Nutrition of Salmonoid Fishes

XII. ISOLEUCINE, LEUCINE, VALINE AND PHENYLALANINE REQUIREMENTS OF CHINOOK SALMON AND INTERRELATIONS BETWEEN ISOLEUCINE AND LEUCINE FOR GROWTH [1,2]

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ABSTRACT Growth studies were conducted with chinook salmon fingerlings (*Oncorhynchus tshawytscha*) to determine the minimal requirements for isoleucine, leucine, valine and phenylalanine. A 41% protein diet containing an indispensable amino acid pattern similar to that found in whole egg protein was used as the experimental diet. Based on the dry diet, the salmon required about 1.6% of leucine, 1.3% of valine and 2.1% of phenylalanine (0.4% supplied by tyrosine). The isoleucine requirement appeared to be influenced by dietary leucine level, being about 0.9% with 1.50% of leucine, about 1.0% with 3.68% of leucine and about 1.1% with 6.00% of leucine. On the other hand, excess dietary isoleucine reduced growth rates when fed in conjunction with a slightly suboptimal level of leucine. Growth of the salmon fingerlings was reduced slightly below that with the control diet when 1.0% of isoleucine and 1.25% of leucine were fed; however, 3.00 and 5.0% levels of isoleucine with 1.25% leucine reduced growth to 67 and 60% of the control, respectively, presumably by increasing the leucine requirement similar to the way excessive leucine increases the isoleucine requirement.

Halver et al. (1) have demonstrated that chinook salmon (*Oncorhynchus tshawytscha*) require the same 10 amino acids indispensable for optimal growth of the rat and the pig. Subsequent experiments with sockeye salmon (*O. nerka*) have confirmed the indispensability of the same 10 amino acids (2). Certain of these amino acids have been quantitated for chinook salmon using 40% protein diets containing an essential amino acid pattern similar to that of whole egg protein. The lysine requirement, reported by Halver et al.[4] was approximately 2.0% of the dry diet, and the methionine requirement, also reported by Halver et al.,[5] was between 0.5 and 0.6% of the dry diet in the presence of 1.0% of cystine. More recently, DeLong et al. (3) observed that the threonine requirement was approximately 0.9% of the dry diet irrespective of 2 water temperatures (8° and 15°C). The purpose of the present studies was to quantitate the requirements for isoleucine, leucine, valine and phenylalanine and to study interrelationships between isoleucine and leucine when one was unbalanced with respect to the other.

EXPERIMENTAL

The experimental diet (table 1) contained casein and gelatin as natural proteins to supply minimal quantities of the amino acids being studied, yet maximal quantities of several other amino acids (table 2). Crystalline amino acid supplements complemented the natural proteins to give an overall pattern of indispensable amino acids similar to that found in whole

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[1] The experimental data in this paper are taken from a thesis submitted by Ronald E. Chance in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry, Purdue University.

[2] Purdue University Agricultural Experiment Station Journal Paper no. 2148.

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[4] Halver, J. E., D. C. DeLong and E. T. Mertz 1958 Threonine and lysine requirements of chinook salmon. Federation Proc., *17*: 1873.

[5] Halver, J. E., D. C. DeLong and E. T. Mertz 1959 Methionine and cystine requirements of chinook salmon. Federation Proc., *18*: 527.

egg protein. The dry solids contained 41% of crude protein (N × 6.25). Isonitrogenous and isocaloric diets were maintained by altering glutamic acid and dextrin with the amino acids in question. The dietary ranges necessary to quantitate the isoleucine, leucine and valine requirements were estimated from preliminary studies with chinook salmon fingerlings, whereas the range used to study the phenylalanine requirement was selected on the basis of the phenylalanine requirements of other species.

TABLE 1

Experimental diet [1]

	g/300 g of diet
Gelatin	20.00
Casein	5.00
Amino acid mixture [2]	20.22
White dextrin	35.78
α-Cellulose flour	7.00
Mineral mixture [3]	4.00
Vitamins [4]	1.00
Corn oil	5.00
Cod liver oil	2.00
Water	200.00

[1] All ingredients except corn oil supplied by Nutritional Biochemicals Corporation. Cleveland.
[2] Amino acid components listed in table 2.
[3] Same as reported by Nicolaides and Woodall (4).
[4] Added via α-cellulose: (in mg) thiamine·HCl, 5; riboflavin, 20; pyridoxine·HCl, 5; nicotinic acid, 75; Ca pantothenate, 50; *i*-inositol, 200; ascorbic acid, 100; biotin, 0.5; folic acid, 1.5. Added via aqueous solution: (in mg) choline·Cl, 500; vitamin B_{12}, 0.01. Added via corn oil: (in mg) menadione, 4; α-tocopheryl acetate, 40.

Complete amino acid analyses were conducted on random samples of casein and gelatin. Eighteen of the amino acids were determined using the automatic ion exchange procedure of Spackman et al. (5). Tryptophan was determined by the chemical method of Spies and Chambers (6, 7) and cystine was estimated from a microbiological assay.[6] Casein and gelatin hydrolyzates for automatic amino acid determinations were prepared by autoclaving 50 mg of sample with 200 ml of 6 N HCl in a sealed glass tube for 20 hours at 120°C and 15 psi. Following hydrolysis the hydrolyzates were filtered through a fine pore sintered glass filter and HCl removed by repeated evaporation. The residues were dissolved in pH 2.2 sodium citrate buffer and stored in polyethylene bottles at −20°C until ready for analysis.

[6] DeLong, D. C. 1958 I. Protein and amino acid requirements of chinook salmon. Ph.D. Thesis, Purdue University, Lafayette, Indiana.

TABLE 2

Amino acid components in 100 g of dry diet

	Supplied by 5 g casein	Supplied by 20 g gelatin	Total supplied by casein and gelatin	Supplied by crystalline supplements [1]	Total α-amino acid in diet [2]
	g	*g*	*g*	*g*	*g*
L-Arginine·HCl	0.18	1.63	1.81	2.05	3.50
L-Histidine·HCl·H_2O	0.13	0.16	0.29	0.96	1.00
L-Isoleucine	0.28	0.21	0.49	2.71	3.20
L-Leucine	0.49	0.52	1.01	2.67	3.68
L-Lysine·HCl	0.41	0.79	1.20	2.08	2.88
L-Methionine	0.17	0.10	0.27	1.38	1.65
L-Cystine	0.02	0.00	0.02	0.94	0.96
L-Phenylalanine	0.25	0.36	0.61	1.91	2.52
L-Tyrosine	0.25	0.10	0.35	1.45	1.80
L-Threonine	0.18	0.31	0.49	1.24	1.73
L-Tryptophan	0.04	0.00	0.04	0.56	0.60
L-Valine	0.34	0.31	0.65	2.27	2.92
L-Alanine	0.14	2.16	2.30		2.30
L-Aspartic acid	0.32	1.06	1.38		1.38
L-Glutamic acid	1.07	1.76	2.83		2.83
Glycine	0.09	5.08	5.17		5.17
L-Hydroxylysine		0.18	0.18		0.18
L-Hydroxyproline		2.53	2.53		2.53
L-Proline	0.51	2.85	3.36		3.36
L-Serine	0.21	0.48	0.69		0.69

[1] Each lot of crystalline α-amino acids was checked for purity by comparing the specific rotation values with those listed in the literature.
[2] Approximates the indispensable amino acid composition (including cystine and tyrosine) of 40 g whole egg protein (3) except for arginine and histidine which have been elevated from 2.56 and 0.84, respectively.

Facilities for maintaining and handling the experimental fish were described previously (8, 9). The chinook salmon fingerlings were selected at random from a central supply tank and placed in screen-top, wooden troughs through which a source of well water (10°C) circulated at the rate of 10 liters/minute. At the end of a 2-week adjustment period undesirable fish were eliminated and the numbers equalized to either 200 or 250/trough. The diet fed during this period was similar in composition and physical consistency to the basal diet used during the experimental period. Also during the adjustment period, a pyridine mercuric acetate solution was added to the water for one hour to eliminate external bacteria on the fish.

The methods and procedures for diet preparation were essentially those reported by Halver (8) and DeLong et al. (10, 11). The best water-to-solids ratio was found to be 2:1 for suitable feeding consistency. Gelatin served as the binding agent giving a semisolid gel diet that could be dispensed to the fish with a garlic press. Diets were prepared in bulk and stored in glass fruit jars at −20°C until periods of use at which time they were stored at 4°C.

Feeding procedures were similar to those reported previously (8). The fish were fed on a rigid schedule 3 times a day, 6 days a week. Dead fish were removed as soon as observed. Each trough was partially drained and cleaned daily to keep fecal material and excess food from accumulating. At each biweekly weighing (detailed weighing procedures described by Halver (9)), the troughs were emptied, scrubbed thoroughly and disinfected with a dilute chlorine solution. Caution was taken to disinfect any equipment common to more than one trough to prevent inadvertent transmittal of diseases.

At the termination of the experiments, 100 g of randomly selected fish were taken from each trough population for proximate analyses as outlined by Wood et al. (12). Similarly, 10 fish were selected at random from each trough for hematological studies in some trials (methods given by Hesser (13)).

RESULTS AND DISCUSSION

The quantitative leucine requirement. Results from this experiment (table 3 and fig. 1) indicated that the leucine require-

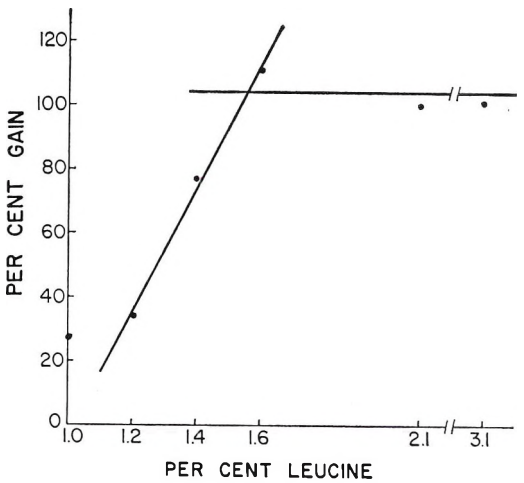

Fig. 1 The leucine requirement of chinook salmon. Growth response as percentage gain based on initial weights at different levels of leucine in the dry diet.

TABLE 3

Growth data for leucine [1]

Leucine in dry diet	No. fish [2]	Initial weight	Gain	Feed/g gain
%		g	%	g
1.00	200(0)	2.44	28	2.29
1.20	200(1)	2.44	34	1.71
1.40	200(2)	2.48	77	1.28
1.60	200(0)	2.48	111	1.11
2.10	200(4)	2.52	100	1.16
3.10	200(1)	2.58	101	1.19
Control diet [3]	200(4)	2.49	91	1.22

[1] Conducted for 8 weeks during the summer of 1959 with spring run salmon from Eagle Creek National Fish Hatchery, Estacada, Oregon.
[2] Numbers in parentheses indicate mortality.
[3] Contained an indispensable amino acid pattern similar to that of whole egg protein.

TABLE 4

Growth data for isoleucine [1] *(exp. 1)*

Isoleucine in dry diet	No. fish [2]	Initial weight	Gain	Feed/g gain
%		g	%	g
0.50	250(17)	1.00	80	2.04
0.70	250(18)	0.94	121	1.44
0.90	250(11)	0.97	142	1.35
1.10	250(16)	0.97	207	1.08
1.30	250(12)	0.96	162	1.28
1.50	250(13)	0.97	170	1.22
2.00	250(19)	0.96	147	1.38
Control diet [3]	250(17)	0.98	136	1.39

[1] Experiment conducted during summer of 1959 with fall run salmon from Willard National Fish Hatchery, Cook, Washington.
[2] Numbers in parentheses indicate mortality.
[3] Contained an indispensable amino acid pattern similar to that of whole egg protein.

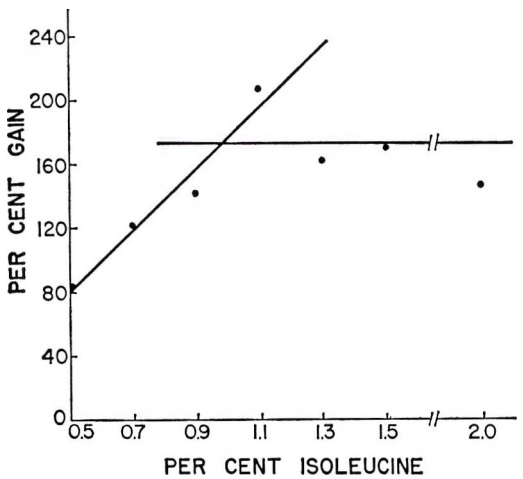

Fig. 2 The isoleucine requirement of chinook salmon studied in the presence of 3.68% of dietary leucine. Growth response as percentage gain based on initial weights at different levels of isoleucine in the dry diet.

ment of chinook salmon fingerlings was approximately 1.6% of the dry diet or 3.9% of the crude protein. When expressed as a percentage of the protein this is in good agreement with the leucine requirement of the pig but less than the leucine requirements for rats and chicks. For example, Eggert et al. (14) reported the leucine requirement for baby pigs was between 4.0 and 5.0%. Similarly, DeLong [7] noted that the minimal leucine requirement of the weanling pig was about 0.6% of the diet or 4.6% of the dietary protein. Rose et al. (15) suggested a minimal leucine requirement of 0.8% for the rat or approximately 7% of the protein in the diet. Similarly, Rama Rao et al. (16) reported the minimal requirement to be 0.7% of a 10% protein diet. The leucine requirement of starting chicks is listed by Bird et al. (17) as 1.4% of the diet or 7.0% of the protein.

The quantitative isoleucine requirement of chinook salmon. Three experiments were conducted to determine the isoleucine requirement at different dietary leucine levels. Results from experiment 1 (table 4 and fig. 2) indicated that the requirement was approximately 1.0% of the dry diet when the leucine level used was the same as that of the control diet (3.68%). Growth of fish was optimal when the intermediate levels of isoleucine, namely, 1.10, 1.30 and 1.50% were fed, but growth was suboptimal at the higher levels of 2.00 and 3.20% (the control diet level).

Four different combinations of isoleucine and leucine were fed to salmon fingerlings in experiment 2 (table 5). As expected, the low levels of isoleucine and leucine (0.50 and 1.00%, respectively) were insufficient to support good growth and feed efficiency. By keeping the isoleucine level at the approximate requirement level of 1.0%, it was confirmed that the leucine requirement did not exceed 1.60% since the 2 groups of fish receiving 1.60 and 2.10% leucine grew at nearly identical optimal rates. The salmon fed 1.00% isoleucine and 6.00% leucine grew more slowly than those fed the optimal levels of these amino acids, indicating the iso-

[7] DeLong, D. C. 1956 I. Classification of the essential amino acids for chinook salmon. II. Quantitative leucine requirement of the weanling pig. M.S. Thesis. Purdue University, Lafayette, Indiana.

TABLE 5

Growth data for isoleucine [1] *(exp. 2)*

Isoleucine and leucine in dry diet	No. fish [2]	Initial weight	Gain	Feed/g gain
%		g	%	g
0.50 Isoleucine, 1.00 leucine	250(18)	1.02	108	1.47
1.00 Isoleucine, 1.60 leucine	250(16)	1.00	176	1.15
1.00 Isoleucine, 2.10 leucine	250(13)	1.02	174	1.12
1.00 Isoleucine, 6.00 leucine	250(18)	1.00	159	1.25
Control diet [3]	250(17)	0.98	136	1.39

[1] Conducted for 10 weeks during summer 1959 with fall run salmon from Willard National Fish Hatchery, Cook, Washington.
[2] Numbers in parentheses indicate mortality.
[3] Contained an indispensable amino acid pattern similar to that of whole egg protein.

leucine requirement was probably increased by the high level of leucine, a level that was reported for some practical hatchery diets (18).

Experiment 3 was designed to study the isoleucine requirement at 2 dietary leucine levels. The low level, 1.50%, represented the approximate minimal requirement for leucine or just slightly less (see above), whereas the high level, 6.00%, represented an excess level that decreased growth when fed in conjunction with the minimal requirement level of isoleucine (see above). Results from this experiment (table 6 and fig. 3) indicated that the minimal isoleucine requirement was approximately 0.9% of the dry diet with the low leucine level and somewhat greater than 0.9% with the high leucine level. The detrimental influence of excess leucine was evident from the slower growth at all levels of isoleucine with the exception of the 1.50% level which was probably sufficient to overcome the adverse effects of excess leucine. From the growth curve of the high leucine regimen the isoleucine requirement appeared to be slightly greater than 0.9%, possibly closer to 1.0%. However, the erratic growth between the 0.9 and 1.3% isoleucine levels may have masked the actual requirement. For this reason it was useful to study other criteria such as the hematology data available on these fish. Plots of the isoleucine levels against the precentage hematocrits (fig. 4) for the 2 leucine regimens demonstrate the effect of excessive dietary leucine. The resulting curves show inflection points at 0.9% isoleucine for the low leucine level and 1.1% isoleucine for the high leucine level.

It was concluded that the minimal isoleucine requirement ranged from 0.9 to 1.1% of the dry diet depending upon the dietary leucine level. The isoleucine requirement appeared to be about 0.9% of the diet in the presence of 1.5% leucine, but was increased about 0.1% with every additional 2.3% of dietary leucine. Expressed in terms of the dietary protein, the requirement ranged from 2.2 to 2.7%, or approximately 2.5%. As a percentage of the protein in the diet, the isoleucine requirement of other species tends to be a little higher. For example, Brinegar et al. (19) reported the requirement of young pigs to be 0.7% of a 22% protein diet or 3.2% of the protein. Becker et al. (20) reported that the isoleucine requirement of pigs was dependent upon the protein level fed. At the 13.35% protein level the requirement was about 0.46 or 3.4% of the protein, whereas the requirement was about 0.65% at the 26.7% protein level or 2.4% of the dietary protein. The requirement of young chicks is listed at 0.6% of a diet containing 20% of protein or 3.0% of the protein (17). Rose et al. (15) suggested the isoleucine requirement for young rats to be 0.5% of a diet containing about 12 to 13% of protein.

The antagonistic effect of excess leucine on the isoleucine requirement of salmon is in agreement with the rat studies by Harper (21) in which the growth-depressing effect of 3.00% leucine added to a 9% casein diet could be alleviated with small supplements of isoleucine. However, Harper was unable to induce an imbalance by feeding the same level of leucine in conjunction with an 18% casein diet. It may be, then, that salmon finger-

TABLE 6

Growth data for isoleucine [1] *(exp. 3)*

Isoleucine in dry diet	No. fish [2]	Initial weight	Gain	Feed/g gain
%		g	%	g
	1.50% of leucine in diet			
0.50	250(4)	0.83	182	1.34
0.70	250(2)	0.80	257	1.25
0.90	250(3)	0.84	299	1.17
1.10	250(3)	0.81	301	1.19
1.30	250(9)	0.81	326	1.16
1.50	250(7)	0.80	302	1.20
Control diet [3]	250(1)	0.78	348	1.22
	6.00% of leucine in diet			
0.50	250(7)	0.79	157	1.30
0.70	250(6)	0.76	233	1.14
0.90	250(11)	0.78	278	1.20
1.10	250(11)	0.79	280	1.22
1.30	250(0)	0.80	314	1.17
1.50	250(1)	0.81	303	1.16
Control diet [3]	250(1)	0.78	348	1.22

[1] Conducted for 10 weeks during summer of 1960 with fall run salmon from Willard National Fish Hatchery, Cook, Washington.
[2] Numbers in parentheses indicate mortality.
[3] Contained an indispensable amino acid pattern similar to that of whole egg protein.

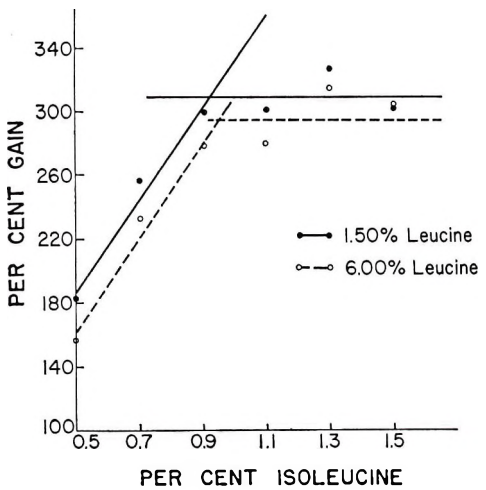

Fig. 3 The isoleucine requirement of chinook salmon studied in the presence of 1.50 and 6.00% dietary leucine. Growth response as percentage gain based on initial weights at different levels of isoleucine in the dry diet.

lings are more sensitive to amino acid imbalances than rats, particularly since the protein needs of salmon are greater (10).

Harper also observed that, in addition to isoleucine, small supplements of valine plus isoleucine were helpful in eliminating the harmful influence of excess leucine. It seems improbable, however, that valine could have been limiting in these animals since the valine level in the control diet was considerably higher than the minimal valine requirement of chinook salmon (see valine quantitation experiment).

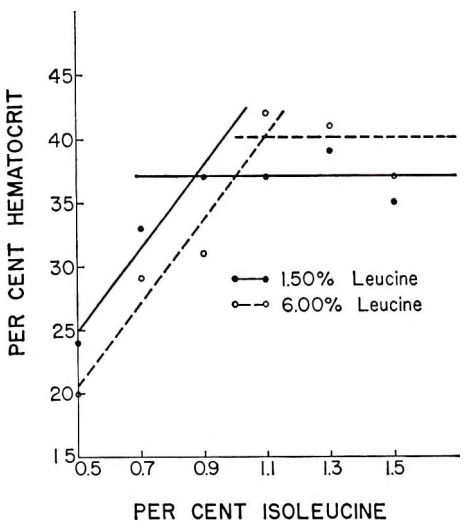

Fig. 4 The isoleucine requirement of chinook salmon studied in the presence of 1.50 and 6.00% dietary leucine. Percentage hematocrits at different levels of isoleucine in the dry diet.

Effect of excess isoleucine on the growth of chinook salmon. In view of the detrimental effects of excess dietary leucine on the isoleucine requirement of salmon, the opposite relationship was studied because of the structural similarity of these 2 amino acids. Interest in this relationship was also stimulated by the data from the isoleucine requirement studies which indicated that higher levels of isoleucine supported slower growth and poorer feed efficiencies. This may have also been responsible for the slower growth observed with the egg protein pattern diets used as controls in the various experiments. To study this relationship 3 levels of isoleucine (1.00, 3.00 and 5.00%) were fed to salmon receiving a slightly suboptimal level of leucine, or 1.25%. The 1.00% of isoleucine plus 1.25% of leucine treatment supported a gain slightly less than that of the egg protein pattern control diet, indicating the leucine level fed was below the requirement (table 7). However, growth with this treatment was 89% of that of fish fed the basal diet, whereas the 3.00 and 5.00% of isoleucine treatments supported growth that was only 67 and 60% of that with the control diet, respectively. This confirmed the suspicion that an imbalance of isoleucine with respect to leucine could be as harmful to good growth as an imbalance of leucine with respect to isoleucine. However, if the leucine level had been at, or close to, the minimal requirement level of 1.60%, this effect might not have been as profound. It seems possible that the leucine requirement may actually be less than 1.60% when a more favorable level of isoleucine is fed simultaneously, such as the minimal requirement level of 1.00% rather than the whole egg protein pattern level of 3.20% — the level present in the diets used for quantitating the leucine requirement.

Brinegar et al. (19) observed a growth depression in young pigs fed 2.08% of isoleucine. This level corresponds to the 3.00% level fed in this experiment providing the ratio between requirement level and level fed is considered. Also, Sauberlich (22) reported that the growth of rats was depressed 50% when a 6% casein control diet was supplemented with 5% of DL-isoleucine; similarly 5% of L-leucine depressed growth 55%.

The quantitative valine requirement. Resulting growth data from this study (table 8 and fig. 5) indicated that the valine requirement of salmon was about 1.3% of the dry diet which corresponds to 3.2% of the dietary protein. In terms of percentage of the protein this value is similar to that listed for other species. The valine requirement for weanling pigs was reported to be about 0.4% of the diet or 3.1% of the protein (23). The minimal requirement for the rat was suggested by Rose et al. (15) as 0.7% of the diet or about 5 to 6% of the protein. Bird et al. (17) list the requirement for chicks at 0.8% of the diet or 4.0% of the dietary protein.

Since valine, isoleucine and leucine are structurally similar, the valine requirement may have been affected by the relatively high levels of isoleucine and leucine (3.20 and 3.68%, respectively) in the egg protein pattern control diet. Thus, the valine requirement of salmon may be less than 1.3% of the dry diet when a more optimal combination of isoleucine and leucine is fed.

TABLE 7

Growth data for isoleucine: leucine ratios [1]

Isoleucine and leucine in dry diet	No. fish [2]	Initial weight	Gain	Feed/g gain
%		g	%	g
1.00 Isoleucine, 1.25 leucine	500(0)	0.69	242	1.32
3.00 Isoleucine, 1.25 leucine	500(13)	0.69	182	1.40
5.00 Isoleucine, 1.25 leucine	500(16)	0.69	164	1.40
Control diet [3]	500(3)	0.69	272	1.31

[1] Conducted for 10 weeks during summer of 1960 with fall run salmon from Willard National Fish Hatchery, Cook, Washington. Data represent average of 2 replicates.
[2] Numbers in parentheses indicate mortality.
[3] Contained an indispensable amino acid pattern similar to that of whole egg protein.

TABLE 8

Growth data for valine [1]

Valine in dry diet	No. fish [2]	Initial weight	Gain	Feed/g gain
%		g	%	g
0.65	500(9)	0.67	206	1.42
0.90	500(11)	0.67	244	1.40
1.15	500(5)	0.67	276	1.35
1.40	500(13)	0.67	290	1.32
1.65	500(8)	0.69	283	1.34
1.90	500(10)	0.67	285	1.32
Control diet [3]	500(3)	0.69	272	1.31

[1] Conducted for 10 weeks during summer of 1960 with fall run chinook salmon from Willard National Fish Hatchery, Cook, Washington. Data represent average of 2 replicates.
[2] Numbers in parentheses indicate mortality.
[3] Contained an indispensable amino acid pattern similar to that of whole egg protein.

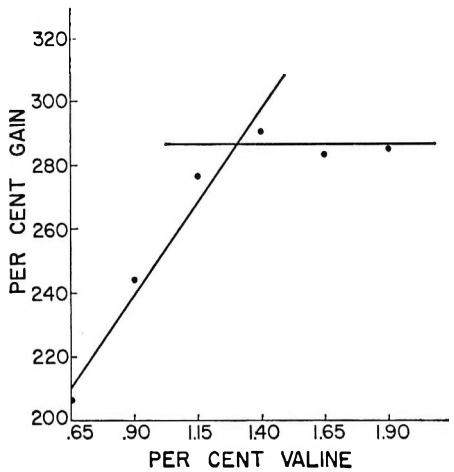

Fig. 5 The valine requirement of chinook salmon. Growth response as percentage based on initial weights at different levels of valine in the dry diet.

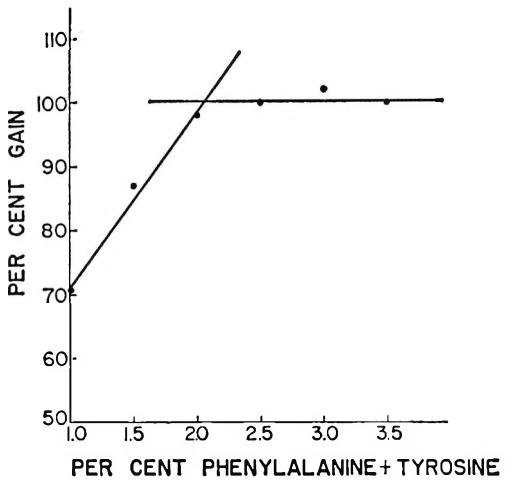

Fig. 6 The phenylalanine (plus tyrosine) requirement of chinook salmon. Growth response based on initial weights at different levels of phenylalanine and tyrosine in the dry diet.

TABLE 9

Growth data for phenylalanine [1]

Phenylalanine in dry diet [2]	No. fish [3]	Initial weight	Gain	Feed/g gain
%		g	%	g
0.96	200(3)	2.60	70	1.37
1.50	200(31) [4]	2.60	87	1.24
2.00	200(2)	2.62	98	1.15
2.50	200(4)	2.62	100	1.17
3.00	200(3)	2.62	102	1.17
3.50	200(4)	2.72	100	1.17
Basal diet [5]	200(4)	2.49	91	1.22

[1] Conducted for 8 weeks during summer of 1959 with spring run salmon from Eagle Creek National Fish Hatchery, Estacada, Oregon.
[2] Includes 0.35% of tyrosine in basal diet.
[3] Numbers in parentheses indicate mortality.
[4] High mortality results from fish jumping from unscreened trough during third week of experiment.
[5] Contained an indispensable amino acid pattern similar to that of whole egg protein.

The quantitative phenylalanine requirement. Results from this experiment indicated that phenylalanine plus tyrosine requirement was approximately 2.1% of the dry diet, or the phenylalanine requirement was 1.7% of the diet in the presence of 0.4% of tyrosine (table 9 and fig. 6). It has been assumed that tyrosine spared a part of the phenylalanine requirement as in other species. Expressed as a percentage of the protein, the combined phenylalanine plus tyrosine requirement was about 5.1%. In comparison, young chicks require 1.4% phenylalanine plus tyrosine in the diet or 7.0% of the dietary protein (17). Similarly, the requirement for the young rat has been reported by Rose and Womack (24) as 0.9% of the diet or about 6 to 7% of the protein. Mertz et al. (25) reported the requirement of weanling pigs to be about 0.5% of the diet or 3.6% of the total protein. Thus the combined phenylalanine plus tyrosine requirement of the chinook salmon fingerlings expressed as a percentage of the dietary protein appeared to be within the range of requirements reported for other species.

