents.

[3] Mean and 95% confidence limit on the mean.

[4] Values are significantly different at the 5% significance level when compared to those obtained with unmodified corn starch.

enlarged ceca and greater food intakes than the other carbohydrates included in the study. The sucrose diet resulted in poor food consumption which was below that obtained from feeding a mixture of fructose and dextrose at the levels equivalent to their levels in sucrose. At the 15% dietary protein level, potato amylose and unmodified potato starch gave significantly better body weight gains than the unmodified corn starch, even when the cecal weights were deducted from the terminal body weights. This is similar to the data obtained at the 6% dietary protein level with casein as the source of protein. At both wheat gluten protein levels, the amylose gave significantly better protein efficiency values than unmodified corn starch.

The unmodified arrowroot starch did not result in large body weight gains in spite of the presence of greatly enlarged ceca. In fact, at both protein levels the protein efficiency values obtained from the unmodified arrowroot starch were significantly lower than the corresponding values obtained from unmodified corn starch. Another similarity to the first study was the cecal enlargement noted in the rats fed either the pregelatinized amioca starch or corn dextrin. The dextrin product containing the higher dextrose level, which was equivalent to 29% reducing sugars as dextrose, gave normal ceca. This again gives an indication that the process used in making the corn dextrin with the lower dextrose content resulted in a product which was more resistant to digestion. The low dextrose corn dextrin used in these studies had been prepared from corn starch which was heated in the presence of "mineral acid." In a recent publication, Leach and Schoch (11) have shown that an 80-fluidity corn starch which had been treated with acid was more resistant to solubilization by dimethyl sulfoxide than unmodified corn starch. These authors noted that poorly soluble starches were resistant to digestion by α-amylose.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to K. W. Schwartz and R. Bendon for their technical assistance in these studies. We are also indebted to I. Coons and to J. Church for their aid in the statistical analyses.

LITERATURE CITED

1. Booher, L. E., I. Behan and E. McMeans 1951 Biological utilization of unmodified starches. J. Nutrition, *45:* 75.
2. Whistler, R. L., and A. M. Belfort 1961 Nutritional value of chemically modified starches. Science, *133:* 1599.
3. Caldwell, M. L., and M. Adams 1950 Action of certain alpha amylases. Adv. Carbohyd. Chem., *5:* 229.
4. Sandstedt, R. M., D. Strahan, S. Ueda and R. C. Abbott 1962 The digestibility of high amylose corn starch compared to that of other starches. The apparent effect of the Ae gene on susceptibility to amylase action. Cereal Chem., *39:* 123.
5. Borchers, R. 1962 A note on the digestibility of the starch of high amylose corn in rats. Ibid., *39:* 145.
6. Harper, A. E., W. J. Monson, D. A. Arata and D. A. Benton 1953 Influence of various carbohydrates on the utilization of low protein rations by the white rat. J. Nutrition, *51:* 523.
7. Harper, A. E., and C. A. Elvehjem 1957 A review of the effects of different carbohydrates on vitamin and amino acid requirements. J. Agr. Food Chem., *5:* 754.
8. Chang, Yet-Oy 1962 Effect of carbohydrates on utilization of protein and lysine by rats. J. Nutrition, *78:* 21.
9. Federer, W. T. 1955 Experimental Design, Theory and Application. Macmillan Company, New York, pp. 18–21.
10. Moinuddin, J. Fischer and H. Wing - Tsit Lee 1958 Effects of feeding diets containing sucrose, cellobiose or glucose on the dry weights of cleaned gastrointestinal organs in the rat. Am. J. Physiol., *192:* 417.
11. Leach, H. W., and T. J. Schoch 1962 Structure of the starch granule. III. Solubilities of granular starches in dimethyl sulfoxide. Cereal Chem., *39:* 318.

Role of the Cecum in the Utilization of Raw Soybean in Chicks[1,2]

ZAFRIRA NITSAN AND EUGENIA ALUMOT
Department of Animal Nutrition, The National and University Institute of Agriculture, Rehovot, Israel

ABSTRACT In previous work, depression of intestinal proteolysis was observed in young chicks fed a raw soybean diet. In the present study nitrogen balances performed on chicks from one to 7 weeks of age revealed smaller differences in nitrogen retention when raw and heated soybean diets were fed than was expected. This difference however, did not improve with age and other parts of the intestinal tract, including the cecum, were therefore tested for proteolytic activity. High proteolytic activity was noted in ceca of chicks up to 4 weeks old fed heated and raw soybean diets; whereas during the sixth week the enzyme activity decreased markedly with heated soybean; a slight depression occurred with the raw soybean diet. An increase of cecal proteolysis was observed when 8-week-old chicks were transferred from a heated to raw soybean diet. The contribution of the cecum to body weight gains, feed efficiency and nitrogen retention was investigated by cecoctomy. Chicks with no cecum that were fed raw soybean gained less weight, showed lower feed efficiency, and retained less nitrogen than sham-operated chicks. With heated soybean the removal of ceca increased the feed utilization somewhat. It appears that cecal proteolysis and subsequent nitrogen absorption compensate partly for inhibited proteolysis in the small intestine.

It is accepted that the main role of the cecum of many animals is cellulose or fiber digestion. Little is known about the function of the cecum in chicks. Radeff (1), Dukes (2) and Halnan (3) mention that the primary role of the fowls' cecum is fiber digestion and liquid absorption.

In a previous work (4), it was reported that raw soybean inhibits the proteolytic activity in the small intestines of chicks. This inhibition was particularly marked during the first 3 weeks of life; proteolysis reached almost normal levels at 6 weeks. This fact led to the study of nitrogen balances at different chick ages to investigate the influence of depressed proteolysis on the protein utilization. Concurrently, proteolytic activity of other parts of the gut were examined.

The present communication presents evidence that the chicks' cecum is involved in protein digestion and in increasing the feed efficiency of a raw soybean diet.

EXPERIMENTAL

The first trial was carried out with 60 White Leghorn male chicks. One-day-old chicks were housed in electrically heated battery brooders with raised wire floors. The chicks were fed sorghum grains for 2 days, and then were divided into 4 equal groups: 2 were fed heated, and 2 were fed raw soybean diets. The percentage composition of the diets was as follows: soybean oil meal (48% protein), 25; sorghum, 61; fish meal (Norwegian, 65% protein), 2; wheat bran 4.6; alfalfa meal, 3.2; calcium carbonate, 1.6; dicalcium phosphate, 1.6; mineral mixture, 0.3; commercial vitamin mixture, 0.3 (the composition of the mineral and vitamin mixtures is given in a previous work (4)); vitamin B_{12} (3 mg/kg), 0.2; antioxidant, 0.1; and coccidiostat,[3] 0.1. The heated soybean oil meal used was commercially processed and toasted. The raw meal was obtained from the same factory. The solvent from the raw extracted flakes was evaporated at room temperature. The crude protein content of the 2 mashes was 21%, 12%

Received for publication January 23, 1963.

[1] Part of a Ph.D. thesis to be submitted by Zafrira Nitsan to the Faculty of Agriculture of the Hebrew University, Jerusalem.

[2] Contribution from the National and University Institute of Agriculture, Rehovot, 1962 series, 510 E.

[3] Bifuron, Hess and Clark, Ashland, Ohio; supplied per 0.907 metric tone of feed: nitrofurazone, 75 g, furazolidone, 8 g, and menadione sodium bisulfite, 1 g.

of which came from the soybean oil meals. Food and water were supplied ad libitum.

Nitrogen balances of chicks fed the 2 diets were measured in 2 replicates from one to 7 weeks of age. All excreta of each group were collected every 24 hours for a period of 6 days each time. After drying the excreta at 80°C in the presence of 1% boric acid to reduce nitrogen losses, the excreta were ground and kept for analysis. Total nitrogen was determined by the Kjeldahl method, and uric acid by the method of Tinsley and Nowakowski (5).

The second trial was conducted to investigate the proteolytic activity in the chicks' cecum. Two groups of 30 one-day-old chicks were used. The management and rations were as in the first trial. The proteolytic activity was measured from one to 6 weeks of age. The chicks were killed by rapid bleeding, 5 chicks at a time. The enzyme preparation and the slight modification of Kunitz's procedure for the determining of proteolytic activity were the same as used previously for the small intestine (6).

The third trial was conducted on 30 White Leghorn male chicks which were raised with a heated soybean diet up to 8 weeks of age. At this age they were changed to a raw soybean diet and their cecal proteolytic activity was measured before, 1, 2, 4 and 8 days after the start of the new diet. The diets used were as follows (in percentage): soybean oil meal (48% protein), 20; sorghum, 67.6; fish meal (Norwegian, 65% protein), 3; alfalfa meal, 2.5; ground shells, 1.8; dicalcium phosphate, 1.5; salt mixture, 0.25 (4); vitamin mixture, 0.25 (4); soybean oil, 3; coccidiostat, 0.1. The soybean oil meals were the same as described above.

In the fourth trial, nitrogen balances were performed on cecoctomized chicks. Eight White Leghorn male chicks, 9 weeks old, were used. The ceca of 4 chicks were ligated at the neck and removed. The other 4 were sham-operated to serve as controls.[4] Following the operation the chicks were fed a heated soybean diet for a recovery period of a few days. There were 2 experimental periods consisting of 5 days each, preceded by a 2-day adjustment period with the following respective diets: in the first, heated, and in the second, raw soybean diet. The diets used were as in the third trial.

Another group of 8, 3-week-old chicks was then operated upon and treated in the same way as the former group. The young chicks, as well as the older ones which were by that time 16 weeks old, were fed semipurified diets, the compositions of which are given in table 1.

Two experiments of 5 days each were run here in the same way as described previously. During each experimental period, food intake and body weights were recorded. Nitrogen balances were measured as described in the first trial.

RESULTS

The nitrogen balances of chicks from one to 7 weeks old are presented in table 2.

The nitrogen retention in chicks fed a raw soybean diet was always lower than

[4] The operating technique was suggested by Dr. S. Hurwitz of the Poultry Department, who performed some of the operations.

TABLE 1

Composition of the semipurified diets for the 3- and 16-week old cecoctomized chicks

	3-week-old group		16-week-old group	
Heated soybean meal (47.6% protein)	50.0	—	36.0	—
Raw soybean meal (45.7% protein)	—	52.3	—	37.5
Glucose	26.4	24.9	33.4	32.7
Potato starch	13.2	12.4	17.2	16.4
Cellulose	3.0	3.0	5.0	5.0
Soybean oil	3.0	3.0	4.0	4.0
Mineral mixture (4)	0.5	0.5	0.5	0.5
Vitamin mixture(4)	0.4	0.4	0.4	0.4
Dicalcium phosphate	2.0	2.0	2.0	2.0
Calcium carbonate	1.5	1.5	1.5	1.5
Total protein, %	23.8	23.9	17.1	17.1

that of those fed heated soybean (table 2). The differences between the 2 groups remained almost the same throughout all the ages examined.

The percentage of uric acid nitrogen from total nitrogen in excreta was always lower (70 to 80%) in the chicks fed raw soybean than in those receiving heated soybean.

The proteolytic activity in the cecum of chicks from one to 6 weeks of age is shown in figure 1.

The results indicate a high proteolytic activity in the first 4 weeks with both diets. At the age of 6 weeks, the proteolytic activity decreased slightly with the raw soybean diet and markedly with the heated soybean diet.

TABLE 2

Nitrogen retention of chicks fed heated and raw soybean diets (15 chicks/group in two replicates)

	Age in weeks									
	1–2		2–3		3–4		4–5		6–7	
	Heated	Raw	Heated	Raw	Heated	Raw	Heated	Raw	Heated	Raw
N in feed[1]	2355	1497	4229	2976	4599	2766	7920	5846	8012	5782
N in excreta[1]	945	688	1872	1478	1917	1648	3483	3153	4206	3479
% N retention[2]	59.6	54.1	55.8	50.5	58.3	40.4	56.0	46.3	47.5	39.9
N uric acid[1]	468	248	930	516	1098	737	1918	1343	2110	1403
% N uric acid from N in excreta	49.5	36.1	49.7	34.9	57.3	44.7	55.1	42.6	50.2	40.3

[1] Mg nitrogen/chick/6-day experiment.

[2] $\frac{(\text{N in feed}) - (\text{N in excreta})}{(\text{N in feed})} \times 100.$

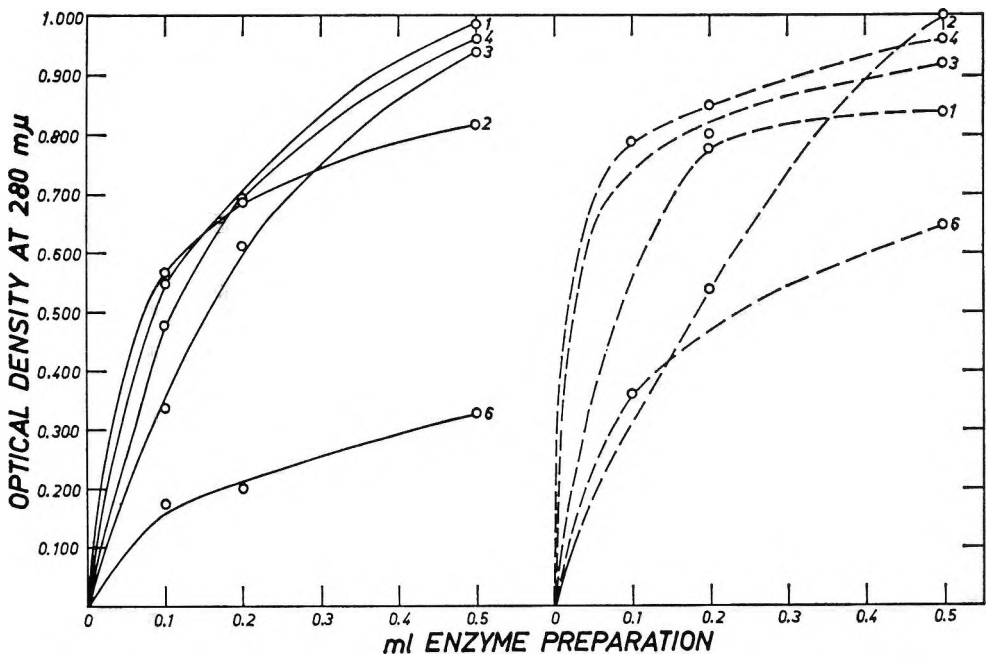


Fig. 1 Proteolytic activity in the cecum of chicks fed heated (left) and raw (right) soybean diets. The numbers at the curves indicate the age of the chicks in weeks. Each point represents an average of 6 chicks.

The 8-week-old chicks in the third trial showed, from the second day of eating raw soybean, a higher cecal proteolytic activity than those continuing with the heated soybean (fig. 2).

The results of the fourth trial (cecoctomized chicks) are presented in tables 3 and 4.

The chicks with no cecum, fed the heated soybean diet, consumed more feed, gained more weight and utilized the feed somewhat more efficiently than those with ceca (tables 3, 4).

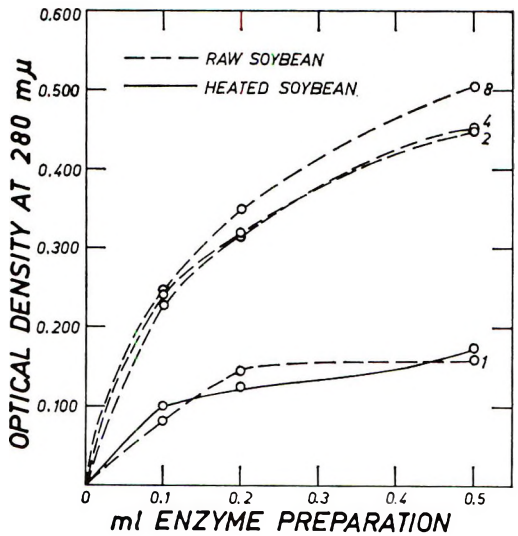


Fig. 2 Proteolytic activity in the cecum after transfer of chicks to raw soybean compared with continuous heated soybean diet. The numbers at the curves indicate days after the start of a raw soybean diet. Each point represents an average of 6 chicks.

On the other hand, the 3- and 9-week-old chicks with no cecum, fed raw soybean, although consuming more feed, showed much lower body weight gains and feed efficiency than those with a cecum. At 16 weeks a change to raw soybean diet caused loss of weight during the 5 days of the experiment to both groups. This loss was 5 times greater in the chicks with no cecum than in the controls.

Nitrogen retention, with heated soybean, was very similar in the chicks with or without a cecum. When raw soybean was fed, the nitrogen retention in all cases was lower than it was with heated soybean. In all cases, this depression was greater in the chicks with no cecum than in the normal ones. The cecoctomized chicks fed the semipurified raw diet suffered, more than those receiving the raw commercial-type diet (tables 3 and 4). The reason for this was probably the greater proportion of raw soybean inhibitor in the semipurified diet.

Here, too, the percentage of uric acid nitrogen from the total nitrogen in excreta was always lower with the raw soybean diet than with the heated. The removal of the ceca does not appear to have any consistent effect on this ratio.

DISCUSSION

Previous work (4) suggested that chicks suffering from depressed proteolysis in the small intestine, fed raw soybean, should retain nitrogen less well than chicks fed heated soy diet. It was further assumed that the difference in nitrogen utilization

TABLE 3

Feed efficiency and nitrogen utilization of chicks with and without ceca, fed heated and raw soybean commercial-type diet (4 chicks/group)

	9 weeks old			
	Heated		Raw	
	With ceca	Without ceca	With ceca	Without ceca
Feed consumption[1]	298	319	231	237
Body wt gains[1]	81	103	34	24
Gain/feed	0.272	0.322	0.147	0.101
N in feed[1]	8.63	9.24	7.70	7.91
N in excreta[1]	4.43	4.82	4.83	5.20
% N retention	48.6	47.8	37.3	34.2
N uric acid[1]	2.19	2.42	2.02	1.97
% N uric acid from N in excreta	49.4	50.2	41.8	37.9

[1] Grams/chick/5-day experiment.