LITERATURE CITED

1. Halver, J. E., D. C. DeLong and E. T. Mertz 1957 Nutrition of salmonoid fishes. V. Classification of essential amino acids for chinook salmon. J. Nutrition, *63:* 95.
2. Halver, J. E., and W. E. Shanks 1960 Nutrition of salmonoid fishes. VIII. Indispensable amino acids for sockeye salmon. J. Nutrition, *72:* 340.
3. DeLong, D. C., J. E. Halver and E. T. Mertz 1962 Nutrition of salmonoid fishes. X. Quantitative threonine requirements of chinook salmon at two water temperatures. J. Nutrition, *76:* 174.
4. Nicolaides, N., and A. N. Woodall 1962 Impaired pigmentation in chinook salmon fed diets deficient in essential fatty acids. J. Nutrition, *78:* 431.
5. Spackman, D. H., W. H. Stein and S. Moore 1958 Automatic recording apparatus for use in chromatography of amino acids. Analyt. Chem., *30:* 1190.
6. Spies, J. R., and D. C. Chambers 1948 Chemical determination of tryptophan. Analyt. Chem., *20:* 30.
7. Spies, J. R., and D. C. Chambers 1949 Chemical determination of tryptophan in proteins. Analyt. Chem., *21:* 1249.
8. Halver, J. E. 1957 Nutrition of salmonoid fishes. III. Water-soluble vitamin requirements of chinook salmon. J. Nutrition, *62:* 225.
9. Halver, J. E. 1957 Nutrition of salmonoid fishes. IV. An amino acid test diet for chinook salmon. J. Nutrition, *62:* 245.
10. DeLong, D. C., J. E. Halver and E. T. Mertz 1958 Nutrition of salmonoid fishes. VI. Protein requirements of chinook salmon at two water temperatures. J. Nutrition, *65:* 589.
11. DeLong, D. C., J. E. Halver and E. T. Mertz 1959 Nutrition of salmonoid fishes. VII. Nitrogen supplements for chinook salmon diets. J. Nutrition, *68:* 663.
12. Wood, E. M., W. T. Yasutake, A. N. Woodall and J. E. Halver 1957 The nutrition of salmonoid fishes. I. Chemical and histological studies of wild and domestic fish. J. Nutrition, *61:* 465.
13. Hesser, E. F. 1960 Methods for routine fish hematology. Prog. Fish-Culturist, *22:* 164.
14. Eggert, R. G., H. H. Williams, B. E. Scheffy, E. G. Sprague, J. K. Loosli and L. A. Maynard 1954 The quantitative leucine requirement of the suckling pig. J. Nutrition, *53:* 177.
15. Rose, W. C., L. C. Smith, M. Womack and H. Shane 1949 The utilization of the nitrogen of ammonium salts, urea and certain other compounds in synthesis of nonessential amino acids *in vivo.* J. Biol. Chem., *181:* 307.
16. Rama Rao, P. B., V. Chalam Metta and B. C. Johnson 1959 The amino acid composition and the nutritive value of proteins. I. Essential amino acid requirements of the growing rat. J. Nutrition, *69:* 387.
17. Bird, H. R., H. J. Almquist, D. R. Clandinin, W. W. Cravens, F. W. Hill and J. McGinnis 1960 Nutrient requirements of poultry, pub. 827. National Research Council — National Academy of Sciences, Washington, D. C.
18. Shanks, W. E., and J. E. Halver 1960 Rapid Analysis of the amino acid content of fish diets. Prog. Fish-Culturist, *22:* 51.
19. Brinegar, M. J., J. K. Loosli, L. A. Maynard and H. H. Williams 1950 The isoleucine requirement for the growth of swine. J. Nutrition, *42:* 619.
20. Becker, D. E., A. H. Jensen, S. W. Terrill, I. D. Smith and H. W. Norton 1957 The isoleucine requirement of weanling swine fed two protein levels. J. Animal Sci., *16:* 26.
21. Harper, A. E. 1958 Balance and imbalance of amino acids. Ann. N. Y. Acad. Sci., *69:* 1025.
22. Sauberlich, H. E. 1961 Studies on the toxicity and antagonism of amino acids for weanling rats. J. Nutrition, *75:* 61.
23. Jackson, H. D., E. T. Mertz and W. M. Beeson 1953 Quantitative valine requirement of the weanling pig. J. Nutrition, *51:* 109.
24. Rose, W. C., and M. Womack 1946 The utilization of the optical isomers of phenylalanine, and the phenylalanine requirement for growth. J. Biol. Chem., *166:* 103.
25. Mertz, E. T., J. N. Henson and W. M. Beeson 1954 Quantitative phenylalanine requirement of the weanling pig. J. Animal Sci., *13:* 927.

Effect of Protein Intake on the Ribonucleic Acid of Liver Cell Sap [1]

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ABSTRACT The RNA content and amino acid-incorporating capacity of rat liver cell sap was studied with 3 groups of animals, (a) rats that had been fed a protein-free diet, (b) rats in the post-absorptive state after receiving a diet of adequate protein content, and (c) rats actively absorbing amino acids from a recent meal of protein. Cell sap prepared from animals of the second dietary group had a higher RNA content than that from either of the other groups, but showed a reduced capacity to incorporate leucine-C^{14} in relation to the amount of RNA present. By centrifugation for 3 hours at 105,000 $\times$ g, a precipitate (post-microsomal fraction) was isolated from the cell sap. The effect of protein intake on the RNA content and amino acid-incorporating capacity of cell sap was shown to be due entirely to changes occurring in this post-microsomal fraction. These changes may be the effect of accumulation in the post-microsomal fraction of breakdown products from the endoplasmic reticulum, which is known to be sensitive to protein intake.

When rats are fasted (1) or are given a protein-free diet (2, 3) a considerable part of the RNA of the liver is lost during the first 2 days of protein deficiency. Thereafter, the RNA content of the liver tends to become constant at a lower level. Munro and Clark (4) concluded that the labile RNA thus lost from the liver is derived from the endoplasmic reticulum, which has been shown by electron microscopy to diminish with protein deficiency (5, 6). Immediately after removing protein from the diet and coincident with the rapid loss of liver RNA, the uptake of labeled precursors of RNA is greatly reduced (7). It has therefore been suggested that fragments of RNA released from the disintegrating endoplasmic reticulum into the cell sap are responsible for diluting the labeled precursors. When protein is once more fed to such animals, uptake of labeled precursors by liver RNA is greatly increased and this has been taken to indicate a cessation of the breakdown of the reticulum (7).

If this interpretation of the response of liver RNA metabolism to protein intake is correct, the RNA of the liver cell sap may show alterations resulting from variations in the rate of breakdown of endoplasmic reticulum with diet. A study was therefore made of the influence of diet on the RNA of cell sap prepared by centrifuging a homogenate of liver for one hour at 105,000 $\times$ g. This was then fractionated by centrifugation for a further 3 hours at 105,000 $\times$ g, which precipitated a pellet, the post-microsomal fraction (8). The results show that the level of protein in the diet does not affect the amount of RNA in the final supernatant fraction from the 3-hour separation procedure or the capacity of this RNA to accept activated amino acids. However, variations in protein intake influence the amount and properties of the RNA in the post-microsomal fraction. It has been concluded that part of the RNA of the post-microsomal fraction may include the products of RNA breakdown coming from other parts of the liver cell. A preliminary report of these observations has appeared (9).

METHODS

Animals and diets. Male albino rats of about 180 g were caged separately and fed the quantities of protein-free or protein-containing diets described previously (10). The total daily food intake per rat provided 8.0 g carbohydrate, and 0.5 g fat for the protein-free diet and 5.6 g carbohydrate, 2.4 g protein and 0.5 g fat for the protein-containing diet; both diets contained ade-

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quate quantities of vitamins and minerals. These diets were fed twice daily in fixed amounts. The morning meal contained carbohydrate, fat and vitamins and the evening meal contained carbohydrate, fat and protein; for animals fed the protein-free regimen, carbohydrate was substituted isocalorically for the protein of this meal. The animals quickly learned to consume each meal promptly. After the animals had been fed the diet for 5 days, they were killed in the morning in the fasting state, the last meal of the protein-containing or protein-free diet having been consumed 18 hours before death except for some of the rats fed the protein-containing diet which were given 2.5 g of casein 1.5 hours before killing. In this way, 3 dietary groups were obtained: (1) rats in a depleted condition as the result of eating a protein-free diet, (2) rats in the post-absorptive state after a diet of adequate protein content, and (3) rats actively absorbing amino acids after a recent meal of protein.

Preparation of liver-cell fractions. Livers from 3 to 4 rats fed each diet were pooled, suspended in 2.5 volumes of the medium of Rendi and Campbell (11) and disintegrated in a Potter-Elvehjem (12) homogenizer. The homogenate was then fractionated to obtain quantitative recovery of the cell sap. Unbroken cells, nuclei and mitochondria were removed by centrifuging for 10 minutes at 6500 × *g*; the supernatant fluid obtained was then centrifuged at 105,000 × *g* for one hour in the Spinco Model L ultracentrifuge (Rotor 40) to precipitate the microsome fraction. This second supernatant fluid is referred to in the text as the "one-hour cell sap." In most of the experiments, the one-hour cell sap was centrifuged for a further 3 hours at 105,000 × *g* to yield a precipitate, the "post-microsomal pellet" and a final supernatant fluid, the "3-hour cell sap." Finally, the surface of the post-microsomal pellet was washed with homogenization medium and the fraction suspended in a small volume of the same medium for use.

In one series of experiments, 2 microsome fractions (light and heavy) were prepared by removing debris, nuclei and mitochondria as above and then centrifuging the homogenate for one hour at 18,000 × *g* to yield a pellet of heavy microsomes and finally centrifuging the supernatant fluid at 105,000 × *g* for one hour to give a sediment of light microsomes (13).

Measurement of protein and RNA content of fractions. Protein was estimated by the procedure of Lowry et al. (14) and RNA by ultraviolet absorption at 260 mμ after treating the fractions with cold 0.4 N $HClO_4$ to remove soluble nucleotides, followed by 2 extractions with 0.4 N $HClO_4$ at 70°C for 20 minutes to extract the RNA. The total RNA content of the cell fractions is expressed per 100 g initial body weight of the animals to allow for differences in the size of the livers at the start of the experimental diets.

Uptake of labeled amino acids by the cell fractions. The one-hour cell sap and the 3-hour cell sap preparations and the suspension of post-microsomal pellet in medium were each adjusted to pH 5.2 by the addition of N acetic acid (15). Such a procedure gives an active preparation of activating enzymes and transfer RNA from cell sap (15). The precipitate formed was separated centrifugally and redissolved in the homogenization medium. Portions of the pH 5 precipitates from the appropriate source containing equal amounts of RNA (about 100 to 200 μg in different experiments) were placed in incubation tubes, to which were added 10 μmoles ATP [2] and 1 μc DL-leucine-C^{14} [3] and the volume made to 1 ml by the addition of homogenization medium. After incubation at 37° for 10 minutes the reaction was terminated by chilling in ice, followed by the addition of 5 volumes of 0.4 N $HClO_4$ at 0°. The precipitate was washed 3 times with cold 0.2 N $HClO_4$, twice with ethanol:ether:chloroform (2:2:1) and finally with ether. Thereafter, the precipitate was allowed to dry in air at room temperature (11).

The dry lipid-extracted residue was then used for the measurement of (a) the total uptake of leucine-C^{14}, (b) the uptake into protein (the activity remaining after extraction with hot $HClO_4$), and (c) the amount of RNA left at the end of incubation. To measure the *total* leucine-C^{14} uptake, the residue left after lipid extraction was dissolved in about 0.1 ml of N NaOH and transferred quantitatively, with wash-

[2] Disodium salt, Sigma Chemical Company, St. Louis.
[3] Radiochemical Centre, Amersham.

ings, to a lens-paper disc on a stainless steel planchet. In confirmation of Garrow and Piper (16), this technique gave a constant self-absorption with samples of differing protein content. To obtain the amount of leucine incorporated into the protein fraction, parallel samples were incubated as above and the dry lipid-free powder obtained after treatment with fat solvents was extracted twice with 0.4 N $HClO_4$ for 20 minutes at 70°. The protein residue remaining after this extraction was dissolved in 0.1 ml N NaOH and plated as above. The absorption of the perchloric acid extract was measured at 260 mμ to give the amount of RNA present at the end of incubation.

The planchets were counted to 1000 counts using an end-window Geiger-Müller counter in the first series of experiments (table 1) and a Nuclear-Chicago windowless counter in subsequent experiments. Activities have been expressed as counts/minute/milligram RNA remaining at the end of incubation.

TABLE 1

Effect of protein intake on the amount of RNA in one-hour cell sap prepared from rat liver and on the capacity of cell sap to accept leucine-C^{14}.

Dietary group [1]	RNA/100 g body wt	Uptake of leucine-C^{14} by pH 5 enzyme [2]
	mg	*count/min/mg RNA*
Protein-depleted	2.41	460
Adequate protein (fasting 18 hr)	3.03	360
Adequate protein (fed protein 2 hr)	2.60	470

[1] The results are the mean data from 5 animals in each dietary group.

[2] A Geiger-Müller end-window counter was used for radioactive assays. Uptake of leucine-C^{14} per milligram RNA was significantly influenced by diet ($P < 0.05$ by analysis of variance). From the residual error variance, the standard error per group was computed to be ± 29; consequently the second dietary group differs significantly from the other two, which are not significantly different from one another.

Chromatography of RNA on Ecteola columns. The RNA from each fraction was extracted by the modified phenol procedure proposed by Hoagland et al. (17). The extracted RNA was applied to a column 3.5 cm by 1.5 cm of Ecteola [4] (capacity 0.41 mEq/g) by the technique of Goldthwait (18). The fractions of RNA were eluted successively with an increasing gradient of NaCl in phosphate buffer (240 ml) followed by 50 ml of M NaCl, then a gradient of NH_4OH in phosphate buffer (200 ml) and finally 50 ml of N NaOH. Approximately 600 μg of RNA were applied to each column and the ultraviolet absorption was measured on 5 ml aliquots of the eluates.

Nucleotide composition of RNA. The nucleotide composition of RNA from cell sap and post-microsomal pellet was examined in relation to the diet previously fed to the rats. Specimens of RNA prepared by the phenol procedure were hydrolyzed in 0.3 N KOH for 18 hours at 37° and the nucleotides were separated by the paper chromatographic method of Lipshitz and Chargaff (19). The amounts of each nucleotide were then measured by ultraviolet absorption at 2 wavelengths on eluates from the paper.[5]

RESULTS AND DISCUSSION

On the basis of previous studies (4, 7), it is believed that part of the RNA in the liver cells of previously well-fed animals becomes unstable when they are given a protein-free diet or are fasted for a few hours. This unstable RNA disappears after a few days of protein depletion or during the period when protein is being absorbed from the alimentary tract after a meal. The unstable RNA involved in these dietary responses is believed to be associated with the endoplasmic reticulum (4). The experiments reported below were carried out on protein-depleted rats, rats fasting after an adequate intake of protein and rats absorbing a meal of protein.

Experiments with one-hour cell sap. Table 1 shows the effect of protein intake on the RNA of cell sap prepared by centrifuging liver homogenates for one hour at 105,000 × *g* ("one-hour cell sap"). The total amount of RNA recovered in the cell sap was somewhat greater in the group of rats fasting after receiving a protein-containing diet than for either the protein-depleted animals or the animals fed protein shortly before death. Rats fasting for a few hours after receiving a diet containing

[4] Ecteola, Serva, Heidelberg.

[5] We are indebted to G. C. Barr for making these results available to us.

protein lose RNA rapidly from their livers (2, 3), and the additional cell sap RNA may represent an accumulation of breakdown products.

When pH 5 enzyme preparations made from cell sap of the different dietary groups were incubated with leucine-C^{14}, uptake was almost exclusively associated with the RNA of the fraction, since it could be extracted with hot $HClO_4$. Incorporation per milligram RNA varied with diet, but in the opposite direction to the changes in the amount of RNA (table 1). It was significantly less for the rats fasting after receiving a protein-containing diet, the decrease being of the same order of magnitude as the increase in the amount of RNA in the cell sap of this group.

It was concluded that the RNA in the one-hour cell sap of animals fasting after receiving a diet adequate in protein is diluted with extra RNA of a kind which does not accept activated amino acids.

Experiments with 3-hour cell sap and post-microsomal pellet. Cell sap prepared by centrifuging a rat liver homogenate for one hour at 105,000 × *g* is known to be heterogeneous (8, 17). By centrifuging one-hour cell sap for 3 hours at 105,000 × *g*, a post-microsomal pellet can be sedimented (8), leaving a supernatant fluid, referred to here as "3-hour cell sap." In view of the effect of protein intake on the RNA of one-hour cell sap, the action of diet on the RNA of each of its components was examined. Table 2 shows that the amount of RNA in the 3-hour cell sap does not vary significantly with diet, but that the RNA sedimented in the post-microsomal pellet is greater for animals fasting after a protein-containing diet than for the other 2 groups. Consequently, diet has a significant influence on the ratio of post-microsomal RNA to 3-hour cell-sap RNA (table 2).

Uptake of leucine-C^{14} by pH 5 fractions prepared from the post-microsomal fraction and from the 3-hour cell sap was examined under the same conditions as those used for studying the uptake of leucine by the pH 5 fraction from one-hour cell sap. Protein intake did not influence the capacity of the pH 5 fraction derived from the 3-hour cell sap to accept leucine-C^{14} (table 3).

The pH 5 precipitate from the post-microsomal fraction also took up the labeled amino acid but to a much smaller extent. This uptake was not the result of contaminating cell sap, since it could not be eliminated by resuspension and reprecipitation at 105,000 × *g*. Since more than 90% of the activity incorporated into the fraction could be extracted with hot $HClO_4$, it was assumed to be associated with post-microsomal RNA, which is also extracted by this procedure. In contrast with the observations with 3-hour cell sap, incorporation by the post-microsomal fraction was affected significantly by diet (table 3). Uptake of leucine was greater for the post-microsomal pellet prepared from the protein-depleted rats than for the other 2 dietary groups, both of which were receiving diets containing protein. It therefore appears probable that the additional RNA in the post-microsomal pellets of the protein-fed groups (table 2) is due to the presence of a different species of RNA which dilutes their capacity to accept leucine-C^{14}. In confirmation of this, the *total* capacity of the post-microsomal RNA

TABLE 2

Effect of protein intake on the amount of RNA in 3-hour cell sap and post-microsomal pellet

Dietary group [1]	RNA in fraction/100 g body wt		
	3-hr cell sap	Post-microsomal	Post-microsomal / 3-hr cell sap [2]
	mg	*mg*	
Protein-depleted	3.19	0.88	0.28
Adequate protein (fasting 18 hr)	3.17	1.31	0.41
Adequate protein (fed protein 2 hr)	3.65	1.23	0.33

[1] The results are the mean data from three animals in each dietary group.

[2] The ratio of post-microsomal RNA to cell sap RNA is significantly affected by diet ($P < 0.02$ by analysis of variance). From the residual error variance, the standard error per group was computed to be ± 0.02; consequently, the second dietary group ratio differs significantly from the other two, which are not significantly different from one another.

TABLE 3

Effect of protein intake on the uptake of leucine-C^{14} by the pH 5 fractions of 3-hour cell sap and post-microsomal pellet [1]

Dietary group [2]	Specific activity of fraction		Total activity of fraction [4]	
	3-hr cell sap	Post-microsomal pellet [3]	3-hr cell sap	Post-microsomal pellet
	count/min/mg RNA		*count/min/total fraction*	
Protein-depleted	4580(143)	970(110)	14,600(455)	850(97)
Adequate protein (fasting)	4500(165)	640(88)	14,300(520)	840(73)
Adequate protein (fed)	4040(118)	670(118)	14,800(450)	830(86)

[1] The conditions of the experiment were identical to those used in table 1 except that a Nuclear Chicago windowless gas-flow automatic counter was used. The numbers in parentheses are the amounts of radioactivity remaining after extraction with hot $HClO_4$ expressed as count/min/mg RNA extracted.

[2] Each entry represents the mean result obtained from 3 experiments, in each of which the livers of 3 to 4 rats fed each diet were pooled.

[3] Diet had a significant effect on the specific activity of the post-microsomal fraction ($P < 0.01$ by analysis of variance). This is due to a significant difference between the specific activity of the first dietary group and that of the other 2 groups, which do not differ from one another.

[4] The total activity of each fraction was obtained by multiplying the specific activity by the amount of RNA in the fraction per 100 g body weight. There were no significant effects of diet on the total activity.

TABLE 4

Comparison of the effect of protein intake on the uptake of leucine-C^{14} by post-microsomal pellet and by two microsomal fractions

Dietary group [1]	RNA recovered [2]		
	Post-microsomal pellet	Light microsomes	Heavy microsomes
	count/min/mg	*count/min/mg*	*count/min/mg*
Protein-depleted	1,120	303	201
Adequate protein (fasting)	422	456	188
Adequate protein (fed)	930	359	215

[1] The results are the mean data from 2 animals fed each diet.

[2] Diet had no significant effect on the incorporating capacity of either of the microsomal fractions.

to accept labeled leucine (obtained by multiplying the quantity of RNA in the pellet by its specific activity after incubation with leucine-C^{14}) was not affected by the dietary conditions (table 3). These changes in the amount and the specific activity of the post-microsomal RNA with different diets are adequate to explain the observations (table 1) made with the one-hour cell sap preparations, which include this material.

Before carrying out further investigations of the properties of the post-microsomal pellet, it was of importance to establish whether this variation in ability to incorporate leucine with changes in diet was confined to the post-microsomal fraction. In table 4, the capacity of post-microsomal RNA to incorporate leucine is compared with that of a heavy and of a light microsome preparation. Uptake by the post-microsomal fraction is greater than by the microsome fractions and is the only cell fraction to show variations with previous diet.

Effect of diet on the molecular heterogeneity of post-microsomal RNA. RNA, extracted from the post-microsomal fraction by the phenol method (17), was resolved by chromatography on columns of Ecteola, using successively a gradient of NaCl in phosphate buffer, M NaCl, a gradient of NH_4OH in phosphate buffer and finally a solution of N NaOH. For the protein-depleted animals, the post-microsomal RNA showed only 2 peaks of ultraviolet absorption, one in the NH_4OH gradient and one in the NaOH eluate, a pattern similar to that shown by microsomal RNA. The RNA extracted from the post-microsomal pellet of animals fed the

TABLE 5

Nucleotide composition of RNA from post-microsomal pellet

Dietary group [1]	Nucleotide content				
	Adenylic acid	Guanylic acid	Cytidylic acid	Uridylic acid	Pseudo-uridylic acid [2]
	%	%	%	%	%
Protein-depleted	18.8	32.1	29.7	17.7	2.0
Adequate protein (fasting)	19.6	31.6	30.1	17.7	1.6
Adequate protein (fed)	18.8	31.3	29.6	18.5	2.0

[1] The results are the mean data from 5 animals in each dietary group.
[2] The effect of diet on the pseudouridylic acid content of post-microsomal RNA is statistically significant ($P < 0.05$). From the residual error variance, the standard error per group was computed to be $\pm$ 0.1; consequently, the second dietary group differs significantly from the other two, which were not significantly different from one another.

protein-containing diet was more heterogeneous, usually showing ultraviolet absorbing material distributed throughout most of the NaCl gradient and the whole of the NH_4OH gradient; this pattern appeared especially in animals fasting after receiving the protein-containing diet. This additional heterogeneous ultraviolet-absorbing material probably represents oligonucleotides and RNA fragments, an observation which is in agreement with our hypothesis (7) that, when an animal is fasted after receiving a diet adequate in protein content, the endoplasmic reticulum breaks down and releases fragments of RNA.

Effect of diet on the nucleotide composition of post-microsomal RNA. Changes in the RNA of the post-microsomal fraction after feeding different diets were also explored by examining the nucleotide composition of the RNA. The post-microsomal RNA contained an appreciable amount of pseudouridylic acid (table 5), which was greater than the content in microsomal RNA but less than that of cell sap RNA (20). The pseudouridylic acid content of post-microsomal RNA changes significantly with diet, being lowest in animals fasting after a protein-containing diet. This dietary group also had the largest amount of RNA in the post-microsomal fraction (table 2), and it may be concluded that the extra RNA is low in pseudouridylic acid. It is therefore relevant to note that an RNA species associated with the membranous portion of the microsomes is devoid of this nucleotide (20). This observation is compatible with the thesis that the variable RNA component of the post-microsomal fraction comes from breakdown of endoplasmic reticulum.

LITERATURE CITED

1. Davidson, J. N. 1947 Some factors influencing the nucleic acid content of cells and tissues. Cold Spring Harbor Symp. Quant. Biol., *12:* 50.
2. Kosterlitz, H. W., and R. M. Campbell 1948 The effect of dietary protein on the turnover of phospholipids, ribonucleic acid, and deoxyribonucleic acid in the liver. J. Biol. Chem., *175:* 989.
3. Munro, H. N., D. J. Naismith and T. W. Wikramanayake 1953 The influence of energy intake on ribonucleic acid metabolism. Biochem. J., *54:* 198.
4. Munro, H. N., and C. M. Clark 1960 The nutritional regulation of ribonucleic acid metabolism in the liver cell. Proc. Nutrition Soc., *19:* 55.
5. Fawcett, D. W. 1955 Observations on the cytology and electron microscopy of hepatic cells. J. Nat. Canc. Inst., *15:* 1475.
6. Bernhard, W., and C. Rouiller 1956 Close topographical relationship between mitochondria and ergastoplasm of liver cells in a definite phase of cellular activity. J. Biophys. Biochem. Cytol., 2 (suppl.): 73.
7. Clark, C. M., D. J. Naismith and H. N. Munro 1957 The influence of dietary protein on the incorporation of ^{14}C-glycine and ^{32}P into the ribonucleic acid of rat liver. Biochim. Biophys. Acta, 23: 587.
8. Goldthwait, D. A. 1959 Metabolic studies in vitro of two ribonucleic acid fractions from rat liver. J. Biol. Chem., 234: 3251.
9. Prosser, E. J. T., A. C. Mallinson and H. N. Munro 1960 The effect of protein intake on the ribonucleic acid of liver cell sap. Biochem. J., 77: 24P.
10. Munro, H. N., and D. J. Naismith 1953 The influence of energy intake on protein metabolism. Biochem. J., 54: 191.
11. Rendi, R., and P. N. Campbell 1959 The role of cytoplasmic ribonucleic acid in the incorporation of amino acids into microsomal proteins. Biochem. J., 72: 435.
12. Potter, V. R., and C. A. Elvehjem 1936 A modified method for the study of tissue oxidations. J. Biol. Chem., *114:* 495.
13. Douglas, T. A., and H. N. Munro 1959 The occurrence of inactive amylase within the

pancreatic cell and its significance. Exp. Cell Res., *16:* 148.

14. Lowry, O. H., H. J. Rosebrough, A. L. Farr and R. J. Randall 1951 Protein measurement with the Folin phenol reagent. J. Biol. Chem., *193:* 265.
15. Keller, E. B., and P. C. Zamecnik 1956 The effect of guanosine diphosphate and triphosphate on the incorporation of labeled amino acids into proteins. J. Biol. Chem., *221:* 45.
16. Garrow, J., and E. A. Piper 1955 A simple technique for counting milligram samples of protein labelled with ^{14}C or ^{35}S. Biochem. J., *60:* 527.
17. Hoagland, M. B., M. L. 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.
18. Goldthwait, D. A. 1959 Isolation and characterization of ribonucleic acid fractions from rat liver. J. Biol. Chem., *234:* 3245.
19. Lipschitz, R., and E. Chargaff 1960 Studies on the fractionation of ribonucleic acid. Biochim. Biophys. Acta, *42:* 544.
20. Goswami, P., G. C. Barr and H. N. Munro 1962 The origin of different types of RNA in rat liver microsomes. Biochim. Biophys. Acta, *55:* 408.

Biochemical Changes in Progressive Muscular Dystrophy

II. PHOSPHORUS METABOLISM IN NORMAL, NUTRITIONAL AND HEREDITARY DYSTROPHIC MUSCLES, LIVERS AND BRAINS OF ANIMALS [1]

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ABSTRACT The distribution pattern of total phosphorus (TP), total inorganic phosphorus (TPi), total acid-soluble phosphorus (TASP), total acid-insoluble phosphorus (TAIP), adenosine triphosphatase, and creatine in the muscle, liver and brain of experimental animals with nutritional (vitamin E) and hereditary dystrophy was studied. ATP and creatine phosphate concentration was greatly reduced in the muscle of dystrophic animals. In contrast, ATPase activity in this tissue increased considerably; in the liver, ATP decreased but the concentration of creatine increased markedly. In the brain of these dystrophic animals, these concentrations did not undergo any significant changes. TP, TPi, TASP and TAIP also increased to various degrees in the muscles of these dystrophic animals, depending upon the severity of the disease and also on the origin of the disease, namely, whether the animal had nutritional or hereditary dystrophy. In the liver of the dystrophic rabbit, TP, TPi, TASP and TAIP showed considerable increases in concentration; on the other hand, in the liver of dystrophic mice, TP, TASP and TAIP decreased and TPi increased. In the brain of rabbits with nutritional dystrophy, the concentration of these was also elevated, but to a much lesser extent; whereas in the brain of severely affected mice, TP and TAIP showed considerable increases with no significant change in TPi and TASP.

Numerous studies on phosphorus metabolism in the muscles of all laboratory animals with nutritional dystrophy show without exception an abnormal phosphorous metabolism in the affected muscle (1–5). Similar studies on the muscles of mice with hereditary dystrophy are few and less informative. Therefore, we have undertaken an investigation of the distribution pattern of phosphorus in various acid-soluble and acid-insoluble fractions of skeletal muscles of rabbits severely affected by a vitamin E-deficient diet. For comparison, we also studied mice with muscular dystrophy, both dietary and hereditary.

High energy phosphate compounds such as adenosine triphosphate (ATP) and creatine phosphate (CP) are important in supplying and storing energy for muscle contraction. Any measurable diminution in the concentrations of ATP and CP in the muscles and livers of affected animals might be construed as a gradual impairment of ATP and CP synthesis in these tissues during the progress of the disease. Variations in the activity of ATPase also have been investigated, since this enzyme may control the contraction and relaxation of muscle (6–7). The results of our experiments are described in the present paper.

MATERIAL AND METHODS

Twenty young white rabbits of New Zealand strain, weighing approximately 1000 g each, were kept separately in metabolic cages and fed a vitamin E-deficient diet; the composition of the diet has been previously described by Srivastava et al. (8). The progress of the disease was followed routinely by measuring the urinary excretion of creatine and creatinine. The same vitamin E-deficient diet was supplied to a control group of 10 rabbits of approximately the same age and weight, except

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that a supplement of an adequate amount of vitamin E, was added.

At regular intervals during the entire period of 26 days (26 to 28 days are generally required for complete development of dystrophy) 2 rabbits from each group (experimental and control) were decapitated. Roughly 1 to 2 g of skeletal muscle (hind leg), liver and brain of each animal were quickly removed, blotted between filter paper, freed of connective tissue, fat, et cetera, and then chilled in ice; 0.5 g of each of these tissues was separately homogenized in 10 ml of 0.85% cold NaCl solution at 0°C in a Virtis homogenizer for 3 minutes at 5000 rpm. An ice bath was used to prevent the temperature from exceeding 2°C. The tissues were homogenized for 30 seconds, then allowed to cool for 30 seconds, and the cycle was repeated until a total homogenization time of 3 minutes had been completed. For the homogenization of liver and brain, a Dounce homogenizer was used. The same quantity of tissue in the same volume of cold NaCl was homogenized at 0°C. To each 5 ml of these homogenates an equal volume of 10% cold trichloroacetic acid was added, the mixture was allowed to stand for 2 hours in a refrigerator and then was centrifuged at 0°C for 10 minutes in an International centrifuge at 3000 rpm. The TCA extract thus obtained was used to determine total inorganic phosphorus (TPi),[3] total acid-soluble phosphorus (TASP), 7-minute hydrolyzable phosphorus and creatine. The TPi was determined directly from the TCA extract; for TASP, an aliquot of this TCA extract was digested with 5 N H_2SO_4, made up to 10 ml and then a part of this was used for phosphorus determination. One milliliter of the homogenate was digested with 36 N H_2SO_4 for total phosphorus (TP); for total acid-insoluble phosphorus (TAIP) determinations, the residue remaining after the addition of TCA to the homogenate was washed twice with 5% cold TCA, and then digested with concentrated 36 N H_2SO_4. The 7-minute hydrolyzable phosphorus was determined by heating an aliquot of the same TCA extract for 7 minutes in a boiling water bath prior to phosphorus analysis (9). In all cases phosphorus was determined by the method of King (10) and creatine was estimated by the diacetyl reaction (11).

In a second series of experiments 20 male mice,[4] 30 to 90 days old, having hereditary dystrophy, were so chosen that 10 of them exhibited a 50 to 60% [5] degree of the disease and the other ten, 90 to 95% [6] dystrophy. Ten male normal littermates of the same age served as controls. All received commercial mouse pellets [7] and water. Two from each of the 2 groups of experimental animals and two from the control group were killed on alternate days. The liver, muscle and brain from these animals were removed and homogenized in an Potter-Elvehjem homogenizer at 0°C and used for the determination of TP, TAIP, TPi, TASP, ATP and creatine.

For ATPase activity determination in the muscles of the rabbits and mice with different degrees of nutritional and hereditary dystrophy, respectively, 1.0 g of muscle was homogenized in 10 ml of 0.25 M sucrose in Virtis homogenizer and then filtered twice through 4 layers of fine cheesecloth. The homogenate was centrifuged at 600 × *g* for 10 minutes in an International centrifuge to remove the unbroken cells, nuclear materials, et cetera, and then centrifuged again at 2000 × *g* at 0°C for 10 minutes. The sediment, consisting largely of mitochondria, was suspended in cold glass-distilled water, subjected to freezing and thawing 3 times and then was used for the determination of ATPase activity. The ATPase activity was measured by the method described by Sarkar et al. (12). Protein was determined by the method of Lang (13).

RESULTS

Changes in concentration of total phosphorus, inorganic phosphorus, acid-soluble

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

[4] Mice of strain no. 129 and genotype of $A^{W}A^{W}C^{ch}$ p/cp, obtained from Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, exhibited various degrees of hereditary muscular dystrophy.

[5] Throughout the text, wherever 50 to 60% or 90 to 95% dystrophy is used, it is implied that the disease has actually developed in each individual animal to that extent. One hundred per cent dystrophy has been arbitrarily chosen to indicate the stage of the diseased condition when the animal dies.

[6] See footnote 5.

[7] Purina Mouse Pellets, Ralston Purina Company, St. Louis.

and acid-insoluble phosphorus in muscle, liver and brain. In table 1 are shown the variations in the distribution of phosphorus into acid-soluble and acid-insoluble fractions as observed in the affected muscles of rabbits and mice with progressive nutritional and hereditary dystrophy, respectively. The concentrations of the different phosphorus compounds observed in the various fractions increased to varying degrees, depending upon the severity and cause of the disease (namely, whether the disease had been caused by nutritional deficiency or heredity). Normally the concentration increased more, expressed as per unit weight of wet tissue, in dystrophic rabbit muscle than in dystrophic mouse muscle. For example, in the skeletal muscle of rabbits exhibiting 90 to 95% of the disease, TP, TPi, TASP and TAIP increased by 45, 60 to70, 50 and 30%, respectively, whereas in the muscles of mice with hereditary dystrophy, having the same degree of severity of the disease as the rabbits, increases of 35, 40, 42 and 33% in TP, TPi, TASP and TAIP, respectively, were observed. Even when the disease had progressed only to 50 to 60%, the concentrations of TP, TPi, TASP and TAIP increased considerably although to a lesser extent than the corresponding changes observed with severely affected muscles.

Changes in the concentrations of TP, TPi, TASP and TAIP in the livers of rabbits and mice with various degrees of nutritional and hereditary dystrophy, respectively, are shown in table 2. The concentrations of all increased in the livers of dystrophic rabbits; for example, TP increased by 20%, TPi by 36%, TASP by 21% and TAIP by 21%, respectively. On the other hand, TP, TAIP and TASP decreased in the livers of the dystrophic mice by 15, 19 and 20%, respectively, whereas TPi increased 22%. In the liver of the 50 to 60% diseased rabbit the relative increases in TP, TPi, TASP and TAIP were 11, 7, 18 and 8%, respectively. In the livers of the mice exhibiting 50 to 60% dystrophy, TP, TASP and TAIP decreased silghtly, whereas TPi increased not so significantly.

The changes in the concentrations of TP, TPi, TASP and TAIP in the brain of animals with 90 to 95% of nutritional and hereditary muscular dystrophy are shown

TABLE 1

Distribution pattern of total phosphorus, total inorganic, total acid-soluble and acid-insoluble phosphorus in the muscles of animals with progressive nutritional and hereditary dystrophy [1]

Animal	Group [2]	Total phosphorus	Total inorganic P + creatinine phosphate	Total acid-soluble phosphorus	Total acid-insoluble phosphorus
		mg/g wet tissue	*mg/g wet tissue*	*mg/g wet tissue*	*mg/g wet tissue*
Rabbits	normal	3.37 ± 0.27 [3] (3.12–3.78) [4]	1.36 ± 0.17 (1.20–1.66)	2.28 ± 0.23 (2.08–2.68)	1.29 ± 0.10 (1.20–1.40)
	dystrophic (50–60%)	4.00 ± 0.33 (3.76–4.60)	1.72 ± 0.18 (1.65–2.08)	2.76 ± 0.28 (2.50–3.19)	1.59 ± 0.08 (1.50–1.68)
	dystrophic (90–95%)	4.83 ± 0.21 (4.61–5.18)	2.42 ± 0.27 (2.08–2.70)	3.25 ± 0.24 (3.10–3.68)	1.69 ± 0.11 (1.56–1.78)
Mice	normal	3.65 ± 0.07 (3.58–3.74)	2.30 ± 0.14 (2.18–2.50)	2.85 ± 0.15 (2.72–3.08)	0.83 ± 0.15 (0.61–0.94)
	dystrophic (50–60%)	4.34 ± 0.07 (4.25–4.40)	2.59 ± 0.24 (2.51–2.95)	3.19 ± 0.10 (3.08–3.32)	1.12 ± 0.04 (1.08–1.18)
	dystrophic (90–95%)	5.06 ± 0.06 (5.00–5.13)	3.14 ± 0.09 (3.08–3.28)	3.65 ± 0.22 (3.50–3.98)	1.18 ± 0.05 (1.13–1.24)

[1] Nutritional dystrophy was produced in rabbits by maintaining them with a vitamin E-deficient diet. Mice with hereditary dystrophy were purchased from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. Each number represents the mean of 5 sets of experiments. Each set consisted of 2 animals.

[2] In using 50 to 60% dystrophy or 90 to 95% dystrophy, it is implied that the disease had actually developed to that extent in each individual animal.

[3] SD.

[4] Numbers in parentheses indicate range.

in table 3. Here, the relative increases in TP, TPi, TASP and TAIP were much less pronounced than those observed in the muscles of the same diseased animals. In the brains of mice TASP changed very slightly and the increase in TPi was also comparatively low; TP and TAIP increased by only 16 and 18%, respectively. TP increased in the brain of severely affected rabbits, by 24%, TPi by 22%, TASP by 36% and TAIP by 12%, respectively.

Changes in the concentrations of 7-minute hydrolyzable phosphorus in liver, brain and muscle and ATPase activity in muscle.

TABLE 2

Distribution pattern of total phosphorus, total inorganic, total acid-soluble and acid-insoluble phosphorus in the liver of animals with progressive nutritional and hereditary dystrophy [1]

Animal	Group	Total phosphorus	Total inorganic phosphorus	Total acid-soluble phosphorus	Total acid-insoluble phosphorus
		mg/g wet tissue	*mg/g wet tissue*	*mg/g wet tissue*	*mg/g wet tissue*
Rabbits	normal	4.49 ± 0.07 [2] (4.42–4.56) [3]	0.81 ± 0.07 (0.73–0.88)	1.90 ± 0.13 (1.72–1.89)	2.61 ± 0.06 (2.56–2.68)
	dystrophic (50–60%)	4.87 ± 0.06 (4.80–4.93)	0.87 ± 0.11 (0.75–0.98)	2.19 ± 0.09 (2.10–2.28)	2.79 ± 0.09 (2.70–2.88)
	dystrophic (90–95%)	5.35 ± 0.13 (5.21–5.47)	1.16 ± 0.10 (1.08–1.28)	2.43 ± 0.15 (2.30–2.60)	3.16 ± 0.12 (3.08–3.83)
Mice	normal	4.65 ± 0.24 (4.40–4.93)	0.88 ± 0.15 (0.75–1.08)	1.45 ± 0.25 (1.23–1.78)	3.35 ± 0.31 (3.13–3.82)
	dystrophic (50–60%)	4.43 ± 0.13 (4.29–4.58)	0.95 ± 0.11 (0.83–1.08)	1.30 ± 0.18 (1.20–1.43)	3.14 ± 0.31 (2.85–3.58)
	dystrophic (90–95%)	4.03 ± 0.27 (3.76–4.38)	1.02 ± 0.12 (0.90–1.16)	1.18 ± 0.15 (1.0 –1.38)	2.72 ± 0.21 (2.53–3.03)

[1] See table 1, footnotes 1 and 2, for experimental conditions.
[2] SD.
[3] Figures in parentheses indicate range.

TABLE 3

Distribution pattern of total phosphorus, total inorganic, total acid-soluble and acid-insoluble phosphorus in the brain of animals with progressive nutritional and hereditary dystrophy [1]

Animal	Group	Total phosphorus	Total inorganic phosphorus	Total acid-soluble phosphorus	Total acid-insoluble phosphorus
		mg/g wet tissue	*mg/g wet tissue*	*mg/g wet tissue*	*mg/g wet tissue*
Rabbits	normal	2.7 ± 0.13 [2] (2.60–2.85) [3]	0.75 ± 0.12 (0.65–0.88)	0.90 ± 0.07 (0.84–0.98)	1.95 ± 0.11 (1.84–2.06)
	dystrophic (50–60%)	2.92 ± 0.09 (2.85–3.02)	0.79 ± 0.11 (0.70–0.92)	0.98 ± 0.09 (0.90–1.08)	2.02 ± 0.15 (1.89–2.18)
	dystrophic (90–95%)	3.36 ± 0.10 (3.30–3.48)	0.88 ± 0.10 (0.78–0.98)	1.15 ± 0.05 (1.1 –1.18)	2.13 ± 0.14 (1.98–2.26)
Mice	normal	3.45 ± 0.35 (3.20–3.98)	0.81 ± 0.13 (0.70–0.98)	1.42 ± 0.16 (1.28–1.58)	1.95 ± 0.13 (1.8 –2.1)
	dystrophic (50–60%)	3.65 ± 0.29 (3.45–4.08)	0.83 ± 0.09 (0.75–0.94)	1.43 ± 0.16 (1.27–1.58)	2.13 ± 0.14 (2.06–2.35)
	dystrophic (90–95%)	3.94 ± 0.21 (3.77–4.22)	0.85 ± 0.08 (0.77–0.94)	1.43 ± 0.13 (1.27–1.58)	2.45 ± 0.19 (2.34–2.76)

[1] See table 1, footnotes 1 and 2, for experimental conditions.
[2] SD.
[3] Figures in parentheses indicate range.

TABLE 4

Changes in ATP concentration in muscle, and 7-minute hydrolyzable phosphorus concentration in liver and brain of animals with progessive nutritional and hereditary dystrophy [1]

Animal	Group	ATP concentration	7-minute hydrolyzable phosphorus concentration	
		Muscle	Liver	Brain
		mg/g wet tissue	*mg/g wet tissue*	*mg/g wet tissue*
Rabbits	normal	0.44 ± 0.04 [2] (0.40–0.48) [3]	0.12 ± 0.03 (0.10–0.16)	0.08 ± 0.01 (0.07–0.09)
	dystrophic (50–60%)	0.30 ± 0.05 (0.27–0.36)	0.09 ± 0.01 (0.08–0.09)	0.08 ± 0.01 (0.07–0.09)
	dystrophic (90–95%)	0.23 ± 0.04 (0.20–0.28)	0.07 ± 0.01 (0.07–0.09)	0.10 ± 0.03 (0.07–0.16)
Mice	normal	0.44 ± 0.03 (0.40–0.48)	0.25 ± 0.04 (0.20–0.28)	0.31 ± 0.05 (0.27–0.38)
	dystrophic (50–60%)	0.35 ± 0.05 (0.30–0.40)	0.22 ± 0.03 (0.20–0.27)	0.31 ± 0.05 (0.27–0.39)
	dystrophic (90–95%)	0.29 ± 0.04 (0.25–0.34)	0.21 ± 0.05 (0.19–0.28)	0.31 ± 0.04 (0.27–0.37)

[1] See table 1, footnotes 1 and 2, for experimental conditions.
[2] SD.
[3] Figures in parentheses indicate range.

TABLE 5

Variation of ATPase activity in the muscle of animals with nutritional and hereditary dystrophy [1]

Animal	Group	Muscle ATPase activity [2]	Increase in ATPase activity
		mg/g protein	%
Rabbits	normal	24	
	dystrophic (90–95%)	34	41
Mice	normal	36	
	dystrophic (90–95%)	47	30.5

[1] Each figure in the table is the mean of 4 sets of experiments; for other experimental conditions, see table 1, footnotes 1 and 2.
[2] Milligrams of phosphorus liberated from ATP.

The results shown in table 4 indicate that in the muscles of severely affected rabbits the ATP concentration decreased as much as 50%, whereas in the muscle of mice with 90 to 95% dystrophy a reduction of only 30% in ATP concentration was observed. In the 50 to 60% diseased state, the ATP concentration decreased by only 20 to 25% in rabbit muscle and 12 to 16% in the muscle of mice, respectively. The 7-minute hydrolyzable phosphorus [8] also decreased in the livers of animals with nutritional and hereditary dystrophy, as shown in table 4; for example, in the livers of 90 to 95% dystrophic rabbits and mice it decreased by 30 to 35%, and 16 to 18%, respectively. In the case of brain, the 7-minute hydrolyzable phosphorus showed no significant change. The variations in the ATPase activities in the muscles of rabbits and mice exhibiting nutritional and hereditary dystrophy are shown in table 5. The activity of the ATP-hydrolyzing enzyme steadily increased as the disease progressed; in fact, an increase of approximately 40% in ATPase activity was observed in the muscles of severely affected rabbits, and of approximately 30% in the muscles of similarly affected mice. In the muscle of 50 to 60% dystrophic rabbits

[8] The 7-minute hydrolyzable phosphorus represents the phosphorus obtained by subtracting the total inorganic phosphorus (which also includes creatine phosphorus) from the "phosphorus" determined after boiling the TCA extract for 7 minutes at 100°C. It represents all the tri-, di- and monophosphates of 4 deoxyribose and ribose nucleotides, di- and triphosphates of pyridine nucleotides (DPN and TPN), IMP, et cetera. In skeletal muscle, ATP actually represents 90 to 92% of the 7-minute hydrolyzable phosphorus (30) and therefore in the text ATP concentration has been used loosely to represent 7-minute hydrolyzable phosphorus concentration in muscle. Since the percentage of ATP in 7-minute hydrolyzable phosphorus of liver and brain is less than 50%, we have used 7-minute hydrolyzable phosphorus instead of ATP to indicate the changes of all high energy phosphate compounds: DPN, TPN, et cetera.

TABLE 6

Changes in creatine concentration in the muscle, liver and brain of animals with progressive nutritional and hereditary dystrophy [1]

Animal	Group	Creatine		
		Muscle	Liver	Brain
		mg/g wet tissue	*mg/g wet tissue*	*mg/g wet tissue*
Rabbits	normal	4.70 ± 0.22 [2] (4.49–4.82) [3]	1.72 ± 0.09 (1.65–1.82)	2.39 ± 0.36 (2.15–2.81)
	dystrophic (90–95%)	2.90 ± 0.19 (2.71–3.08)	3.54 ± 0.40 (3.15–4.08)	2.50 ± 0.19 (2.33–2.72)
Mice	normal	3.33 ± 0.25 (3.18–3.70)	0.05 ± 0.01 (0.04–0.06)	2.55 ± 0.26 (2.18–2.98)
	dystrophic (90–95%)	2.65 ± 0.23 (2.50–2.98)	0.28 ± 0.08 (0.20–0.38)	2.58 ± 0.28 (2.12–2.92)

[1] See table 1, footnotes 1 and 2, for experimental conditions.
[2] SD.
[3] Figures in parentheses indicate range.

and mice, the ATPase activities increased by 15 to 18% and 10 to 12%, respectively.

Changes in the concentrations of creatine in liver, brain and muscle. Changes in the concentrations of creatine in the muscle, liver and brain of rabbits and mice, with different degrees of progress of the disease, are shown in table 6. The results show that the concentration of creatine varied from organ to organ of the same animal and also among the same organs of the 2 species of animals. In all normal animals the largest amount of creatine was observed in the skeletal muscle and the lowest concentration in the liver, as reported by Lipmann (14). In brain, the creatine concentration was less than observed in muscle but it was greater than that of the liver. Its concentration in the liver of normal mice was extremely low but the creatine concentration in the liver of dystrophic mice steadily increased as the disease progressed. This steady increase was also observed for the liver of the dystrophic rabbit under similar conditions. On the other hand, there was a gradual decrease in the concentration of creatine in the muscles of all animals with progressive dystrophy. This has been also observed for rabbit muscles by other investigators (15). Its concentration in brain did not show any significant change. These results are shown in table 6.

DISCUSSION

The results of our ATP measurements in the muscles of animals with nutritional dystrophy agree fairly well with those of Grigoreva and Medovar (16) who also noted a 50% decrease in ATP concentration in dystrophic rabbit muscle. Zymaris et al. (17), on the other hand, observed a diminution of ATP concentration by 30 to 40% in muscle of dystrophic mice. Bonetti et al. (18) also reported a decrease in the ATP concentration in the muscles of patients with progressive muscular dystrophy. The relative decrease in ATP concentration may be attributed to the increased ATPase activity in the muscles of such dystrophic animals; a regular increase in the ATPase activity of muscles of animals during the progress of the disease was noted by us. A 15% increase of ATPase activity in the muscles of dystrophic mice was also reported by Pennington (19). The increased hydrolysis of ATP in dystrophic muscles probably accounts to a large extent for the increased concentration of inorganic phosphorus in such muscles. Similarly the decrease in the concentration of creatine phosphate in dystrophic muscles could also be partly responsible for the increase in TPi concentration. However, about 50% of muscle creatine is present as creatine phosphate (14), and therefore variations in the concentration of creatine should be considered as reflecting changes in creatine phosphate in dystrophic animals. The creatine concentration in muscle steadily decreases with the progress of the disease, although its concentration increases in the liver. This may be attributed to a gradual loss of the ability of the muscle to retain

creatine. This is not due to lack of enzyme, since the activity of creatine phosphokinase is not significantly changed in such muscles (20, 21), but rather appears to be the effect of unavailability of a sufficient amount of ATP for carrying out the phosphorylation. This is plausible since the ATP concentration is reduced by 30 to 50% in the muscles of severely affected animals. In this connection it has been observed that the creatine concentration in dystrophic muscle also decreases by 30 to 50%, a reduction which parallels roughly the reduction of ATP in such muscles.

There may be other explanations for the inability of the muscle to retain creatine. For example, our previous work (8) showed that the protein content in the muscle of severely affected animals decreased by 28 to 30% and it is possible that the remaining muscle mass was not sufficient to absorb and retain the excess of creatine synthesized by the liver and transferred to the muscle.

Many investigators have noted previously an increase in the O_2 consumption of the muscles of rabbits maintained with a vitamin E-deficient diet (22–24). Since, according to Houchin (25), tocopherol acts as an inhibitor of succinoxidase, and since respiration is coupled with phosphorylation, an increased rate of ATP synthesis in the muscles of animals maintained with a vitamin E-deficient diet might be expected. However, this explanation may not be adequate as it is now believed by some that tocopherol acts as a cementing material for maintaining cytochromes *b* and c in correct spatial relationship for optimal electron transfer (25–27). In animals maintained with a vitamin E-deficient diet, an impaired electron transport chain is to be expected and therefore an adequate synthesis of ATP should not occur (25–27). In the present study the ATP concentration was actually reduced by 30 to 50% in such muscles. This decrease in ATP concentration also might have been caused by the increased ATPase activity in these affected muscles. The inability of dystrophic muscle to phosphorylate creatine thus appears to be the result of a deficiency in ATP.

The increase of total phosphorus in the acid-soluble fraction can be explained largely on the basis of a relative increase in the concentration of inorganic phosphorus (TPi) in the muscles of affected animals owing to the hydrolysis of ATP and other similar compounds. It is also possible that an increase in hexose-6-phosphate, fructose-1,6-diphosphate and other such similar phosphorus compounds in the affected muscles might also be responsible, to some extent, for the increase of total phosphorus in the acid-soluble fraction. Actually, an increase of phosphorus in the barium-insoluble fraction representing hexose mono- and diphosphates, and glyceric acid phosphate was observed by Calvert et al. (4) for breast muscle of dystrophic chicks. The apparent increase in TPi, as observed for muscles of affected animals, is somewhat less than that which might be predicted if the reduction of creatine (30 to 40%) in such muscles is taken into consideration. Since in the skeletal muscle, one-half of the total creatine is present as creatine phosphate (14), a reduction in creatine in dystrophic muscle would obviously cause a diminution in TPi, as the method of measuring the latter includes the creatine "phosphorus." However, the increase in TPi, as noted in the livers of dystrophic animals, should have increased slightly, since the creatine concentration in the livers of dystrophic animals actually increased two- to threefold over the value observed in the livers of corresponding normal littermates. In the brain of dystrophic animals the creatine concentration remained unaltered and therefore the changes observed in TPi represent true changes.

As observed by many other investigators in this field, we have also noted an increase in total phosphorus in the muscle of rabbits and mice with nutritional and hereditary dystrophy, respectively, but it is more difficult to explain this increase. It might be due to the increase in various inorganic phosphorus compounds in the acid-soluble and acid-insoluble fractions. From our previous work (8) and that of others (28, 29) it appears that the nucleic acid content increases considerably (two- to threefold) in the muscle of dystrophic animal; there also appears to be an increase in lipid content in such muscles. These compounds contain phosphorus and appear in the acid-

insoluble fraction, and therefore can possibly explain the increase in phosphorus observed in acid-insoluble fraction. There is a loss of roughly 30% of muscle protein, and this might partly account for the increase in phosphorus concentration in the different fractions. However, whether these explanations are adequate to explain the cause of such a considerable increase in the total phosphorus cannot be stated, and further experiments will be needed.

LITERATURE CITED

1. Goettsch, M., I. Lonstein and J. J. Hutchinson 1939 Muscle phosphorus in nutritional muscular dystrophy in rabbits. J. Biol. Chem., *128:* 9.
2. Wagitendonk, W. J., and H. Lamfrom 1945 Changes in the distribution of acid-soluble phosphorus in the muscle during a deficiency of the anti-stiffness factor of guinea pigs. J. Biol. Chem., *158:* 421.
3. Shabanova, I. A. 1953 Carbohydrate-phosphorus metabolism in the muscle of rats in experimental muscular dystrophy. Biochimiya, *18:* 385.
4. Calvert, C. C., R. A. Monroe and M. L. Scott 1961 Studies on phosphorus metabolsim in dystrophic chicks. J. Nutrition, *73:* 355.
5. Fitch, C. D., and J. S. Dinning 1959 Phosphate metabolism in nutritional muscular dystrophy and hyperthyroidism. Proc. Soc. Exp. Biol. Med., *100:* 201.
6. Perry, S. V. 1961 The biochemistry of muscle. Ann. Rev. Biochem., *30:* 486.
7. Needham, D. M. 1960 Biochemistry of Muscular Action in Structure and Function of Muscle, vol. 2, ed., G. H. Bourne. Academic Press, Inc., New York, p. 55.
8. Srivastava, U., A. Devi and N. K. Sarkar 1964 Biochemical changes in progressive muscular dystrophy. I. Nucleic acid and nucleotide metabolism in normal and dystrophic muscles, livers and brains of rabbits and mice exhibiting nutritional and hereditary dystrophy. Exp. Cell. Res., in press.
9. Umbriet, W. W., R. H. Burris and J. F. Stantter 1959 Analysis of Phosphorylated Intermediates in Manometric Techniques. Burgess Publishing Company, Minneapolis, p. 269.
10. King, E. J. 1932 The colorimetric determination of phosphorus. Biochem. J., *26:* 292.
11. Hawk, P. B., B. L. Oser and W. H. Summerson 1947 Muscular tissue. In: Practical Physiological Chemistry, ed. 13. McGraw Hill Book Company, New York, p. 287.
12. Sarkar, N. K., A. E. Szent-Györgyi and L. Verga 1950 Adenosine triphosphatase activity of the glycerol extracted muscle fibers. Enzymologia, *14:* 267.
13. Lang, C. A. 1958 Simple micro-determination of Kjeldahl nitrogen in biological material. Anal. Chem., *30:* 1692.
14. Lipmann, F. 1941 Metabolic generation and utilization of phosphate bond energy. In: Advances in Enzymologia, eds., F. F. Nord and C. H. Werkman, Interscience Publishers Inc., New York, p. 117.
15. Heinrich, M. R., and H. A. Mattill 1949 The creatine content of the liver in the muscular dystrophy of vitamin E deficiency. J. Biol. Chem., *178:* 911.
16. Grigoreva, V. A., and E. N. Medovar 1959 Studies on the components of the adenylic system in skeletal and cordial muscles in experimental muscular dystrophy. (Russian text) Ubr. Biochemi. Zl., *31:* 351.
17. Zymaris, C. M., A. Saifer and B. W. Volk 1960 Phosphate turnover in dystrophic muscle. Specific activities of acid-soluble nucleotides in hind leg muscle of mice with dystrophia muscularis. Nature, *188:* 323.
18. Bonetti, E., F. N. Taschi and M. Levi 1954 Acid-soluble phosphorus fraction in progressive muscular dystrophy. Sperimentale, *104:* 315.
19. Pennington, R. J. 1961 Biochemistry of dystrophic muscle. Mitochondrial succinate — tetrazolium reductase and adenosine triphosphatase. Biochem. J., *80:* 649.
20. Read, W. O., and S. Nehorayan 1959 Effect of vitamin E deficiency on creatine phosphokinase of heart and skeletal muscle. Am. J. Physiol., *196:* 1286.
21. Read, W. O. 1961 Creatine phosphorylkinase in muscles of dystrophic mice. Proc. Soc. Exp. Biol. Med., *109:* 696.
22. Friedman, I., and H. A. Mattill 1941 The oxygen consumption of skeletal muscle from animals deprived of vitamin E. Am. J. Physiol., *131:* 595.
23. Houchin, O. B., and H. A. Mattill 1942 The oxygen consumption, creatine, and chloride content of muscles from vitamin E-deficient animals as influenced by feeding tocopherol. The influence of parenteral administration of X-tocopherol phosphate on the metabolic processes in dystrophic muscle. J. Biol. Chem., *146:* 301, 309.
24. Kaunitz, H., and A. M. Pappenheimer 1943 Oxygen consumption in vitamin E deficiency. Am. J. Physiol., *138:* 328.
25. Houchin, O. B. 1942 The in vitro effect of tocopherol and its phosphate derivative on oxidation in muscle tissue. J. Biol. Chem., *146:* 313.
26. Lehman, I. R., and A. Nason 1956 The role of lipides in electron transport. 1. Properties of a diphosphopyridine nucleotide-cytochrome c reductase from rat skeletal muscle. J. Biol. Chem., *222:* 497.
27. Nason, A., and I. R. Lehman 1956 The role of lipides in electron transport. II. Lipide cofactor replaceable by tocopherol for the enzymatic reduction of cytochrome c. J. Biol. Chem., *222:* 511.
28. Dinning, J. S., and P. L. Day 1957 Vitamin E deficiency in the monkey. II. Tissue

concentrations of nucleic acids and creatine. J. Nutrition, *63:* 393.

29. Young, J. M., Jr., and J. S. Dinning 1951 A relationship of vitamin E to nucleic acid metabolism. J. Biol. Chem., *193:* 743.

30. Masters, Y. F., P. C. Johnson, W. H. Mosley, P. B. McCay and R. Caputto 1960 Incorporation of radioactive phosphate into organic phosphates of tocopherol-deficient and control rabbit muscle. Am. J. Physiol., *199:* 295.

Phenylalanine Requirements with Different Levels of Tyrosine [1,2]

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ABSTRACT The quantitative relationship between phenylalanine and tyrosine was investigated. The subjects were 22 young college men and women. They were fed a semipurified diet containing an amino acid mixture which supplied the essential amino acids in amounts present in 20 g of egg protein plus arginine, histidine, cystine and tyrosine. When nitrogen equilibrium was attained the subjects were shifted to a diet of the same composition except that phenylalanine and tyrosine were omitted. This diet was then supplemented with crystalline tyrosine at 3 levels, zero, 200, and 400 mg, and the amount of phenylalanine required for N equilibrium at each level was determined. Nitrogen balance periods for the different levels of tyrosine and phenylalanine studied were usually 6 days long. Two criteria of adequacy were used, the Leverton "zone of equilibrium" and the zero balance used by Rose as the equilibrium point. With no tyrosine in the diet both criteria indicated a requirement of 600 to 700 mg phenylalanine for the women and 900 to 1000 mg for the men. At 200 mg tyrosine both women and men maintained nitrogen balances that were within the "equilibrium zone" and most of the women were storing nitrogen. With 400 mg tyrosine most of the women stored nitrogen at 300 to 400 mg phenylalanine; the men required 600 to 700 mg to attain the "equilibrium zone" or store nitrogen, or both. Under the conditions of this study 200 mg tyrosine had a replacement value of 35 to 40% for the women and approximately 50% for the men. The replacement value of 400 mg tyrosine was approximately 50% for both women and men.

The early investigations of Rose et al. (1, 2) on the amino acid needs of the human species established phenylalanine as one of the 8 amino acids that must be supplied in the diet for the maintenance of nitrogen equilibrium in the adult male. As a result of quantitative studies, these workers (3), using young men as subjects, proposed a tentative minimal daily requirement of 1.1 g of L-phenylalanine. This amount of phenylalanine represents the highest level found necessary for the attainment of a positive nitrogen balance in the absence of tyrosine. That tyrosine can be readily formed from phenylalanine was demonstrated by Moss and Schoenheimer (4). This relationship led Rose and Wixom (5) to postulate that the presence of tyrosine in the diet may exert a sparing action upon the phenylalanine requirement. In a study of 2 young men, having phenylalanine requirements of 0.8 to 1.1 g respectively, in the absence of tyrosine, these workers found that the addition of liberal amounts of tyrosine reduced the phenylalanine requirement 70 to 75%. Tolbert and Watt (6) found a range of 834 to 1184 mg/day in the phenylalanine requirement of 6 young women with a 10-g nitrogen intake and 45 mg of tyrosine from the basal diet. Positive nitrogen balances were maintained in 3 subjects in which 66 to 70% of the phenylalanine was replaced by tyrosine.