TABLE 4

Feed efficiency and nitrogen utilization of chicks with and without ceca fed semipurified heated and raw soybean diets

	3-week-old group				16-week-old group			
	Heated		Raw		Heated		Raw	
	With ceca	Without ceca	With ceca	Without ceca	With ceca	Without ceca	With ceca	Without ceca
Feed consumption[1]	127	160	131	141	544	577	448	428
Body wt gains[1]	51	78	21	1	117	197	−7.5	−37.0
Gain/feed	0.401	0.487	0.160	0.007	0.215	0.341	−0.016	−0.086
N in feed[1]	5.02	6.34	4.99	5.37	15.44	16.39	14.71	10.75
N in excreta[1]	2.27	2.79	3.27	4.17	8.84	9.77	9.58	8.14
% N retention	54.8	56.0	34.4	22.3	42.7	40.4	34.9	24.3
N uric acid[1]	1.09	1.41	1.03	1.25	4.56	4.86	2.65	2.72
% N uric acid from N in excreta	48.0	50.5	31.5	30.0	51.6	49.7	27.7	33.4

[1] Grams/chick/5-day experiment.

between the 2 would decrease with age, as the proteolysis in the small intestine approaches normal levels. These assumptions were not proved in this study. The nitrogen retention with the raw diet was always lower, but not as low as expected, and it did not improve with age (up to 7 weeks). This can be explained now by the fact that high proteolysis was observed in the cecum of chicks.

If we compare the magnitude of the cecal enzyme proteolytic activity up to 4 weeks of age with that of the small intestine as reported by Alumot and Nitsan (4), we can see that the former is even higher. (Proteolytic activity expressed as average optical density at 280 mμ of 0.1 ml enzyme preparations was 0.485 and 0.280 in the cecum and small intestine, respectively.)

The contribution of this proteolytic capacity to the total nitrogen utilization is probably due to the amounts of undigested protein reaching the cecum. In the chicks fed heated soybean diet, the main bulk of protein is digested and absorbed in the small intestine, and the enzyme activity observed in the cecum does not influence the total nitrogen retention, as seen from the experiments with the cecoctomized chicks. However, when raw soybean is fed, protein digestion is inhibited in the small intestine and undigested protein reaches the cecum. It is digested there and absorbed through the walls of the cecum or the colon. These results confirm the work of Carrol et al. (7) who reported that, in rats fed heated soybean diet, most of the nitrogen was absorbed in the small intestine, while, with a raw soybean diet, much of the nitrogen was absorbed in the large intestine.

High proteolytic activity persists in the cecum of chicks continuously fed raw soybean at the age when it markedly decreased with heated soybean diet (fig. 1). This activity increases from low to high values for some days when 8-week-old chicks are transferred suddenly to raw soybean diet. It was noted by the authors[5] that, at the same time, inhibition of proteolysis occurs in the small intestine. In

[5] Unpublished results.

this way, cecal proteolysis partly compensates for this undesired effect.

The cecal compensation of proteolysis improves nitrogen retention. These results can explain first, the unexpected small depression in nitrogen retention in chicks fed raw soybean diet, and second, the fact that in the case of raw soybean, the cecoctomy greatly reduced feed efficiency and nitrogen retention.

More information is necessary on the possible disturbances in nitrogen metabolism caused by raw soybean and the utilization of the nitrogen absorbed from the cecum and colon. Such information may help in understanding that raw soybean protein is digested at a rate only 5% less than that of heated soybean, whereas its biological value is 18% lower (Mitchell et al. (8)). It may also explain the results of Hayward et al. (9), Evans et al. (10), and Carrol et al. (7), who felt that the lowered digestibility caused by raw soybean could not fully account for its considerable growth depressant effect.

ACKNOWLEDGMENTS

The authors wish to thank Professor A. Bondi for his continuous interest and helpful suggestions, and gratefully acknowledge the technical assistance of C. Genige, E. Mandel and the workers of the Poultry farm.

LITERATURE CITED

1. Radeff, T. 1928 Über die Rohfaserverdauung beim Huhn und die hierbei dem Blinddarm zukommende Bedeutung. Biochem. Z., *193:* 192.
2. Dukes, H. H. 1955 The Physiology of Domestic Animals. Comstock Publishing Company, a division of Cornell University Press, Ithaca, New York.
3. Halnan, E. T. 1949 The architecture of the avian gut and tolerance of crude fibre. Brit. J. Nutrition, *3:* 245.
4. Alumot, E., and Z. Nitsan 1961 The influence of soybean antitrypsin on the intestinal proteolysis of the chick. J. Nutrition, *73:* 71.
5. Tinsley, J., and T. Z. Nowakowski 1957 The determination of uric acid particularly in avian excreta. The Analyst, *82:* 971.
6. Nitsan, Z., and E. Alumot 1960 Proteolytic activity of chicks' intestines. Bull. Res. Counc. Israel, *9A:* 23.
7. Carrol, R. W., G. W. Hensley and W. R. Graham, Jr. 1952 The site of nitrogen absorption in rats fed raw and heated soybean meals. Science, *115:* 36.
8. Mitchell, H. H., T. S. Hamilton and J. R. Beadles 1945 The importance of commercial processing for the protein value of food products. 1. Soybean, coconut and sunflower seed. J. Nutrition, *29:* 13.
9. Hayward, J. W., H. Steenbock and G. Bohstedt 1936 The effect of heat as used in the extraction of soybean oil upon the nutritive value of the protein of soybean oil meal. Ibid., *11:* 219.
10. Evans, R. J., J. McGinnis and J. L. John 1947 The influence of autoclaving soybean oil meal on the digestibility of the proteins. Ibid., *33:* 661.

Amino Acid Requirements of Children: Minimal Needs of Tryptophan, Arginine and Histidine Based on Nitrogen Balance Method

ITSIRO NAKAGAWA, TETSUZO TAKAHASHI, TAKESHI SUZUKI AND KATSUMI KOBAYASHI

Department of Nutrition and Biochemistry, Institute of Public Health, Tokyo, Japan

ABSTRACT Minimal requirements for tryptophan, arginine and histidine of eleven 10- to 12-year-old boys were estimated by using the nitrogen balance method. Urinary excretion of creatine, creatinine, riboflavin and N-MNA was also measured. All children maintained a constant body weight and positive nitrogen balance without any accompanying clinical symptoms throughout the periods of arginine and histidine deprivation. With respect to tryptophan, all children maintained a positive balance at a level of 0.12 g, but some of them could not maintain a positive balance at the level of 0.06 g. The results obtained in all of our experiments with 10 amino acids in children have been summarized.

Minimal requirements of children for 8 essential amino acids, as measured by the nitrogen balance method, have been established (1–4). The present paper deals with the requirements of arginine and histidine and a re-examination of the minimal requirement for tryptophan, although the latter has been discussed in the previous paper (1).

EXPERIMENTAL PROCEDURE AND RESULTS

Eleven healthy 10- to 12-year-old boys served as experimental subjects (table 1). The general procedures followed in the experiment were described in detail in the preceding papers (1–4). The composition and daily intake of essential and nonessential amino acid mixtures are shown in table 2. The mixture of isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine was given at 1.5 times the amount which appeared to be the minimal requirement.

Experiment 1. In the first paper of this series (1) the minimal requirement of tryptophan was estimated to be 0.25 g daily. But this intake level was not necessarily sufficient to be defined as minimal,

Received for publication January 3, 1963.

TABLE 1

Age, height, weight, basal metabolism and energy intake of subjects

Exp. no.	Subject	Age	Body height	Body wt	Basal metabolism	Average daily energy intake[1]
		years, months	*cm*	*kg*	*Cal./kg/day*	*Cal./kg*
1	U. H.	12 1	137.5	32.9	32	55
	T. D.	12 0	142.9	34.6	36	55
	N. M.	11 4	142.6	31.8	30	56
	F. K.	10 11	139.2	30.4	37	61
2	K. T.	12 0	151.0	39.1	30	47
	K. K.	12 0	144.7	32.9	27	59
	H. S.	10 10	144.5	34.3	34	58
3	U. D.	11 1	147.3	33.5	29	55
	H.T.	9 7	120.3	21.1	36	64
	N. M.	11 5	142.9	31.7	30	54
	H. I.	11 9	131.8	25.8	36	63

[1] Calories derived from the amino acid mixture are not included.

TABLE 2
Composition and daily intake of amino acid mixture

Component	Daily intake	Nitrogen content
	g	*g*
Essential amino acids		
L-Isoleucine	1.50	0.160
L-Leucine	2.20	0.235
L-Lysine	2.40	0.460
L-Methionine	1.20	0.113
L-Phenylalanine	1.20	0.102
L-Threonine	1.50	0.176
L-Tryptophan	0.40	0.055
L-Valine	1.40	0.167
L-Histidine	0.75	0.203
Nonessential amino acids		
L-Alanine	8.60	1.325
L-Arginine	10.08	3.242
L-Aspartic acid	10.03	1.055
L-Glutamic acid	10.03	0.955
L-Sodium glutamate	11.66	0.965
Glycine	7.17	1.331
L-Proline	4.30	0.523
L-Serine	6.45	0.860
Total		12.000

because the intake was examined only at a level of 0.25 g. In the present experiment tryptophan in the amino acid mixture was given at different levels. Four healthy boys served as experimental subjects for 19 days. Following the 3 days of normal diet consumption, a basal diet derived from amino acid mixture, cornstarch, corn oil. butter fat, vitamin and mineral mixture was fed. Both diets were isonitrogenous and isocalorigenic. Tryptophan in the amino acid mixture was administered at levels of 0.40, zero, 0.12 and 0.06 g. The results obtained are shown in table 3 and figure 1. All children maintained a positive nitrogen balance at an intake level of 0.40 g of tryptophan. When tryptophan was totally excluded, maintaining the total nitrogen at a constant level by the substitution of isonitrogenous nonessential amino acid, the nitrogen balance became negative. Then the requirement for maintaining a positive balance was examined at level of 0.12 and 0.06 g. All children maintained positive balance at a level of 0.12 g, and subjects U. H. and N. M. kept positive also at the level of 0.06 g, but subjects T. D. and F. K. were not able to maintain a positive balance at the level of 0.06 g. Body weight was maintained at about a constant level in all children throughout the experimental period. As anticipated, the excretion of riboflavin and N'-methylnicotinamide (N-MNA) increased with the deficiency of tryptophan.

Experiment 2. This experiment was carried out on 3 subjects for 15 days to determine the requirement of arginine. Following the 3 days of normal diet consumption, a basal diet was given. Arginine in the amino acid mixture was fed at a level of 10.08 g. Then arginine in the amino acid mixture was excluded totally for 7 days. The subjects maintained a constant body weight and positive nitrogen balance (table 4 and figure 2).

Experiment 3. Four healthy 10- to 12-year-old boys served as subjects for examining the requirement of histidine. Following the 3 days of normal diet and the 4 days of basal diet (including 0.75 g of histidine) consumption, histidine was to-

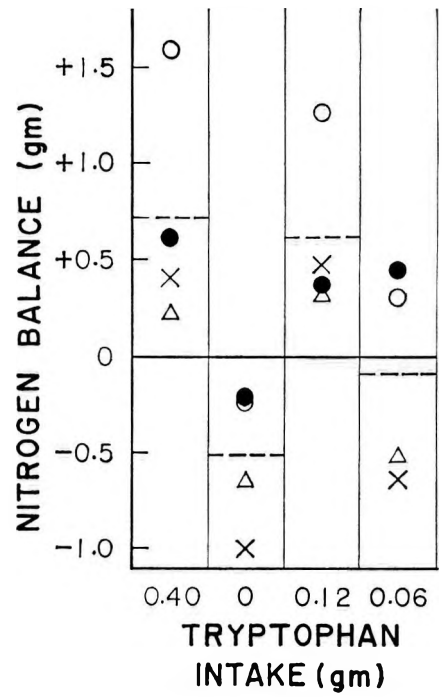


Fig. 1 Tryptophan intake and nitrogen balance. The dotted line denotes the mean levels of daily nitrogen balance of all subjects at each period. ○, represents the mean nitrogen balance of subject U. H. at each period; ●, represents that of subject N. M.; ×, represents that of subject T. D.; △, represents that of subject F. K.

TABLE 3

Nitrogen balance and urinary excretion of creatinine, creatine, riboflavin and N-MNA with different levels of tryptophan intake

Subject	Period	Body wt	Daily tryptophan intake	Daily N intake	Average daily N output		Average daily N balance	Average daily urinary excretion			
					Urine	Feces		Creatinine	Creatine	Riboflavin	N-MNA
	days	*kg*	*g*	*g*	*g*	*g*	*g*	*mg*	*mg*	*μg*	*mg*
U.H.		32.9[1]						884[2]	0[2]		
	3	33.0	0.40	12.14	9.80	0.75	+1.59	760	26	726	5.0
	4	33.4	0	12.12	11.58	0.77	−0.23	727	160	744	6.0
	4	32.6	0.12	12.11	10.10	0.74	+1.27	774	90	816	5.1
	5	32.4	0.06	12.13	10.98	0.84	+0.31	809	54	836	3.9
T. D.		34.6[1]						965[2]	0[2]		
	3	34.8	0.40	12.15	10.85	0.89	+0.41	884	39	755	6.8
	4	35.0	0	12.13	12.30	0.82	−0.99	624	160	1321	9.0
	4	34.4	0.12	12.12	11.03	0.61	+0.48	909	79	775	4.2
	5	33.9	0.06	12.13	11.78	0.98	−0.63	887	57	792	7.1
N. M.		31.8[1]						861[2]	0[2]		
	3	32.1	0.40	12.14	10.58	0.94	+0.62	832	40	961	5.4
	4	31.8	0	12.13	11.59	0.75	−0.21	886	204	1048	7.6
	3	31.7	0.12	12.11	10.87	0.86	+0.38	824	110	1110	5.6
	4	31.1	0.06	12.13	11.05	0.63	+0.45	851	185	1094	7.1
F. K.		30.4[1]						800[2]	22[2]		
	3	30.7	0.40	12.14	11.10	0.80	+0.24	744	42	793	5.7
	4	30.9	0	12.13	11.98	0.78	−0.63	609	174	1394	8.7
	4	30.3	0.12	12.11	11.14	0.63	+0.34	772	100	950	7.2
	5	29.7	0.06	12.13	11.73	0.90	−0.50	915	137	1006	7.0

[1] Initial body weight.

[2] Average of urinary excretion for 3 days, when consuming a normal diet.

TABLE 4

Nitrogen balance and urinary excretion of creatinine, creatine, riboflavin and N-MNA with different levels of arginine intake

Subject	Period	Body wt	Daily arginine intake	Daily N intake	Average daily N output		Average daily N balance	Average daily urinary excretion			
					Urine	Feces		Creatinine	Creatine	Riboflavin	N-MNA
	days	*kg*	*g*	*g*	*g*	*g*	*g*	*mg*	*mg*	*μg*	*mg*
K. T.		39.1[1]						1056[2]	0[2]		
	5	39.6	10.08	12.16	11.18	0.80	+0.18	979	210	780	7.2
	7	39.4	0	12.12	10.89	0.76	+0.47	1061	66	935	6.3
K. K.		32.9[1]						910[2]	0[2]		
	5	33.6	10.08	12.16	10.95	0.70	+0.51	859	102	566	6.9
	7	33.9	0	12.12	10.91	0.82	+0.39	862	97	842	5.8
H. S.		34.3[1]						858[2]	0[2]		
	5	34.7	10.08	12.14	10.64	0.71	+0.79	805	128	568	6.7
	7	35.2	0	12.12	10.58	0.75	+0.79	804	89	679	6.1

[1] Initial body weight.
[2] Average of urinary excretion for 3 days, when consuming a normal diet.

TABLE 5

Nitrogen balance and urinary excretion of creatinine, creatine, riboflavin and N-MNA with different levels of histidine intake

Subject	Period	Body wt	Daily histidine intake	Daily N intake	Average daily N output		Average daily N balance	Average daily urinary excretion			
					Urine	Feces		Creatinine	Creatine	Riboflavin	N-MNA
	days	*kg*	*g*	*g*	*g*	*g*	*g*	*mg*	*mg*	*μg*	*mg*
U. D.		33.5[1]						852[2]	0[2]		
	4	34.0	0.75	12.11	11.31	0.65	+0.15	846	9	442	7.0
	15	34.2	0	12.11	11.07	0.77	+0.27	925	98	588	6.5
H. T.		21.1[1]						523[2]	169[2]		
	4	21.7	0.75	12.07	10.15	0.80	+1.12	516	66	634	5.5
	15	21.8	0	12.12	10.84	0.62	+0.66	511	211	771	4.6
N. M.		31.7[1]						722[2]	0[2]		
	4	31.6	0.75	12.13	10.22	0.94	+0.97	760	0	689	5.9
	13	31.5	0	12.11	10.97	0.82	+0.32	822	63	920	4.8
H. A.		25.8[1]						640[2]	161[2]		
	4	25.9	0.75	12.09	10.76	0.95	+0.38	785	163	794	6.5
	15	26.0	0	12.10	10.76	0.72	+0.62	742	148	772	4.0

[1] Initial body weight.
[2] Average of urinary excretion for 3 days, when consuming a normal diet.

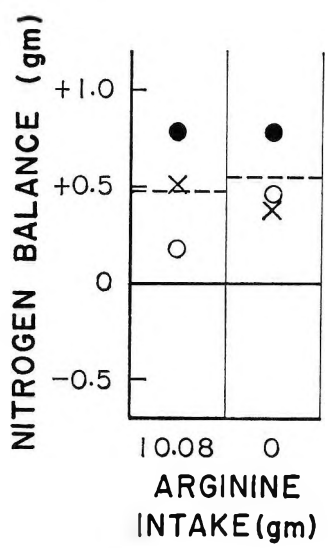


Fig. 2 Arginine intake and nitrogen balance. The dotted line denotes the mean levels of daily nitrogen balance of all subjects at each period. ○, respresents the mean nitrogen balance of subject K. T. at each period; ●, represents that of subject H. S.; ×, represents that of subject K. K.

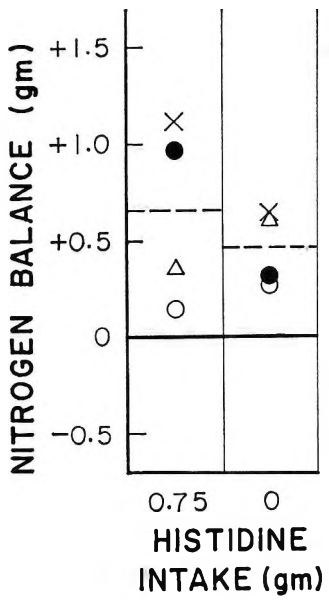


Fig. 3 Histidine intake and nitrogen balance. The dotted line denotes the mean levels of daily nitrogen balance of all subjects at each period. ○, represents the mean nitrogen balance of subject U. D. at each period; ●, represents that of subject N. M.; ×, represents that of subject H. T.; △, represents that of subject H. A.

tally excluded for 15 days, maintaining the total nitrogen at a constant level of 12 g by the substitution of isonitrogenous nonessential amino acid. All children maintained constant body weight and positive nitrogen balance throughout the experimental period. No clinical symptoms were observed (table 5 and figure 3).