In a study on the phenylalanine requirement of women, Leverton et al. (7) investigated the amount needed for nitrogen equilibrium with diets providing zero, 100, 200, 450 and 900 mg of tyrosine. Based on the results of this study, Leverton has suggested 220 mg of L-phenylalanine as a tentative minimal daily requirement for young women when the diet also supplies 900 mg of L-tyrosine. With the particular levels of phenylalanine and tyrosine used in Leverton's study, there appeared to be no clear-cut quantitative relationship between the 2 amino acids, but the sparing action of tyrosine was evident. Leverton et al., in light of the information obtained in the study just cited, concluded that further investigation was needed, espe-

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[2] Received support under the RRF program NC-49.

cially studies using intakes within a range of 220 to 620 mg phenylalanine and 100 to 450 mg of tyrosine.

The extent to which tyrosine can substitute for phenylalanine has recently become of special interest in the metabolic disturbance associated with phenylketonuria in infants and children.

EXPERIMENTAL

The purpose of this investigation was to study further the quantitative relationship between phenylalanine and tyrosine. Four separate studies were conducted between January and March, one in each of the years 1959, 1960, 1961 and 1962. A different group of subjects served each year with 4 to 6 in a group. The number studied was 22, of which 13 were college women and 9 college men. The age of the women ranged from 19 to 27 years; height, 152 to 175 cm; weight, 50.4 to 77.2 kg; and surface area from 1.48 to 1.94 cm^2. The age range for the men was 20 to 29 years; height, 167 to 182 cm; weight, 59.0 to 84.4 kg; and surface area, 1.68 to 2.02 cm^2. Records obtained from the college health department, and examinations made at a local clinic indicated that all subjects could be considered normal.

At the beginning of each study there was an adjustment period in which subjects were given a weighed diet of natural foods. The use of 2 semipurified diets followed. All 3 diets were planned to furnish approximately 9 g of nitrogen.

Semipurified diet 1 consisted of an amino acid mixture, which supplied the essential amino acids in the amounts contained in 20 g of egg protein, plus the addition of arginine, histidine, cystine, and tyrosine. The major part of the nitrogen was furnished by glycine, diammonium citrate, and glutamic acid in the proportion of 40% from each of the first 2 compounds and 20% from the last named. Semipurified diet 2 was the same as semipurified diet 1 except that tyrosine and phenylalanine were omitted from the amino acid mixture. These 2 amino acids were added as supplements. Small amounts of canned fruit (pineapple and peaches) and orange juice were used to make the diet more palatable. The mineral mix proposed by Leverton (8) was the source of the required minerals, and vitamin needs were met by the use of one vitamin capsule[3] daily. Cornstarch wafers and pudding, anhydrous milk fat, jelly, pure sugar candy and bottled soft drinks were supplied to bring calories to the desired level. Calories were based on the estimated needs of the subjects and adjusted to maintain constant weight by varying the amounts of the above items or varying the amount of milk fat used in the preparation of the pudding. The amino acids, with the exception of cystine and tyrosine, which were mixed with the milk fat, were dissolved in water. As the water solution was sour in taste, sugar was added to make it more palatable. Approximately equal amounts were fed at each of the 3 meals. One-half of the mineral mix was incorporated in the wafers and the other half was dissolved in lemon juice.

The subjects were maintained with the diet of natural foods until equilibrium was reached. Sometimes it was necessary to lower the nitrogen intake to shorten the time necessary to attain this state. This was especially true of the men who had a history of high protein intake before serving as subjects for the study. In the 1959 study some of the subjects reported discomfort on the first day of change to a semipurified diet. In an effort to avoid this, a slight modification of procedure was made in the later studies. A breakfast of natural foods, which was low in protein, was fed on the day of introduction of semipurified diet 1 and the experimental diet was begun at the noon meal.

The diets used following which nitrogen balances were determined were the semipurified diets 1, and semipurified diet 2 without added tyrosine and with supplements of 200 and 400 mg tyrosine and varying amounts of phenylalanine. Although only urinary and fecal losses were considered in establishing the balances, it is recognized that integumental losses are often appreciable. Five-day periods to test the different levels of tyrosine and phenylalanine were used in the first study and 6-day periods in subsequent ones. All

[3] Composition/capsule: vitamin A, 1.5 mg; vitamin D, 12.5 μg; thiamine·HCl, 2.5 mg; riboflavin, 2.5 mg; ascorbic acid, 50 mg; nicotinamide, 20 mg; Ca pantothenate, 5 mg; pyridoxine·HCl, 0.5 mg; vitamin B_{12}, 2 μg.

TABLE 1

Nitrogen balances of women tested with different levels of tyrosine and phenylalanine

Subjects	Without tyrosine						Tyrosine, 200 mg				Tyrosine, 400 mg				Semi-purified diet [1] [1]
	Phenylalanine, mg						Phenylalanine, mg				Phenylalanine, mg				
	300	400	500	550	600	700	200	300	400	500	300	400	500	600	
	g/day	*g/day*	*g/day*	*g/day*	*g/day*	*g/day*	*g/day*	*g/day*	*g/day*	*g/day*	*g/day*	*g/day*	*g/day*	*g/day*	*g/day*
LA	−0.70	−0.52			0.35			−0.58	−0.31						0.15
TB	−0.82		−0.40		0.73		−0.49	−0.44							−0.16
EE		−0.87	−0.56		−0.26			−0.84	−0.84	0.37					−0.33
HG	−0.81	−0.63			0.01		−0.81	−0.47							0.78
JC					−0.44							0.34	0.09		0.40
WZ					−0.26							0.14	−0.10		0.72
ET					−0.34							−0.25		0.10	0.16
RH					1.03	0.13			0.76		1.34	0.27			1.16
KW					0.43	0.48			0.07		0.89	0.94			0.82
DF					−0.01	0.38			0.33		−0.02	1.26			1.14
MR			−0.47	−0.26					−0.36	0.33					0.28
JP			−0.48	−0.49					−0.76	−0.33					0.12
CR			−0.58	−0.42					−0.72	0.17					0.22
Mean	−0.78	−0.67	−0.50	−0.39	+0.12	±0.33	−0.65	−0.58	−0.23	+0.14	+0.74	+0.45	−0.005	0.10	+0.42

[1] Egg protein pattern; tyrosine, 900 mg; phenylalanine, 1260 mg.

TABLE 2

Nitrogen balances of men tested with different levels of tyrosine and phenylalanine

Subjects	Without tyrosine						Tyrosine, 200 mg		Tyrosine, 400 mg				Semi-purified diet 1 [1]
	Phenylalanine, mg						Phenylalanine, mg		Phenylalanine, mg				
	500	550	700	800	900	1000	400	500	400	500	600	800	
	g/day	*g/day*	*g/day*	*g/day*	*g/day*	*g/day*	*g/day*	*g/day*	*g/day*	*g/day*	*g/day*	*g/day*	*g/day*
ER		−0.89	−0.45				−0.63	−0.11					−0.75
JO	−0.92	−0.70	−0.53				−0.79	−0.32					−0.37
DD		−0.42	−1.35				−0.05	−0.02					−0.57
DoS			−0.94	−0.64					−0.58		−0.23	0.08	−0.52
RL			−1.33	−0.66					−0.76		−0.85	−0.85	−0.09
DS			−1.09	−0.63					−0.41		−0.19	−0.27	−1.12
WW					−0.58	−0.17	−0.19			−0.29	0.16		0.15
DL					−0.02	0.25	0.43			0.03	0.23		−0.29
RR					0.09	0.32	−0.14			−0.22	0.22		−0.05
Mean	−0.92	−0.67	−0.95	−0.64	−0.17	+0.13	−0.23	−0.15	−0.58	−0.16	−0.11	−0.35	−0.40

[1] Egg protein pattern; tyrosine, 900 mg; phenylalanine, 1260 mg.

subjects received the semipurified diet for 2 periods. When results were doubtful, more than one period was used for given levels of supplementation of semipurified diet 2.

DISCUSSION AND RESULTS

In considering the results of the investigation, 2 criteria of adequacy were applied, the Leverton "zone of equilibrium" [4] and the zero balance used by Rose as the equilibrium point. The authors feel, however, for this particular study that the use of the "equilibrium zone" is more meaningful as a basis for comparison with the earlier study of Leverton (7) on the relationship of the tyrosine level to the phenylalanine requirement.

The mean nitrogen balances of the subjects consuming semipurified diet 1, and semipurified diet 2 supplemented with the different levels of tyrosine and phenylalanine are shown in tables 1 and 2 and figures 1 and 2. The figures are designed to make possible the use of both criteria mentioned above.

Applying the criterion of Leverton (4) most of the subjects attained nitrogen equilibrium with semipurified diet 1 (in which the mixture of essential amino acids was equivalent to that of 20 g of whole egg protein) in 6 to 12 days. Three of the men required a third period of 6 days to establish equilibrium. It was necessary to increase the calorie level of these 3 men to have them attain or closely approach the zone of equilibrium. With the zero balance as the criterion, most of the women, likewise, were in nitrogen equilibrium, but this was true of only one of the 9 men.

With the semipurified diet, lacking both tyrosine and phenylalanine (except for the 12 to 15 mg furnished by the fruits and orange juice), the response to additions of phenylalanine varied with the different levels of tyrosine. At the zero level of tyrosine both criteria indicated that all subjects were in negative nitrogen balance at a level below 550 mg phenylalanine. At this level two of the 10 women fell within the "zone of equilibrium," but

[4] Subjects whose N excretion was within 95 to 105% of the intake were considered to be in N equilibrium.

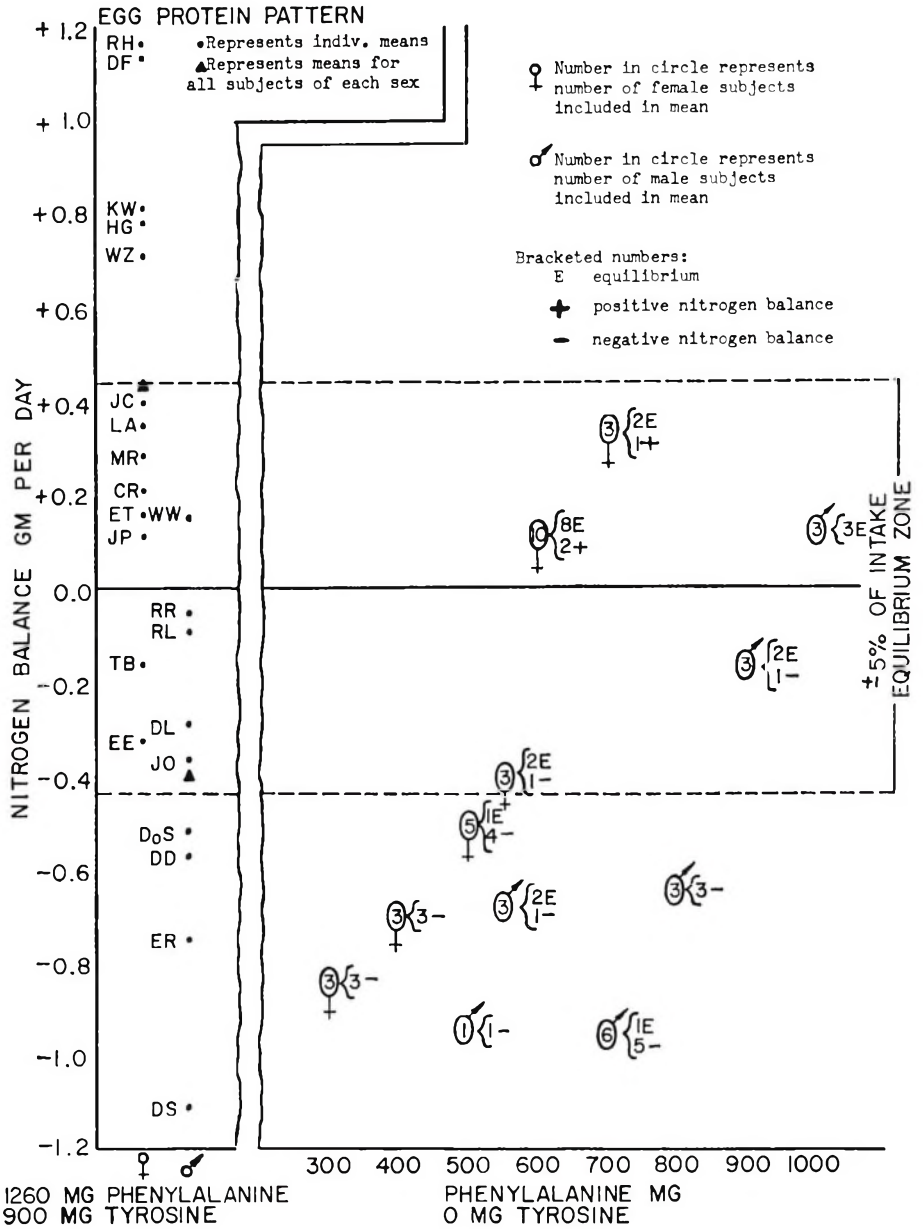

Fig. 1 Nitrogen balances with semipurified diet 1 (900 mg tyrosine, 1260 mg phenylalanine) and semipurified diet 2 without tyrosine and supplemented with varying levels of phenylalanine.

all were in a negative state with zero balance as the criterion. Both criteria indicated that all of the men were in negative nitrogen balance with less than 900 mg of phenylalanine. At this level of phenylalanine 2 of the 3 men fell in the "equilibrium zone" and one was in negative nitrogen balance. Two out of three men were in a negative state with zero balance as the criterion. When the level of phenylalanine was increased to 1000 mg, all 3 men observed at this level were within the equilibrium zone and two were on the positive side of zero balance.

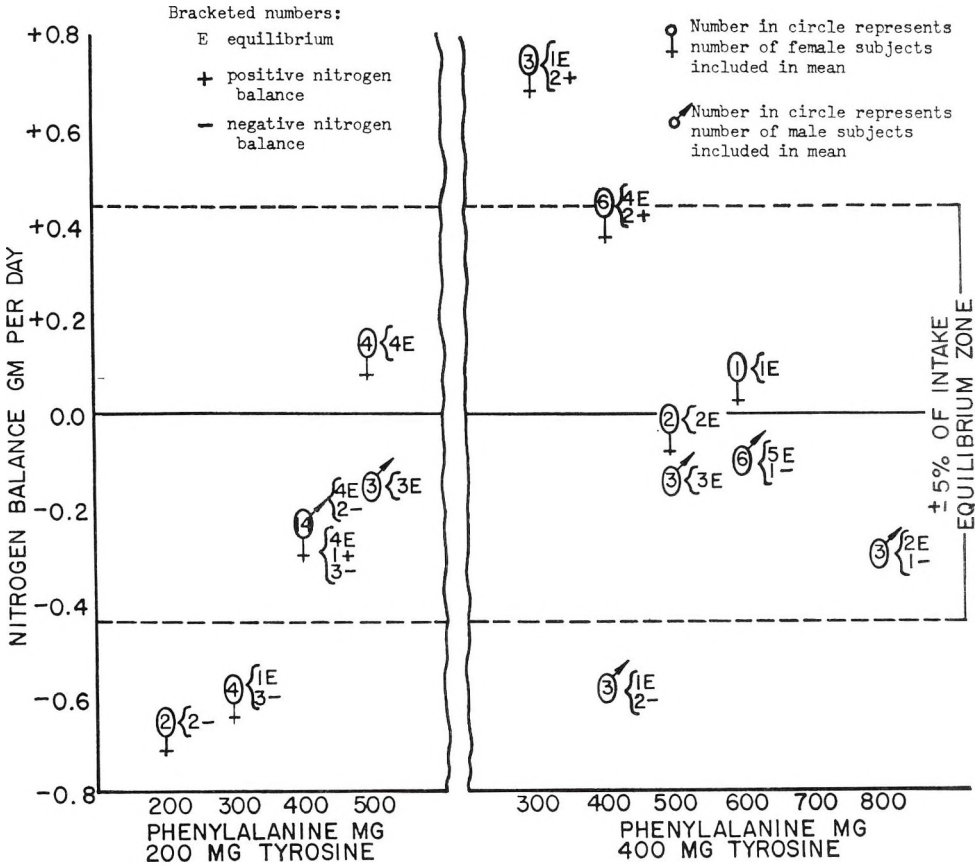

Fig. 2 Nitrogen balances with semipurified diet 2 supplemented with 200 and 400 mg tyrosine and varying levels of phenylalanine.

Differences in the phenylalanine requirement of men and women at the 200-mg level of tyrosine were not apparent when the "equilibrium zone" constituted the criterion for adequacy. Five out of 8 women and 4 out of 7 men were in nitrogen equilibrium with 400 mg of phenylalanine. Raising the level of phenylalanine to 500 mg did not markedly change the results for either men or women. With 400 mg tyrosine, most of the women were in a positive state with reference to zero balance with intakes of 300 to 400 mg phenylalanine, but only one-half of the men were able to attain this state at 600 mg. However, 5 of the 6 men at this level and the 3 men at the 500 level were within the "equilibrium zone." One of the men (RL) failed to attain equilibrium by either criterion at any of the levels of tyrosine or phenylalanine studied. This subject had a high fecal excretion, and maintenance of calorie balance was difficult. His mean weight did not vary greatly from period to period, but there were notable fluctuations from day to day. Short-term calorie deficits may have contributed to the large negative balances observed.

CONCLUSIONS

The results of this study suggest the following conclusions. With no tyrosine in the diet women require 600 to 700 mg phenylalanine to come within the nitrogen "equilibrium zone"; men, 900 to 1000 mg. At 600 mg, one-half of the women stored nitrogen and at 700 mg all did so. One of the 3 men was storing nitrogen at 900 mg phenylalanine and 2 out of 3 were in positive nitrogen balance at 1000 mg.

With 200 mg tyrosine both women and men maintained nitrogen balances within the "equilibrium zone" with 400 to 500 mg phenylalanine. Most of the women and one of the 3 men stored nitrogen at these levels of phenylalanine. With 400 mg tyrosine the results indicated that women can store nitrogen at 300 to 400 mg phenylalanine; men require 500 to 600 mg phenylalanine to bring them into the "equilibrium zone" or to attain positive nitrogen balance, or both.

The observations indicate that 200 mg tyrosine show a phenylalanine replacement value of 35 to 40% for the women and approximately 50% for the men. The replacement value of 400 mg tyrosine was approximately 50% for both women and men. Both replacement levels are lower than those reported by Rose and Wixom (5) and used by Tolbert and Watts (6) with subjects receiving more liberal amounts of tyrosine.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Mrs. Dorothy Deethardt, Mrs. Joan Aspelin and Mrs. Joyce Schreiber for their valuable technical assistance. They also extend thanks to the subjects for their interest and fine cooperation.

LITERATURE CITED

1. Rose, W. C., W. G. Haines, J. E. Johnson and D. T. Turner 1943 Further experiments on the role of amino acids in human nutrition. J. Biol. Chem., *148:* 457.
2. Rose, W. C., B. E. Leach, M. J. Coon and G. F. Lambert 1955 The amino acid requirement of man. IV. The role of leucine and phenylalanine. J. Biol. Chem., *193:* 613.
3. Rose, W. C., B. E. Leach, M. J. Coon and G. F. Lambert 1955 The amino acid requirements of man. IX. The phenylalanine requirement. J. Biol. Chem., *213:* 913.
4. Moss, A. R., and R. Schoenheimer 1940 The conversion of phenylalanine to tryrosine in normal rats. J. Biol. Chem., *135:* 415.
5. Rose, W. C., and R. L. Wixom 1955 The amino acid requirements of man. XIV. The sparing effect of tyrosine on the phenylalanine requirement. J. Biol. Chem., *217:* 95.
6. Tolbert, B., and J. H. Watts 1963 Phenylalanine requirement of women consuming a minimal tyrosine diet and sparing effect of tyrosine on phenylalanine requirement. J. Nutrition, *80:* 103.
7. Leverton, R. M., N. Johnson, J. Ellison, D. Geschwender and F. Schmidt 1956 The quantitative amino acid requirements of young women. IV. Phenylalanine with and without tyrosine. J. Nutrition, *58:* 341.
8. Leverton, R. M., M. R. Gram, M. Chaloupka, E. Brodovsky and M. Mitchell 1956 The quantitative amino acid requirements of young women. I. Threonine. J. Nutrition, *58:* 59.

All-vegetable Protein Mixtures for Human Feeding

XV. STUDIES IN DOGS ON THE ABSORPTION OF GOSSYPOL FROM COTTONSEED FLOUR-CONTAINING VEGETABLE PROTEIN MIXTURES [1,2]

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ABSTRACT The results of the present study indicate that in the dog and under the conditions used, the cottonseed pigment, gossypol, is excreted in the feces almost quantitatively, particularly for total gossypol. Fecal free gossypol is about 3.5 times greater than intake, suggesting that some of the bound gossypol is liberated during passage through the gastrointestinal tract. The pigment was identified by maximal absorption curves, for the original compound and as the dianilido gossypol and phloroglucinol derivatives. Other chemical and biological tests also indicated that the fecal pigment is gossypol. Furthermore, typical gossypol reactions could not be demonstrated in fecal extracts of dogs fed cottonseed-free diets, and upon gossypol additions to the diet gossypol appeared in the feces. Several possible dietary factors were studied to learn the conditions under which gossypol is excreted with the feces. None of the factors studied, such as type and intake of calories, and different fats, altered the pattern of gossypol excretion in feces.

The use of cottonseed flour in the feeding of monogastric animals, including man, has been limited by the presence of the pigment gossypol in the cottonseed. Although during processing of cottonseed for oil extraction, most of the gossypol is either extracted or inactivated, the amounts remaining following one type of cottonseed processing are high and could produce toxic symptoms if fed in sufficient amounts. The toxicity, however, is variable since chicks, rats and other experimental animals grow at normal rates when consuming the amounts of gossypol found to be toxic to swine, making this animal the most sensitive to the toxicity of the pigment. Furthermore, reduced growth is not always a good indication of gossypol toxicity since much of the reduced growth encountered with cottonseed meal rations now appears to have been the result of a lysine deficiency (2). No explanation concerning the variability in toxicity has been given (1, 2).

During the development of INCAP Vegetable Mixture 9, which contains 38% cottonseed flour (3), extensive nutritional tests were carried out in several experimental animals (4, 5) including dogs (6) and humans (7). Since no adverse effects were noticed during prolonged feeding of the mixture, it became important to determine the fate of the pigment once it was consumed. The results reported by Smith (8, 10) and Clawson et al. (9) have indicated that gossypol is present in the tissues of swine. In a recent study by Smith (8), the pigment from swine tissue was isolated and identified chemically. In the present study, results will be presented showing that in dogs the gossypol consumed is excreted, to a very large extent, in the feces. Some possible factors affecting the absorption were also studied.

MATERIALS AND METHODS

In the first experiment, 3 dogs 10 months of age and weighing 8.90, 11.98 and 8.44 kg were fed approximately 5.5 g of protein and 125 kcal/kg body weight/day. The protein fed was exclusively from Vegetable Mixture 9,[3] and the intake of calories was adjusted by feeding a nitrogen-free diet consisting of cornstarch, 20;

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[1] This investigation was supported by grants RF-NRC-1 from the National Research Council (U.S.) and by Public Health Service Research Grant no. A-981, from the National Institutes of Health.
[2] INCAP Publication I-310.
[3] Ground corn, 58; cottonseed flour, 38; torula yeast, 3; calcium hydroxide, 1%; vitamin A, 4500 IU/100 g.

dextrose, 40; sugar, 24; cellulose, 3; hydrogenated vegetable fat, 10; mineral mixture, 2; and cod liver oil, 1%, supplemented with 4 ml of a complete vitamin mixture (11). The animals were fed once a day, at 8:00 A.M.

The calculated quantity of Vegetable Mixture 9 for each dog was mixed with 800 ml of water, and 1% Rhozyme H-39[4] was added to make the cooked mixture more fluid. The suspension was cooked for 0, 8, 16 and 24 minutes, with constant stirring. After cooling, an amount of the nitrogen-free diet was added to bring the intake of calories to the desired level and then fed to the animals. One balance period of 7 days' duration was carried out for each cooking time. The animals were weighed daily and feces and urine were collected twice daily and stored at 4°C. The urine was collected in dark-colored bottles containing 2 cm^3 of concentrated acetic acid. The nitrogen balance results have been reported previously.[5] A representative aliquot of homogenized feces was freeze-dried for estimation of free and total gossypol (12) and another sample was taken for moisture analysis. The entire collection of feces of each dog was dried for studies on the extraction and identification of gossypol.

The dry feces were extracted with either acetone-water (80:20) or chloroform containing 10% concentrated hydrochloric acid with constant shaking for one hour. After filtering, the chloroform extract was concentrated in a current of air and then chromatographed through either a Florosil[6] or a silicic acid column. The extract was eluted first with chloroform, then with chloroform containing 10% ethyl ether and then with chloroform containing 80% methanol. The maximal absorption of the different fractions and their aniline and phloroglucinol derivatives was determined in a DU Beckman spectrophotometer.

Reaction with hydrogen peroxide. The gossypol extract was treated with a 10% sodium hydroxide solution; hydrogen peroxide (20 volumes) was added drop by drop, in the presence of gossypol, a violet color developed.

Reaction with phloroglucinol. The chloroform elution was treated with a phloroglucinol solution in ethyl alcohol and with concentrated hydrochloric acid. On standing, a purple color developed. The maximal absorption of the developed color was determined in a DU spectrophotometer.

Balance test in dogs. In another experiment, 4 dogs weighing at the start of the experiment 14.0, 10.1, 12.0 and 9.5 kg were used. Six grams of protein from Vegetable Mixture 9 and 120 kcal/kg body weight/day were fed. To adjust the intake of calories, a nitrogen-free diet of the composition previously described was used. The fats added to this diet at a level of 10% were: a) hydrogenated vegetable fat; b) butter fat, and c) purified cottonseed oil. Two balance periods of 4 days' duration were carried out with each type of fat and gossypol analysis as well as nitrogen balance were determined with each fat.

Three additional experiments were carried out to learn whether certain dietary factors affect gossypol absorption. Four dogs were used to study the effect of intake of 2 levels of calories, 140 and 85 kcal/kg body weight/day. In each case, one dietary treatment consisted of providing all calories as carbohydrate compared with a second treatment in which 10% of the calories were provided by fat. The average weight of the dogs, when fed the higher level of calories was 7.79 kg and when fed the lower level, the average weight was 11.2 kg. Protein intake was from Vegetable Mixture 9 and was held at a constant rate of 6 g/kg/day.

In another experiment using 3 dogs with an average weight of 13.5 kg, the effect of cooking Vegetable Mixture 9 in a glass container, as compared with cooking it in a metal container, on gossypol absorption was studied. The intake of calories and of protein were 110 kcal and 6.0 g/kg body weight/day, respectively.

In a third experiment 3 dogs with an average weight of 9.90 kg were fed 6 g protein/kg/day from Vegetable Mixture 9 and 140 kcal/kg/day for 8 days in 2 four-day balance periods. During the first 3 days, of the first 4-day balance, the dogs were given 50 mg of free gossypol/day, in

[4] Rohm and Haas, Philadelphia, Pa.

[5] Bressani, R., L. G. Elías, R. Jarquin and J. E. Braham 1964 All-vegetable protein mixtures for human feeding. XIII. Effect of cooking mixtures containing cottonseed flour on free gossypol content. J. Food Sci., in press.

[6] Floridin Company, Tallahasee, Florida.

capsules, and no additional gossypol was fed for the last 4-day balance period. Collection, treatment and analysis of feces in these experiments was performed as previously described.

RESULTS

Table 1 presents the results of the first test in dogs. Free gossypol intake varied from 22 to 34 mg/dog/day, and the intake of total gossypol ranged from 689 to 780 mg/dog daily. The excretion of free gossypol in the feces averaged approximately 3.3 times higher than the intake. However, total gossypol in the feces was essentially the same as the intake. A decrease in free gossypol and in fat content in the feces was observed with respect to feed cooking times.

The possibility existed that the determination for free gossypol was giving high values because of contamination with other fecal pigments. Therefore, studies were carried out to identify gossypol. Figure 1 shows the absorption curves of acetone-water extracts of the feces and of a solution of pure gossypol. The upper part of the figure shows the derivative of gossypol

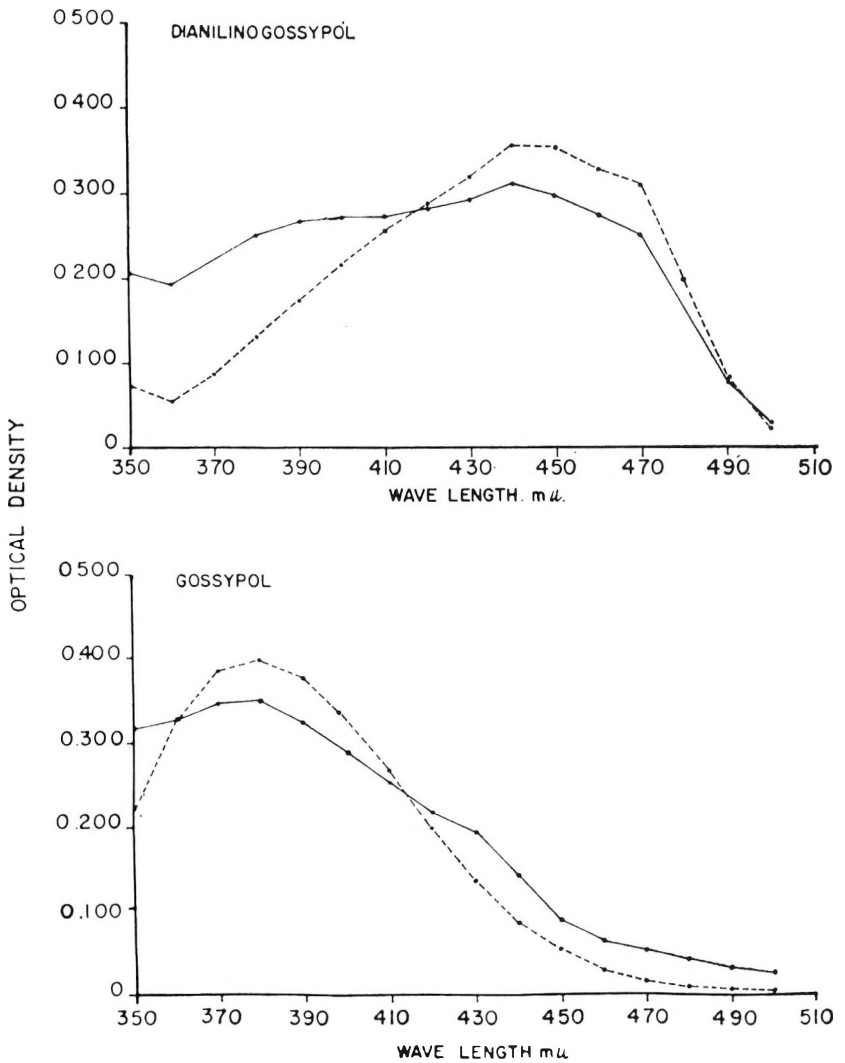

Fig. 1 Acetone-water extracts of dog feces. —— feces extract; – – – – pure gossypol.