DISCUSSION

In the first paper (1), the minimal requirement of tryptophan was estimated to be 0.25 g, but this was re-examined, because the experiment was carried out at only one intake level. In the present experiment, all children maintained a positive balance at a level of 0.12 g, although 2 of them showed a negative balance at a level of 0.06 g.

The necessity of arginine for adults remains in question. Holt et al. (5) considered it to be necessary for adults, because the reduction in the number of spermatozoa in the seminal fluid was induced by the removal of arginine. But the work of Rose et al. (6) appeared not to support this observation.

In our experiment with children, the effect of the removal of arginine on the spermatogenesis has not been taken into consideration, but all of them maintained positive nitrogen balance and a constant level of body weight throughout the period of arginine deprivation. Moreover, no clinical symptoms appeared.

With respect to histidine, Albanese et al. (7) considered it to be indispensable for infants, and stated that histidine deficiency induced an "indican reaction" in the urine, as measured by the Sharlit method (8). Although we could not confirm their observations, our children maintained a constant body weight and positive nitrogen balance throughout the deprivation period of 15 days without any accompanying clinical symptoms. Both in the arginine deprivation period and the histidine deprivation period, the urinary excretion of N-MNA tended to decrease and that of riboflavin increased only to a slight degree on the average, but from the viewpoint of daily variation, it cannot be defined as significant.

Finally, we conclude the series of experiments dealing with the requirements

TABLE 6

Summary of amino acid requirements of children

Amino acid	No. of subjects	Age range in years and months	Value defined tentatively as minimum
			g/day
L-Isoleucine	3	11 0 — 11 6	1.00
L-Leucine	3	10 11 — 11 2	1.50
L-Lysine	5	10 7 — 11 0	1.60
L-Methionine	4	10 9 — 11 3	0.80
L-Phenylalanine	4	10 9 — 12 3	0.80
L-Threonine	4	10 4 — 12 6	1.00
L-Tryptophan	4	10 11 — 12 1	0.12
L-Valine	4	11 6 — 12 3	0.90

of children for essential amino acids by presenting the results accumulated in the entire study in table 6.

ACKNOWLEDGMENTS

We wish to express our appreciation to Taeko Ishihara, Youko Soeno, Tadahiro Nojiri and Terumi Sagara for their technical assistance.

LITERATURE CITED

1. Nakagawa, I., T. Takahashi and T. Suzuki 1960 Amino acid requirements of children. J. Nutrition, *71:* 176.
2. ——— 1961 Amino acid requirements of children: Isoleucine and leucine. Ibid., *73:* 186.
3. ——— 1961 Amino acid requirements of children: Minimal needs of lysine and methionine based on nitrogen balance method. Ibid., *74:* 401.
4. Nakagawa, I., T. Takahashi, T. Suzuki and K. Kobayashi 1962 Amino acid requirements of children: Minimal needs of threonine, valine and phenylalanine based on nitrogen balance method. Ibid., *77:* 61.
5. Holt, L. E., Jr., A. A. Albanese, L. B. Shettles, C. Kajdi and P. M. Wangerin 1942 Studies of experimental amino acid deficiency in man. I. Nitrogen balance. Federation Proc., *1:* 116.
6. Rose, W. C., R. L. Wixom, H. B. Lockhart and G. F. Lambert 1955 The amino acid requirements of man. XV. The valine requirement; summary and final observations. J. Biol. Chem., *217:* 987.
7. Albanese, A. A., L. E. Holt, Jr., J. E. Frankstar and V. Irby 1944 Observations on a histidine deficient diet in man. Bull. Johns Hopkins Hosp., *74:* 251.
8. Sharlit, H. 1932–33 A method for the quantitative estimation of indoxyl compounds in urine. J. Biol. Chem., *99:* 537.

Dietary Protein and Fertility of Male Chickens[1]

G. H. ARSCOTT AND J. E. PARKER
Department of Poultry Science, Oregon State University, Corvallis, Oregon

ABSTRACT Results from an experiment with 3 groups of 8 White Leghorn male chickens each fed rations containing 16.9, 10.7 and 6.9% of protein over a 33-week period show that decreasing the protein in the diet had no adverse effect on semen volume. Decreasing the quantity and quality of protein within the limits of this trial also had no adverse effect on fertilizing capacity of semen, as determined by artificial insemination; in fact a significant difference ($P < 0.05$) in favor of the lowest protein level was noted. Hatchability of fertile eggs was unaffected by protein level. Males on the high protein ration ate more feed and gained more in body weight than those on the lower protein rations.

Even though fertility can be determined in the domestic fowl with greater precision than in most other species, relatively little research with respect to the effect of nutrition on reproduction in the male fowl has been reported. Prolonged feeding of diets deficient in vitamin E (1) and in vitamin A (2)[2] decreased the reproductive efficiency of male fowls. Schumacher et al. (3) and Ferrand and Bohren (4) have shown that rations low in carotenoids but adequate in vitamin A have little if any effect on fertilizing capacity of chicken semen. Limiting feed intake 42 to 72% was observed by Parker and McSpadden (5) to be detrimental to fertility of male chickens. Whether this adverse effect was due to restriction of protein, calories, minerals or vitamins was not determined. The purpose of this experiment was to determine the effect of protein intake on fertility of male chickens.

EXPERIMENTAL

Twenty-four dubbed White Leghorn cockerels, approximately 8 months of age were housed in individual cages on January 3, 1961. All males were fed a control ration (ration 1, table 1) until April 18 when they were divided into 3 groups

Received for publication January 26, 1963.

[1] Technical paper no. 1621, Oregon Agricultural Experiment Station.

[2] Parker, J. E., and B. J. McSpadden 1941 Factors influencing fertility in domestic fowls. Proc. Assoc. Southern Agr. Workers, 42: 214 (abstract).

TABLE 1

Composition of experimental diets

	Ration		
	1	2	3
	%	%	%
Corn, yellow, ground	75.75	89.0	65
Animal fat[1]		1	10
Cellulose[2]			15
Soybean meal, solvent-extracted (44% protein)	13.75		
Fish meal, (70% protein)	3		
Alfalfa meal, dehydrated (20% protein)	3	3	3
Whey, dried, (58% lactose)		2	2
Limestone flour	1.75	1.5	1.5
Bonemeal, special steamed	1.75	2.5	2.5
Salt, iodized	0.5	0.5	0.5
Vitamin-trace mineral premix[3]	0.5	0.5	0.5
Total	100.00	100.00	100.00

[1] See footnote 3 in text.
[2] See footnote 4 in text.
[3] Nopcosol M-6 (Nopco Chemical Company, Richmond, Calif.), supplies per 454 g of mixture: vitamin A, 500,000 USP units; vitamin D_3, 200,000 ICU; vitamin E, 100 IU; riboflavin, 0.35 g; D-pantothenic acid, 0.3 g; niacin, 2 g; choline, 20 g; vitamin B_{12}, 0.4 mg; Mn, 5.448 g; Fe, 1.816 g; Cu, 0.1816 g; I, 0.109 g; butylated hydroxytoluene, 11.34 g.

and fed the 3 experimental diets shown in table 1. The experiment was concluded December 5, 1961 after a 33-week treatment period.

Ration 1 was a modified corn-soybean meal layer diet containing 16.9% of protein by analysis (N × 6.25), 1392 Cal. of metabolizable energy (ME)/454 g and 1.35% of calcium by calculation. Ration 2 differed from ration 1 in that soybean meal and fish meal were removed and 1% of animal fat[3] and 2% of whey added with an appropriate adjustment made in the calcium-phosphorus level and in the amount of corn used. This ration contained 10.7% of protein and 1470 Cal. of ME/454 g. In ration 3 an attempt was made to decrease protein still further but maintain the energy level of this diet virtually the same as ration 1 by the use of 15% of cellulose[4] and 10% of fat. This ration contained 6.9% of protein and 1379 Cal. of ME/454 g. All rations and water were provided ad libitum. Body weights and feed consumption data were obtained at 28-day intervals. Fourteen hours of incandescent light were provided for all birds throughout the experiment.

To determine fertility the males were ejaculated by the massage technique one week before the feeding of the experimental rations and at 4 to 6 week intervals thereafter. To insure a more reliable measure of the male's semen-producing capabilities all were ejaculated 2 days before the collection of the experimental ejaculates. After determining semen volume, 3 battery-maintained White Leghorn pullets were artificially inseminated from each ejaculate using a dose of 0.05 ml of undiluted semen. The insemination of the 24 groups of 3 females was done on a rotational basis so that the fertility of semen from a given male was checked on a different group of females each time. Eggs were saved for incubation on the second through the ninth day, inclusive, following each insemination. Fertility of eggs was determined by candling the eggs after 7 or more days of incubation. All "clear" eggs were broken out and examined macroscopically for evidence of embryo development. Hatchability data were obtained for eggs fertilized by the fourth and fifth inseminations only. A total of 2381 eggs were incubated in the experiment. The semen volume and fertility data were treated statistically.

RESULTS AND DISCUSSION

A summary of results is shown in table 2. No significant differences were obtained on volume of semen produced by the White Leghorn cockerels fed the various protein levels, although the males fed the ration containing 16.9% of protein produced slightly greater volumes than males fed the lower protein diets (fig. 1). No adverse effects on fertilizing capacity of semen were observed with males fed the lower protein diets; in fact, the data show a significant difference ($P < 0.05$) in favor of males receiving a diet containing

[3] Calogen (Swift and Company, Inc., Portland, Oregon), stabilized with Tenox R which is composed of 20% of citric acid (anhydrous), 20% of butylated hydroxyanisole and 60% of propylene glycol.
[4] Solka Floc, BW-100 (Brown Company, Berlin, New Hampshire).

TABLE 2

Influence of protein level on certain reproductive characters, body weights and feed consumption of male chickens

Treatment[1]	Average data					
	Semen[2] vol/male	Fertility[2]	Hatchability[3] fertile eggs	Body weights		Feed[4]/ male
				Initial	Gain	
	ml	%	%	*kg*	*g*	*kg*
16.9% protein	0.62	75.4(7)[5]	85.9	2.570	472	2.607
10.7% protein	0.55	82.6(8)	85.6	2.583	227	2.402
6.9% protein	0.56	88.9(7)	83.4	2.601	214	2.453
L.S.D. ($P < 0.05$)	nsd[6]	11.0				

[1] Started April 18, 1961; ended December 5, 1961.
[2] Avg 7 collections.
[3] Avg 2 hatches.
[4] Avg 8 28-day periods.
[5] Figures in parentheses represent survivors.
[6] No significant differences.

6.9% as compared with 16.9% of protein. Examination of the fertility data shown in figure 1 suggests that the difference in fertility of the males fed the lowest protein diet could be related to the fact that these males also had higher fertility during the preliminary period when all males were fed a common diet. However, covariance analysis of these data show these treatment differences remain significant even after adjustment for variation in initial fertility.

No differences between treatments were evident for hatchability of fertile eggs. The males fed the high protein ration consumed more feed and gained more in body weight than those fed the low protein diets. There was no tendency for males fed the low protein rations to increase their protein intake by consuming more feed. Two males died during the experiment — one male on the highest and one on the lowest protein level.

The failure of the lower protein rations to adversely affect volume and fertilizing capacity of semen indicates that protein quantity *within the levels tested* was not a significant factor in this experiment. It is evident from these results that levels of protein much lower than the 15% minimum (6) normally consumed by breeder males pen-mated to hens, have no adverse effects on reproductive performance. These observations are in accord with the report of Leveille and Fisher (7) on the nitrogen requirement for maintenance in the adult rooster. From their data a maintenance requirement equivalent to about 7.0% protein may be calculated. No data were presented by these investigators, however, on reproductive performance. No explanation for the significant improvement in fertility noted with the low protein diet presently can be made. Confirmation of this observeration is neeed as is further study involving even lower protein levels.

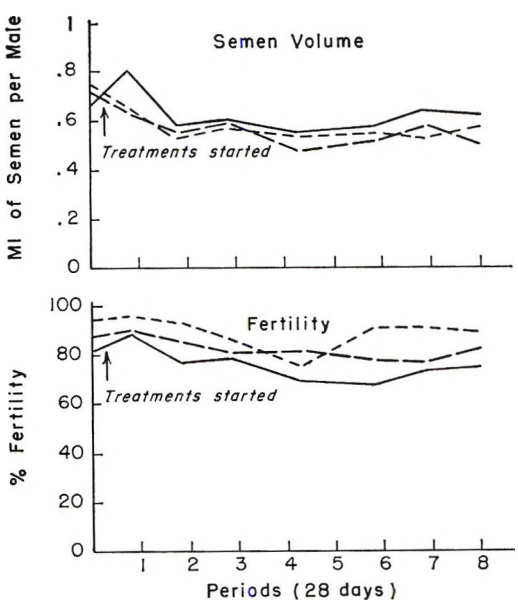


Fig. 1 Influence of protein level on volume and fertility of male semen. Legend: 16.9% protein, ———; 10.7% protein, — —; 6.9% protein, – – – –.

An examination of the calculated amino acid content of the diets used in relation to the male's amino acid maintenance requirements[5] as reported by Leveille et al. (8) shows that ration 1 (16.9% protein) exceeds these requirements for all amino acids (table 3). Ration 2 (10.7% protein) has a lower methionine value. Ration 3 (6.9% protein) has lower arginine and methionine-cystine values and a slightly lower threonine value. No attempt was made to relate these amino acid levels to the minimal maintenance levels[6] reported by the above-mentioned workers, whose levels are considerably below those noted here even for the most limiting amino acids. These levels were determined by removing individual amino acids one at a time from the diet. To the authors' knowledge no experimental data are presently available that demonstrate the adequacy of the resulting mixture with nonprotein-depleted birds from the standpoint of maintaining a positive nitrogen balance. Thus, it appears that protein quality *within the amino acids levels encountered in this experiment* was of limited importance from the standpoint of reproductive efficiency.

[5] Defined as the lowest level of an amino acid that will maintain the same level of nitrogen excretion as observed with the starting diet (9). The authors are aware of the limitations involved in relating such maintenance requirements as determined in nitrogen balance trials of 3 to 6 days duration with amino acid levels in diets fed to males over eight 28-day periods. To their knowledge no other data is presently available that might be more appropriate.

[6] Defined as the lowest level of a specific amino acid that will maintain a positive nitrogen balance (9).

TABLE 3

Amino acid content of the diets used in relation to maintenance requirements

Amino acid	Maintenance requirement[1]	Ration 1		Ration 2		Ration 3	
		Maintenance requirement[2]	Amount present	Maintenance requirement[3]	Amount present	Maintenance requirement[4]	Amount present
	mg/kg/day	%[5]	%[5]	%	%	%	%
Arginine	120	0.36	0.89	0.38	0.39	0.39	0.29
Histidine	0	0	0.35	0	0.19	0	0.15
Lysine	29	0.09	0.88	0.09	0.32	0.09	0.25
Leucine	124	0.37	1.34	0.39	0.87	0.4	0.65
Isoleucine	72	0.21	0.77	0.23	0.4	0.23	0.3
Methionine	71[6]	0.21	0.28	0.22	0.17[7]	0.23	0.13
Cystine	42	0.12	0.26	0.13	0.15	0.14	0.12
Phenylalanine	26[6]	0.08	0.71	0.08	0.39	0.08	0.29
Tyrosine	78	0.23	0.58	0.25	0.38	0.25	0.29
Threonine	74	0.22	0.56	0.23	0.3	0.24	0.23
Tryptophan	19	0.06	0.18	0.06	0.08	0.06	0.06
Valine	61	0.18	0.68	0.19	0.31	0.2	0.24

[1] Based on data of Leveille et al. (6).
[2] Based on average body weight of 2.836 kg and daily feed consumption of 95 g.
[3] Based on average body weight of 2.723 kg and daily feed consumption of 86 g.
[4] Based on average body weight of 2.791 kg and daily feed consumption of 86 g.
[5] Per cent of diet.
[6] Requirement of methionine and phenylalanine in presence of cystine and tyrosine, respectively.
[7] Figures underlined show levels present in diet below requirement.

LITERATURE CITED

1. Adamstone, F. B., and L. E. Card 1934 The effects of vitamin E deficiency on the testis of the male fowl (*Gallus domesticus*). J. Morphol., 56: 339.
2. Paredes, J. R., and T. P. Garcia 1959 Vitamin A as a factor affecting fertility in cockerels. Poultry Sci., 38: 3.
3. Schumacher, A. E., H. M. Scott, J. S. Hughes and W. J. Peterson 1944 The role of carotenols in growth and reproduction in the fowl. Ibid., 23: 529.
4. Ferrand, R. H., Jr., and B. B. Bohren 1948 The effect of carotenols on fertilizing capacity of fowl sperm. Ibid., 27: 759.
5. Parker, J. E., and B. J. McSpadden 1943 Influence of feed restriction on fertility in male domestic fowls. Ibid., 22: 170.
6. National Research Council 1960 Nutrient requirements of domestic animals, no. 1. Nutrient requirements of poultry, pub. 827. National Academy of Sciences — National Research Council, Washington, D. C.
7. Leveille, G. A., and H. Fisher 1958 Amino acid requirements for maintenance in the adult rooster. I. Nitrogen and energy requirements in normal and protein-depleted animals receiving whole egg protein and amino acid diets. J. Nutrition, 66: 441.
8. Leveille, G. A., R. Shapiro and H. Fisher 1960 Amino acid requirements for maintenance in the adult rooster. IV. The requirements for methionine, cystine, phenylalanine, tyrosine and tryptophan: the adequacy of the determined requirements. Ibid., 72: 8.
9. Leveille, G. A., and H. Fisher 1959 Amino acid requirements for maintenance in the adult rooster. II. The requirement for glutamic acid, histidine, lysine and arginine. Ibid., 69: 289.

Serum and Liver Protein Metabolism in Protein-depleted Dogs[1,2]

R. W. WANNEMACHER, JR., T. J. RUSSELL AND J. B. ALLISON
Bureau of Biological Research, Rutgers, The State University, New Brunswick, New Jersey

ABSTRACT Protein-depletion in the dog results in a decrease in liver protein and ribonucleic acid which could be correlated with body nitrogen loss. The drop in liver protein concentration may be the result of a decreased synthesis and concentration of soluble cytoplasmic and nuclear proteins. Analyses of blood serum suggested that as the dog lost body nitrogen, there was a decrease in the concentration and synthesis of serum albumin and beta globulin. In the protein-depleted dog there was evidence, also, for an increase in the relative half-life of all serum protein fractions, which did not appear to be correlated with the rates of synthesis of the various fractions. By using the current concepts of protein synthesis, it is possible to explain some of these changes in serum and liver protein in the protein-depleted dogs through an amino acid-ribonucleic acid-ribonuclease interaction.

Protein-depletion results in a decrease in liver protein and ribonucleic acid (RNA), (1–3). Previous work by Allison et al. (4) has demonstrated a relationship between ribonuclease activity, RNA and protein concentration of the liver cell. Therefore, the present experiments were performed to test this relationship between ribonuclease and RNA in the protein-depleted animals. It was also pertinent to determine what cellular fraction decreased in protein concentration and if this change could be correlated with the utilization of S^{35} methionine by the various fractions of the cell. Previous reports have demonstrated that protein-depletion results in a reduction of serum albumin in man (5), in the dog (6), and in the rat (7). The serum globulin, on the other hand, tends to remain constant in dogs fed a low protein diet (8). Although preliminary data in both man (9) and rat (7) have suggested that the decrease in serum albumin was the result of a slower synthesis of this protein, little information has been accumulated concerning the metabolism of serum globulin synthesis during protein deprivation. Since serum albumin has been associated with the "protein reserve" of the organism (10), it appears worthwhile to compare its synthesis with that of those globulins which do not decrease during mild protein depletion. In as much as the liver is the site of formation for most serum proteins (11), an attempt will be made to correlate, in part, the change in composition of this organ with the rate of synthesis of certain serum proteins.

METHODS

Six male, adult, Beagle dogs were fed a protein-free diet, described by Allison et al. (12). They were maintained with this diet for 9 weeks during which time continuous urine and fecal collections were taken to determine the amount of body nitrogen lost, assuming that the non-protein-depleted animals had an overall protein content of 16%. At weekly intervals 10 ml of blood were removed from each dog, and at zero, 4 and 8 weeks after feeding the diet they were injected intravenously with 0.8 mg of DL-methionine-S^{35} (0.05 mc)/kg of body weight. Blood samples were removed at 6 hours, 1, 3, 7, 10 and 14 days after injection of the radioactive material, and 24 hours post-injection liver biopsies were performed by means of a laparotomy. Samples were taken before the injection of radioactive material to determine the amount of residue S^{35} left in the tissues.

The liver tissues were broken down to various cellular fractions by differential

Received for publication February 13, 1963.
[1] The work was supported in part by U. S. Public Health Service (4341) and by Gaines Division of General Foods.
[2] The work with radioactive material was performed in the Ruth Estrin Goldberg Memorial Isotope Laboratories.

centrifugation. The nuclei were separated into acid-soluble and -insoluble fractions by the method of Busch et al. (13), and the mitochondria, microsome and cell sap were separated in 0.25 M sucrose medium by a modification of the procedure of Hogeboom et al. (14). The cell sap was brought to pH 5.2 and centrifuged to remove the pH 5 fraction of Hoagland et al. (15). The labeled proteins of the tissues and cellular fractions were extracted, purified and analyzed for radioactivity and protein content by the methods previously described (7). The liver tissues were analyzed for the nucleic acid phosphorus concentrations by the procedure of Schmidt and Thannhauser (16).

The serum from the various blood samples was separated electrophoretically into different protein fractions by means of paper electrophoresis as described by Allison et al. (4). The resulting paper strips were dyed with alcoholic bromphenol blue and run through a recording densitometer. A scanner was used to record the radioactivity associated with each electrophoretic fraction. These data were then calculated as the percentage of total serum protein radioactivity that appeared in each fraction.

RESULTS

The specific activity of liver proteins increased as the dogs lost body nitrogen (table 1). This increase in activity can be explained by a decrease in the sulfur free amino acid pool of the serum and liver of the protein-depleted animals (7), thereby resulting in an increased concentration of S^{35} in the metabolic pool. The distribution of S^{35} concentration between the various cellular particles can be expressed as a percentage of the total liver protein radioactivity in each fraction. This distribution (table 1) indicates that the nuclear and soluble cytoplasmic proteins decreased in radioactivity with increasing loss in body nitrogen. Such a decrease in isotope concentration suggests a slower synthesis of these proteins in the depleted dog. A similar result was noted by Wannemacher (7) in the liver of protein-depleted rats. The decrease in total liver protein of the dog was also reflected by a decrease in the above mentioned protein fractions, suggesting that the lower liver

TABLE 1

Distribution of S^{35} in the various cellular protein fractions of liver from protein-depleted dogs

Liver protein fraction	Specific activity			% Distribution of radioactivity in protein fractions			Protein concentration		
	Nitrogen loss			Nitrogen loss			Nitrogen loss		
	0%	14%	23%	0%	14%	23%	0%	14%	23%
	count/min/mg protein						*mg protein/g wet tissue*		
Total	298 ± 12[1]	500 ± 19	1211 ± 89				254 ± 10	231 ± 8	184 ± 11
Nuclei									
HCl-insoluble	276 ± 24	607 ± 31	1060 ± 112	33 ± 2	30 ± 2	27 ± 2	75 ± 6	58 ± 6	44 ± 4
HCl-soluble	288 ± 31	630 ± 28	1168 ± 94	14 ± 1	11 ± 1	10 ± 1	29 ± 2	25 ± 2	15 ± 1
Mitachondria	276 ± 32	804 ± 18	1168 ± 85	11 ± 1	16 ± 1	20 ± 1	30 ± 4	32 ± 3	31 ± 5
Microsome	353 ± 38	987 ± 52	1294 ± 152	11 ± 1	11 ± 3	16 ± 1	19 ± 3	22 ± 2	19 ± 1
pH5 (15)	312 ± 23	897 ± 37	878 ± 132	5 ± 1	6 ± 1	6 ± 1	12 ± 1	11 ± 1	10 ± 1
Soluble	183 ± 11	365 ± 35	639 ± 86	26 ± 2	23 ± 1	22 ± 1	93 ± 8	83 ± 6	65 ± 7

[1] SE.

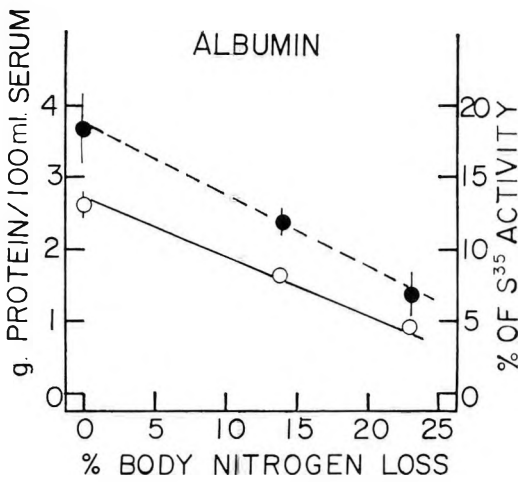


Fig. 1 The effect of body nitrogen loss upon serum albumin concentration (open circles) and upon the percentage of total S^{35} activity in the serum albumin fraction at 6 hours after the injection of methionine-S^{35} (solid circles). The vertical line through the circle is the standard error of the mean except for those values which were less than the diameter of the circle.

protein concentration of the depleted animals could be the result of a slower synthesis of nuclear and soluble cytoplasmic proteins. Associated with these changes in liver protein was a decrease in RNA/DNA ratio from 6.50 ± 0.25 in controls to 4.85 ± 0.24 in dogs which had lost 23% of their body nitrogen.

The data in figure 1 illustrate the decrease in serum albumin protein (open circles) with increasing loss in body nitrogen. Accompanying this change in albumin concentration was a decrease in the amount of radioactivity associated with this fraction (closed circles). These data also suggest that there was a slower rate of synthesis of serum albumin associated with protein depletion leading to a decreased concentration of this fraction, results similar to those reported by Wannemacher (7) for depleted rats.

As the dogs lost body nitrogen there was a slight increase in α_1-globulin, and a decrease in α_2-, and β-globulin (fig. 2, open circles). These changes in serum

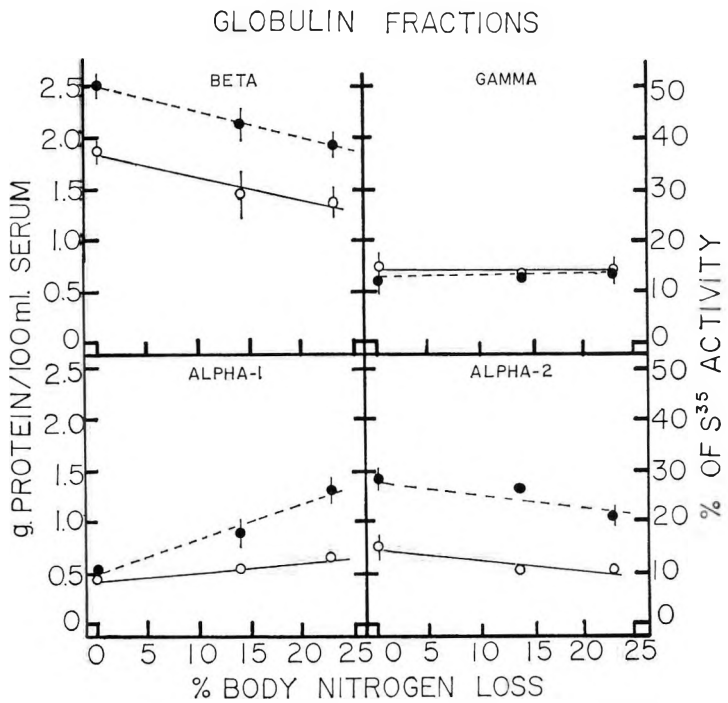


Fig. 2 The effect of body nitrogen loss upon the concentration of the various serum globulins (open circles) and upon the percentage of total methionine-S^{35} (solid circles). The vertical line through the circle is the standard error of the mean except for those values which were less than the diameter of the circle.

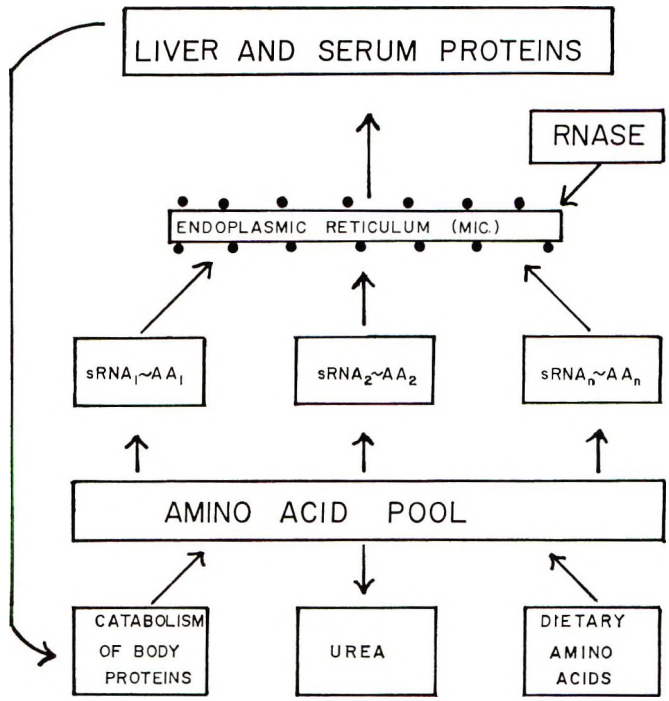


Fig. 3 A hypothetical scheme for the synthesis of serum and liver proteins.

globulins can be correlated with the percentage of radioactivity and the relative rate of synthesis of each electrophoretic fraction (closed circles). Thus, the data suggest that following protein-depletion there was an increase in the rate of synthesis of α_1-globulin and a decrease in α_2-, and β-globulin fractions. The concentration and rate of synthesis of gamma globulin did not appear to be affected by loss in body nitrogen (fig. 2). According to Miller and Bale (11) serum gamma globulin is synthesized mostly in nonhepatic tissue.

At lower nitrogen intakes in rat (17) and dog (18) there is a decrease in the half-life of the serum proteins. The data in table 2 illustrate a similar change in dogs fed a protein-free diet. As the dogs lost body nitrogen there was an increase in half-life which may be interpreted as a decrease in catabolism of the serum protein fractions. This change in half-life was observed even in proteins that were not changing or even increasing in concentration. From these observations it may be concluded that in protein-depleted dogs the serum protein concentration is a function of the rate of synthesis of the individual fraction and does not necessarily reflect the changes in overall catabolism of the organism.

Protein-depletion appears to have an effect upon the serum proteins which are synthesized mainly by the liver. From the observations presented in this and other papers an hypothesis has been presented to describe the synthesis of certain serum and liver proteins (19). Dietary proteins as well as the "labile proteins" of the body

TABLE 2

The half-life (T/2) of various serum protein fractions of protein-depleted dogs

Serum protein fraction	Nitrogen loss, 0%	Nitrogen loss, 14%	Nitrogen loss, 23%
	days	*days*	*days*
Total	28.6 ± 1.1[1]	74.0 ± 3.1	68.0 ± 2.9
Albumin	21.6 ± 0.8	77.3 ± 6.0	62.7 ± 5.8
Alpha$_1$	18.7 ± 2.3	26.6 ± 2.9	36.0 ± 3.2
Alpha$_2$	21.3 ± 3.5	26.9 ± 3.0	33.0 ± 3.7
Beta	34.8 ± 5.6	87.4 ± 7.2	76.1 ± 6.8
Gamma	31.3 ± 1.9	41.7 ± 1.7	58.2 ± 2.2

[1] SE.

contribute to the metabolic amino acid pool of the tissues (see fig. 3). Amino acids are removed from the pool by soluble RNA and transported to the ribosomal RNA at which point they are synthesized into various proteins. When the animal is fed a protein-free diet, the amino acid pool is decreased (20) which results in a decreased utilization of messenger RNA. Possibly, the enzyme ribonuclease is only able to combine with RNA which is not in combination with amino acids (21). Thus, the ever present ribonuclease could hydrolyze the RNA that is not utilized for protein synthesis. It should be emphasized, however, that the hypothesis is an over-simplification of a mechanism involving a number of types of RNA and RNase and the correlation between these and protein biosynthesis. There is a possibility, too, that an inhibitor to RNA hydrolysis may play an important role in the conduct of RNA concentrations in cells. Such a hypothesis would explain the decreased concentration of microsomal RNA in a protein-depleted animal, and further evidence (3,22) suggests that it is mostly the membrane RNA of the microsome that decreases with protein-free diet. Peters (23) and Campbell (24) have demonstrated further that serum albumin is synthesized on this membrane RNA of the microsomes, thus suggesting a mechanism which could control the synthesis rate of serum albumin in the protein-depleted organism. It should be emphasized, also, that an hypothesis designed to explain protein biosynthesis in the liver need not apply to other tissues.

LITERATURE CITED

1. Kosterlitz, H. W. 1947 The effect of changes in dietary protein on the composition and structure of liver cell. J. Physiol., *106:* 194.
2. Campbell, R. M., and H. W. Kosterlitz 1948 Relationship between losses in labile liver cytoplasm and urinary N excretion. Biochem. J., 43: 416.
3. Munro, H. N., and C. M. Clark 1960 The nutritional regulation of ribonucleic acid metabolism in the liver cell. Proc. Nutrition Soc., *19:* 55.
4. Allison, J. B., R. W. Wannemacher, Jr. and R. A. Gomez-Brenes 1961 Nitrogen intakes and ribonuclease activity in normal and tumor-bearing rats. J. Nutrition, *74:* 176.
5. Scrimshaw, N. S., and M. Behar 1961 Protein malnutrition in young children. Science, *133:* 2039.
6. Allison, J. B., R. W. Wannemacher, Jr., T. Russell and J. R. McCoy 1958 The uptake of S^{35} from methionine by plasma proteins in protein-depleted and tumor-bearing dogs. Cancer Res., *18:* 395.
7. Wannemacher, Jr., R. W. 1961 Incorporation of S^{35} from L-methionine in tumor-bearing and protein-depleted rats. Proc. Soc. Exp. Biol. Med., *107:* 277.
8. Allison, J. B., and R. W. Wannemacher, Jr. 1957 Repletion of Depleted Protein Reserves in Animals in Amino Acid Malnutrition, ed., W. H. Cole. Rutgers University Press, New Brunswick, New Jersey.
9. Gitlin, D., J. Craviato, S. Frenk, E. L. Montano, R. Romos-Galvan, F. Gomez and C. A. Janeway 1958 Albumin metabolism in children with protein malnutrition. J. Clin. Invest., 37: 682.
10. Allison, J. B., and W. H. Fitzpatrick 1960 Dietary Proteins in Health and Disease. Charles C Thomas. Springfield, Illinois, p. 36.
11. Miller, L. L., and W. F. Bale 1954 Synthesis of all plasma fractions except gamma globulins by the liver. The use of zone electrophoresis and lysine-e-C^{14} to define the plasma proteins synthesized by the isolated perfused liver. J. Exp. Med., *99:* 125.
12. Allison, J. B., R. W. Wannemacher, Jr. and J. F. Migliarese 1954 Diet and the metabolism of 2-aminofluorene. J. Nutrition, 52: 415.
13. Busch, H., J. R. Davis and D. C. Anderson 1958 Labeling of histones and other nuclear proteins in L-lysine-u-C^{14} in tissues of tumor-bearing rats. Cancer Res., *19:* 1030.
14. Hogeboom, G. H., W. C. Schneider and M. J. Strulick 1952 Cytochemical studies. V. On the isolation and biochemical properties of liver cell nuclei. Ibid., *196:* 11.
15. Hoagland, M. B., M. J. Stephenson, J. F. Scott, L. I. Hecht and P. C. Zamecnik 1958 A soluble ribonucleic acid intermediate in protein synthesis. J. Biol. Chem., *231:* 241.
16. Schmidt, G., and S. J. Thannhauser 1945 A method for the determination of desoxyribonucleic acid, ribonucleic acid and phosphoproteins in animal tissues. Ibid., *161:* 83.
17. Jeffay, H., and R. J. Winzler 1958 The metabolism of serum proteins. II. The effect of dietary protein on the turnover of rat serum proteins. Ibid., *231:* 111.
18. Yuile, C. L., F. V. Lucus, J. P. Olson and A. B. Shapiro 1959 Plasma protein turnover and tissue exchange. Influence of dietary protein and protein depletion. J. Exp. Med., *109:* 173.
19. Allison, J. B., R. W. Wannemacher, Jr., W. L. Banks, W. H. Wunner and R. A. Gomez-Brenes 1962 Dietary proteins correlated

with ribonuclease, ribonucleic acid, and tissue proteins. J. Nutrition, *78:* 333.
20. Thompson, P. E., L. M. Schun, L. M. Henderson and C. A. Elvehjem 1950 The influence of fasting and nitrogen deprivation on the concentration of free amino acids in rat tissue. J. Biol. Chem., *182:* 47.
21. Schweet, R., J. B. Bishop and A. Morris 1961 Protein synthesis with particular reference to hemoglobin synthesis. Lab. Invest., *10:* 992.
22. Goswami, P., G. C. Bair and H. N. Munro 1962 The origin of different types of ribonucleic acid in rat liver microsomes. Biochem. Biophys. Acta, *55:* 408.
23. Peters, T. Jr., 1962 The biosynthesis of rat serum albumin. II. Intracellular phenomena in the secretion of newly formed albumin. J. Biol. Chem., *237:* 1186.
24. Campbell, P. N. 1961 The biosynthesis of rat serum albumin. Fifth International Congress of Biochemistry Symposium II:1.