TABLE 1

Gossypol intake and fecal excretion of dogs fed a cottonseed-corn mixture cooked for several periods of time [1]

Gossypol [2]	Cooking time,[3] min				
	0	8	16	24	0
Intake					
Free (A)	31	30	26	22	34
Total	709	689	704	715	780
Fecal					
Free (B)	108	91	86	83	103
Total	703	687	757	735	805
Free B/A	3.48	3.04	3.31	3.78	3.03

[1] Average of 3 dogs.
[2] Mg/dog/day.
[3] Each cooking period was tested in feeding tests lasting 7 days.

with aniline. Both curves show a peak of maximum absorption at 440 mμ. The lower part of the figure shows the curves of the acetone-water extracts read directly in the spectrophotometer. Both curves show a peak of maximal absorption at 370 to 380 mμ. Figure 2 shows the absorption curve of gossypol extract chromatographed on a silicic acid column. Curves A and B corresponding to the chloroform eluates and curve D corresponding to pure gossypol, gave a peak of maximal absorption of 370 to 380 mμ. Figure 3 shows the absorption curves of the feces extract chromatographed on Florisil, eluted with different solvents and reacted with aniline. All the solutions studied gave a maximal peak of absorption at 440 mμ. Figure 4 shows the absorption curves of the phloroglucinol derivatives of pure gossypol, and of the feces extract. The maximal absorption for both solutions was at 550 mμ. Further evidence showing that the largest amount of gossypol ingested is present in

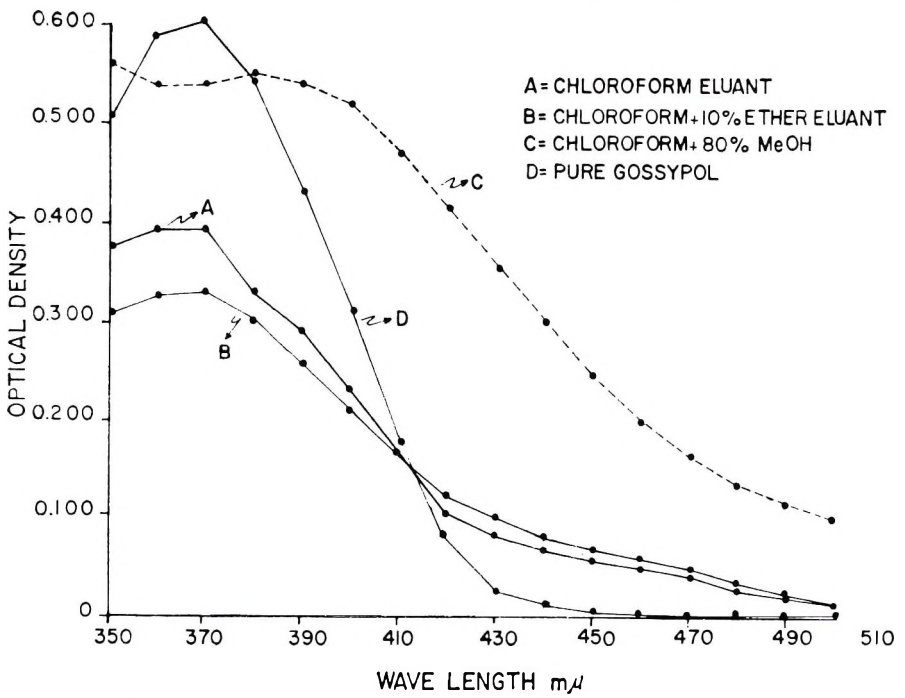

Fig. 2 Gossypol extracts from dog feces. A = chloroform eluant; B = chloroform + 10% ether eluant; C = chloroform + 80% MeOH; D = pure gossypol.

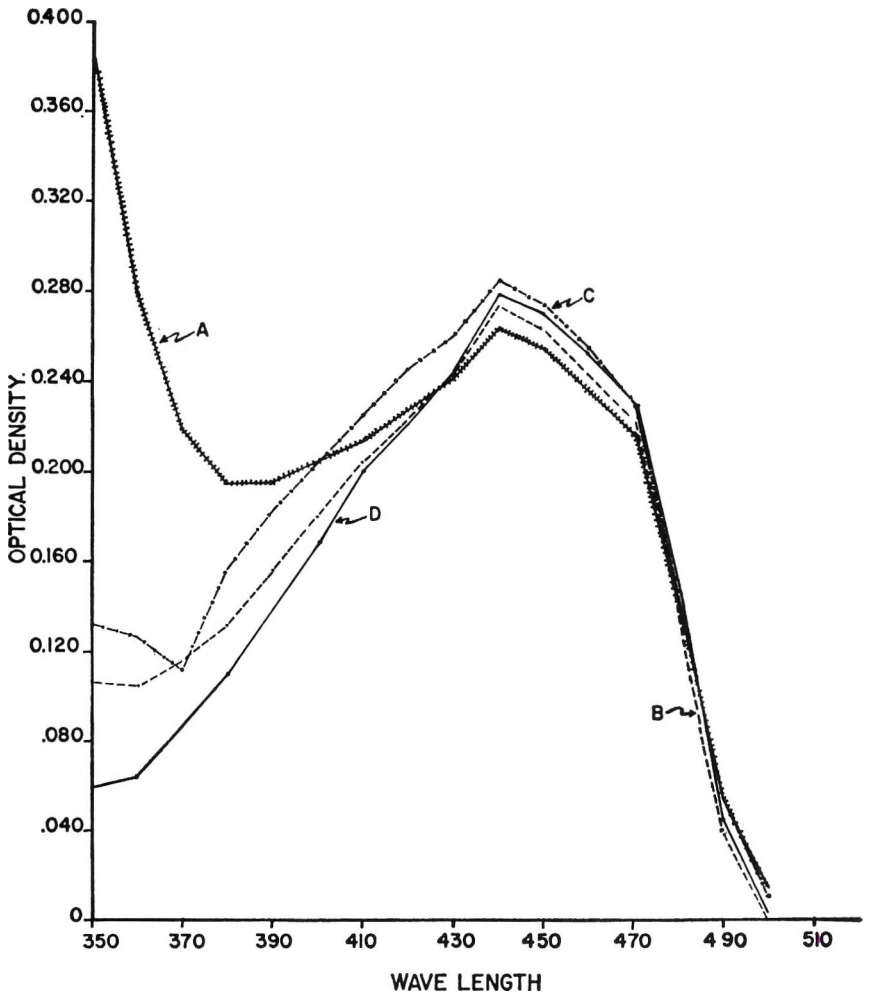

Fig. 3 Dianilino gossypol derivatives of $CHCl_3$ feces extract absorbed on Florisil. -|-|-|-|- Florisil eluant $CHCl_3$ (A); – – – – Florisil eluant $CHCl_3$ (B); –·–·–·– original extract in $CHCl_3$ (C); ——— pure gossypol in $CHCl_3$ (D).

the feces is indicated in figure 5. In this experiment, 4 dogs used previously were fed a casein diet for 4 days, followed by the casein diet plus 100 mg of gossypol daily, to 2 dogs from crude cottonseed oil and to the other two from ground cottonseed, for 4 days and finally, the casein diet. The feces were collected daily and analyses for gossypol were performed on freeze-dried samples from 2-day pools. The graph shows that when no gossypol was fed, little gossypol was present in the feces. A peak excretion of gossypol occurred 2 to 4 days after initial gossypol feeding and then decreased to low values when gossypol was withdrawn. On the basis of 400 mg of gossypol, about 75 to 80% was recovered in feces over the entire period. Only one dog gave zero fecal gossypol values at the end of the study. The results suggest a lag in gossypol excretion.

Table 2 shows the results of qualitative reactions of gossypol. All reactions carried out in the fecal extract gave positive results. The extract of the feces, and the feces were fed to hens and the eggs collected. After storage, eggs from hens fed pure gossypol, the fecal extract, a fraction from the silicic acid column and the dry feces showed discoloration of the egg yolk.

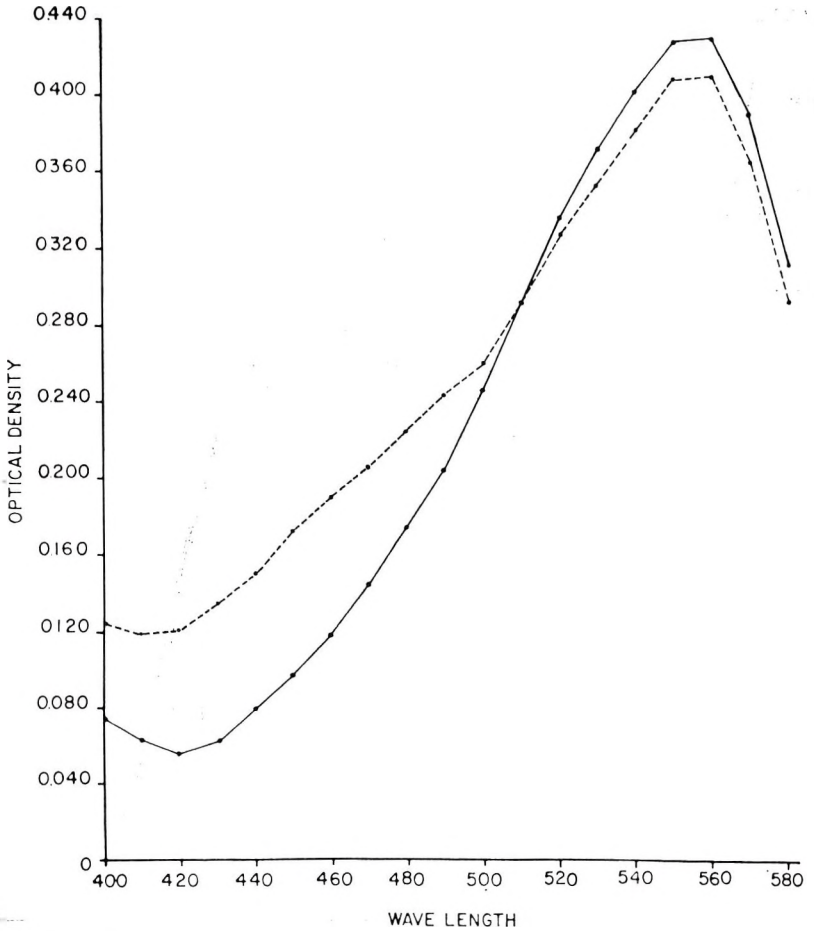

Fig. 4 Phloroglucinol derivative of feces extract and pure gossypol. —— pure gossypol; - - - - feces extract $CHCl_3$ eluant.

TABLE 2

Qualitative reactions on feces and fecal extracts of dogs fed cottonseed flour

Test	Reaction
Fecal extract	
+ NaOH + H_2O_2	+
+ aniline	+
+ phloroglucinol	+
+ $SbCl_3$	+
fed to hen	egg yolk discoloration
Feces fed to hen	egg yolk discoloration

Table 3 presents the results of the experiment where 3 types of fat were tested to learn their effect on gossypol absorption. The excretion of free gossypol was essentially the same with any of the fat used and it was, as in previous studies, about 3.4 times greater than the intake. Total gossypol excretion was 83% of the intake when hydrogenated vegetable fat was fed. It was, however, totally excreted when butter fat or refined cottonseed oil were fed. Nitrogen retention was better when the fat used was purified cottonseed oil.

Several possible factors studied to learn the mechanism by which gossypol was being excreted, are summarized in table 4. One variable studied was a diet with all calories coming from carbohydrate as against one in which part of the calories was contributed by fat. These treatments were tested at 2 levels of intake of calories, 140 and 85 kcal/kg body wt/day, respectively. The results, however, indi-

TABLE 3

Effect of type of fat on nitrogen balance and gossypol excretion in dogs

Type of fat in diet	Nitrogen balance [1]				
	Intake	Fecal	Urine	Absorption	Retention
	mg/kg/day		*mg/kg/day*	*mg/kg/day*	
Hydrogenated vegetable fat	973	230	490	743	253
Butter fat	977	230	498	747	249
Refined cottonseed oil	976	216	477	760	283

Type of fat in diet	Period	Gossypol balance [2]				
		Free		Total		Fecal fat
		Intake	Excreted	Intake	Excreted	
		mg/kg/day		*mg/kg/day*		*mg/kg/day*
Hydrogenated vegetable fat	1	2.8	9.1	59.8	50.0	0.15
	2	2.8	9.2	60.1	49.8	0.18
Butter fat	1	2.7	8.9	46.8	47.9	0.16
	2	2.7	10.0	47.0	49.3	0.14
Cottonseed oil	1	2.8	8.8	47.2	44.8	0.11
	2	2.8	10.6	47.1	48.9	0.13

[1] Average of 4 dogs and 2 four-day balance periods.
[2] Average of 4 dogs.

TABLE 4

Effect of type and intake of calories and of gossypol feeding on fecal content of free and total gossypol [1]

Treatment	Intake of calories	Free gossypol		Total gossypol	
		Intake	Fecal	Intake	Fecal
	kcal	*mg/kg/day*		*mg/kg/day*	
All calories from CHO	140	3.3 [2]	14.0	87.3	77.9
10% Calories from fat	140	3.3 [2]	14.0	87.6	73.9
All calories from CHO	85	2.7 [2]	11.3	65.7	50.4
10% Calories from fat	85	2.7 [2]	11.9	65.9	49.3
Cooking in metal container	110	1.9 [3]	4.6	63.4	45.4
Cooking in glass container	110	2.1 [3]	6.2	60.7	49.5
With gossypol	140	6.5 [3]	16.6	89.3	84.5
Without gossypol	140	2.7 [3]	14.8	85.2	70.7

[1] Protein intake: 6 g/kg/day.
[2] Average of 4 dogs and 2 four-day balance periods.
[3] Average of 3 dogs and 2 four-day balance periods.

cated no difference in excretion of free and total gossypol between the 2 treatments within intake of calories. The recoveries of ingested total gossypol at the 140- and 85-kcal intake levels was 87 and 76%, respectively. In another study, the food mixture containing cottonseed flour was cooked for 10 minutes in a glass container in one case, and in a metal container in another. This was done in view of previous results[7] which indicated that iron decreases gossypol content in cottonseed flour-containing mixtures. The results were again not different between treatments and about 78% of the ingested total gossypol was recovered in the feces. As in previous experiments, the amount of free gossypol in feces was about 4 times the amount ingested. The table also shows that addition of gossypol to the diet resulted in an increase of gossypol in the feces.

[7] See footnote 5.

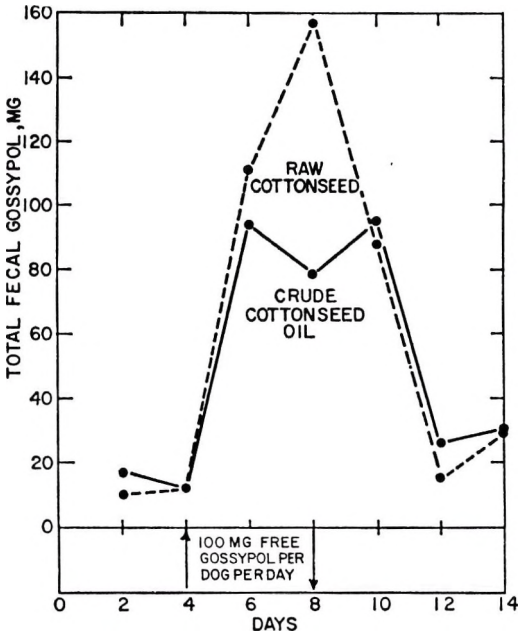

Fig. 5 Fecal excretion of gossypol in dogs fed a casein diet and free gossypol (average 2 dogs/group). —— Crude cottonseed oil; – – – – raw cottonseed.

DISCUSSION

During the time the problem of cottonseed and gossypol has been studied, it became apparent that not all monogastric animals have the same degree of susceptibility towards gossypol (1, 2). Although different animals may have different detoxification mechanisms, it is possible that a reason for differences in resistance to gossypol toxicity is the amount of gossypol absorbed by the animal through the intestinal tract.

The results of the present study indicate that in the dog and under the conditions used, the cottonseed pigment gossypol is excreted in the feces. The excretion is in the majority of the cases quantitative for total gossypol, whereas more free gossypol appears in the feces when compared with intake. At present, the only available explanation may be that certain compounds of gossypol are susceptible of being hydrolyzed in the intestinal tract, therefore resulting in higher values for free gossypol. That the higher values are not entirely due to an error in the method of analysis due to contamination is indicated by: a) the almost quantitative relationship between the amounts of total gossypol in food and feces, b) by low optical density readings of fecal extracts of dogs fed diets without cottonseed and subsequent increases when either cottonseed flour, crude cottonseed oil and pure gossypol are given, and c) by the maximal absorption curves of the fecal extracts and derivatives which were the same as those from pure gossypol and its derivatives. Furthermore, the pigment isolated from the feces is gossypol as indicated from the last point mentioned, as well as from the qualitative and biological tests which were positive for gossypol.

Holley et al. (13) reported on the toxicity of cottonseed flour and of gossypol in rabbits. It was indicated that gossypol was absorbed slowly and that the habit of coprophagy apparently increased, by some unknown mechanism, the sensitivity of the rabbit of gossypol.

The results presented on some of the possible factors which could affect excretion of gossypol indicated that no difference was found between treatments and that most of the gossypol ingested was in the feces. Therefore, it is not possible at this time to suggest a mechanism or conditions which would cause gossypol to be totally excreted. At this time, 3 possibilities can be offered. One is that the amounts of gossypol ingested are small, thus easily not absorbed. A second one is that during cooking, gossypol reacts with organic compounds or iron salts forming high molecular weight substances or chelates which, because of their size, are not easily absorbed through the intestinal tract. These high molecular weight compounds or chelates are decomposed later in the large intestine, resulting in fecal levels of free gossypol in food slightly larger than intakes. In all studies so far carried out, the relationship between free gossypol in food and feces is about 3.3 to 3.7 times greater in feces, and that in several studies the difference between the amount of total gossypol in feces is about 10 to 15 mg less in feces than in intake. And the third possibility, which is now being studied is that the gossypol is absorbed, transported to the liver and excreted with the bile into the intestinal tract. This mechanism could explain the higher free gossypol as well as

the slightly lower total gossypol values observed in feces as compared with the intakes of the 2 forms of the pigment. The results presented here are of practical interest, particularly if the conditions favoring excretion of gossypol are defined, so that they can be applied to other animals and increase the use of cottonseed flour in both human and animal feeding.

LITERATURE CITED

1. Altschul, A., ed. 1958 Processed Plant Protein Foodstuffs. Academic Press, Inc., New York.
2. Cottonseed protein for animal and man. Proceedings of a conference, sponsored jointly by Southern Utilization Research and Development Division, United Nations Children's Fund and National Cottonseed Products Association. November 14–16, 1960. New Orleans, Louisiana.
3. Bressani, R., L. G. Elías, A. Aguirre and N. S. Scrimshaw 1961 All-vegetable protein mixtures for human feeding. III. The development of INCAP Vegetable Mixture Nine. J. Nutrition, *74:* 201.
4. Bressani, R., A. Aguirre, L. G. Elías, R. Arroyave, R. Jarquín and N. S. Scrimshaw 1961 All-vegetable protein mixtures for human feeding. IV. Biological testing of INCAP Vegetable Mixture Nine in chicks. J. Nutrition, *74:* 209.
5. Bressani, R., L. G. Elías and N. S. Scrimshaw 1962 All-vegetable protein mixtures for human feeding. VIII. Biological testing of INCAP Vegetable Mixture Nine in rats. J. Food. Sci., *27:* 203.
6. Bressani, R., J. E. Braham, R. Jarquín and L. G. Elías 1962 Mezclas de proteïnas vegetales para consumo humano. IX. Evaluación del valor nutritivo de las proteínas de la mezcla vegetal INCAP 9 en diversos animales de experimentación. Arch. Venezol. Nutr., *12:* 229.
7. Scrimshaw, N. S., M. Béhar, D. Wilson, F. Viteri, G. Arroyave and R. Bressani 1961 All-vegetable protein mixtures for human feeding. V. Clinical trials with INCAP Mixtures 8 and 9 and with corn and beans. Am. J. Clin. Nutrition, *9:* 196.
8. Smith, F. H. 1963 Isolation of gossypol from tissue of porcine livers. J. Am. Oil Chemists' Soc., *40:* 60.
9. Clawson, A. J., F. H. Smith and E. R. Barrick 1960 Determination of gossypol and iron in the livers of pigs fed rations containing cottonseed meals of varying gossypol content. J. Animal Sci., *19:* 1254.
10. Smith, H. A. 1957 The pathology of gossypol poisoning. Am. J. Pathol., *33:* 353.
11. Manna, L., and S. M. Hauge 1953 A possible relationship of vitamin B_{13} to orotic acid. J. Biol. Chem., *202:* 91.
12. American Oil Chemists' Society 1945–1950 Official and Tentative Methods of the American Oil Chemists' Society, ed. 2. Chicago.
13. Holley, K. T., W. W. Harms and R. W. Storherr 1955 Cottonseed meal in swine and rabbit rations. Georgia Experimental Station, Mimeograph Series N. S. 12. Athens, Georgia.

Influence of Dried-grass Silage and Silage Fractions on the Basal Metabolic Rate of Rats [1]

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ABSTRACT Seven metabolism trials were conducted to determine the influence of ground dried-grass silage (DGS), or fractions of DGS on the basal metabolic rate (BMR) of rats. Incorporation of 20% of each of 5 samples of DGS, ensiled in 4 different years, into the diets increased BMR above that of rats fed a control ration. Increased BMR was observed in growing and mature male and female rats fed diets containing DGS; however, a much greater increase in BMR occurred in males than in females. Ground grass hay of similar botanical composition to that of the grass silage had no effect on BMR when it made up 20% of the diet. Histological examination of the thyroids of rats fed 2 samples of 1957 DGS revealed increases in the height of the follicular epithelial cells. DGS which had been extracted first with petroleum ether then with absolute ethanol was more active in increasing BMR than the unextracted DGS. Extraction of the petroleum ether-absolute ethanol extracted DGS with 80% ethanol removed most of the BMR stimulating activity. Hot aqueous extraction of the residue of the 80% ethanol extraction removed the remainder of the activity.

Unusually high plasma protein-bound iodine values in dairy cattle were reported by Asplund et al. (2) to be associated with the feeding of grass silage. Since this appeared to be related to thyroid activity, it prompted an investigation of the influence of grass silage on basal metabolic rate (BMR), which is influenced by thyroid activity. In the present work dried-grass silage (DGS) or various fractions of grass silage were incorporated into the diets of rats and the effect on BMR was determined by measurement of the rate of oxygen uptake.[5,6]

EXPERIMENTAL

Grass silage used in this work was ensiled in June of 1956, 1957, 1959 and 1960 at the West Virginia University Dairy Farm. The forage was a mixture of wheat, vetch, orchard grass and alfalfa. The 1956 and 1957 grass silage was ensiled from direct-cut grass which was chopped, treated with 0.4% of the preservative, sodium metabisulfite, and packed in upright, circular commercial silos. In 1959 and 1960 the grass was wilted, chopped and treated with 5% beet pulp instead of sodium metabisulfite. As indicated in table 1 grass silage will be designated according to the year it was ensiled. An exception to this is the 1957 silage which is designated 1957a and 1957b to account for removal from the silo in September 1957 and February 1958, respectively. All samples of grass silage were dried in a forced-air oven at 65°C immediately after removal from the silos. The samples were ground in a Wiley mill having a 40-mesh screen and stored in closed metal containers until mixed into the various diets.

Samples of grass hay were obtained in June 1958 from a field adjacent to and having similar botanical composition to that of the grass from which silage had been made. The field-cured hay was dried at 65°C, ground and stored as described for the grass silage.

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[5] Asplund, R. O., G. A. McLaren and I. D. Porterfield 1959 Thyroid stimulation in rats as a result of feeding grass silage. J. Animal Sci., *18:* 1565 (abstract).

[6] McLaren, G. A., D. G. Crow, W. R. Lewis and I. D. Porterfield 1961 Effects of fractions of grass silage on thyroid activity. J. Animal Sci., *20:* 943 (abstract).

TABLE 1

Forages incorporated into rat diets

Trial	Forage	Forage designation in ration tables	Date of cutting or ensiling	Date of removal from silo
1	1956 Grass silage	1956 DGS [1]	June 1956	February 1957
1,2	1957 Grass silage	1957a DGS	June 1957	September 1957
1,2	1957 Grass silage	1957b DGS	June 1957	February 1958
3	Grass hay [2]	hay	June 1958	—
4	1959 Grass silage	1959 DGS	June 1959	February 1960
5,6,7	1960 Grass silage	1960 DGS	June 1960	October 1960

[1] DGS indicates dried-grass silage.
[2] Field-cured.

Ground DGS obtained from grass ensiled in 1956, 1957 and 1959 and the grass hay were incorporated at a level of 20% in the diets of rats used in metabolism trials 1, 2, 3 and 4. The composition of these diets are shown in tables 2 and 3. Diets fed to rats in trials 1, 2 and 3 contained approximately 20% protein. The semipurified diets fed in trial 4 contained approximately 7.6% protein. Four rats fed the control diet in trial 2 were injected twice a week with 0.2 USP units of thyroid-stimulating hormone.

Trials 5, 6 and 7 were conducted to determine the influence of various fractions of grass silage on the BMR of rats.

The diets fed in all trials consisted of a constant portion and a variable portion which amounted to 80 and 20% of the diets, respectively. The constant portion, the same for all diets fed in any trial, contained all of the supplementary vitamins and minerals and most of the protein and energy required by the rat. In every trial one group of rats received a control diet consisting of 80% constant portion plus 20% of an artificial roughage of cornstarch, protein (purified soybean protein or casein) and cellulose. The artificial roughage was added to make these diets approximately equivalent in gross energy, crude fiber and protein to the treatment diets which contained 20% of ground DGS or hay. In those diets fed in trials 5, 6 and 7 in which a grass silage extract was incorporated into the diet, the variable portion was the same as in the control diet with the exception that the amount of cornstarch was reduced by an amount equal to the dry matter content of the concentrated grass silage extract.

Albino rats of the Wistar strain were used in all trials except trial 4 in which Hooded Norway rats of the Lane-Allen strain were used. The rats were kept in individual cages in an air conditioned room maintained at 22°C. Rats within each trial were fed either a control or treatment diet during a 3- or 4-day preliminary period prior to the start of the metabolism trial. The food intake was equalized during each preliminary period and was kept constant during the metabolism period.

The fractions incorporated into diets of rats on trials 5, 6 and 7 were obtained from ground 1960 DGS. The fractionation procedure which is diagrammed in figure 1 involved continuous, cold extraction of 2 kg of ground DGS for 36 hours each with petroleum ether and absolute ethanol. An aliquot of the DGS (Fraction 1) that had been extracted with petroleum ether-ethanol was saved for assay and the remainder placed in a percolator and 20 volumes of cold 80% ethanol percolated through for 36 hours. The 80% ethanol extract was concentrated to a volume of 400 ml (Fraction 4). Each milliliter, which contained 30 mg of dry matter, was equivalent to 5 g of DGS. A portion of the residue of the 80% ethanol extraction (Fraction 3) was dried and used for assay purposes and the remainder was refluxed twice for periods of 8 hours each with 10 volumes of distilled water. Filtration of this extract mixture yielded Fraction 6 which was concentrated in vacuo. The residue of the aqueous extraction (Fraction 5) was refluxed for 8 hours with 10 volumes of 0.1 N sulfuric acid. Filtration of the mixture yielded the acid ex-

TABLE 2

Composition of diets fed to rats in trials 1, 2 and 3

	Control diet	Silage diet	Hay diet
	%	%	%
Basal ration			
Yellow cornmeal	46.4	46.4	46.4
Soybean meal [1]	16.0	16.0	16.0
Dried whole milk	16.0	16.0	16.0
Brewer's yeast	0.7	0.7	0.7
NaCl	0.4	0.4	0.4
$CaCO_3$	0.4	0.4	0.4
Fish liver oil [2]	0.1	0.1	0.1
Total	80.0	80.0	80.0
Artificial roughage			
Cellulose [3]	10.0	—	—
Cornstarch	8.0	—	—
Purified soybean protein [4]	2.0	—	—
Natural roughages			
Ground 1956 DGS	—	20.0	—
or			
Ground 1957a DGS	—	—	—
or			
Ground 1957b DGS	—	—	—
Ground dried mixed grass hay	—	—	20.0

[1] Solvent-extracted.
[2] "Vital" Fish Liver Oil (containing not less than 2250 IU vitamin A and 600 IU vitamin D/g), Midland-Western, Inc., Reading, Pennsylvania.
[3] Solka Floc, Brown Company, Berlin, New Hampshire.
[4] Drackett Assay Protein C-1, Archer-Daniels-Midland, Minneapolis.

TABLE 3

Composition of diets fed to rats in trial 4

	Control diet	1959 DGS diet
	%	%
Cornstarch	75.4	63.9
Casein	7.2	5.2
Methionine	0.15	0.15
Salt mix [1]	3.2	3.2
Choline chloride	0.15	0.15
Corn oil [2]	4.0	4.0
Fish liver oil [3]	2.4	2.4
Glucose — vitamin mix [4]	1.0	1.0
Cellulose [5]	6.5	—
Ground 1959 DGS	—	20.0

[1] Salt mix USP XIV, Nutritional Biochemicals Corporation, Cleveland.
[2] Five milligrams α-tocopherol and 0.5 mg menadione/100 g of diet were dissolved in 10 ml of absolute ethanol and mixed with the corn oil.
[3] "Vital" Fish Liver Oil (containing not less than 2250 IU vitamin A and 600 IU vitamin D/g), Midland-Western, Inc., Reading, Pennsylvania.
[4] B-vitamin mixture of Harper et al. (5) was mixed with glucose so that one gram of glucose-vitamin mixture contained sufficient vitamins for 100 g of diet.
[5] Solka Floc, Brown Company, Berlin, New Hampshire.

tract (Fraction 8) and the residue of the acid extraction (Fraction 7).

The composition of diets containing the various silage fractions is shown in table 4. These diets contained approximately 12.6% protein. In the preparation of diets containing a silage extract as the variable factor, a known volume of extract was added to the amount of starch required in the diet. The starch-extract mixture was dried at 65°C and incorporated into the particular diet.

At the end of each trial the rats were fasted for 16 hours and the BMR was determined by indirect calorimetry.

The BMR of rats in trial 2 was determined after 7 and 60 days. The BMR of rats used in all other trials was determined only at the end of the feeding period.