Mineral Utilization in the Rat

I. EFFECTS OF VARYING DIETARY RATIOS OF CALCIUM, MAGNESIUM AND PHOSPHORUS

R. M. FORBES
Department of Animal Science, University of Illinois, Urbana

ABSTRACT Forty weanling male albino rats were used in a 28-day growth and mineral balance factorially designed experiment to investigate effects of 2 levels each of Ca (0.4 and 0.8%), Mg (142 and 420 ppm) and P (0.19 and 0.50%). Weight gain and feed intake were severely restricted by adding Ca to the low P diets irrespective of Mg supply. Obvious deficiency of Mg was produced only by addition of both Ca and P to low Mg diets; the major interference with Mg utilization was a reduction in net absorption leading to a high Ca:Mg ratio in the tissue gained. Addition of dietary Ca lowered blood serum Mg but did not affect the percentage of Mg in the bone ash. Moderate to severe kidney calcification was produced by addition of P to low Mg diets, the effect being greatest in presence of low Ca.

Stating with precision the requirement by an animal for a nutrient is an exceedingly difficult problem whose ramifications are often overlooked in the face of the practical necessity of establishing standards by which to measure nutritive adequacy of diets. Aside from animal variables such as stage and rate of development, the problem is complicated by dietary variables, prime among which are the interrelationships that may exist between nutrients. Studies of mineral interrelationships date from the early observations that low calcium rations inhibit phosphorus balance and continue to the more recent investigations revealing fundamental interdependencies between Cu and Mo, Zn and Ca, Mg and P, Ca and Mg, Cd and Zn, to name but a few.

In view of our earlier experience showing Ca to affect Mg requirement of the rat (1) and that of O'Dell et al. (2) demonstrating a Mg and P relationship, we selected as the objective of this experiment an investigation of interrelationships between ratios of Ca, P and Mg as they affect utilization of these minerals by the young rat. Criteria of utilization were weight gain, nutrient balance, tissue analysis and visually detectable symptoms of abnormality.

METHODS

Forty weanling male albino rats, of the Sprague-Dawley strain, averaging 60 g body weight, were housed individually in stainless steel cages for 28 days. Feed intake was measured and was limited to 12 g daily, and deionized water was supplied ad libitum. Excreta were collected during the second and fourth weeks. At the end of the experiment femurs, diet and excreta were analyzed for Ca, Mg and P; kidneys and blood serum were analyzed for Ca and Mg. The cation analyses were made chelometrically, using EDTA as titrant (3). Except for blood serum, all samples were ashed, phosphorus was removed with $Zr(NO_3)_2$ and trace minerals by extraction of their diethyldithiocarbamates with CCl_4 (4). Phosphorus analyses were made by the AOAC[1] colorimetric procedure. Analysis of variance was used to test the data statistically. Differences referred to in the text possess a probability value of less than 0.01 unless otherwise noted.

The ingredients of the basal diet are shown in table 1. By analysis the diet contained 0.40% Ca, 0.19% P, and 142 ppm Mg. Seven modifications of the basal diet were made by additions, singly and in all possible combinations, of Ca, P and Mg so that total concentrations were 0.80%, 0.50% and 420 ppm, respectively, in a factorial design. The supplements were added as 1% $CaCO_3$, 1.33% NaH_2PO_4·

Received for publication February 18, 1963.

[1] Association of Official Agricultural Chemists 1960 Official Methods of Analysis, ed. 9. Washington, D. C.

TABLE 1

Composition of basal diet

	%
Casein[1]	12.0
DL-Methionine	0.4
Corn oil	10.0
Glucose	67.03
Cellulose[2]	3.0
Vitamin mix 691[3]	5.0
Vitamin A and D mix[4]	0.5
Salts[5]	2.07

[1] Vitamin-Free Test Casein, General Biochemicals Corporation, Cleveland.
[2] Woodflock, Brown Company, Chicago.
[3] Forbes and Vaughan ('54).
[4] 2,000 IU vitamin A and 250 IU vitamin D/g.
[5] The salt mixture contained in per cent: NaCl, 17.88; K_2CO_3, 24.17; $CaHPO_4$, 21.27; $CaCO_3$, 32.38; $FeSO_4 \cdot 7H_2O$, 1.93; $MgCO_3$, 1.38; $MnSO_4 \cdot H_2O$, 0.58; $CuSO_4 \cdot 5H_2O$, 0.29; KI, 0.02; $ZnCO_3$,0.10.

H_2O, and 0.11% $MgCO_3$ at the expense of glucose. The rats were randomly assigned to the 8 treatment groups, 5 animals per treatment.

RESULTS

The data on general response of the animals are shown in figure 1. The differences in gain are closely related to differences in total feed intake. The relationship between the 28-day gain (Y) and feed intake (X) may be expressed by the equation $Y = 0.57\ X - 73$, the linear correlation coefficient being 0.96. The main effect of Ca addition was to decrease weight gain. This was an effect most noticeable in rats fed the low P diets. Magnesium addition increased weight gain, an effect more noticeable in the presence of high Ca and low P than in other situations ($P = 0.05$). Phosphorus addition increased weight gain, notably in the cases involving high Ca diets and independent of Mg level.

Figure 1 also describes the general appearance of the animals at the end of the experiment. The typical signs of Mg deficiency appeared uniformly in only the group receiving supplementary Ca and P, and low in Mg. In this group the erythema of the ears first appeared on the eighth day of the experiment and persisted for about a week. After this time facial edema and blotchy appearance of the face appeared and persisted throughout the experiment.

Blood serum Ca averaged 12.6 mg/100 ml and was not affected by dietary treatment. Blood serum Mg was lowest, 0.76 mg/100 ml in the animals showing frank Mg deficiency symptoms, and averaged 1.07 mg/100 ml in rats fed low Mg diets and 1.86 mg/100 ml in rats fed higher Mg diets. Supplemental dietary P decreased serum Mg of rats fed the low Mg diet but did not affect that of animals fed the high Mg diet. Supplemental dietary

Figs. 1–6 The minus (−) signs denote the lower level and the plus (+) signs denote the higher level of mineral in the diet. In the right-hand portion of figures 2, 4 and 5 the total bars represent the percentage of absorption, the dotted areas represent urinary excretion, and the solid areas represent positive balance.

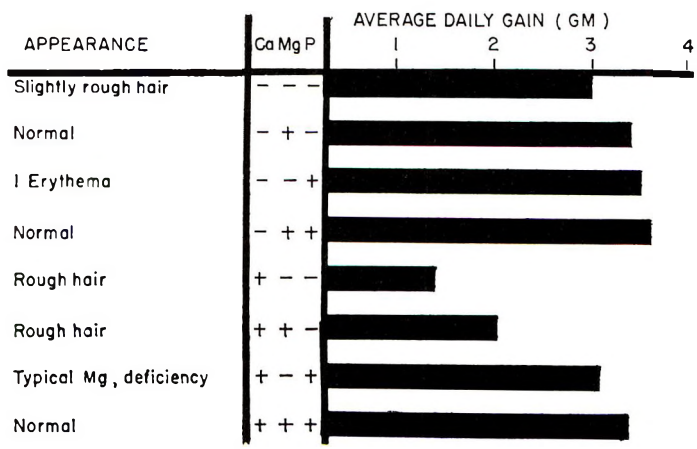


Fig. 1 Geneal appearance and average daily gain.

Ca lowered serum Mg from 1.63 to 1.30 mg/100 ml.

The effects of dietary treatment on Mg utilization are shown in figure 2 in which the data are presented both in terms of net gain and in percentage absorption, urinary excretion and balance. Increasing Mg intake increased both the percentage absorbed and the percentage excreted in the urine of animals fed the low Ca diet. However, the Mg intake was sufficiently increased so that total Mg gained was increased by the Mg addition and particularly so in the case of the high P, low Ca diet.

Addition of Mg was particularly advantageous to Mg storage by rats fed the high Ca diets, largely an effect due to poor storage of Mg by animals fed high Ca, low Mg diets. The only animals showing negative Mg balance were those fed high Ca, low Mg, low P diets, and these animals did not show typical Mg deficiency symptoms. On the other hand, animals with typical Mg deficiency symptoms were essentially in Mg balance.

The data shown in figure 3 demonstrate that Mg did not influence the percentage of ash in the dry fat-free femur, but that its addition did increase the concentration

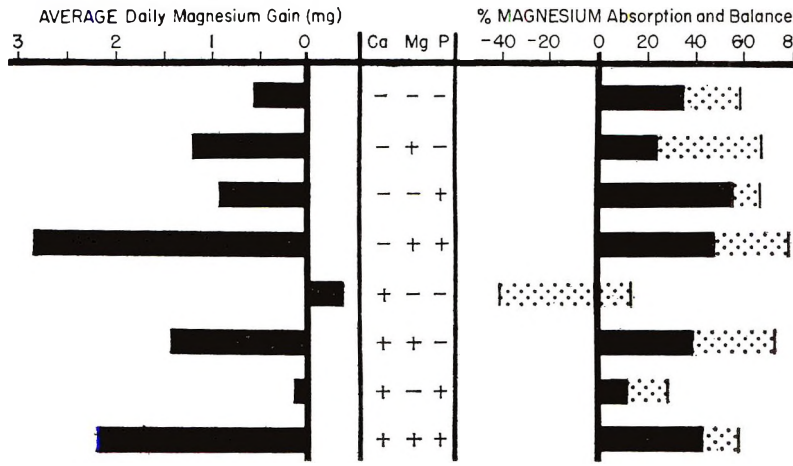


Fig. 2 Magnesium absorption, urinary excretion, and balance.

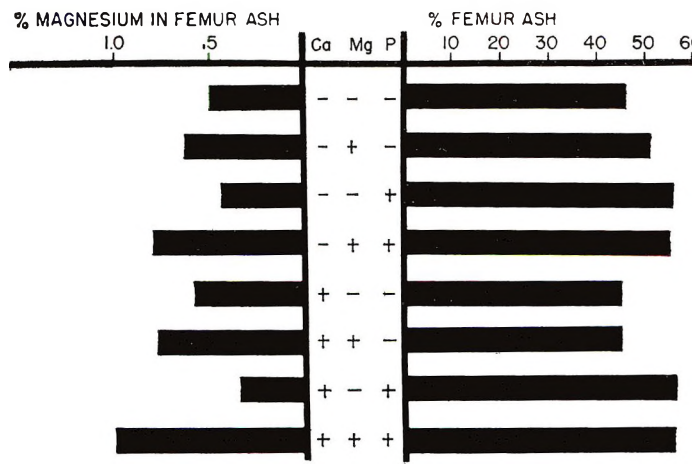


Fig. 3 Magnesium and ash in femur.

of Mg in the ash in the expected manner, the response being most marked in animals fed the higher level of phosphorus. In spite of having a normal ash percentage on the dry, fat-free basis, the bones of the rats exhibiting Mg deficiency were quite brittle.

Figures 4 and 5 illustrate the effects of the dietary variables on Ca and P utilization. Both Ca and P gain were increased by addition of P without respect to Mg supply. The addition of Ca increased Ca gain only in the presence of supplemental P, did not affect P gain in the presence of added P but did depress P gain in animals fed the low P diets. The reciprocal relationship between Ca and P supplementation and the percentage of urinary excretion of these elements is evident in the figures. The absolute amounts of Ca and P in the urine, as influenced by supplemental Ca and P, are shown in table 2.

Figure 6 illustrates the preventive effect of an adequate supply of Mg on calcification of kidneys, and that kidney calcification occurred in rats fed a low Mg diet only in the presence of a P supplement.

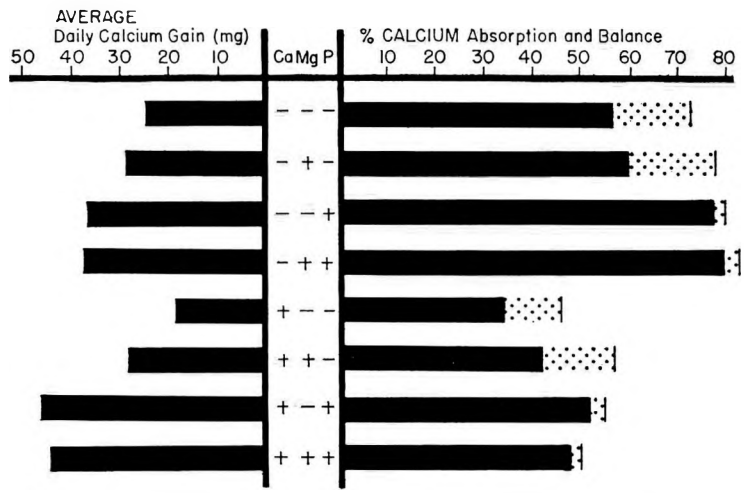


Fig. 4 Calcium absorption, urinary excretion, and balance.

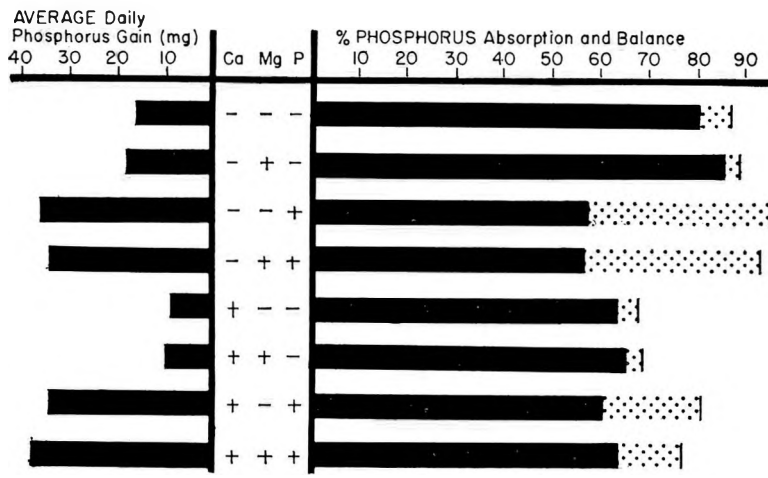


Fig. 5 Phosphorus absorption, urinary excretion, and balance.

TABLE 2

Calcium and phosphorus excretion in the urine

Treatments	Mineral excretion in urine		Ca:P ratio
	Calcium	Phosphorus	
	mg/rat/day	*mg/rat/day*	
Low Mg[1]	4.57	8.57	0.53:1
High Mg[1]	5.85	7.85	0.75:1
Low Ca, low P[2]	8.28	0.71	12:1
Low Ca, high P[2]	1.14	21.99	0.05:1
High Ca, low P[2]	9.00	0.51	18:1
High Ca, high P[2]	2.28	6.00	0.38:1

[1] Including all combinations of Ca and P.
[2] Including both levels of Mg.

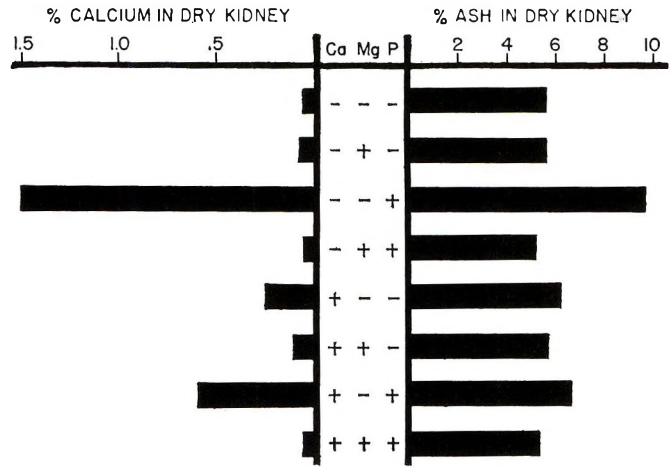


Fig. 6 Calcium and ash in kidneys.

DISCUSSION

The data obtained in this experiment illustrate some of the problems involved in establishing a dietary requirement. In particular they demonstrate the influence on the Mg metabolism of the rat of the amounts and ratios of Ca and P in the diet as well as of the total feed intake.

The lower level of Mg chosen for this experiment was selected on the basis of previous experience (1) to provide a sufficient supply for maintenance of normal weight gain, and the larger Mg amount was designed to promote normal performance in terms of bone and blood serum values and to prevent kidney calcification in diets otherwise optimum for Mg utilization. The data obtained show how drastically the addition of Ca to a diet slightly deficient in P can impair the general performance of the animals irrespective of their Mg supply even though the Ca:P ratio is within usually acceptable limits. The well-known effect of Ca in precipitating a frank Mg deficiency was produced only when the P supply was adequate, and hence the Ca:P ratio suitable, to permit a reasonable rate of gain. That similar rates of gain obtained with no greater Mg intake and with lower intakes of Ca and P did not produce Mg deficiency symptoms may be ascribed to the fact that absorption and net retention of Mg in the latter situation was greater than when the larger amounts of Ca were supplied. Actually the ratio of Ca:Mg gain in the severely deficient animals was 160:1 as opposed to 40:1 in animals fed the basal diet. This change in ratio was due both to a larger Ca gain in the deficient animals and a smaller Mg gain. The average daily feed intake of animals in these two groups was

10.1 g and 10.4 g. The Mg concentration of the gain of the deficient animals was 90 ppm vs. 250 ppm for animals receiving equivalent nutrients except for Ca. The major factor accounting for this was the lesser net absorption of Mg by animals receiving the higher Ca diet; the mechanism accounting for this remains to be determined.

The data may also be interpreted as showing that Mg deficiency is precipitated by increasing the P content of the ration. O'Dell et al. have clearly shown this for the guinea pig (5). In the present experiment the amount of P added was not excessive and in fact was necessary to permit reasonable rates of gain; it has been our experience that the faster gaining animals are those which may be expected to exhibit the more marked Mg deficiency symptoms.

The incidence of severe to moderate calcification of kidneys of rats receiving the lower level of Mg with added Ca or P, or with both, is in line with expectations but as yet defies explanation. Comparison of the Ca, Mg, and P content of the urines does not reveal a basis for prediction of calcification. Presumably, then, one must look beyond these criteria for factors predisposing to deposition of calcium in the kidney tissue. Studies such as those of Lindner et al.[2] detailing deterioration of enzymic activity and RNA content of kidneys of Mg-deficient rats are significant in this respect.

Magnesium nutriture did not markedly affect urinary excretion of Ca or of P, as shown in table 2. In rations containing no additions of Ca or P the ratio of these elements in the urine was about 12:1; in presence of P supplements the ratio was 0.05:1; in presence of Ca supplements the ratio was 18:1, and in presence of both Ca and P supplements the ratio was 0.38:1. The reciprocal relationship noted here is similar to that found by Vermeulen (6), but we found kidney calcification to occur over a wide range of ratios (dependent on Mg supply), while Vermeulen found foreign body uroliths to appear only at urinary Ca:P ratios approximating unity.

LITERATURE CITED

1. McAleese, D. M., and R. M. Forbes 1961 The requirement and tissue distribution of magnesium in the rat as influenced by environmental temperature and dietary calcium. J. Nutrition, *73:* 94.
2. O'Dell, B. L., E. R. Morris, E. E. Pickett and A. G. Hogan 1957 Diet composition and mineral balance in guinea pigs. Ibid., *63:* 65.
3. Malmstadt, H. V., and T. P. Hadjiioannou 1959 Automatic titration of calcium or magnesium in blood serum. Clin. Chem., *5:* 50.
4. ——— 1959 Rapid and accurate automatic tritration method for determination of calcium and magnesium in plant materials with EDTA titrant. J. Agr. Food Chem., *7:* 103.
5. O'Dell, B. L., E. R. Morris and W. O. Regan 1960 Magnesium requirement of guinea pigs and rats. Effect of calcium and phosphorus and symptoms of magnesium deficiency. J. Nutrition, *70:* 103.
6. Vermeulen, C. W. 1959 Effects of high calcium intakes on urine in animals. Federation Proc., *18:* 1096.

[2] Lindner, A., T. Kutkam, W. O. Smith and D. Baxter 1962 Changes in enzymes and RNA in kidneys of magnesium deficient rats. Federation Proc., *21:* 310 (abstract).

Effectiveness of Selenium and Noneffectiveness of Sulfur Amino Acids in Preventing Muscular Dystrophy in the Turkey Poult[1]

E. D. WALTER AND LEO S. JENSEN
Department of Poultry Science, Washington State University, Pullman, Washington

ABSTRACT Torula yeast-glucose monohydrate diets deficient in vitamin E were used in 2 experiments conducted to study the influence of selenium, ethoxyquin and sulfur amino acids on the incidence of skeletal and gizzard muscular dystrophy in turkey poults. Three groups of 10 Broad Breasted Bronze poults were used per treatment. A high incidence of gizzard dystrophy was observed in the groups fed the basal diets calculated to be only slightly deficient in sulfur amino acids. Skeletal muscles were also affected, but to a lesser extent. Supplementation of the ration with cystine (0.15%) or methionine (0.4%) did not alter the development of dystrophy. Similarly, low levels of ethoxyquin (0.025%) or selenium (0.01% or 0.1 mg/kg) proved ineffective. Ethoxyquin at a high level (0.3%) reduced the incidence of both skeletal and gizzard muscular dystrophy, but did not provide complete protection. Complete prevention was obtained, however, with a higher level of selenium (1 mg/kg) or α-tocopheryl acetate (20 IU/kg). Anemia and reduced albumin-globulin ratios observed with the selenium and vitamin E-deficient diets could be overcome as effectively with selenium (0.1 ppm) as with vitamin E. Ethoxyquin also was effective, but only at a high level (0.3%).

In the first studies on nutritional muscular dystrophy in chicks Dam et al. (1) showed that this disorder could be prevented by either 0.01% α-tocopheryl acetate or 0.5% L-cystine. Later Machlin and Shalkop (2), using a casein diet low in cystine and vitamin E, reported that muscular dystrophy could be prevented by adding vitamin E, cystine or methionine. Scott and Calvert (3) recently demonstrated that with vitamin E-deficient diets, meeting only one-half of the chick's requirement for methionine and one-fifth of its requiremnt for cystine, muscular dystrophy could be prevented by the addition of only 0.15% L-cystine, whereas a somewhat higher level of methionine (0.38%) was required. Selenium has been found relatively ineffective for muscular dystrophy prevention in chicks (4). Levels of 0.1 mg/kg of diet proved ineffective, whereas 1.5 mg/kg reduced the incidence but did not prevent it. The antioxidant, ethoxyquin, was effective in preventing muscular dystrophy in chicks only when a high level (0.1%) was used (5).

The most characteristic vitamin E deficiency syndrome in young turkeys is gizzard myopathy, described by Jungherr and Pappenheimer (6). A skeletal muscle dystrophy has been described in turkey poults fed a casein-gelatin purified diet containing 10% ethyl linoleate or vitamin E free lard.[2] Vitamin E or ethoxyquin prevented its occurrence, but the levels required were not given.

Exudative diathesis, another well defined vitamin E deficiency syndrome in chicks, has been reported in turkey poults fed a torula yeast ration (7). Only a mild edema occurred, however, and then only if the maternal diets of the poults used were deficient in dried brewer's yeast, a selenium source. Adding vitamin E to the maternal diet reduced the incidence. Reduced albumin/globulin ratios were observed only when the diets of the poults and that of the dams were not supplemented with vitamin E and dried brewer's yeast.

Experiments reported herein were conducted to determine the effect of selenium, antioxidants, and sulfur amino acids on

Received for publication February 2, 1963.

[1] Scientific paper no. 2313, Washington State Experiment Stations, Pullman. Project no. 1706.

[2] Ferguson, T. M., and J. R. Couch 1961 Muscular dystrophy in turkeys. Federation Proc., 20: 297 (abstract).

the development of skeletal and gizzard muscular dystrophy in the turkey poult.

PROCEDURE

Experiment 1. Four hundred eighty day-old Broad Breasted Bronze poults from dams fed practical rations were randomly distributed into groups of 10 and placed in wire-floor battery brooders. Basal diets are described in table 1. Eight treatments were used with each of 2 experimental diets (table 2).

Body weights were recorded at weekly intervals. After 2 weeks of age, all dead birds were examined for skeletal and gizzard muscular dystrophy, similar observations being made on all birds remaining at the end of the 4 week experimental period.

Prior to killing the birds at 4 weeks, 1-ml blood samples were obtained by cardiac puncture from three birds selected at random from each experimental group. The blood was subsequently pooled for hematological and electrophoretic studies. Erythrocyte count, hematocrit, and hemoglobin content were determined. Plasma samples were used for electrophoresis analysis in a Spinco Model R paper electrophoresis apparatus. A barbiturate buffer of ionic strength 0.075 and pH 8.6 was used in the electrophoretic procedure[3] carried out for 16 hours at a constant current of 2.5 ma using 0.006 ml of plasma. The percentages of albumin and globulin were estimated by scanning with a Spinco Analytrol and calculating the area under the curve of each component.

Experiment 2. Two hundred seventy day-old Broad Breasted Bronze poults were reared to one week of age with a torula yeast ration deficient in vitamin E and selenium (diet 1, table 1). At one week of age they were randomly divided into 27 battery brooder compartments of 10 birds each. Nine treatments were studied (table 3).

Daily records of mortality were kept and all dead birds were examined for skeletal and gizzard muscular dystrophy. Birds surviving at 6 weeks of age were killed for similar observations.

At 4 and 6 weeks of age, pooled blood samples from 3 poults per group (where possible) were used for hematological and electrophoretic analysis, as previously described.

TABLE 1

Composition of diets

	Diet 1	Diet 2
	%	%
Torula yeast	67.00	67.00
Glucose monohydrate[1]	27.25	24.15
Gelatin	—	4.00
Glycine	0.60	—
L-Arginine·HCl	0.30	—
Limestine, ground	2.00	2.00
Choline chloride	0.35	0.35
Vitamin mixture[2]	1.50	1.50
Mineral mixture[3]	1.00	1.00

[1] Cerelose, Corn Products Company, Argo, Illinois.
[2] The vitamin mixture supplied the following/kg of diet: (in milligrams) Ca pantothenate, 16.5; menadione, 2.2; pyridoxine·HCl, 5.5; riboflavin, 4.4; thiamine·HCl, 11.0; folic acid, 1.1; biotin, 0.22; vitamin B_{12}, 0.02; vitamin A, 66,000 IU; vitamin D_3, 1760 ICU; glucose to make up 1.5% of ration.
[3] The mineral mixture supplied the following in g/100 g of diet: iodized salt, 0.50; $MnSO_4$, 0.24; $ZnSO_4$, 0.008; $CoCl_2 \cdot 6H_2O$, 0.002; $CuSO_4 \cdot 5H_2O$, 0.01; glucose to make up 1% of ration.

RESULTS AND DISCUSSION

Feeding the nonsupplemented basal diets, calculated to be only slightly deficient in sulfur amino acids (0.48% methionine and 0.32% cystine), produced a high incidence of gizzard muscular dystrophy (table 2). Skeletal muscles were also affected, but to a lesser extent. Scoring for skeletal muscle dystrophy was confined to birds showing distinct white muscle striations, although additional birds in the dystrophic groups showed a pale-colored breast musculature. Adding methionine (0.4%) improved growth ($P < 0.05$) but did not alter the incidence of dystrophy. However, selenium which was relatively ineffective in preventing dystrophy in chicks (4) was completely effective in the turkey poult. Ethoxyquin (0.025%) was ineffective in the absence and presence of supplemental methionine.

Growth with basal diet 2, containing 4% gelatin, was superior to that obtained with diet 1 and mortality was also lower, but the incidence of dystrophy was similar. On the other hand, some factor present in gelatin (diet 2) produced an improvement in hemoglobin level, red blood

[3] Spinco Division, Beckman Instruments, Inc., Stanford Industrial Park, Palo Alto, California.

TABLE 2

Influence of selenium, ethoxyquin, vitamin E and methionine on body weight, incidence of muscular dystrophy and certain hematological values in turkey poults at 4 weeks of age

Treatment[1]	Avg body wt, 4 weeks	Mortality		Incidence of dystrophy		Hematocrit	Hemoglobin	Red blood cells	Albumin/ globulin ratio
		0–2 weeks	2–4 weeks	Skeletal muscle	Gizzard				
	g	*%*	*%*	*%*	*%*	*ml/100 ml*	*g/100 ml*	*millions*	
Basal diet no. 1	167	—	60	27	77	31	6.4	0.96	0.17
+ selenium[2]	266	—	10	0	0	36	11.1	1.98	0.75
+ ethoxyquin[3]	158	—	70	20	77	32	8.1	1.54	0.41
+ vitamin E[4]	236	13	—	0	0	37	10.4	2.30	0.62
+ methionine[5]	172	3	40	28	86	22	5.4	1.25	0.24
+ methionine + selenium	273	7	7	0	0	36	10.9	2.53	0.62
+ methionine + ethoxyquin	169	3	87	10	59	28	7.9	1.26	0.34
+ methionine + vitamin E	283	7	—	0	0	36	10.0	2.00	0.78
Basal diet no. 2	212	—	17	20	90	36	9.5	2.13	0.47
+ selenium	254	—	—	0	0	34	10.5	2.26	0.73
+ ethoxyquin	209	—	17	7	90	33	10.2	2.13	0.62
+ vitamin E	242	7	—	0	0	35	10.6	2.21	0.69
+ methionine	235	20	20	13	83	34	9.9	1.73	0.50
+ methionine + selenium	267	7	—	0	0	38	12.2	2.21	0.85
+ methionine + ethoxyquin	262	7	37	14	86	36	11.1	1.71	0.82
+ methionine + vitamin E	266	3	—	0	0	36	11.2	2.48	0.82

[1] Three groups of 10 poults used/treatment.
[2] Selenium as selenous acid (1 mg/kg).
[3] Ethoxyquin, 0.025%.
[4] Alpha-tocopheryl acetate, 20 IU/kg.
[5] DL-Methionine, 0.4%.

cell count, and albumin/globulin ration (table 2).

The anemia observed in poults fed basal diet 1, with or without methionine was accompanied by low albumin/globulin ratios. Selenium (1 mg/kg) and vitamin E (20 IU/kg) were equally effective in maintaining a satisfactory hematological picture and albumin/globulin ratio. These results are in accord with those of Creech et al. (7) where the selenium was supplied by dried brewer's yeast. However, in their studies reduced albumin/globulin ratios were obtained only when the maternal diets were also deficient in selenium (no dried brewer's yeast) and vitamin E. Ethoxyquin (0.025%) afforded some beneficial effect on blood hemoglobin levels and erythrocyte counts, the values being intermediate between those of the basal rations and those receiving vitamin E or selenium.

A somewhat higher incidence of gizzard and skeletal muscular dystrophy was obtained with diet 1 in experiment 2 than was observed in experiment 1 (table 4). The fact that the birds were kept on test an additional two weeks probably accounted for this. Selenium at 1 mg/kg was again completely effective in preventing both skeletal and gizzard muscular dystrophy, whereas the lower levels provided little, if any, protection. However, the 0.1 mg/kg level markedly improved body weight and viability and maintained a normal blood picture (table 3).

TABLE 3

Effect of selenium, ethoxyquin and sulfur amino acids on hemoglobin, erythrocyte counts and albumin-to-globulin ratio in turkey poults

Treatment	Hematocrit		Hemoglobin		Red blood cells		Albumin/globulin ratio	
	4 Weeks	6 Weeks	4 Weeks	6 Weeks	4 Weeks	6 Weeks	4 Weeks	6 Weeks
	ml/100 ml		*g/100 ml*		*millions*			
Basal diet no. 1								
+ selenium	22	13	7.1	2.7	1.50	1.14	0.27	0.27
0.01 mg/kg	27	14	7.5	2.9	1.86	0.75	0.35	0.25
0.1 mg/kg	29	33	8.2	8.2	2.16	2.24	0.47	0.83
1 mg/kg	32	33	9.6	8.0	2.29	2.38	0.53	1.15
+ L-cystine, 0.15%	19	—	4.6	—	0.78	—	0.26	—
+ DL-methionine, 0.4%	17	—	4.8	—	1.19	—	0.32	—
+ ethoxyquin								
0.025%	30	—	8.5	—	1.44	—	0.38	—
0.3%	33	34	9.5	8.2	2.38	2.19	0.34	0.59
+ gelatin, 8%	32	34	8.3	8.2	1.81	2.18	0.52	0.79

TABLE 4

Effect of sulfur amino acids, gelatin and various levels of selenium and ethoxyquin on body weight, mortality and incidence of muscular dystrophy in turkey poults

Treatment[1]	Avg body wt 4 weeks	Mortality		Incidence of dystrophy	
		1–4 weeks	1–6 weeks	Skeletal muscle	Gizzard
	g	%	%	%	%
Basal diet no. 1	164	30	97	30	97
+ selenium, 0.01 mg/kg	178	17	87	50	93
+ selenium, 0.1 mg/kg	245	10	20	23	83
+ selenium, 1 mg/kg	282	0	0	0	0
+ L-cystine, 0.15%	179	47	90	27	93
+ DL-methionine, 0.4%	165	57	100	37	97
+ ethoxyquin, 0.025%	163	52	100	17	97
+ ethoxyquin, 0.3%	199	17	40	7	33
+ gelatin (8% of ration)[2]	240	18	35	40	87

[1] Three groups of 10 poults used/treatment.
[2] Gelatin was incorporated into the ration at the expense of glucose monohydrate.

Adding 0.15% cystine, which when added to that present in the basal diet would more than meet the turkey poult's requirement for sulfur-containing amino acids, did not influence the incidence of dystrophy. In experiment 2, methionine again proved ineffective. Ethoxyquin at the higher level (0.3%) reduced mortality and incidence of both skeletal and gizzard muscular dystrophy, but did not provide complete protection. From a hematological standpoint, the antioxidant fed at this level was equally as effective as selenium (table 3).

Gelatin was again effective in preventing anemia in experiment 2, but still did little to reduce the incidence of dystrophy, even though fed at a higher level (8%). The hematological results obtained with gelatin were comparable to those obtained with 0.1 mg/kg of selenium (table 3). Neutron activation analysis of the gelatin showed that it contained 1.2 ± 0.2 mg selenium/kg. At 8% of the ration, gelatin would thus contribute a minimum of 0.08 mg/kg of selenium to the ration.

Mortality was high with the basal diet and the diets supplemented with sulfur amino acids, a low level of ethoxyquin, or selenium at the 0.01 mg/kg level (table 3). The mortality probably was due, at least in part, to anemia. Some hemorrhaging, varying in severity, was observed, particularly on the thighs in approximately 25% of the birds on these treatments.

Excess pericardial fluid was also noted in a small percentage of these birds, perhaps indicative of the lowered albumin/globulin ratios obtained. There was little evidence of exudative diathesis even of the mild form reported in turkey poults (7). When mild edema did occur, it was most frequent in birds also showing symptoms of skeletal muscular dystrophy and may have been an effect of muscular degeneration. The absence of even mild edema in most turkey poults having serum albumin-to-globulin ratios even lower than those reported for chicks suffering from severe exudative diathesis, suggests that capillary damage may not be associated with a vitamin E deficiency in the poult or the extent of the damage is much less severe.

In both experiments, there was evidence that the sulfur-containing amino acids accentuated the anemic condition which developed in the absence of supplemental selenium or vitamin E. The effect of both methionine and cystine in this connection in experiment 2 (table 3) was particularly striking.

The development of muscular dystrophy in turkeys by nutritional means varies considerably from that in the chick. Not only is a deficiency of sulfur-containing amino acids not necessary for dystrophy production in poults, but selenium also appeared to be completely effective in its prevention. The latter warrants further investigation, however, with respect to vitamin E carryover in view of the report by Calvert et al. (9) of a synergistic effect between selenium and vitamin E in the chick.

LITERATURE CITED

1. Dam, H., I. Prange and E. Sondergaard 1952 Muscle degeneration (white striation of muscles) in chicks reared on vitamin E deficient low fat diets. Acta Pathol. Microbiol. Scand., *31:* 172.
2. Machlin, L. J., and W. T. Shalkop 1956 Muscular degeneration in chickens fed diets low in vitamin E and sulfur. J. Nutrition, *60:* 87.
3. Scott, M. L., and C. C. Calvert 1962 Evidence of a specific effect of cystine in the prevention of nutritional muscular dystrophy in vitamin E-deficient chicks. Ibid., *77:* 105.
4. Nesheim, M. C., and M. L. Scott 1961 Nutritional effects of selenium compounds in chicks and turkeys. Federation Proc., *20:* 674.
5. Machlin, L. J., R. S. Gordon and K. H. Meisky 1959 The effect of antioxidants on vitamin E deficiency symptoms and production of liver "peroxide" in the chicken. J. Nutrition, *67:* 333.
6. Jungherr, E., and Alwin M. Pappenheimer 1937 Nutritional myopathy of the gizzard in turkey. Proc. Soc. Exp. Biol. Med., *37:* 526.
7. Creech, B. G., G. L. Feldman, T. M. Ferguson, B. L. Reid and J. R. Couch 1957 Exudative diathesis and vitamin E deficiency in turkey poults. J. Nutrition, *62:* 83.
8. Goldstein, J., and M. L. Scott 1956 An electrophoretic study of exudative diathesis in chicks. Ibid., *60:* 349.
9. Calvert, C. C., M. C. Nesheim and M. L. Scott 1962 Effectiveness of selenium in prevention of nutritional muscular dystrophy in the chick. Proc. Soc. Exp. Biol. Med., *109:* 16.