The rate of oxygen consumption was determined in an apparatus similar to that described by Visscher et al. (8) except that a desiccator was used as the rat cham-

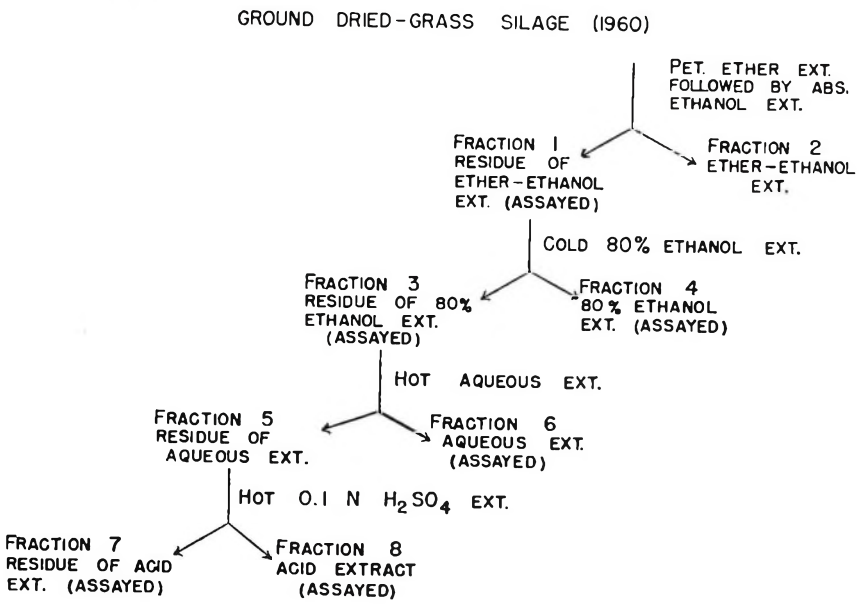

Fig. 1 Flow-diagram of dried-grass silage fractionation.

TABLE 4

Composition of diets fed to rats in trials 5, 6 and 7

	Diet designation as variable component								
	Control	1960 DGS	DGS Fraction 1	DGS Fraction 3	DGS Fraction 4 (1×)	DGS Fraction 4 (3×)	DGS Fraction 6	DGS Fraction 7	DGS Fraction 8
Trial	5,6,7	5	5	6	6	6	7	7	7
	%	%	%	%	%	%	%	%	%
Constant constituents [1]	80.0	80.0	80.0	80.0	80.0	80.0	80.0	80.0	80.0
Cornstarch	11.7				11.58	11.34	10.4	2.7	9.7
Casein	1.8				1.80	1.80	1.8	1.8	1.8
Cellulose [2]	6.5				6.50	6.50	6.5		6.5
Ground 1960 DGS [3]		20.0							
DGS Fraction 1 [4]			20.0						
DGS Fraction 3				20.0					
DGS Fraction 4					0.12 [5]	0.36 [6]			
DGS Fraction 6							1.3		
DGS Fraction 7								15.5	
DGS Fraction 8									2.0

[1] Eighty grams constant constituents contained in grams each of the following: cornstarch, 51.1; casein, 12.6; methionine, 0.3; salt mix USP XIV, 4.0 (Nutritional Biochemicals Corp., Cleveland); corn oil, 4.0, containing 5 mg *a*-tocopherol; fish liver oil, 3.0 (see footnote 3, table 3); B-vitamin mix, 5.0, Rama Rao et al. (6).

[2] Alpha cellulose, Nutritional Biochemicals Corporation, Cleveland.

[3] Ground 1960 dried-grass silage (DGS) was used in trials 5, 6 and 7.

[4] See figure 1 for fractionation procedure.

[5] Four milliliters of concentrated 80% ethanol extract of DGS contained 0.12 g dry matter; equivalent to 20 g DGS.

[6] Twelve milliliters of concentrated 80% ethanol extract of DGS contained 0.36 g dry matter; equivalent to 60 g DGS.

ber. The bottom of the desiccator contained soda lime for the absorption of carbon dioxide. For determining oxygen consumption rate a fasted rat was placed on a porcelain desiccator plate in the desiccator and sufficient time allowed for the rat to become relaxed. At this time the cover was placed on the desiccator and pure oxygen, saturated with water by passing through a wash bottle, was passed through the desiccator for 10 minutes to replace the air with oxygen. The animal chamber and gas-washing bottle were maintained in a constant temperature bath at 30.0°C ± 0.1. Following this a 50-cm^3 hypodermic syringe connected to the desiccator by means of rubber tubing was filled with oxygen to the 30-cm^3 mark. The intake and outlet tubing were then clamped to make this a closed system. Measurements on rats in trials 4 through 7 were made using a 100-cm^3 syringe and oxygen was filled to the 80-cm^3 mark. When the pressure of the desiccator became equal to that of the atmosphere, as indicated by the change in the manometer attached to the desiccator, a stop watch was started and as the animal used oxygen the piston of the syringe was moved inwardly to equalize the pressure. The time required for the animal to use 30 cm^3 of oxygen in trials 1, 2 and 3, or 80 cm^3 in trials 4 through 7, was recorded. The temperature of the desiccator, atmospheric pressure and weight of the rat were also recorded. The BMR was calculated from the oxygen consumption by assuming a respiratory quotient of 0.82. Calculations were made according to Brody (3) and are expressed as kilocalories per 24 hours per kilogram of body weight$^{0.75}$.

At the completion of the 60-day feeding period of trial 2 the thyroid glands of the male rats were removed, sectioned and stained with hematoxylin and eosin. The follicular cell height was measured according to the procedure of Armstrong and Hansel (1).

All data were subjected to analysis of variance according to Snedecor (7).

RESULTS AND DISCUSSION

Metabolism data presented in tables 5 and 6 indicate that rats fed diets containing 20% of either 1956 DGS, 1957a DGS, 1957b DGS, 1959 DGS or 1960 DGS exhibited significant increases ($P < 0.05$) in BMR above the rate of rats fed control diets. Increased BMR as the result of feeding diets containing 20% DGS occurred in growing and mature male and female albino rats and in mature male Hooded-Norway rats. The BMR was increased in male rats fed DGS-containing diets for 7 days, but the increase was greater after 60 days of feeding. Although the increases in BMR of female rats fed DGS were significant ($P < 0.05$) in all groups except those fed the 1957a DGS for 7 days, the increases as indicated in table 7 were less than 10%. In contrast with this the average increase in BMR of male rats fed the 5 samples of DGS was 28% above the controls. The percentage of dietary protein, which was approximately 20.0, 7.2 and 12.6% in trials 1 through 3, 4, and 5, respectively, did not influence the qualitative effect of DGS on BMR.

Injection of thyroid-stimulating hormone twice weekly into rats fed control diets in trial 2 for 7 days induced increases in BMR of about the same magnitude as in rats fed DGS. However, injection of thyroid-stimulating hormone twice weekly for 60 days resulted in a greater increase in BMR than resulted from DGS feeding.

The lack of change in BMR of rats fed ground grass hay suggests that the active substance(s) is not present in grass but occurs during ensiling. The influence of DGS on BMR may be due to the action of a compound(s) synthesized during the silage fermentation or to the release of an active compound(s) from larger molecules through enzymatic action. It appears improbable that sodium metabisulphite used in ensiling the 1956 and 1957 grass silage, and beet pulp used in ensiling the 1959 and 1960 grass silage, were responsible for the influence of DGS on BMR. However, the results reported do not preclude this possibility.

Histological examination of the thyroids of male rats in trial 2 revealed changes in the appearance of the follicles. Although more small follicles occurred in thyroid sections of rats fed DGS or injected with thyroid-stimulating hormone, the change in height of the follicular epithelium cell

was striking. The height of the follicular epithelium of the thyroids of rats fed the control diets was 5.5 μ compared with 7.4, 7.5 and 7.0 μ for the follicular epithelium of rats fed the ration containing 1957a DGS, 1957 DGS, or that received thyroid-stimulating hormone, respectively. The difference between means was highly significant ($P < 0.01$). This increase in follicular epithelial cell height on rats fed

TABLE 5

Effect of feeding ground dried-grass silage (DGS) and hay on the BMR of rats (trials 1, 2, 3 and 4)

Trial	Diet designation	No. of rats/ treatment	Days on treatment	Range of rat weights		BMR [1]	
				Male	Female	Male	Female
				g	*g*		
1				(352–429)	(184–223)		
1	Control	4	7			66	81
1	1956 DGS	4	7			93 [2]	86 [2]
1	1957a DGS	4	7			79 [2]	82
2				(113–138)	(102–122)		
2	Control	4	7			57	78
2	1957a DGS	4	7			70 [3]	84 [3]
2	1957b DGS	4	7			72 [3]	84 [3]
2	Control + TSH [4]	4	7			71 [3]	75
2				(254–300)	(146–183)		
2	Control	4	60			56	87
2	1957a DGS	4	60			77 [3]	117 [3]
2	1957b DGS	4	60			83 [3]	96 [3]
2	Control + TSH [4]	4	60			96 [3]	102 [3]
3				(397–456)			
3	Control	4	7			58	
3	1958 hay	4	7			57	
4				(357–411)			
4	Control	8	75			55	
4	1959 DGS [5]	8	75			62 [6]	

[1] Kilocalories/24 hr/$kg^{0.75}$.
[2] $D = 2.2$ at $P < 0.05$.
[3] $D = 5.1$ at $P < 0.05$.
[4] 0.2 USP units of thyroid-stimulating hormone (TSH) administered intraperitoneally twice a week.
[5] Albino rats were used in all trials except trial 4 in which Hooded-Norway rats were used.
[6] $P < 0.05$.

TABLE 6

Effect of feeding ground dried-grass silage and silage fractions on the BMR of mature male rats (trials 5, 6 and 7) [1]

Trial	Diet designation	No. of rats/ treatment	Days on treatment	BMR [2]
5	Control	6	15	64
5	1960 DGS [3]	6	15	70 [4]
5	DGS Fraction 1, residue of ether-ethanol extraction	6	15	77 [4]
6	Control	8	8	61
6	DGS Fraction 3, residue of 80% ethanol extraction	8	8	67 [5]
6	DGS Fraction 4 (1 ×), 80% ethanol extraction [6]	8	8	69 [5]
6	DGS Fraction 4 (3 ×), 80% ethanol extraction [7]	8	8	79 [5]
7	Control	7	11	55
7	DGS Fraction 6, aqueous extract	7	11	67 [8]
7	DGS Fraction 7, residue of acid extraction	7	11	56
7	DGS Fraction 8, acid extraction	6	11	56

[1] Range of weights of rats used in trials 5, 6 and 7 was 270 to 418 g.
[2] Kilocalories/24 hr/$kg^{0.75}$.
[3] Ground 1960 dried-grass silage (DGS) was used in trials 5, 6 and 7.
[4] $D = 4.2$ at $P < 0.05$.
[5] $D = 3.1$ at $P < 0.05$.
[6] 0.12 g of dry matter in the concentrated ethanol extract.
[7] 0.36 g of dry matter in the concentrated ethanol extract.
[8] $P < 0.01$.

TABLE 7

Summary of changes in basal metabolic rate of rats in trials 1 through 7

Treatment [1]	No. of rats/ treatment	Days on treatment	Change in BMR	
			Male	Female
			%	%
1956 DGS	4	7	+41	+ 6
1957a DGS	8	7	+21	+ 4
1957b DGS	4	7	+26	+ 8
Control + TSH [2]	4	7	+25	0
1957a DGS	4	60	+38	+35
1957b DGS	4	60	+48	+10
Control + TSH [2]	4	60	+71	+29
1958 Hay	4	7	0	—
1959 DGS	8	75	+13	—
1960 DGS	6	15	+ 9	—
DGS Fraction 1 [2]	6	15	+20	—
DGS Fraction 3	8	8	+10	—
DGS Fraction 4 (1 ×)	8	8	+13	—
DGS Fraction 4 (3 ×)	8	8	+29	—
DGS Fraction 6	7	11	+22	—
DGS Fraction 7	7	11	0	—
DGS Fraction 8	6	11	0	—

[1] DGS indicates dried-grass silage; TSH, thyroid-stimulating hormone.
[2] All DGS fractions made from 1960 DGS.

DGS suggests that the active substance(s) in DGS may act directly on the thyroid, which in turn influences BMR.

Preliminary work on the fractionation of DGS has revealed that extraction of DGS with petroleum ether followed by absolute ethanol results in a fraction having greater capacity to increase BMR. The report of Grosvenor and Turner (4) that a relatively low level of estradiol benzoate increased thyroid hormone secretion would tend to preclude the possibility that the inhibitory substances removed from DGS by the organic solvent are estrogens. Extraction of the DGS that had been extracted with petroleum ether-absolute ethanol with cold 80% ethanol removed most of the substance(s) that increased BMR in rats. Concentration of the 80% ethanol extract of DGS (Fraction 4) resulted in a preparation more than 160 times as active in increasing the BMR of rats than the unextracted 1960 DGS. Although most of the activity of the DGS was removed in the 80% ethanol extraction (Fraction 3), it still retained some of the factor(s) which increased BMR. Hot aqueous extraction of this residue removed the remainder of the activity. This was evident since the extraction of the residue of the aqueous extraction (Fraction 5) with hot 0.1 N H_2SO_4 yielded a fraction that did not increase the BMR of rats. Failure to obtain an active fraction after extraction with the hot acid solution suggests that the active substance(s) does not occur in the bound state in DGS.

LITERATURE CITED

1. Armstrong, D. T., and W. Hansell 1956 The effect of age and plane of nutrition on growth hormone and thyrotropic hormone content of pituitary glands of Holstein heifers. J. Animal Sci., *15:* 640.
2. Asplund, R. O., G. A. McLaren, H. O. Henderson and I. D. Porterfield 1959 Unusual magnitude and variation of plasma protein-bound iodine values of dairy cattle. J. Dairy Sci., *42:* 1718.
3. Brody, S. 1945 Bioenergenetics and Growth. Reinhold Publishing Corporation, New York.
4. Grosvenor, C. E., and C. W. Turner 1959 Effect of estrogen upon thyroxine secretion rate in intact female rats. Proc. Soc. Exp. Biol. Med., *101:* 194.
5. 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. J. Nutrition, *51:* 523.
6. Rao, P. B. R., V. C. Metta and B. C. Johnson 1959 The amino acid composition and the nutritive value of protein. I. Essential amino acid requirements of the growing rat. J. Nutrition, *69:* 387.
7. Snedecor, G. W. 1956 Statistical Methods, ed. 5. The Iowa State College Press, Ames.
8. Visscher, M. B., E. B. Brown and N. Lifson 1950 Experimental Physiology. Burgess Publishing Company, Minneapolis, Minnesota.

Mineral Utilization in the Rat

III. EFFECTS OF CALCIUM, PHOSPHORUS, LACTOSE AND SOURCE OF PROTEIN IN ZINC-DEFICIENT AND IN ZINC-ADEQUATE DIETS

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ABSTRACT A total of 96 weanling male albino rats was used in a study of effects of Ca, P, lactose, and substitution of isolated soy protein for whole egg white protein on growth and mineral utilization of normal and of Zn-deficient animals. Dietary Ca decreased weight gain, Zn absorption, femur Zn concentration, Mg absorption and balance, percentage Ca absorption and balance and percentage P absorption. It increased milligrams of Ca and P retained, percentage femur ash, and percentage Fe absorbed. Dietary P increased kidney calcification, percentage P absorbed and milligrams of P retained, while decreasing percentage P balance. Substitution of soy protein for whole egg white protein affected (increased) weight gain only of animals fed Zn-deficient diets. It decreased concentration of femur ash Zn, Ca and P absorption and balance, Mg, Zn, and Fe absorption. It increased kidney calcification. Lactose increased kidney calcification, percentage femur ash, Ca absorption and balance and Mg absorption. The main effects cited above were influenced by strong interactions, particularly between Ca and soy protein, indicating that the Ca effects were greater in the presence of soy protein. This is of practical importance in the consideration of Ca effects on Zn utilization.

The investigation reported here represents an extension of a previous study (1) of the effects on mineral utilization of adding Ca, Mg, and P to rat diets deficient in Mg and P. In the present study the basal diet contained normally adequate concentrations of these minerals, and the effects of adding Ca, P, and lactose and of substitution of isolated soy protein for whole egg white was investigated with zinc-deficient and with zinc-adequate diets.

The dietary variables were selected in view of the current interest in effects of Ca and P on Mg and Zn utilization (2–5), effects of lactose on mineral utilization (6–8) and effects of isolated soy protein on utilization of zinc and other trace minerals (9–11).

PROCEDURE

A total of 96 weanling male albino rats of the Sprague-Dawley strain was employed. They were received at 2-week intervals in groups of 24 and were kept on experiment for 28 days. The animals were kept in individual stainless steel cages throughout the experiment with deionized water always available to them. During the first 14 days, food intake was controlled, and a 7-day collection of excreta was made during the second week. Ad libitum feeding was permitted during the last 14 days and all feed intakes were recorded. At the end of the experiment animals were decapitated, and blood serum and tissue samples were obtained and preserved by freezing until analyses could be made.

Four diets were designed to contain 12% protein, either from egg white[1] or from isolated soy protein[2] supplemented with 3.4% DL-methionine. All diets contained 10% corn oil and 0.5% cod liver oil containing 0.6 mg vitamin A/g and 250 USP units of vitamin D/g. The carbohydrate was supplied either as glucose or as a mixture of glucose and lactose, the latter carbohydrate making up 25% of the diet. The basal salt mixture, added to all diets at a level of 2.89%, contained: (in per cent) NaCl, 12.7; K_2CO_3, 17.3; $FeSO_4 \cdot 7H_2O$, 1.27; $MnSO_4 \cdot H_2O$, 0.41; $CuSO_4 \cdot$

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[1] Spray-dried whole egg white, Nutritional Biochemicals Corporation, Cleveland.

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

TABLE 1

Mineral content of diets

			P	Ca	Mg	Fe	Zn−	Zn+
			%	%	%	%	*ppm*	*ppm*
Egg protein	Glucose	—	0.36	0.39	0.042	0.0056	1.6	9.6
		P	0.73	0.39	0.042	0.0056	1.6	9.6
		Ca	0.36	0.78	0.042	0.0056	1.6	9.6
		P+Ca	0.73	0.78	0.042	0.0056	1.6	9.6
	Lactose	—	0.36	0.39	0.042	0.0056	1.6	9.6
		P	0.73	0.39	0.042	0.0056	1.6	9.6
		Ca	0.36	0.78	0.042	0.0056	1.6	9.6
		P+Ca	0.73	0.78	0.042	0.0056	1.6	9.6
Soy protein	Glucose	—	0.57	0.40	0.042	0.0056	6.7	14.7
		P	0.91	0.40	0.042	0.0056	6.7	14.7
		Ca	0.57	0.78	0.042	0.0056	6.7	14.7
		P+Ca	0.91	0.78	0.042	0.0056	6.7	14.7
	Lactose	—	0.57	0.40	0.042	0.0056	6.7	14.7
		P	0.91	0.40	0.042	0.0056	6.7	14.7
		Ca	0.57	0.78	0.042	0.0056	6.7	14.7
		P+Ca	0.91	0.78	0.042	0.0056	6.7	14.7

$5H_2O$, 0.20; KI, 0.013; $CaHPO_4$, 47.0; $4MgCO_3 \cdot Mg(OH)_2 \cdot nH_2O$, 4.8; $NaH_2PO_4 \cdot H_2O$, 16.0. This mixture was designed to provide adequate levels of all mineral elements except zinc. A vitamin-glucose mixture of the following composition was added to all diets at a level of 5.0% : (in mg) thiamine·HCl, 440; riboflavin, 264; pyridoxine·HCl, 176; Ca panthothenate, 704; biotin, 9; niacin, 1100; folic acid, 22; vitamin B_{12}, 9 (0.1% in mannitol), menadione, 14; and (in grams) choline chloride, 66; glucose, 2117.

The 4 diets described above were each modified by addition of $CaCO_3$, $NaH_2PO_4 \cdot H_2O$ or both to give a total of 16 diets which were fed to a total of 48 rats in 2 periods (A and B). These 16 diets were then supplemented with $ZnCO_3$ and fed in similar fashion to 48 rats in 2 periods (C and D). Each diet was thus fed to a total of 3 rats. The diet designations and their analyzed mineral composition are shown in table 1.

Chemical analyses were conducted by the following methods: Ca and Mg by EDTA titration, employing Calcon and Erichrome Black T as indicators (12) and using an automatic titrator with constant rate delivery of titrant, following removal of P and interfering cations by use of zirconium nitrate and diethyldithiocarbamate (12), tungstic acid (13) or cyclohexanediaminetetraacetic acid on acetate-saturated Dowex 21K (14); P by the AOAC colorimetric procedure,[3] Zn by the method of Butts et al. (15); and Fe by the method of Moss and Mellon (16). Prior to analysis bones were dry-ashed after extraction for 24-hour periods with ethyl alcohol and with petroleum ether. Diets, kidneys and excreta were ashed with HNO_3 and H_2O_2. The ashed materials were taken up in a minimum of dilute HCl and made to a convenient volume with deionized water.

Statistical analysis of the data was carried out by standard analysis of variance procedures for a 2^4 factorial design.

RESULTS

At the end of a 4-week growth period rats fed the egg white diets in periods A and B showed the scaly paws and poor hair coat typical of Zn deficiency. A few of the animals fed soy protein diets showed these same symptoms but in milder form. No Zn deficiency symptoms were observed in animals fed in periods C and D.

Since the Zn deficiency produced in periods A and B markedly affected the performance of the animals in terms of feed intake and weight gain, the data for these

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

periods are presented separately from those of periods C and D in which Zn-adequate diets were fed.

Table 2 shows the 4-week gain and feed intake data and the results of the femur and kidney analyses. Each figure in the table represents the average obtained from 3 rats. The mean main effects and significant interactions are shown in table 3. The correlation between feed intake and weight gain was 0.993, considering all of the data for both levels of zinc. The various treatments had specific effects on feed intake and weight gain as indicated in table 3. These measures of performance were markedly decreased by addition of calcium to both the Zn-deficient and Zn-adequate diets and were increased by the substitution of isolated soy protein for egg white protein only in the case of the frankly Zn-deficient diets. The Ca effect on intake and gain was much greater with the soy protein diets than with the egg protein diets at both Zn levels. Although the addition of P did not in general affect gain and intake, it did decrease these in rats fed the Zn-adequate diets containing soy protein.

The main effect of lactose on gain was not significant but soy protein prevented the growth depression obtained with lactose in egg protein diets.

With respect to percentage ash and parts per million Zn in the ash of femurs, there were no differences due to treatment among rats fed the Zn-deficient diets, al-

TABLE 2

Twenty-eight-day weight gain, feed intake, bone and kidney composition in 4-week study

	Zinc	Glucose				Lactose			
		—	P	Ca	P + Ca	—	P	Ca	P + Ca
Egg protein diets									
Wt gain, g	—	19	19	19	18	16	21	12	8
	+	90	90	81	73	65	74	70	81
Feed, g	—	149	138	147	143	137	144	130	126
	+	248	254	240	234	219	239	221	241
Femur ash, %	—	54.7	53.4	56.1	55.6	54.3	53.3	57.5	57.4
	+	57.7	57.7	60.9	60.0	58.8	58.5	61.6	62.8
Femur ash Zn, ppm	—	313	293	292	283	318	384	320	496
	+	371	383	385	297	392	367	301	323
Kidney Ca, %	—	0.017	0.065	0.021	0.022	0.024	1.80	0.029	0.232
	+	0.041	0.104	0.041	0.401	0.096	3.69	0.094	1.95
Kidney Mg, %	—	0.073	0.094	0.068	0.070	0.068	0.168	0.072	0.071
	+	0.072	0.082	0.074	0.094	0.081	0.219	0.088	0.193
Soy protein diets									
Wt gain, g	—	76	60	34	38	68	66	45	39
	+	94	80	64	66	97	88	75	56
Feed, g	—	237	202	172	175	225	207	187	179
	+	273	237	223	230	273	262	234	205
Femur ash, %	—	58.1	58.1	58.6	57.2	56.2	59.3	59.0	54.9
	+	59.0	58.0	60.2	60.3	60.8	60.2	61.8	59.2
Femur ash Zn, ppm	—	312	333	247	325	378	308	340	307
	+	386	359	240	230	379	355	251	239
Kidney Ca, %	—	0.236	1.45	0.037	2.43	1.08	4.65	0.051	2.02
	+	0.790	2.51	0.045	1.92	1.07	4.27	0.525	2.21
Kidney Mg, %	—	0.084	0.136	0.065	0.148	0.111	0.212	0.069	0.142
	+	0.100	0.199	0.081	0.149	0.117	0.265	0.097	0.188

TABLE 3

Effects of dietary variables on 28-day weight gain, feed intake, bone and kidney composition

	Zn	Control group mean	Mean main effects				Significant interactions			
			P	Ca	Lactose	Soy	P × lactose	Lactose × soy	P × soy	Ca × soy
Weight gain, g	−	19	2	−17 [a]	0	37 [a]				−24 [a]
	+	90	−3	−14 [a]	−4	0		14 [a]	−14 [a]	−21 [a]
Feed intake, g	−	149	−8	−23 [a]	−3	58 [a]				−35 [a]
	+	248	−4	−22 [b]	−5	4			−27 [b]	−32 [b]
Femur ash, %	−	54.7	no significant effect							
	+	57.7	−0.5	2 [a]	1.2 [a]	0.2				−23 [a]
Femur ash Zn, ppm	−	313	no significant effect							
	+	371	−19	−91 [a]	−5	−47 [a]				−78 [a]
Kidney Ca, %	− [c]	0.017	1.39	−0.56	0.70	1.22	0.97			
	+	0.044	1.80 [a]	−0.68 [a]	1.01 [a]	0.87 [a]	1.58 [a]			
Kidney Mg, %	− [c]	0.073	0.054	−0.030	0.022	0.035	0.029			
	+	0.072	0.085 [a]	−0.021	0.050 [a]	0.036 [a]	0.071 [a]			

[a] Significant at 1% level.
[b] Significant at 5% level.
[c] Data in this row result from single pooled sample for each treatment.

though differences in these measurements were produced by dietary variables in the Zn-adequate diets. In rats fed the latter diets, the femur ash was increased by Ca and by lactose. The Ca effect was smaller in the presence of soy protein than in its absence. The concentration of Zn in the femur ash was definitely decreased by adding Ca or soy protein to the diets, and the effect of the Ca addition was magnified in the presence of soy protein. Actually, only in the presence of Ca did soy protein additions accompany a decrease in femur Zn concentration.

An important effect of some of the dietary variations relates to Ca and Mg deposition in the kidneys. The trend of the results is the same in both Zn-deficient and Zn-adequate diets, but in the former instance analyses were performed on pooled kidneys and statistics could not be applied. In the latter case individual pairs of kidneys were analyzed and the statistical analysis was conducted. On the average, soy protein, lactose and P each increased mineralization of the kidneys, whereas Ca had the opposite effect. The lactose × P combination was more than additive in its effect on kidney calcification. Kidney Mg concentration was less affected by dietary variables than was Ca, but the correlation coefficient between kidney Ca and Mg was 0.70 and was highly significant.

The data obtained relative to mineral absorption and balance are shown in table 4 and the mean main effects and significant interactions are shown in table 5.

The main effects of the dietary variables on Ca absorption and balance were qualitatively similar irrespective of Zn nutriture. Increasing dietary Ca from 0.4 to 0.8% reduced the percentage Ca absorbed and balance by 17 to 20% although the weight of Ca retained by the Zn-adequate animals was increased 84 mg. It is only in these latter animals that weight of Ca retained can be used as a criterion since equal feed intake was obtained only in this case. The main effect of feeding lactose was to increase the percentage Ca absorbed and balance by 7 to 14%, whereas soy protein feeding decreased these values by 7 to 9%. In no instance was the main effect of P significant on Ca absorption and balance.

TABLE 4

Mineral absorption and balance data

	Zn	Egg protein diets								Soy protein diets							
		Glucose				Lactose				Glucose				Lactose			
		—	P	Ca	P + Ca	—	P	Ca	P + Ca	—	P	Ca	P + Ca	—	P	Ca	P + Ca
Ca absorbed, %	—	93	91	73	67	96	96	92	87	80	81	62	57	97	95	80	73
	+	90	93	78	71	94	97	84	78	83	83	60	57	93	93	77	70
Ca balance, %	—	92	89	72	66	94	94	86	86	79	80	61	56	95	92	78	72
	+	87	90	72	69	92	94	71	75	80	81	58	55	89	90	71	68
Weekly Ca balance, mg	+	188	194	305	302	189	203	313	293	206	202	252	235	222	233	325	298
P absorbed, %	—	94	95	81	83	88	93	85	88	88	92	72	76	93	93	76	79
	+	93	96	85	87	91	94	81	86	90	91	72	82	90	93	75	78
P balance, %	—	58	43	68	42	60	36	72	49	52	26	48	32	58	43	57	38
	+	61	33	81	42	63	35	78	45	47	29	52	37	44	34	57	33
Weekly P balance, mg	+	124	136	162	172	121	144	153	183	149	142	166	185	164	172	178	156
Mg absorbed, %	—	87	84	77	73	85	86	84	87	77	73	58	53	91	86	76	65
	+	90	89	86	81	89	90	84	81	77	74	68	59	86	87	72	65
Mg balance, %	—	43	43	41	40	46	40	37	50	58	34	43	36	58	57	45	28
	+	45	44	35	44	43	40	31	44	44	46	39	33	39	43	40	32
Zn absorbed, %	—	77	74	67	63	57	55	49	53	59	66	46	48	83	79	51	45
	+	91	91	88	85	87	89	84	83	65	66	50	49	77	80	57	52
Fe absorbed, %	—	42	34	50	39	61	64	67	55	−4	9	19	26	27	−20	23	46
	+	29	37	44	51	38	39	57	63	13	13	26	15	11	23	13	17
Feed inatke, g	—	36	37	34	35	36	33	29	33	49	48	39	45	49	46	45	41
	+	56	56	56	56	53	56	54	55	56	54	56	55	54	56	55	53

TABLE 5

Effects of dietary variables on mineral absorption and balance

	Zn	Control group mean	Mean main effects				Significant interactions				
			P	Ca	Lactose	Soy	P × Ca	P × soy	Ca × lactose	Ca × soy	Lactose × soy
Feed intake, g	−	36	0	−4 [a]	−1	11 [a]					
	+	56	no significant effects								
Ca absorbed, %	−	93	−3 [b]	−17 [a]	14 [a]	−9 [a]	−3 [c]		9 [a]	−5 [c]	
	+	90	−2	−20 [a]	8 [a]	−8 [a]	−7 [b]			−6 [b]	8 [a]
Ca balance, %	−	92	−3	−17 [a]	3 [a]	−8 [a]			8 [a]		
	+	87	0	−21 [a]	7 [a]	−7 [a]					8 [a]
Weekly Ca balance, mg	+	188	−5	85 [a]	24 [a]	−2				−48 [a]	43 [a]
P absorbed, %	−	94	3 [a]	−12 [a]	2 [c]	−5 [a]			4 [c]	−8 [a]	
	+	93	4 [a]	−12 [a]	−1	−5 [a]				−5 [a]	
P balance, %	−	58	−21 [a]	4 [c]	6 [a]	−10 [a]				−9 [a]	8 [a]
	+	61	−24 [a]	10 [a]	1	−13 [a]		15 [a]		−7 [c]	
Weekly P balance, mg	+	124	9	26 [a]	5	15 [a]				−22 [c]	
Mg absorbed, %	−	87	−3 [c]	−12 [a]	10 [a]	−11 [a]				−13 [a]	9 [a]
	+	90	−3 [b]	−11 [a]	4 [a]	−13 [a]				−8 [a]	9 [a]
Mg balance, %	−	43	−5 [c]	−7 [a]	3	2		−14 [a]		−12 [b]	
	+	45	1	−6 [a]	−2	−1		−6 [b]			
Zn absorbed, %	−	77	1	−16 [a]	−4 [c]	2				−16 [a]	27 [a]
	+	91	−1	−12 [a]	3 [b]	−25 [a]				−15 [a]	12 [a]
Fe absorbed, %	−	42	−4	14 [a]	14 [a]	−36 [a]					
	+	29	−3	10	−4	−28 [a]					

[a] Significant at 1% level.
[b] Significant at 2.5% level.
[c] Significant at 5% level.