Vitamin D and the Intestinal Absorption of Iron and Cobalt[1]

T. MASUHARA[2] AND B. B. MIGICOVSKY
Animal Research Institute, Research Branch, Canada Department of Agriculture, Ottawa, Ontario

ABSTRACT The influence of vitamin D and dietary level of calcium on absorption of Fe and Co by chicks was investigated. Differences in absorption were estimated by administering the isotopes Fe^{59} and Co^{60} orally and intraperitoneally and measuring the quantity of isotope in the bone, blood and liver. Neither vitamin D nor dietary calcium level had any effect on the intraperitoneal dose. Vitamin D increased the amount of both isotopes which appeared in the blood, liver and bone when the isotopes were administered orally to chicks fed a low calcium diet. No effect was observable when the dietary calcium was high. Dietary calcium level influenced the absorption of Fe and Co. Absorption was greater when the calcium level of the diet was high. The significance of these results with respect to the nature of the vitamin D effect on absorption of cations is discussed.

Consideration of the biochemical role of vitamin D has been based largely on its association with calcium metabolism. The bulk of the experimental work has dealt with calcium absorption or bone mineralization. It now appears possible that the movement of calcium across the intestinal barrier may be an effect of vitamin D activity that is independent of changes brought about at the epiphysis two hours after vitamin D treatment as observed by Belanger and Migicovsky (1).

The calcium absorption effect has been observed repeatedly by numerous investigators using a variety of *in vitro* and in vivo techniques. Consequently, a biochemical role for the vitamin was sought in the calcium absorption mechanism. Since recent investigations have demonstrated that the influence of vitamin D on absorption is not specific for calcium, it appears possible that the effect of vitamin D on the intestinal barrier is of a general nature.

The influence of vitamin D on the absorption of a number of different cations has been reported by several investigators in recent years. Sobel and Burger (2) observed that vitamin D increased the levels of lead in blood and bone. Meintzer and Steenbock (3) reported that the absorption of magnesium was depressed in rats fed a low vitamin D diet. An effect of vitamin D on strontium absorption was shown by Greenberg (4) and Mraz and Bacon (5). Worker and Migicovsky (6, 7) extended the list of cations, absorption of which come under the influence of vitamin D, to include in addition to calcium and strontium, beryllium, magnesium, barium, zinc and cadmium. They also observed that mercury absorption was not affected by the vitamin. Wasserman (8) further extended the list to include cobalt and to a minor extent, cesium, and also reported that absorption of sodium, potassium, copper, iron and zinc was not under the influence of vitamin D.

Studies of the effect of vitamin D on the absorption of different cations have been continued in this laboratory and this report includes studies with iron and cobalt.

MATERIALS AND METHODS

White Rock chicks were used in these experiments. In the first experiment one-day-old chicks received a low calcium rachitogenic diet AOAC (9) for 3 weeks, at which time they were visibly rachitic, and were then separated into 2 large groups. One group continued to be fed the same diet and the second group received the same diet supplemented with 2000 IU

Received for publication February 4, 1963.

[1] Contribution no. 135 from the Animal Research Institute, Research Branch, Canada Department of Agriculture, Ottawa, Canada.

[2] Postdoctorate Fellow, National Research Council, Ottawa, Canada, 1961–62. Present address: Institute for Hard Tissues, Tokyo Medical and Dental University, 3-1 Yushima, Bunkyo-Ku, Tokyo, Japan.

of vitamin D/100 g of feed and were also given 2 oral doses of 5000 IU of vitamin D over a period of 2 days. After a 24-hour fast one-half the number of birds in groups 1 and 2 was given an oral tracer dose of approximately 2 μc iron-59. The other half was given an oral tracer dose of approximately 2 μc cobalt-60. Following the isotope dose, groups of 5 birds were killed at time intervals of 0.5, 1.5, 3, 4.5, 6 and 8 hours. Blood, liver and left tibia were taken from each bird for measurement of isotope concentration.

In addition to the above oral isotope dosing, 2 groups of 5 birds were taken from each of the vitamin D-free and vitamin D-treated groups, and were given iron-59 or cobalt-60 intraperitoneally. These birds were killed 4.5 hours after the isotope dose, and the same tissues were taken for analysis. The reason for this part of the experiment was to establish that if a difference occurred following the oral dose of vitamin D it was not due to the movement of the isotope after it crossed the intestinal barrier. Therefore, if vitamin D exerted no effect on the intraperitoneal dose, and did exert an effect on the oral dose, one could conclude that the vitamin D effect was at the intestinal barrier.

In the second experiment the same procedure was followed as in the first except that the chicks were separated into 2 large groups when one day old and were fed either a low calcium (0.6%) or a high calcium (2.0%) rachitogenic diet. After 3 weeks, one-half the number of chicks in each group received the same vitamin D treatment as did the birds of experiment 1, and also iron-59 or cobalt-60 orally and intraperitoneally. In this experiment all the chicks were killed 24 hours after the isotope treatment and only the left tibiae were sampled.

Measurement of iron-59 and cobalt-60. Gamma emission of the dosing solutions and tissues were measured in a Nuclear Chicago Model DS5-5 scintillation well counter (sodium iodide, thallium-activated crystal) connected through a Model 1810 γ-ray spectrometer. Appropriate corrections were made for background radiation.

Two milliliters of blood were taken from each bird and distilled water was added to make a volume of 5 ml.

Liver was weighed and thoroughly homogenized in a Potter-Elvehjem homogenizer with water and made up to a volume of 5 ml.

Bone was cleaned of adhering tissue, the marrow was removed, and the bones were ashed. The ash from each bone was dissolved in 5 ml of 20% hydrochloric acid.

RESULTS

The objective of this study was twofold: to learn whether vitamin D affected the absorption of iron and cobalt, and whether these cations behaved similarly to calcium with respect to adaptation to a high calcium diet as observed by Nicolaysen and Eeg-Larsen (10) and by Migicovsky and Jamieson (11).

The influence of short-term vitamin D treatment on an oral dose of iron-59 and cobalt-60 with respect to the level of the isotope in blood, liver and bone, at varying intervals after isotope administration is shown in figures 1 to 6. Analysis of variance showed that for data in figures 1 to 4 the variance for the vitamin D effect is significant at $P < 0.01$. The vitamin D effect in figure 5 is significant at $P < 0.05$ and in figure 6 is significant at $P < 0.1$. Table 1 shows that the vitamin had no effect on the amount of an intraperitoneal dose of either iron-59 or cobalt-60 which appeared in bone 4.5 hours after dosage. The amount of cobalt-60 in liver and blood appeared to be elevated in the absence of vitamin D at this time.

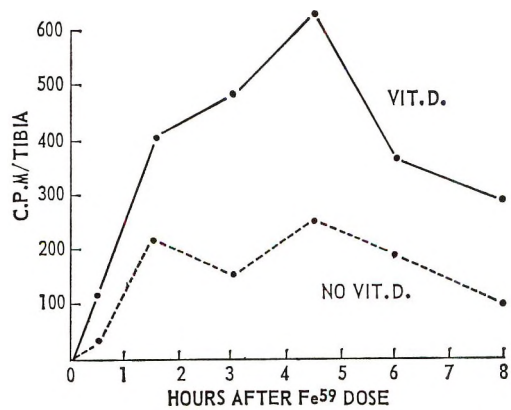


Fig. 1 Effect of vitamin D on the level of radioactivity in the tibia after an oral dose of Fe^{59} (145,225 count/min).

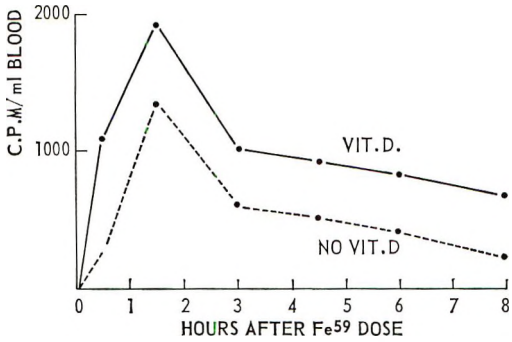


Fig. 2 Effect of vitamin D on the level of radioactivity in blood after an oral dose of Fe^{59} (145,225 count/min).

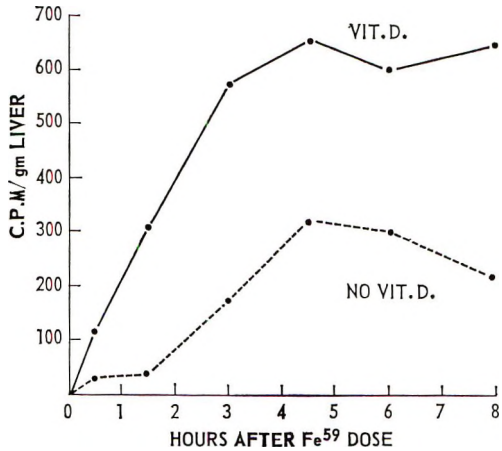


Fig. 3 Effect of vitamin D on the level of radioactivity in liver after an oral dose of Fe^{59} (145,225 count/min).

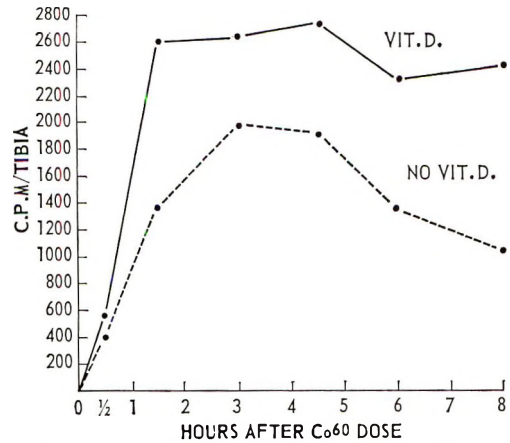


Fig. 4 Effect of vitamin D on the level of radioactivity in the tibia after an oral dose of Co^{60} (318,690 count/min).

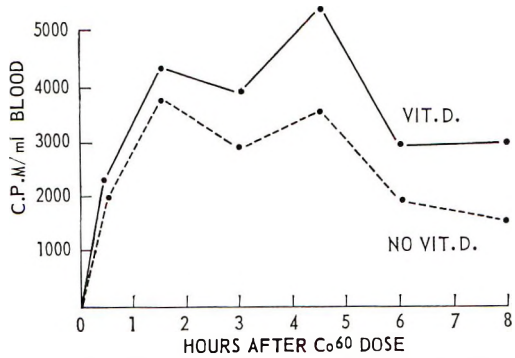


Fig. 5 Effect of vitamin D on the level of radioactivity in blood after an oral dose of Co^{60} (318,690 count/min).

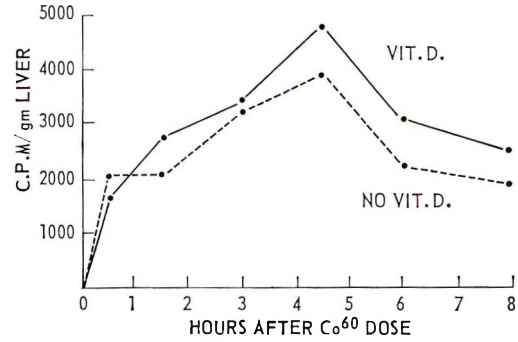


Fig. 6 Effect of vitamin D on the level of radioactivity in liver after an oral dose of Co^{60} (318,690 count/min).

The data illustrate quite conclusively that absorption of both iron and cobalt was increased by short-term vitamin D treatment and that the effect was evident within one-half hour after oral administration of the cations. In this respect they both behaved like calcium. The effect of the dietary calcium regimen on the influence of vitamin D on absorption of iron and cobalt is illustrated in tables 2 and 3. In this second experiment chicks received either low or high calcium diets prior to vitamin D treatment and iron-59 or cobalt-60 administration intraperitoneally and orally. The data confirm that vitamin D did not influence the movement into bone of iron-59 and cobalt-60 when the cations were given intraperitoneally. When the isotopes were administered orally vitamin D exerted an effect in chicks fed a low calcium diet. A previous dietary history of high calcium reduced or eliminated the vitamin D effect. This observation is simi-

TABLE 1

Effect of vitamin D on the amount of an intraperitoneal dose of iron-59[1] and cobalt-60[2] in blood, liver and tibia

Treatment	Blood		Liver		Tibia	
	Fe^{59}	Co^{60}	Fe^{59}	Co^{60}	Fe^{59}	Co^{60}
	count/min/ml		*count/min/g*		*count/min/tibia*	
No vitamin D	3052 ± 322[3]	4063 ± 1829	3560 ± 924	3396 ± 247	2517 ± 561	1847 ± 376
Vitamin D	3543 ± 1678	2372 ± 368	3582 ± 1950	1827 ± 228	2293 ± 119	1762 ± 371

[1] 229,215 count/min.
[2] 361,965 count/min.
[3] All results are expressed as averages ± SE of mean.

TABLE 2

Effect of dietary calcium and vitamin D on the amount of oral[1] and intraperitoneal dose[1] of Fe^{59} in tibia

Diet	Fe^{59} orally		Fe^{59} intraperitoneally	
	No vitamin D	Vitamin D	No vitamin D	Vitamin D
	count/min/tibia		*count/min/tibia*	
Low Ca	262 ± 127[2]	775 ± 97	685 ± 196	516 ± 152
High Ca	534 ± 131	747 ± 107	720 ± 211	610 ± 182

[1] 94,600 count/min.
[2] All results are expressed as averages ± SE of mean.

TABLE 3

Effect of dietary calcium and vitamin D on the amount of oral[1] and intraperitoneal dose[1] of Co^{60} in tibia

Diet	Co^{60} orally		Co^{60} intraperitoneally	
	No vitamin D	Vitamin D	No vitamin D	Vitamin D
	count/min/tibia		*count/min/tibia*	
Low Ca	267 ± 56[2]	672 ± 63	1191 ± 191	1586 ± 152
High Ca	515 ± 111	648 ± 69	1461 ± 373	1444 ± 185

[1] 266,400 count/min.
[2] All results are expressed as averages ± SE of mean.

lar to the effect of vitamin D on calcium absorption as recorded by Migicovsky and Jamieson (11) and Nicolaysen and Eeg-Larsen (10).

The effect of dietary calcium level on the absorption of iron and cobalt was different than the effect on calcium absorption. Migicovsky and Jamieson (11) reported that in the absence of vitamin D the dietary level of calcium had little or no effect on absorption of calcium, whereas in the presence of vitamin D calcium absorption capacity was greater in chicks fed a low calcium diet than in chicks fed a high calcium diet. The results shown in tables 2 and 3 indicate that in the absence of vitamin D absorption capacity for iron and cobalt was greater in chicks fed a high calcium diet. In the presence of vitamin D, dietary level of calcium had no effect.

DISCUSSION

The view that vitamin D has a specific influence on absorption of calcium has been discarded. The observations of Worker and Migicovsky (6, 7) and of Wasserman (8) established that vitamin D exerts an effect on the absorption of all of the divalent cations tested, except copper and mercury, and has a slight effect on the monovalent cation cesium.

The cations which appear to be influenced by vitamin D are beryllium, magnesium, calcium, strontium, barium, lead, zinc, cadmium, iron, cobalt, and to a minor extent cesium. It is possible that further work will add other cations to

this list. This leads one to contemplate the nature of the vitamin D absorption effect. Several ideas present themselves and many have been suggested by numerous investigators. (a) The action of the vitamin at the skeletal level provokes production of endogenous factor, which affects absorption, as proposed by Nicolaysen and Eeg-Larsen (10). This theory would be more reasonable if the absorption effect of vitamin D was specific for calcium. The basis for this theory was the observation that when the animal adapted to a low calcium regimen the calcium absorption effect of the vitamin was markedly increased. This same phenomenon occurs with respect to the absorption of iron and cobalt. It is difficult to understand why increased metabolic activity at the skeletal level should provoke increased absorption of iron or cobalt. (b) Vitamin D or a metabolic product thereof may act as a carrier for the cations across the intestinal barrier. (c) Vitamin D alters the chemical or physical composition of the intestinal mucosa, or both, so that passage of the cations is facilitated. This change may be similar to that which occurs at the epiphysis as observed by Belanger and Migicovsky (1) and Cipera et al. (12). The latter idea appears to be the most probable at this stage of our knowledge. These theories including those mentioned by Wasserman (8) all present possibilities although current efforts to demonstrate their validity may prove to be false labor.

ACKNOWLEDGMENTS

We wish to thank P. Richard, A. Meunier and L. J. Carter, of the Institute's Small Animal Laboratory, for technical assistance and for feeding and care of the chicks. Thanks are also due to F. Pacha who provided us with considerable assistance with the radioassays.

One of us (T.M.) wishes to express appreciation to the National Research Council of Canada for a Postdoctoral Fellowship during the tenure of which this work was undertaken.

LITERATURE CITED

1. Belanger, L. F., and B. B. Migicovsky 1958 Early changes at the epiphysis of rachitic chicks following administration of vitamin D. J. Exp. Med., *107:* 821.
2. Sobel, A. E., and M. Burger 1955 Calcification. XIII. The influence of calcium, phosphorus and vitamin D on the removal of lead from blood and bone. J. Biol. Chem., *212:* 105.
3. Meintzer, R. B., and H. Steenbock 1955 Vitamin D and magnesium absorption. J. Nutrition, *56:* 285.
4. Greenberg, D. M. 1945 Studies in mineral metabolism with the aid of artificial radioactive isotopes. VIII. Tracer experiments with radioactive calcium and strontium on the mechanism of vitamin D action in rats. J. Biol. Chem., *157:* 99.
5. Mraz, F. R., and J. A. Bacon 1960 Influence of excessive amounts of vitamin D_3 on Sr^{89} metabolism in the rat. Proc. Soc. Exp. Biol. Med., *104:* 1.
6. Worker, N. A., and B. B. Migicovsky 1961 Effect of vitamin D on the utilization of beryllium, magnesium, calcium, strontium and barium in the chick. J. Nutrition, *74:* 490.
7. ——— 1961 Effect of vitamin D on the utilization of zinc, cadmium and mercury in the chick. Ibid., *75:* 222.
8. Wasserman, R. H. 1962 Studies on vitamin D_3 and the intestinal absorption of calcium and other ions in the rachitic chick. Ibid., *77:* 69.
9. Association of Official Agricultural Chemists 1940 Official and Tentative Methods of Analysis, ed. 5. Washington, D. C.
10. Nicolaysen, R., and N. Eeg-Larsen 1953 The biochemistry and physiology of vitamin D. Vitamins and Hormones, *11:* 29.
11. Migicovsky, B. B., and J. W. S. Jamieson 1955 Calcium absorption and vitamin D. Canad. J. Biochem. Physiol., *33:* 202.
12. Cipera, J. D., B. B. Migicovsky and L. F. Belanger 1960 Composition of epiphyseal cartilage. I. Changes in hexosamine and acetone extractable contents of epiphyseal cartilage of rachitic chicks following administration of vitamin D_3. Ibid., *38:* 807.

Some Metabolic Effects of Methionine Toxicity in the Rat[1]

GEORGE J. KLAIN, DAVID A. VAUGHAN AND LUCILE N. VAUGHAN
Arctic Aeromedical Laboratory, APO 731, Seattle, Washington

ABSTRACT Rats consuming a 15% casein diet containing 4.0% of DL-methionine developed the following metabolic alterations in the liver: increased activities of arginase, tryptophan pyrrolase, glutamic-oxalacetic and pyruvic transaminase, decreased levels of DPN and slightly increased levels of total and neutral fat. However, the fat content approached a normal level at the end of the tenth week of the experimental period. Urinary excretion of a number of amino acids was also markedly increased. Supplementation of the high methionine diet with 4.0% of glycine decreased the activities of liver arginase and GOT, and increased the activity of GPT. The levels of some amino acids in the urine were also decreased by the supplemental glycine. The data indicate that ingestion of excess methionine leads to a severe disorder in nitrogen metabolism and that the supplemental glycine counteracts some of these metabolic derangements.

The following metabolic disturbances have been reported to be associated with growth depression in the rat, as a result of an excessive intake of dietary methionine: decreased retention of nitrogen in the body, accompanied with a marked loss in body fat (1); decreased activity of plasma pseudocholinesterase (2); increased urinary excretion of total nitrogen and creatinine (3); increased levels of plasma globulins (4) and increased levels of fat in the liver (2). In addition, excessive dietary methionine has been found to produce an hypertrophy of the kidney (5), splenic hemosiderosis (6) and pancreatic damage (7).

However, it has been observed that methionine toxicity can be overcome by supplemental glycine and arginine, either singly or in combination (1, 2, 8)[2] and reduced by supplemental ethanolamine or serine (8).

The work described herein was conducted to obtain more information with respect to metabolic lesions underlying methionine toxicity and to further investigate the antagonistic effect of glycine on this condition.

METHODS

Male, Sprague-Dawley rats, ranging in weight from 180 to 220 g were used in all experiments. They were housed in individual wire cages, and both the experimental diets and water were given ad libitum.

The basal diet consisted of 15% crude casein, 77% sucrose, 4% corn oil, 4% USP salt mixture XIV; the vitamin mixture supplied 2,000 units of vitamin A, 222 units of vitamin D, 11.1 mg of α-tocopherol, and the following in mg/100 g of diet: ascorbic acid, 100; inositol, 11.1, choline chloride, 166.5; menadione, 5; *p*-aminobenzoic acid, 11.1; niacin, 10; pyridoxine hydrochloride, 2.22; riboflavin, 2.22; thiamine hydrochloride, 2.22; Ca pantothenate, 40.3; also 44 μg of biotin, 200 μg of folic acid and 3 μg of vitamin B_{12}. Methionine toxicity was produced by feeding the basal diet supplemented with 4.0% DL-methionine for a period of 2 to 10 weeks, the methionine replacing an equal amount of sucrose by weight. The methionine used was of the highest purity.[3] In addition to glycine, nicotinic acid, a known methyl group acceptor, was tested as a supplement that might possibly alleviate methionine toxicity.

Received for publication February 4, 1963.

[1] The views expressed are those of the authors and do not necessarily represent official Air Force policy. The experiments were conducted according to the "Rules Regarding Animal Care" as established by the American Medical Association. (AFR 160-94.)

[2] Cohen, H. P., and C. P. Berg 1951 Response of rats to diets high in methionine. Federation Proc., 10: 172 (abstract).

[3] Purchased from Nutritional Biochemicals Corporation, Cleveland.

At the end of the specified feeding periods of time the animals were decapitated, the liver was immediately excised and chilled in chipped ice. The following methods were applied for the determination of the selected liver components: tryptophan pyrrolase (TP), the method of Knox and Auerbach (9); total fat, the method of Handler (10); phospholipids, the method of Chen et al. (11); cholesterol, the method of Searcy and Berquist (12); the extraction and measurement of pyridine nucleotides, the method of Ciotti and Kaplan (13); arginase, the method of Brown and Cohen (14); glutamic-pyruvic (GPT) and glutamic-oxalacetic (GOT) transaminases, the method of Wróblewski and LaDue (15) and LaDue et al. (16), respectively. The results of the enzyme assays were calculated as micromoles of product per gram of wet tissue per unit of time.

Individual urine collections were carried out in standard metabolism cages over a 2-day period, and the urine samples were kept frozen until analyzed. Urinary amino acids were separated by two-dimensional paper chromatography according to Berry and Cain (17) and the quantitative estimation of the separated amino acids was carried out according to the procedure of Bode (18). Excretion of some amino acids by many of the rats was apparently very low, and was not, therefore, determined quantitatively. Excretion of only those amino acids is reported for which complete values for each rat were available.

RESULTS AND DISCUSSION

The marked growth depression commonly observed as a result of methionine toxicity is shown in table 1 (group 2). Supplementation of the high methionine diet with 2% of glycine alleviated the growth depression somewhat (group 4), whereas supplementation with 4.0% of glycine counteracted the effects of excess methionine. In contrast, the equimolar level of nicotinic acid did not provide any protection against methionine toxicity (group 8), and it caused a slight inhibition in growth even when added to the basal diet (group 7).

Several metabolic changes resulting from an excessive intake of methionine were studied in the second experiment, and the data obtained are presented in table 2. The levels of total and neutral fat, and the activities of the 4 enzymes studied increased, whereas the level of DPN markedly decreased. The levels of the other components, however, were not altered (group 2 vs. 1). The supplemental glycine decreased the activities of arginase and GOT and further increased the activity of GPT, whereas the activity of TP and the levels of the other liver components remained unchanged (group 3 vs. 2). In addition, urinary excretion of alanine, aspartic acid, glutamic acid, glycine, histidine, methionine and lysine was also increased by excess methionine, and, again decreased by the supplemental glycine (group 3 vs. 2). However, it is

TABLE 1

Growth response of rats fed excess methionine with or without other dietary supplements (exp. 1)

Group	Addition to basal diet	Avg Δ body wt	Food intake
		g/2 weeks	*g/day*
1	None	34.2 ± 1.2[1]	15.2 ± 0.41
2	4.0% DL-Methionine	−49.2 ± 2.3	7.1 ± 0.25
3	2.0% Glycine	34.0 ± 1.4	14.6 ± 0.36
4	4.0% DL-Methionine + 2.0% glycine	19.5 ± 1.9	10.1 ± 0.27
5	4.0% Glycine	35.6 ± 1.8	16.1 ± 0.29
6	4.0% DL-Methionine + 4.0% glycine	30.8 ± 2.1	14.7 ± 0.36
7	3.3% Nicotinic acid	23.5 ± 1.6	10.2 ± 0.18
8	4.0% DL-Methionine + 3.3% nicotinic acid	−53.3 ± 3.1	7.3 ± 0.21

[1] SE of mean for 10 animals.

TABLE 2

Effect of excessive intake of methionine, with or without supplemental glycine, on selected liver and urinary components (exp. 2)

Liver components	Group and diet		
	1 Basal	2 Basal + 4.0% DL-methionine	3 Basal + 4.0% DL-methionine + 4.0% glycine
Average Δ body wt (g/3 weeks)	54.8 ± 3.2[1]	−42.1 ± 4.3[2]	45.0 ± 5.2
Total fat, %	5.11 ± 0.41	7.91 ± 0.39[2]	7.62 ± 0.45[2]
Neutral fat, %	1.45 ± 0.21	4.08 ± 0.18[2]	3.98 ± 0.24[2]
Phospholipids, %	3.34 ± 0.18	3.52 ± 0.15	3.32 ± 0.10
Total cholesterol, %	0.32 ± 0.02	0.31 ± 0.01	0.32 ± 0.02
DPN, μg/g	368 ±46	252 ±31[2]	239 ±50[2]
DPNH, μg/g	211 ±28.1	230 ±34.4	219 ±29.3
TPN, μg/g,	19 ± 1.2	21 ± 1.8	18 ± 2.3
TPNH, μg/g	171 ±46.2	163 ±37.3	182 ±28.5
Arginase, mmoles urea/g/hour	42.3 ± 2.8	71.1[2] ± 6.4[2]	59.6 ± 4.9[2]
Tryptophan pyrrolase, μmoles kynurenine/g/hour	2.4 ± 0.08	3.5[2] ± 0.04[2]	3.6 ± 0.19[2]
Glutamic-oxalacetic transaminase, μmoles DPN/g/min	119.2 ± 5.2	156.3 ± 3.1[2]	146.1 ± 2.8[2]
Glutamic-pyruvic transaminase, μmoles DPN/g/min	39.4 ± 3.6	56.8 ± 4.0[2]	65.3 ± 2.3[2]
Urinary amino acids, mg/24 hours			
Alanine	0.32 ± 0.06	0.55 ± 0.08[2]	0.38 ± 0.04
Aspartic acid	0.11 ± 0.02	0.36 ± 0.04[2]	0.16 ± 0.05
Glutamic acid	0.69 ± 0.07	1.45 ± 0.08[2]	0.78 ± 0.09
Glycine	0.46 ± 0.05	0.63 ± 0.04[2]	25.6 ± 1.42[2]
Histidine	0.84 ± 0.07	1.89 ± 0.12[2]	1.01 ± 0.13
Methionine	0.59 ± 0.04	60.4 ±10.51[2]	74.6 ±11.23[2]
Lysine	0.24 ± 0.03	0.75 ± 0.12[2]	0.30 ± 0.09
Threonine	0.17 ± 0.02	0.19 ± 0.04	0.18 ± 0.05
Valine	0.08 ± 0.01	0.07 ± 0.01	0.11 ± 0.02

[1] Average values for at least 5 animals.
[2] Difference from group 1 ($P < 0.05$).

possible that the high value for urinary methionine or glycine, or both, is partly due to contamination of the urine by feed.

Because excess methionine causes a slight accumulation of fat in the liver, the lipid content and the activities of GOT and GPT were followed for 10 weeks in another experiment, as indicated in table 3. The data show that the levels of total and neutral fat gradually increased, reaching the highest level around the fourth week and thereafter declined slowly. From the data presented in table 2 it appears that the oxidation of neutral fat in the livers of animals fed excess methionine is impaired due to the lack of DPN. However, the data in table 3 indicate that the animals were able to overcome this metabolic block in the later stages of toxicity and utilize the accumulated fat.

The activities of GOT and GPT were again significantly higher in the animals fed excess methionine than in the controls, both at the end of the fifth and the tenth week of the experimental period. The relationship between excess methionine and changes in the activities of the enzymes studied remains obscure. However, it is possible that an extensive breakdown of body tissues, as indicated by increased levels of urinary amino acids, is one of the factors responsible for this phenomenon. The results of the enzyme assays plus an increased urinary excretion of a number of amino acids indicate that ingestion of excess methionine leads to a severe and complex disorder in nitrogen metabolism in the animal. Recently, it was reported from this laboratory (19) that the livers of rats fed an amino acid-

TABLE 3

Effect of excessive intake of methionine on liver lipids, GOT and GPT[1] (exp. 3)

Week	Diet	Δ Body wt	Total fat	Phospholipids	Cholesterol	Neutral fat	GOT	GPT
		g	%	%	%	%	*μmoles DPN/g/min*	
0	Basal		5.42 ± 0.12	3.10 ± 0.18	0.39 ± 0.02	1.92 ± 0.14	104 ± 2	28 ± 2
5		80.1 ± 7.3	5.51 ± 0.21	3.41 ± 0.25	0.32 ± 0.02	1.78 ± 0.10	137 ± 6	39 ± 2
10		144.3 ± 8.9	5.62 ± 0.14	3.20 ± 0.24	0.35 ± 0.04	2.07 ± 0.25	172 ± 19	35 ± 4
1	Basal + 4.0%	− 32.4 ± 2.8	5.26 ± 0.04	3.42 ± 0.29	0.33 ± 0.05	1.51 ± 0.14	122 ± 5	39 ± 3
2	DL-Methionine	− 51.8 ± 4.6	6.54 ± 0.19	3.13 ± 0.14	0.30 ± 0.01	3.11 ± 0.17	145 ± 3	39 ± 2
3		− 58.2 ± 3.1	6.92 ± 0.18	3.23 ± 0.12	0.29 ± 0.04	3.40 ± 0.14	173 ± 8	43 ± 1
4		− 61.0 ± 5.6	8.57 ± 0.05	3.34 ± 0.18	0.30 ± 0.09	4.93 ± 0.11	151 ± 11	46 ± 2
5		− 67.2 ± 2.6	7.79 ± 0.10[2]	3.30 ± 0.24	0.36 ± 0.03	3.33 ± 0.09[2]	159 ± 12[2]	55 ± 3[2]
6		− 71.2 ± 8.6	7.02 ± 0.16	3.30 ± 0.09	0.34 ± 0.07	3.38 ± 0.03		
7		− 65.7 ± 7.4	6.78 ± 0.18	3.32 ± 0.04	0.34 ± 0.11	3.12 ± 0.16	157 ± 18	53 ± 5
8		− 67.6 ± 5.8	6.29 ± 0.21	3.20 ± 0.09	0.35 ± 0.03	2.77 ± 0.19		
9		− 75.2 ± 10.1	6.20 ± 0.20	3.44 ± 0.18	0.34 ± 0.01	2.42 ± 0.21		
10		− 70.4 ± 6.4	5.70 ± 0.13	3.40 ± 0.21	0.33 ± 0.09	1.95 ± 0.24	229 ± 9[2]	64 ± 6[2]

[1] Average values for at least 5 animals.
[2] Difference from the basal group — same week ($P < 0.05$).

imbalanced diet exhibited increased activities of arginase, GOT and GPT. Ingestion of a diet containing excess methionine, which can be perhaps regarded as an extreme case of amino acid imbalance, produces similar alterations in these 3 enzymes.

It is realized that the metabolic effects described have been produced with a mixture of 2 compounds, the D- and L-enantiomers of a racemic mixture. Since the course and rates of metabolism of enantiomorphs often differ, as pointed out by Cohen et al. (8), use of the L-methionine might have produced metabolic effects quantitatively different from those described.

Although the ability of glycine to overcome the growth effects of excess methionine has been amply demonstrated, the mechanism involved remains to be determined. However, it appears that glycine counteracts some of the derangements in nitrogen metabolism resulting from ingestion of excess methionine, as indicated by growth, a decreased activity of liver arginase, and a decreased excretion of some urinary amino acids.

LITERATURE CITED

1. Brown, J. H., and J. B. Allison 1948 Effects of excess dietary *dl*-methionine and/or *l*-arginine on rats. Proc. Soc. Exp. Biol. Med., 69: 196.
2. Roth, J. S., J. B. Allison and L. J. Milch 1950 The sulfur balance of rats fed excess DL-methionine plus glycine or DL-alanine. J. Biol. Chem., *186:* 113.
3. Roth, J. S., and J. B. Allison 1949 The effect of feeding excess glycine, L-arginine and DL-methionine to rats on a casein diet. Proc. Soc. Exp. Biol. Med., 70: 327.
4. Brown, J. H. 1949 Influence of methionine and thiouracil on nitrogen balance index and organ weights of adult rats. Ibid., 71: 612.
5. Roth, J. S., and J. B. Allison 1950 The effects of feeding excess DL-methionine and choline chloride to rats on a casein diet. J. Biol. Chem., *183:* 173.
6. Van Pilsum, J. F., and C. P. Berg 1950 The comparative availabilities of mixtures of L- and DL- modifications of the essential amino acids for growth in the rat. Ibid., *183:* 279.
7. Kaufman, N., J. V. Klavins and T. D. Kinney 160 Pancreatic damage induced by excess methionine. Arch. Pathol., 70: 331.
8. Cohen, H. P., H. C. Choitz and C. P. Berg 1958 Response of rats to diets high in

methionine and related compounds. J. Nutrition, 64: 555.
9. Knox, W. E., and V. H. Auerbach 1955 The hormonal control of tryptophan peroxidase in the rat. J. Biol. Chem., *214:* 307.
10. Handler, P. 1948 The influence of thyroid activity on the liver and plasma lipids of choline- and cystine-deficient rats. Ibid., *173:* 295.
11. Chen, P. S., T. Y. Taribara and H. Warner 1956 Micro-determination of phosphorus. Analytical Chem., *28:* 1756.
12. Searcy, R. L., and L. M. Berquist 1960 A new color reaction for the quantitation of serum cholesterol. Clin. Chim. Acta, 5: 192.
13. Ciotti, M. M., and N. O. Kaplan 1957 Methods of Enzymology, ed., S. P. Colowick and N. O. Kaplan, vol. 3. Academic Press, Inc., New York, p. 890.
14. Brown, G. W., and P. P. Cohen 1959 Comparative biochemistry of urea synthesis. I. Methods for the quantiative assay of urea cycle enzymes in liver. J. Biol. Chem., 234: 1769.
15. Wróblewski, F., and J. A. LaDue 1956 Serum glutamic-pyruvic transaminase in cardiac and hepatic disease. Proc. Soc. Exp. Biol. Med., *91:* 569.
16. LaDue, J. S., F. Wróblewski and A. Karmen 1954 Serum glutamic-oxalacetic transaminase activity in human acute transmural myocardial infarction. Science, *120:* 497.
17. Berry, H. K., and L. Cain 1951 Quantitative study of urinary and salivary amino acids using paper chromatography. University of Texas, pub. 5109. Austin, Texas, p. 71.
18. Bode, F. 1955 Eine Vereinfachung und Verbesserung der Methode zur quantitativen Bestimmung von Aminosäuren und Peptiden mittels des Ninhydrin-Kupferkomplexes. Biochem. Ztschr., 326: 433.
19. Klain, G. J., D. A. Vaughan and L. N. Vaughan 1962 Interrelationships of cold exposure and amino acid imbalances. J. Nutrition, 78: 359.