A number of interactions between these variables are apparent, but in this case there are differences in response of the animals dependent on their Zn nutriture. For example, in periods A and B, the lactose × Ca interaction was significant as a result of the lactose effect being greater in the presence of added Ca than in its absence. Actually, the Ca effect was greatly minimized by the presence of lactose. The soy protein effect was also minimized in the presence of lactose and was absent in presence of lactose at the lower Ca level. In periods C and D, on the other hand, the lactose × Ca interaction was not evident, but a strong soy protein × lactose interaction was evidenced by the fact that lactose almost entirely abolished the soy protein effect of decreasing Ca absorption and balance.

As in the case of Ca absorption and balance, the presence of Zn did not affect the qualitative response in terms of P absorption and balance to dietary treatment. Increasing dietary Ca decreased the percentage of P absorbed by 13% but this was accompanied by a decreased urinary excretion of P so that the net effect was an increase of 7 to 16% in P balance. The observed decrease in the percentage absorption and balance of P by rats fed soy protein diets probably occurred because the soy diets contained a higher concentration of total P than did the egg protein diets. Some of the P in the soy diets was present as phytate-P, which is less well utilized by the rat than is inorganic P. Nonetheless, the milligram P balance was greatest in animals fed the soy protein diets.

Deliberate addition of P to the diets resulted in a small but significant increase in the percentage P absorbed and a large decrease in percentage balance. The difference between effects on absorption and on balance was a result of increased urinary excertion of P sufficient to cause no difference in the milligram P balance despite the greater amount absorbed.

With respect to depressing the percentage P absorbed, the combination of soy protein and Ca had a greater effect than the sum of these variables individually. In terms of the percentage P balance, the combination of soy protein and Ca also yielded a lesser balance than the sum of their individual effects, again indicating a synergism between these, and pointing out that the effect of Ca and soy protein is more than additive in depressing P absorption and balance. In terms of the percentage balance, increased Ca did not overcome effects of increased soy protein.

The magnesium absorption was qualitatively influenced by the imposed variables in the same manner as was Ca. The presence of soy protein and of supplemental Ca depressed Mg absorption 14%, whereas lactose increased it 4 to 11%. The effect of P was to decrease Mg absorption slightly, an effect not observed with respect to Ca absorption. Lactose increased Mg absorption, its effect being evident only in presence of the soy protein diets. The decreased Mg absorption of rats fed soy protein diets was greatest when Ca was also fed as a supplement, the combined effect of these variables was more than additive.

The magnesium balance was depressed 17% by supplemental Ca, but not by lactose or by soy protein when the main effects are studied, since the changes in absorption brought about by these factors were offset by compensatory changes in urinary excretion. A depressing effect of P on Mg balance occurred in the presence of soy protein. In fact, soy protein alone increased Mg balance, P alone did not affect it, but their combination depressed Mg balance with respect to the control group. The soy × Ca interaction was also synergistic in its effect of decreasing Mg balance.

The percentage Zn absorption was decreased 27% by soy protein and 13 to 21% by Ca. Although the soy effect was present at both Ca levels, the Ca effect was present in only the presence of soy protein. Lactose itself was without significant effect, but its presence minimized the decrease of Zn absorption observed with soy protein diets.

The Fe absorption, like that of Zn, was decreased in the presence of soy protein, but unlike that of Zn, was increased by supplemental Ca. No significant amounts of iron were observed in the urine.

DISCUSSION

The stimulus of soy protein to feed intake and weight gain of rats fed the low-Zn diets is doubtless a result of the larger amount of Zn absorbed from these diets. The egg protein diets contained only 1.6 ppm Zn, and the soy protein diets contained 6.7 ppm, yet the apparent absorption of Zn from both of these treatments approximated 60%. All those animals receiving the egg diet low in Zn showed the scaly dermatitis typical of Zn deficiency in the rat. This was also true of several of those receiving the soy protein-low Zn diet and they did not grow as well as those receiving the Zn supplement in periods C and D.

The facts that dietary Ca depressed weight gain and feed intake and concentration of Zn in the femur ash, and that these effects were observed mainly in the presence of soy protein may be ascribed to the combined influence of Ca and soy protein on Zn absorption. The depression in Zn absorption accompanying the presence of both Ca and soy protein was greater than that in the presence of either variable alone. The Ca effect was significant only in the presence of soy protein. This observation contributes to our understanding of the practical observation that in some situations Ca exaggerates Zn deficiency symptoms more markedly than in others (5). More specificially, it appears that the effect of Ca on Zn utilization is to depress absorption and that a third dietary variable, probably involving phytic acid (17) mediates this interaction. Support for this is found in the publications of

Oberleas et al. (19).[4] These investigators have observed that Zn deficiency in swine was produced by adding Ca to a casein diet only if phytic acid were also added. They have also reported that the precipitate formed in a phytic acid solution at pH 6.0 by the addition of both Ca and Zn is greater than that formed by addition of either cation separately. The inference then drawn is that the Ca-phytic acid disturbance of Zn utilization is at the level of the gastrointestinal tract and not in the tissues. The present data support this interpretation.

None of the dietary treatments affected the Ca (36.6%) or the Mg (1.01%) concentration in the femur ash. The percentage ash in the femur was markedly depressed in the animals fed the 1.6 ppm Zn diets, but this was probably a reflection of total nutrient deficiency since the feed intake of these animals was so low. Among animals receiving supplemental Zn, Ca and lactose, each increased the percentage femur ash, but the Ca effect was minimized in the presence of soy protein. This is in agreement with the balance data, showing the milligrams of Ca to follow the same pattern. Although the addition of Ca did decrease weight gain of the animal, it increased the percentage ash in the femur and the milligrams of Ca gain.

The data relative to mineral absorption and balance show that inclusion of soy protein inhibited the percentage utilization of Ca, P, Mg, Zn and Fe as compared with that exhibited by rats receiving egg white diets. The effect of soy protein on Zn utilization by animals has been recorded previously (9, 11) and is thought to be a result of the phytic acid content of the isolated soy protein (17, 18). The effect on P utilization observed in this experiment is probably a result of 2 factors, the higher total P level of the soy protein diets and the relative lower availability of phytate-P. The effects on Ca, Mg and Fe absorption have yet to be explained and, indeed, with respect to Fe are in opposition to the data of Davis et al. (10).

Inclusion of lactose in this experiment improved absorption and balance of Ca, and the absorption but not the balance of Mg, and was without a consistent effect on Zn absorption. These effects are in agreement with our earlier investigations (19) and extend them by showing these effects to be modified by the protein source used in the diets. For example, lactose had only a slight effect in the egg protein diet but did increase Ca absorption and balance when added to the soy protein diet. In a similar manner, the effect of soy protein of decreasing Zn absorption was minimized by the presence of lactose.

Although Ca reduced the percentage absorption and balance of Ca, the milligrams of Ca retained were increased. This observation is verified by the increased absolute amount of P retained as a result of Ca addition. The effect of Ca in increasing Ca and P retention was not so great in the presence of soy protein as in presence of egg white. Although Ca reduced absorption of P, it increased the balance by decreasing urinary P excretion. The combined effect of Ca and soy protein was not additive with respect to P absorption and balance, mainly by virtue of the lesser effect of Ca in the soy diets. The effects of Ca on Mg absorption and balance was to decrease it, an effect which was exaggerated in the presence of soy protein as evidenced by the strong negative interaction between soy protein and Ca.

Phosphorus addition did not affect absorption or balance of Ca, of Zn or milligrams of P retained. It did, however, increase the percentage of P absorption and markedly decreased the percentage P balance by increasing urinary P. Phosphorus also had a small effect in decreasing absorption and balance of Mg, the effect on balance being greatest in the case of P addition to soy protein diets.

Supplemental Ca in this experiment was shown to decrease feed intake and gain over the 4-week period, to increase Ca and P retained, to decrease Zn and Mg absorbed and Mg balance, and to decrease the concentration of Zn in the femur ash. Possibly this effect of Ca on intake and gain was mediated by its effect on Zn nutriture of the animals — the regression of percentage Zn in femur ash on weight gain being highly significant, the concen-

[4] Oberleas, D., B. L. O'Dell and M. E. Muhrer 1962 Interaction of Ca and phytate in zinc availability J. Animal Sci., 21: 1008 (abstract).

tration of absorbable Zn in the various diets supplemented with Zn varying from 7.2 to 11.8 ppm, and the regression of weight gain on absorbable Zn being highly significant, with the regression of weight gain on Mg balance not significant. In an earlier paper we reported that the Zn requirement of the young rat is 8.2 to 8.6 ppm of absorbable Zn (9) lending support to the thesis that a limitation in absorbable Zn is the most probable reason for the Ca effects on performance in this study.

The Ca × soy protein interaction was highly significant for decreasing weight gain over the 4-week period and also resulted in strong reduction of the milligram Ca balance, milligram and percentage P balance and percentage Zn absorbed during collection periods (C and D) in which feed intake was constant and hence was not a complicating factor. The observation that the effect of soy protein of decreasing 4-week weight gain was less evident in lactose diets is in agreement with the effect of these variables on Ca and Zn absorption, soy protein decreasing these much more in the absence of lactose than in its presence.

The above considerations indicate that the main detrimental effects of soy protein and of Ca and the beneficial effects of lactose are a result of the effects of these variables on Zn utilization.

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LITERATURE CITED

1. Forbes, R. M. 1963 Mineral utilization in the rat. I. Effects of varying dietary ratios of calcium, magnesium and phosphorus. J. Nutrition, *80:* 321.
2. Alcock, N., and I. MacIntyre 1962 Interrelation of calcium and magnesium absorption. Clin. Sci., 22: 185.
3. Toothill, J. 1963 The effect of certain dietary factors on the apparent absorption of magnesium by the rat. Brit. J. Nutrition, *17:* 125.
4. Morris, E. R., and B. L. O'Dell 1963 Relationship of excess calcium and phosphorus to magnesium requirement and toxicity in guinea pigs. J. Nutrition, *81:* 175.
5. Forbes, R. M. 1960 Nutritional interactions of zinc and calcium. Federation Proc., *19:* 643.
6. Hegeness, F. W. 1959 Galactose ingestion and urinary excretion of calcium and magnesium. J. Nutrition, 69: 142.
7. Vaughan, O. W., and L. J. Filer 1960 Enhancing action of certain carbohydrates on the intestinal absorption of calcium in the rat. J. Nutrition, *71:* 10.
8. Dupuis, Y., and P. Fournier 1959 Effets de l'administration de divers glucides sur l'utilization calcique et sur la calcémie du jeune rat préablement carencé en calcium. C. R. Acad. Sci., 248: 2246.
9. Forbes, R. M., and M. Yohe 1960 Zinc requirement and balance studies with the rat. J. Nutrition, 70: 53.
10. Davis, P. N., L. C. Norris and F. H. Kratzer 1962 Interference of soybean proteins with the utilization of trace minerals. J. Nutrition, 77: 217.
11. Smith, W. H., M. P. Plumlee and W. M. Beeson 1962 Effect of source of protein on zinc requirement of the growing pig. J. Animal Sci., *21:* 399.
12. Malmstadt, H. V., and T. P. Hadjiioannou 1959 Rapid and accurate automatic titration method for determination of calcium and magnesium in plant material with EDTA as titrant. Agr. Food Chem., 7: 418.
13. Dunstone, J. R., N. P. Madsen and H. R. Bell 1960 The determination of calcium and magnesium in rat liver. Analyst., 85: 519.
14. Carlson, R. M., and C. M. Johnson 1961 Chelometric titration of calcium and magnesium in plant tissue. Method for elimination of interfering ions. Agr. Food Chem., *9:* 460.
15. Butts, P. G., A. R. Gahler and M. G. Mellon 1951 Determination of metals in industrial wastes. Metal Finishing, 49: 50.
16. Moss, M. L., and M. G Mellon 1942 Colorimetric determination of iron with 2,2′-bipyridyl and with 2,2,′2″-terpyridyl. Ind. Eng. Chem., *14:* 862.
17. O'Dell, B. L., and J. E. Savage 1960 Effect of phytic acid on zinc availability. Proc. Soc. Exp. Biol. Med., *103:* 304.
18. Oberleas, D., M. E. Muhrer and B. L. O'Dell 1962 Effects of phytic acid on zinc availability and parakeratosis in swine. J. Animal Sci., *21:* 57.
19. Forbes, R. M. 1961 Excretory patterns and and bone deposition of zinc, calcium and magnesium in the rat as influenced by zinc deficiency, EDTA and lactose. J. Nutrition, 74: 194.

Metabolism of 4,7,10,13,16-Docosapentaenoic Acid in the Essential Fatty Acid-deficient Rat [1]

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ABSTRACT Three groups of male rats of the Sprague-Dawley strain, in advanced stages of essential fatty acid deficiency, were given orally 150 mg/animal/day, of safflower oil, 50 mg/animal/day, of ethyl arachidonate and of methyl 4, 7, 10, 13, 16-docosapentaenoate, respectively. After a supplementation period of 52 days, the animals were killed by exsanguination, the livers were excised and the fatty acids of liver lipids analyzed. Growth, dermal symptoms and fatty acid composition showed that docosapentaenoic acid possessed almost the same degree of essential fatty acid activity as arachidonic acid. The fatty acid composition analyses also showed that docosapentaenoic acid was converted to arachidonic acid, and, together with other considerations, provided evidence for the degradation of unsaturated fatty acids via a concerted chain shortening-reduction process which may be represented as follows: 22:4,7,10,13,16 → [20:2,5,8,11,14] → 20:5,8,11,14.

Docosapentaenoic acid with double bonds in the 4,7,10,13,16 positions may be synthesized in the rat according to the main pathway of interconversions of polyunsaturated fatty acid (1, 2). Polyunsaturated fatty acids that possess essential fatty acid (EFA) activity have been characterized as having all-*cis* methylene interrupted double bonds, numbering from the 6 position from the terminal end (methyl group) of the molecule (3). Accordingly, it was demonstrated that 4,7,10,13,16-docosapentaenoate exhibited EFA activity (4).

Since 4,7,10,13,16-docosapentaenoic acid is synthesized from arachidonic acid, the question arises whether it or arachidonic acid plays the principle role in essential fatty acid activity. The present investigation shows that 4,7,10,13,16-docosapentaenoic acid corrects an EFA deficiency almost as well as arachidonic acid, and that it is converted to arachidonic acid in the rat. These observations are discussed in relation to present knowledge on EFA activity and interrelationships in the metabolism of polyunsaturated fatty acids.

EXPERIMENTAL PROCEDURE

Methyl 4,7,10,13,16-docosapentaenoate was prepared from egg yolk lipids by using a combination of low temperature fractional crystallization, urea fractionation and silver nitrate-silicic acid as described by Privett and Nickell (5). The final preparation was approximately 97% pure 22:5 by gas-liquid chromatography (GLC), and was completely devoid of methyl linoleate and methyl arachidonate. Structural analysis of the final preparation by reductive ozonolysis (6) showed that it consisted of 11% of the 5,8,11,14,17 isomer, and 89% of the 4,7,10,13,16 isomer.

Ethyl arachidonate was obtained commercially[2] and was 95% pure by GLC. High quality commercial safflower oil, containing 95% linoleic acid, was used.

Isolation and analysis of polyunsaturated fatty acids from liver tissues was carried out as described by Privett et al. (6).

Male rats of the Sprague-Dawley strain in advanced stages of EFA deficiency from having been fed a fat-free diet ad libitum for 9 months, were used in this study. All animals had severe symptoms of EFA deficiency at the start of the experiment. The fat-free diet had the following percentage composition: vitamin-test casein, 16; sucrose, 74; α-cellulose, 4; salt mixture, 4;[3] a mixture of vitamins in casein, 1; and casein containing chloride, 1 (7). Vitamins A and E were mixed into the diet in a diethyl ether solution; the ether was removed by evaporation under reduced pressure.

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[2] A gift from the Hoffmann-LaRoche Company, Nutley, New Jersey.

[3] Wesson, L. G. 1932 A modification of the Osborn-Mendel salt mixture containing only inorganic constituents. Science, 75: 339. Salt Mixture-W, obtained from Nutritional Biochemicals Corporation, Cleveland.

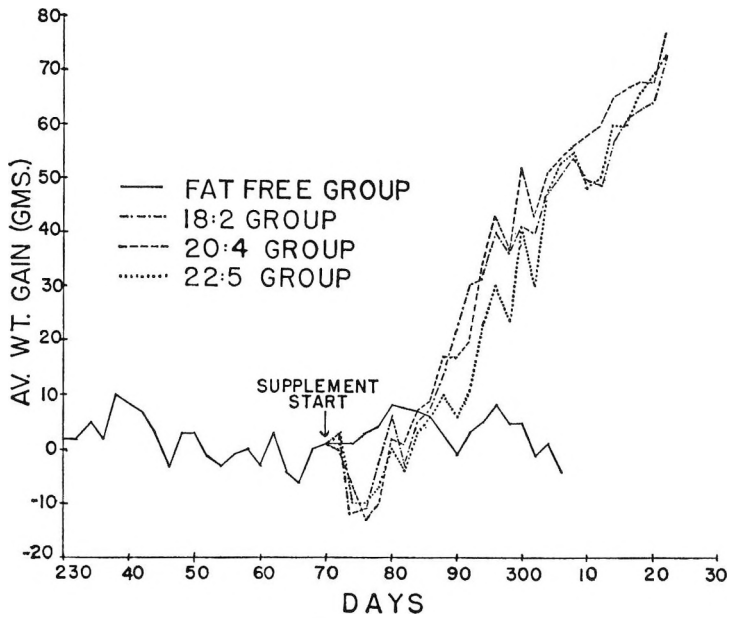


Fig. 1 Data showing growth rates of the 4 groups of animals. The 18:2 group received 150 mg of safflower oil; the 20:4 group, 50 mg of ethyl arachidonate; and the 22:5 group, 50 mg of methyl 4,7,10,13,16-docosapentaenoate/animal/day, at start of supplementation.

Four groups of 5 animals each were used in the experiment. One was given an oral administration of 50 mg/animal/day of ethyl arachidonate; another, 50 mg/animal/day of the methyl docosapentaenoate preparation; and a third group, 150 mg/animal/day of safflower oil. The fourth group continued to be fed the basic fat-free diet. The experiment was concluded after 52 days with the exhaustion of the supply of the arachidonate and docosapentaenoate supplements. The animals receiving the supplements continued to gain weight throughout the supplementation period, as shown in figure 1, but only one animal in the fourth group, the fat-free group, survived to the end of the experiment.

The animals were killed by exsanguination under light anesthesia, and the livers, as well as other organs, were excised. The liver lipids were extracted immediately, esterified with methanol and the fatty acid composition determined as described previously (8).

RESULTS

The animals used in this study did not gain any weight for 4 months prior to the start of the experiment. Thus, had the fat-free group of animals survived, they would not have gained weight during the additional period of the experiment. There was no significant difference in the increase in growth made by the animals of the 3 supplemented groups at the end of the experiment (table 1). The dermal symptoms of EFA deficiency were not completely cured in these groups, but they were greatly improved and, undoubtedly, would have been completely cured had sufficient supplements been available to continue the experiment.

The fatty acid composition of the livers of the animals of the 3 supplemented groups are presented in table 2. An analysis of the livers of a group of animals fed the fat-free diet for 6 months are included for comparison. The results also show that the animals in the supplemented groups had started to recover from the EFA deficiency, as evidenced by a decrease in the eicosatrienoic acid and an increase in eicosatetraenoic acid. Isolation and analysis of the 20:4 and the 22:5 acids from the docosapentaenoate-supplemented group showed that the 20:4 acids consisted exclusively of arachidonic acid, and that the 22:5 acid consisted of 5% of the 5,8,11,14,17-isomer and 95% of 4,7,10,13,16-isomer.

TABLE 1

Growth of essential fatty acid-deficient rats

Group	Avg wt at start of experiment	Avg wt at completion of experiment	Avg wt gain
	g	*g*	*g*
Fat-free	260 ± 13.5 [1]	—	—
Safflower	275 ± 12.0	348 ± 15.0	73 [2]
Arachidonate	240 ± 2.2	319 ± 25.8	79 [2]
Docosapentaenoate	226 ± 9.1	306 ± 22.4	80 [2]

[1] SD.
[2] Based on a weight gain of zero for the fat-free group; weight gains are significant at the 98% level of probability by the *t* test.

TABLE 2

Fatty acid composition of liver lipids

Fatty acid [1]	Safflower oil	Arachidonate 20:4	Docosa-pentaenoate	Fat-free
	%	%	%	%
14:0	0.9 ± 0.1 [2]	1.0 ± 0.3	0.5 ± 0.1	0.8
15:0	0.2 ± 0.2	0.4 ± 0.1	0.4 ± 0.1	tr
16:0	22.7 ± 1.6	21.3 ± 2.4	22.3 ± 2.3	21.4
16:1	9.4 ± 1.1	10.1 ± 0.8	9.8 ± 1.2	11.7
17:0	0.5 ± 0.1	1.1 ± 0.1	1.4 ± 0.2	0.7
18:0	12.1 ± 0.8	12.0 ± 2.5	12.6 ± 1.4	11.8
18:1	30.2 ± 2.3	30.1 ± 3.8	27.8 ± 2.1	39.1
18:2	4.5 ± 1.1	0.3 ± 0.2	0.7 ± 0.7	1.1
18:3	—	—	tr	—
20:1	0.2 ± 0.2	0.3 ± 0.1	0.4 ± 0.4	0.5
20:3	2.2 ± 0.3	1.5 ± 0.7 [3]	3.0 ± 0.7 [3]	11.2
20:4	13.9 ± 0.7	18.5 ± 4.3	8.6 ± 0.5	1.3
(?)	tr	tr	—	—
(?)	0.2 ± 0.1	0.7 ± 0.1	—	0.4
22:5	2.2 ± 0.3	2.6 ± 0.2	11.3 ± 2.8	tr
22:6	0.4 ± 0.2	tr	1.1 ± 0.6	tr

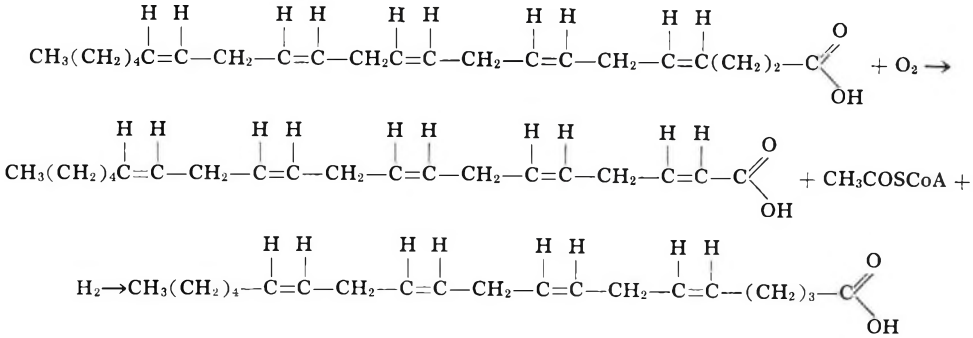
[1] The first figure represents the number of carbon atoms, the second the number of double bonds.
[2] SD.
[3] The level of significant difference of 20:3 in these 2 groups is at 92% probability by the *t* test.

DISCUSSION

There is little direct evidence that arachidonic acid is converted to 4,7,10,13, 16-docosapentaenoic acid by tracer experiments (9), but such a conversion is generally accepted from theoretical considerations (1, 2). Evidence for this conversion in the present studies is the absence of docosapentaenoic acid in the EFA-deficient animal and its presence in animals which have been fed either safflower oil or ethyl arachidonate.

The data on the depression of eicosatrienoic acid indicated that methyl docosapentaenoate acid was not as efficient as ethyl arachidonate for correcting an EFA deficiency in rats. Although the difference in the EFA activity was not great in this experiment, it was significant at the 92% level of probability on the basis of the lowering of the eicosatrienoic acid. However, more elaborate experiments must be performed to estimate quantitatively the difference in the effectiveness of these compounds.

The results (table 2) showed that 4,7, 10,13,16-docosapentaenoic acid was converted to arachidonic acid, because the elevation of 20:4 in the tissues of this group approached that of the arachidonate-fed group. The mode of this conversion may be visualized as a combination of chain shortening and reduction of the double bond in the 2-position of the 2-carbon shorter intermediate. However, the process may be a combined one in which the intermediate with a double bond in the 2-position exists only transitorily, if at all, because fatty acids with double bonds in the 1-, 2- or 3-position have not been detected in animal tissues. The steps in the process may be represented as follows:



An analogy for the reduction of a double bond in conjugation with a carbonyl group may be seen in the case of the synthesis of prenoic acids (10). That fatty acids with a double bond in the 1- or 3-position may likewise be reduced in a scheme similar to that above is indicated since they, also, are not found in animal tissues.

The mode of oxidative degradation of saturated fatty acids has been well established (11). Unsaturated fatty acids also undergo oxidative degradation as readily as saturated fatty acids in the animal body, but the pathway has not been delineated (1, 11). One may also speculate, on the basis of the present study, that unsaturated fatty acids are degraded by the mechanism shown above or in a combined reaction in which hydrogen peroxide has a role in a catalase-type reaction. Either pathway may be criticized on the basis that oleic and linoleic acid, for example, should give a series of short-chain monoenoic and dienoic fatty acids. However, intermediates in a fast process would not only be present in extremely small amounts, but might exist for only a very short time should the reaction be intermittent or controlled. Fatty acids with double bonds in the 1-, 2- or 3-position may be considered as intermediates in a similar manner in the degradation of polyunsaturated fatty acids in which the first double bond is in position 5 from the carboxyl group.

Since arachidonic acid is converted to docosapentaenoic acid, these fatty acids may be considered to be in equilibrium with each other. Thus, a regulatory type of mechanism may govern the relationship between many polyunsaturated fatty acids as opposed to a competitive mechanism. A competitive mechanism is based primarily on the basis that under certain conditions linolenic acid appears to inhibit the conversion of linoleic acid to arachidonic acid (12–15). However, inconsistent with this view is the observation that administration of linolenic acid to rats fed a fat-free diet accelerated the depletion of arachidonic acid from the tissues (8).

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LITERATURE CITED

1. Mead, J. F. 1960 Metabolism of the unsaturated fatty acids. Lipide Metabolism, ed., K. Bloch. John Wiley and Sons, Inc., New York, chapt. 2, p. 41.
2. Mead, J. F., and D. R. Howton 1958 Interconversions of unsaturated fatty acids. Essential Fatty Acids, ed., H. M. Sinclair. Academic Press, Inc., New York, p. 15.
3. Thomasson, H. J. 1953 Biological standardization of essential fatty acids (a new method). Internat. Ztschr. Vitaminforsch., *25:* 62.
4. de Jongh, H., and H. J. Thomasson 1956 Essential fatty acid activity of docosapolyenoic acids from brain glycerophosphatides. Nature, *178:* 1051.
5. Privett, O. S., and E. C. Nickell 1963 Preparation of highly purified fatty acids via liquid-liquid chromatography. J. Am. Oil Chemists' Soc., *40:* 189.
6. Privett, O. S., M. L. Blank and O. Romanus 1963 Isolation analysis of tissue fatty acids by ultramicro-ozonolysis in conjunction with thin-layer chromatography and gas-liqiud chromatography. J. Lipid Res., *4:* 260.
7. Aaes-Jørgenson, E., and R. T. Holman 1958 Essential fatty acid deficiency. 1. Content of polyenoic acids in testes and heart as an indicator of EFA status. J. Nutrition, *65:* 633.
8. Blank, M. L., and O. S. Privett 1963 Studies on the metabolism of *cis, trans*-isomers of methyl linoleate and linolenate. J. Lipid Res., *4:* 470.

9. Mead, J. F., and D. R. Howton 1957 Metabolism of essential fatty acids. VII. Conversion of α-linolenic acid to arachidonic acid. J. Biol. Chem., *229:* 575.
10. Christophe, J., and G. Popjak 1961 Studies on the biosynthesis of cholesterol. XIV. The origin of prenoic acids from allyl pyrophosphates in liver enzyme systems. J. Lipid Res., *2:* 244.
11. Green, D. E., and S. J. Wakil 1960 Enzymatic mechanisms of fatty acid oxidation and synthesis. Lipide Metabolism, ed., K. Bloch. John Wiley and Sons, Inc., New York, p. 1.
12. Mohrhauer, H., and R. T. Holman 1963 Alteration of the fatty acid composition of brain lipids by varying levels of dietary essential fatty acids. J. Neurochem., *10:* 523.
13. Mohrhauer, H., and R. T. Holman 1963 Effect of linolenic acid upon the metabolism of linoleic acid. J. Nutrition, *81:* 67.
14. Marco, G. S., L. J. Machlin, E. Emery and R. S. Gordon 1961 Dietary effects of fats upon fatty acid composition of the mitochondria. Arch. Biochem. Biophys., *94:* 115.
15. Machlin, L. J. 1962 Effect of dietary linolenate on the proportion of linoleate and arachidonate in liver fat. Nature, *194:* 868.

Chromium, Lead, Cadmium, Nickel and Titanium in Mice: Effect on Mortality, Tumors and Tissue Levels [1]

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ABSTRACT About 700 mice were given throughout their lives 5 ppm cadmium, lead, chromium, nickel or titanium in drinking water while fed a diet deficient in cadmium and low in other metals. Tissue concentrations comparable to those of man were observed. Sex differences appeared. Mortality of females was unaffected. Mortality of males on cadmium, lead and nickel was increased compared with that of the chromium group, and of those on cadmium and lead compared with the controls. Longevity of the oldest 10% of both sexes was less in lead and titanium groups, and of males in cadmium group, compared with controls. No metal was carcinogenic; incidence of tumors in males on cadmium and lead, and in females given nickel was decreased. Body weights at death were greater in titanium, chromium and lead groups. Large increments of titanium, moderate increments of cadmium and nickel, and lesser increments of chromium and lead were observed in 5 organs, compared with controls. Higher concentrations of cadmium and titanium occurred in younger mice and the other metals did not increase markedly in tissues with age. All metals except chromium exhibited one or more signs of innate toxicity.

We have reported the effects of small doses of chromium, lead, cadmium, nickel and titanium in drinking water on the growth and survival of mice up to 21 months of age in experiments attempting to duplicate human concentrations (1). Chromium and titanium increased growth rates in both sexes; in males chromium lessened early mortality, whereas cadmium and lead increased mortality at older ages. This report is concerned with our total experience for the lifetime of these animals, regarding mortality, gross causes of death, effects on incidence of tumors and organ accumulations of metals.

MATERIAL AND METHODS

The experimental conditions of the relatively metal-free environment has been reported in detail (1). White Swiss mice of the Charles River strain numbering 697 were exposed from the time of weaning to drinking water containing the essential trace metals, manganese, cobalt, copper, zinc and molybdenum. The diet of seed rye flour, powdered skim milk and corn oil was low in most of the metals under study and devoid of cadmium (1). It also contained only a trace of arsenic, 0.05 μg/g. Iron and vitamins were added. Plastic cages, stainless steel covers and drinking tubes, polyethylene water bottles and wood chips of low metallic content largely prevented environmental exposures to extraneous trace metals. One of the following as the acetate or oxalate[3] was added to the drinking water at 5 ppm metal: cadmium, divalent lead, trivalent chromium, divalent nickel and tetravalent titanium, and given for life to 50 or more animals of each sex. Experiments continued until all animals had died; the total elapsed time was 36 months. No animals were bred.

Dead animals were weighed and dissected, gross anatomical lesions were recorded and abnormal tissues fixed in Bouin's solution, sectioned and stained. Microscopic examinations were made on 257 sections. Although mice were autopsied as they died and each series was run concurrently, cannibalism and gross autolytic changes occurring over week-ends made many carcasses unfit for analysis. The figures shown in the tables, 251 males and

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[3] Reagent grade chemicals were used, stored in solution in polyethylene bottles.

222 females, are those of available autopsied mice, whereas data on mortality comprise all 697 animals.

Organs large enough for analysis (heart, lungs, kidneys, liver, spleen) were frozen in polyethylene bottles and, after a convenient interval, 3 to 6 of each group were pooled according to age at death, dried at 110°C, ashed in a muffle furnace at 400°C and analyzed for as many of the metals as the size of each sample allowed. Controls for each metal included tissues of animals given another metal. Microanalytical chemical methods of Saltzman (2, 3) and Sandell (4) were used as previously reported (5–9). Nearly 1000 analyses were made. All data are given in terms of wet weight and a value of 0.0 indicates that the metal was not detected by the method used. Statistics were analyzed by chi-square or Student's t test.

Water was weighed to the nearest gram at frequent intervals. Unavoidable wastage was caused by, 1) slight variations in environmental temperature, 2) movement of the drinking tube by playing mice, and 3) the habit of stuffing bedding, food and feces into the drinking tube with consequent irregular leakage. Food was allowed ad libitum and was not weighed because of considerable wastage in powdered form.

RESULTS

Major causes of death. The gross anatomical lesions in the 473 mice autopsied were divided into 4 categories: tumors, internal hemorrhage, infection and others, plus unknown (visible renal or hepatic lesions, splenomegaly, dilatation of the intestines or no major abnormalities). In table 1 are shown the causes of death. The only significant change was the reduction of visible tumors in a cadmium, lead or nickel group. In the control females there were 9 pulmonary, 3 mammary and 2 thyroid carcinomas, and one each in liver, peritoneal cavity, skin, ovary, ureter and of unknown origin with extensive metastases, as well as 2 leukemic infiltrations of spleen. Only 3 mice given nickel had tumors, all pulmonary ($P < 0.01$). Two to 5 mammary tumors and 4 to 5 lung tumors occurred in each of the other metal-fed groups.

In the control males there were 8 lung carcinomas, 1 sarcoma in the liver and 2 leukemic infiltrations of spleen. Only 2 tumors appeared in the lead and cadmium groups. One mouse given lead, one given titanium and none given cadmium had a pulmonary tumor. The incidence of these tumors was 13.5% in 222 autopsied females and 12.5% in 164 males, excluding

TABLE 1

Gross causes of death in mice [1] given various metals: significant differences from controls

	Controls	Cadmium		Lead		Nickel		Titanium		Chromium
	No.	No.	P value [2]	No.	P value	No.	P value	No.	P value	No.
Males										
Tumors	11(5) [3]	1	< 0.005	1(1)	< 0.01	7(4)		4(3)		6(3)
(Lung)	8	0	< 0.01	1		5		1	< 0.05	6
Hemorrhage	7	16		12		14		10		12
Infection	5	4		15		9		5		11
Other and unknown	21	27		11		11		21		10
Total no.	44	48		39		41		40		39
Females										
Tumors	22(8)	10(2)		11		3(3)	< 0.01	7(2)		9(4)
(Lung)	9	5		4		3		5		4
Hemorrhage	9	9		5		8		3		2
Infection	11	6		10		12		9		11
Other and unknown	18	14		3		10		13		7
Total no.	60	39		29		33		32		29

[1] Autopsied mice.
[2] P = probability according to chi-square analysis of differences from controls being due to chance.
[3] Numbers in parentheses are deaths from tumor before 600 days of age.

those given cadmium and lead. By chi-square analysis of these larger groups, the reduction of male pulmonary tumors associated with cadmium and lead was significant ($P < 0.005$).

The ages at death of the 30 male mice with tumors were studied to ascertain whether early mortality from cadmium or lead could affect the appearance of neoplasms. Whereas 16 deaths from tumor (53.3%) occurred before 600 days of age, one in the lead group died during this interval ($P < 0.05$). Of the 14 deaths from tumor after 600 days of age, one was in the cadmium group ($P < 0.05$). At the half-lives of each group none of the 8 deaths from tumors were in the cadmium- or lead-fed animals. At 500 days of age 46 males were alive in these 2 groups; tumors occurred in only two ($P < 0.05$). In the females 2 of the 21 deaths from tumor in the cadmium and lead groups occurred before 600 days of age, compared with 17 (27.4%) in the others ($P < 0.025$).[4] No deaths of nickel-fed mice occurred after 547 days of age ($P < 0.005$).

The typical lung tumor was an adenocarcinoma, of which 45 were observed out of 51 lung tumors. Eleven mammary tumors, 8 sarcomata, 4 splenomegalias of leukemic origin, 4 thyroid, 3 hepatic, 3 skin, 2 ovarian, 2 ureteral and 1 each in various other sites, totaled 92. The incidences of all tumors were: males 11.9%; females 27.8%.

There were no obvious changes in other causes of death, such as pneumonitis (19 cases), pyelonephritis (12 cases), mediastinal infections (4 cases), hepatic cystic or parenchymal degeneration (7 cases). Of 12 kidneys examined in the cadmium and lead groups, 8 showed hyalinized glomeruli, thickened basement membrane and a reduction of lumen to wall of arterioles; lesser alterations were seen in 3 others.

Mortality. Survival curves are shown in figures 1–6. Because the curves of 5 groups of male animals are rather straight, or slightly concave, unlike "normal" survival curves, indicating early mortality, a deficiency in the basic diet for males may have been present. As the rate of growth of mice fed chromium is increased (1) and the survival curve of males more nearly resembles a "normal" one, males fed each metal were compared with the chromium as well as to the control groups (figs. 3–6).

Compared with the chromium group, mortality rates were significantly higher in males given cadmium, lead and nickel. Males fed cadmium and lead also had higher mortalities at one or more intervals than did the controls. Significant differences in the groups on metals did not appear in females at any age (figs. 1, 2). Although mice of both sexes given titanium died at somewhat earlier intervals, changes were relatively small (figs. 2, 3).

To ascertain whether lifespan was affected, the ages at death of the last surviving 10% of animals in each group were compared with the controls. Longevity was less in each group fed a metal (table 2), and was significantly lower in males fed lead, cadmium, and titanium and in females given lead. Males receiving titanium lived 80.5%, cadmium 85.1%, chromium 86.8%, and lead 90.4% as long as controls, or 92 to 187 days less. Females given titanium and those receiving lead lived 91.5% as long as controls. There was no sex difference in mean lifespans of controls treated in this manner. A few animals lived more than 1000 days; 4 controls, three fed chromium and three given nickel. Of the titanium group the last surviving male died 231 days and the last surviving female 118 days before the oldest control animals. The relative order of decreased longevity was: males, titanium > cadmium > lead > chromium; females, titanium ≅ lead > cadmium.

Body weights at death. Mean weights of animals dying at less than one year, 1 to 2 years and more than 2 years of age were calculated. At ages after one year animals of both sexes taking nickel weighed somewhat less, by 4 to 13%, and those fed the other 4 metals, more than did the controls. The differences were small in females, 3 to 11%. In males dying under one year of age, mean weights compared with controls were: cadmium, 113%; lead, 122% ($P < 0.01$); chromium, 123% ($P < 0.025$); titanium 138%, ($P <$

[4] Percentages of deaths from tumor in both sexes occurring at various ages were: < 400 days, 3.1%; 400 to 499 days, 12.8%; 500 to 599 days, 23.4%; 600 to 799 days, 43.6%; 800+ days, 17.1%. The assumption is made that autolyzed and cannabalized mice not examined were randomly selected and did not influence the incidence of tumors.

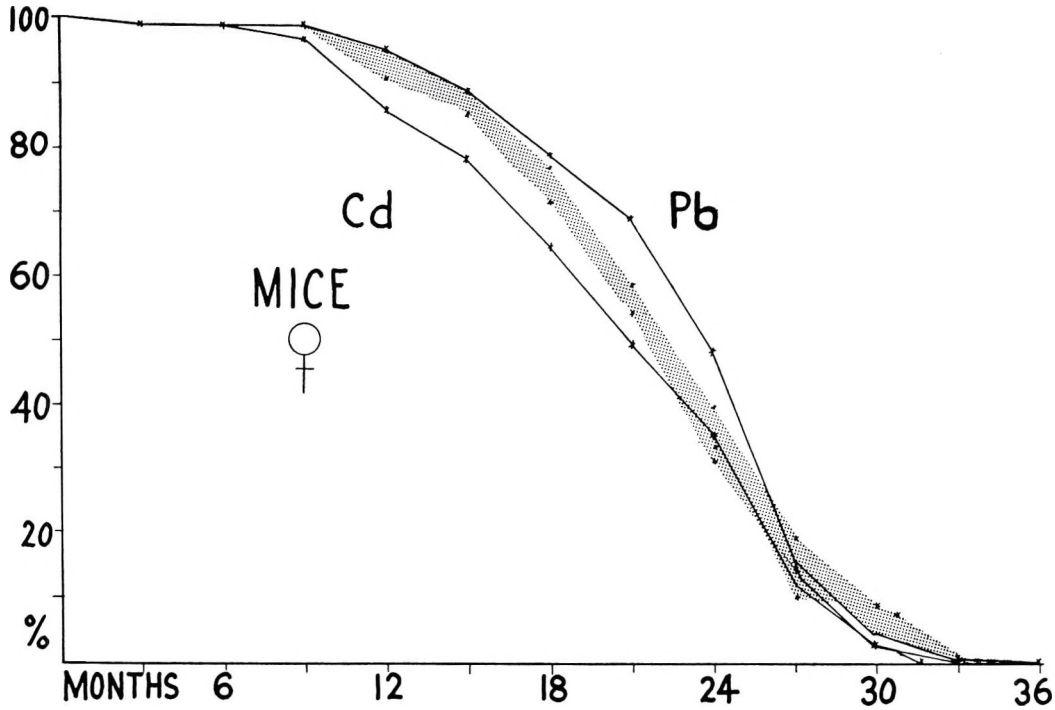

Fig. 1 Survival of female mice. The shaded area represents the curves of 54 mice given nickel, 54 given chromium, and 88 controls. Curves for 60 fed cadmium and 52 fed lead are shown separately. There were no significant differences at any 3-month period, except between lead and cadmium at 21 months, where $P < 0.05$ by chi-square analysis.

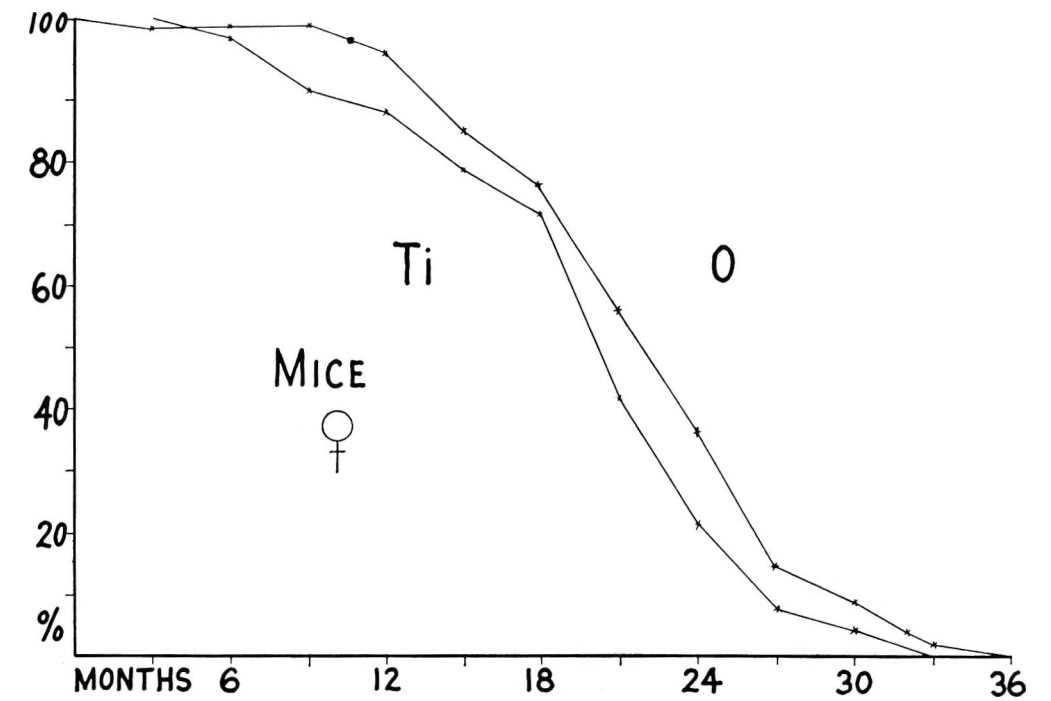

Fig. 2 Survival of 53 female mice given titanium and of 88 controls (O). No significant differences appear at any interval.

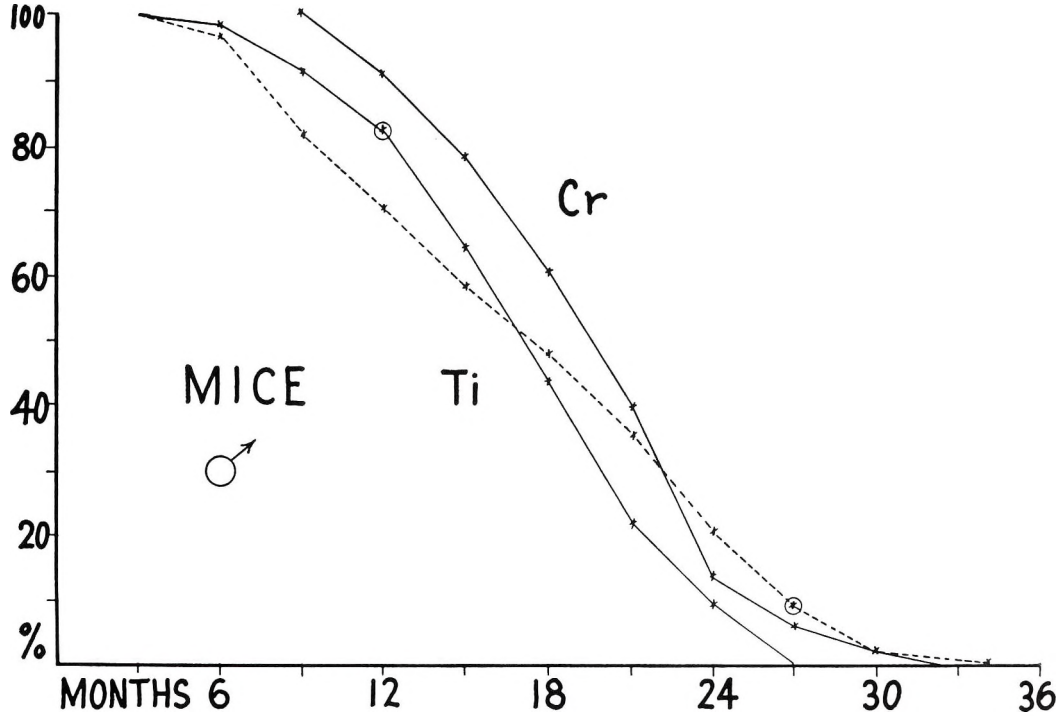

Fig. 3 Survival of 54 male mice given titanium, 54 given chromium and 61 controls (dashed line). The circles represent significant differences between titanium-fed animals and the controls; at 12 months. $P < 0.05$, at 27 months, $P < 0.025$. Chromium-fed mice differed from the controls significantly up to 17 months of age. Note that all given titanium had died by 27 months of age.

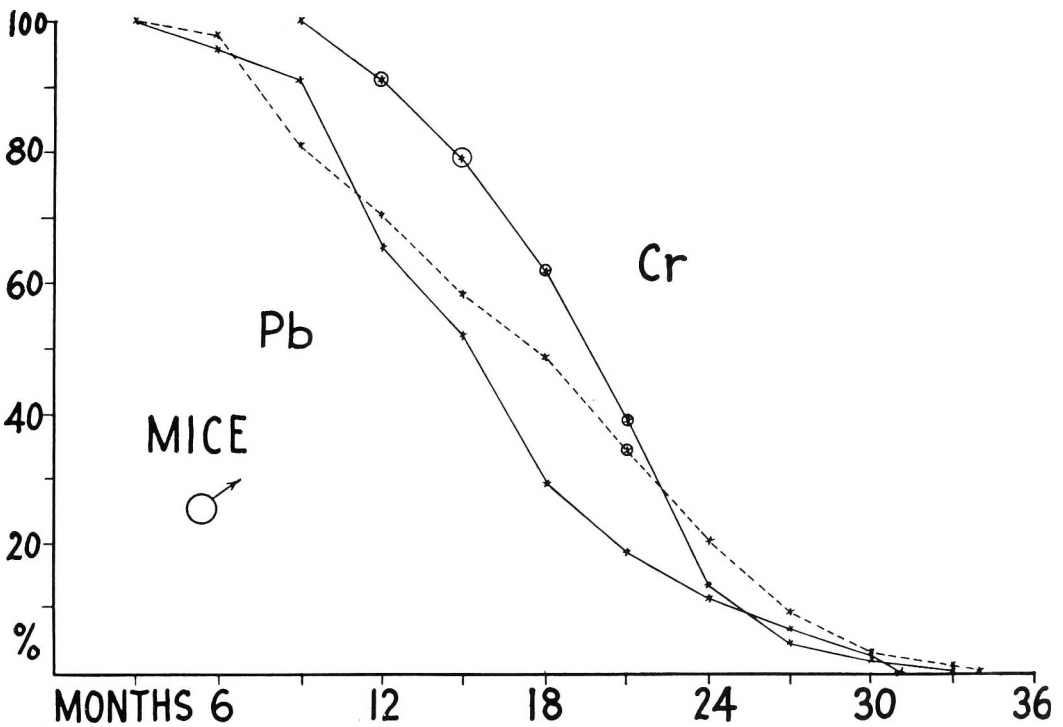

Fig. 4 Survival of 52 male mice given lead compared with 61 controls (dashed line) and 54 given chromium. The circles represent significant differences; at 12, 15 and 18 months, $P < 0.001$, at 21 months, $P < 0.025$. The half-life of lead-fed animals was 146 days less than those fed chromium.

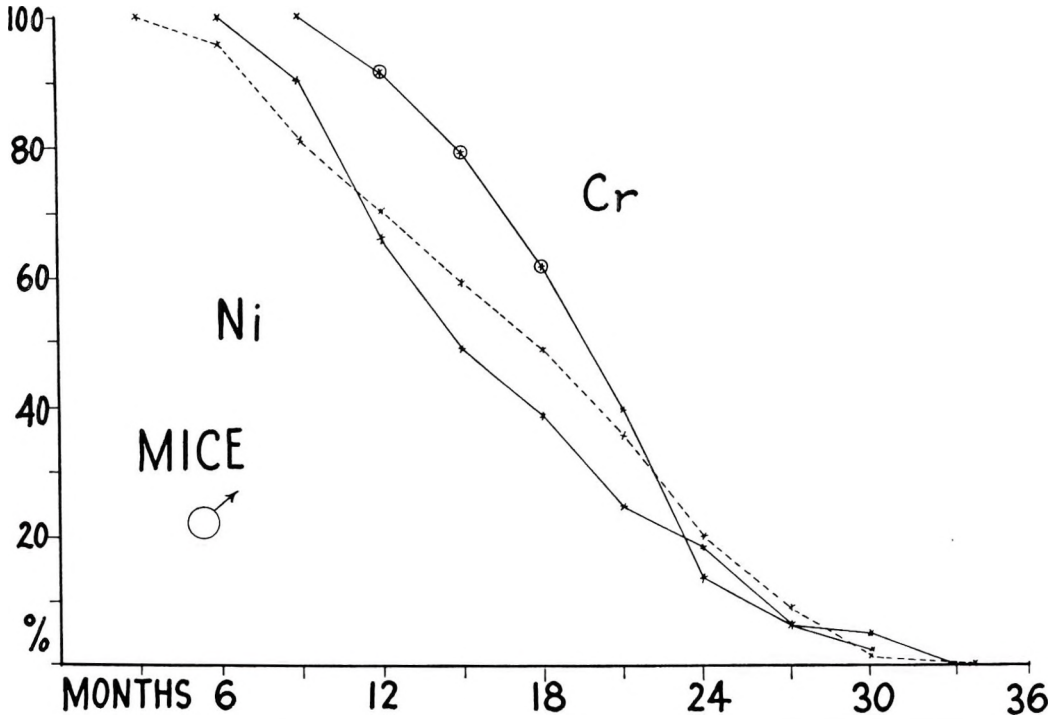

Fig. 5 Survival of 50 male mice given nickel, compared with 61 controls (dashed line) and 54 on chromium. The circles represent significant differences from the nickel-fed animals; at 12 and 15 months, $P < 0.0005$, at 18 months, $P < 0.02$. The half-life of the nickel group was 160 days less than that of the chromium group.

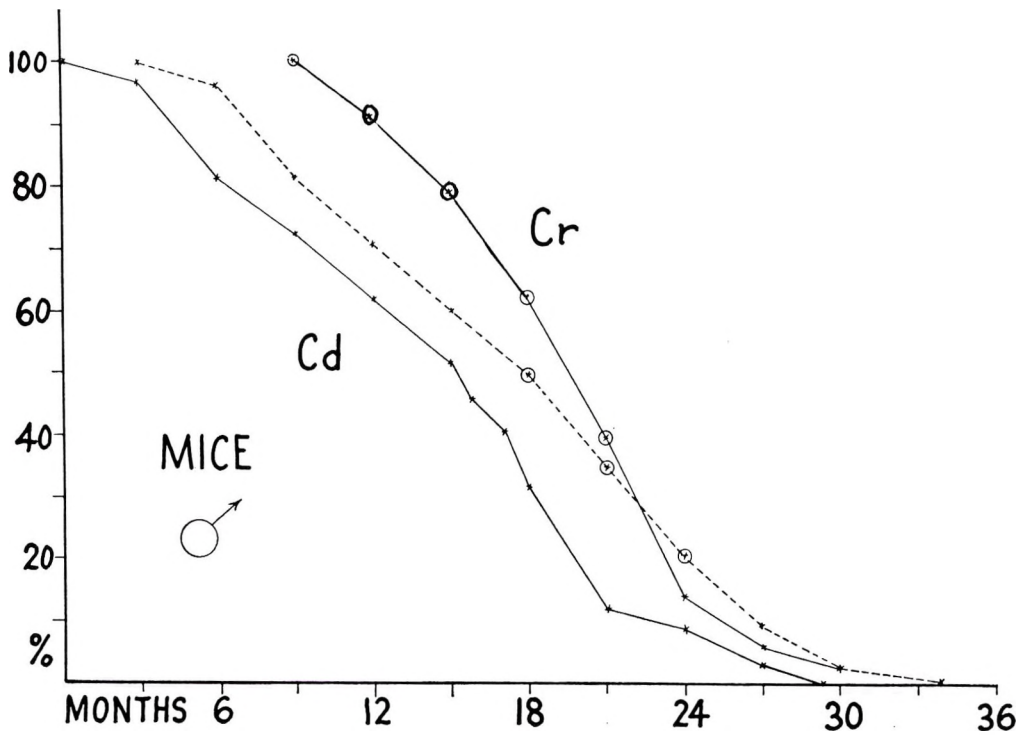

Fig. 6 Survival of 65 male mice given cadmium, compared with 61 controls and 54 fed chromium. The circles represent significant differences from the cadmium-fed animals; from 9 to 21 months, $P < 0.001$, for chromium; at 18 and 21 months for controls, $P < 0.01$; at 24 months, $P < 0.02$. Mice given cadmium had a half-life 133 days less than those fed chromium.

TABLE 2

Longevity of mice given trace metals: ages at which 75% were dead, mean ages at death of last surviving 10%, and ages of last survivors (days)

	Males				Females			
	No.	75% dead	Mean age	Maximal age	No.	75% dead	Mean age	Maximal age
Control	61	693	957	1035	88	777	966	1084
Nickel	50	623	896	995	54	750	929	1042
Lead	52	576	865	934	52	786	888	938
P value [1]			~ 0.025				~ 0.01	
Chromium	54	680	831	1016	54	740	940	1059
P value			< 0.05					
Cadmium	65	570	814	883	60	758	904	955
P value			< 0.005					
Titanium	54	618	770	804	53	702	884	966
P value			< 0.005				< 0.05	

[1] *P* is the probability of the difference from the controls being due to chance by Student's *t* test. The male titanium group also differed from the nickel ($P \sim 0.01$) and the lead ($P < 0.005$) groups. A few animals lived more than 912 days (30 months): males, 5 controls, 1 fed lead, 1 fed chromium, 2 fed nickel; females, 7 controls, 2 fed lead, 2 fed cadmium, 3 fed chromium, 4 fed nickel and 2 fed titanium.

0.005); and nickel, 103%. At 1 to 2 years of age, they were: chromium, 129%; nickel, 96% and titanium 109%, but differences were not significant at this or older ages.

Males in each group had lost 22 to 30% and females 28 to 37% of their body weights at the time of death. Relative weights, however, were of the same order as in living animals, those given titanium and chromium weighing the most and those given nickel the least (1).

Accumulation of trace metals. In tables 3–5 are shown the mean concentrations of the various metals in 5 organs according to sex, for mice 100 to 800 days or more of age, compared with values in the literature for adult man. At these low levels of dosage, no metal accumulated markedly with age; on the contrary, higher concentrations of cadmium and titanium appeared in younger mice. Table 6 indicates the levels in the organs of highest concentrations according to 3 ages.

Cadmium: Renal concentrations of 1.3 to 2.8 μg/g were observed in 4 males 134 to 174 days of age, 6.14 μg/g in 6 about one year of age, and 0.90 to 5.8 μg/g in 34 up to 2 years of age. In females the pattern was similar with a range of 0.86 to 6.5 μg/g. The mean renal concentrations at older ages was a third of that of younger mice. Cadmium was undetected in controls and in mice given other metals, except when animals raised commercially were analyzed, in which cases 0.01 to 0.3 μg/g were observed in kidney.

Lead: The food contained approximately 0.2 μg/g, an amount reflected in organ concentrations of controls almost always less than 1.0 μg/g; no accumulation with age was noted. Renal and hepatic ranges were 0.0 to 0.7 and 0.0 to 0.8 μg/g, respectively. Three groups of control mice were apparently devoid of lead. When given the metal, concentrations in spleen increased somewhat with age, but not in kidney (table 6). Males showed 0.52 to 3.3 μg/g and females 0.1 to 1.0 μg/g in liver, with 0.65 to 2.15 and 0.28 to 3.39 μg/g, respectively, in kidney. Thus there was some overlapping of values between controls and treated animals.

Chromium and nickel: These metals behaved similarly, with a predilection for spleen and heart and with little tendency to accumulate in other organs in amounts much greater than the controls. In male mice, concentrations of nickel in kidney and liver with ranges of 0.0 to 3.7 and 0.0 to 2.5 μg/g, respectively, decreased almost regularly from 300 to 755 days of age; in females the phenomenon occurred only in liver where the range was 0.0 to 2.3 μg/g. Taking chromium for 2 years did not cause significantly higher levels than one in any organ; the range of values in heart was 0.58 to 6.2 μg/g.

Titanium: High concentrations in liver and kidney were observed only in animals

TABLE 3

Organ concentrations of cadmium and lead in mice and man [1]

	No.	Kidney	Liver	Heart	Lung	Spleen	Mean [2]	Metal in food and water [3]
		μg/g wet wt	*μg/g wet wt*	*μg/g wet wt*	*μg/g wet wt*	*μg/g wet wt*		*ppm*
				Cadmium				
Mice								5.0
Males	44	2.94	0.63	0.20	0.34	0.66	0.93	
Females	35	2.65	0.66	0.62	0.42	0.27	0.92	
% present [4]		100	83	75	73	92		
Control mice [5]								0.0 [6]
Males	69	0.0 [6]	0.0	0.0	0.0	0.0	0.0	
Females	34	0.0	0.0	0.0	0.0	0.0	0.0	
Adult man	145	32.0	1.8	0.0	< 0.5	0.0	0.43	0.01
% present		100	98	3	50	13		
				Lead				
Mice								5.19
Males	41	1.25	1.37	1.03	1.17	1.42	1.25	
Females	23	1.35	0.39	0.81	0.49	2.68	1.14	
% present		100	83	92	67	9(		
Control mice [5]								0.19
Males	118	0.51	0.37	0.58	0.33	0.53	0.46	
Females	121	0.27	0.25	0.82	0.33	0.70	0.47	
% present		81	69	80	67	92		
Adult man	145	1.2	1.5	< 0.05	0.67	0.27	1.10	0.12
% present		100	100	56	100	97		

[1] The levels for adult man are the means obtained by spectrographic methods from Tipton and Cook (10), subjects from the U.S. Mainland, and show general levels in tissues. The ranges in all cases were wide.
[2] The means of the averages for each organ are shown as indexes of relative tissue concentrations and are not to be construed as representing other than aids to the reader in comparing treated with control groups.
[3] Food and water values are measured levels for mice and approximations based on analyses for man (5–9).
[4] Percentage of organs analyzed in which the designated metal was detected.
[5] Controls represent all animals not given the metal indicated.
[6] No metal detected by analysis.

less than 260 days of age, with 3 to 15 μg/g in the young and 0.2 to 1.5 μg/g in the old. Heart, lung and spleen had much titanium, up to 32.5, 18.0 and 18.0 μg/g, respectively, in the young and up to 9.3, 6.7 and 9.9 μg/g in the older animals. Controls usually had little, 0.0 to 1.9 μg/g. In an attempt to establish a "standard," organs of wild field mice were compared; levels were mainly in the same range as laboratory mice given the metal, with a range of 0.4 to 7.98 μg/g.

Influence of one divalent metal on accumulation of another. Organ concentrations of a metal present in the basal diet were compared when another divalent metal was or was not given. Cadmium-fed animals had concentrations of lead in kidney, liver and lungs similar to controls; lead did not affect nickel in liver. Limitations in amounts of tissue prevented further such evaluations.

Intake of trace metals. There were no significant differences in the amounts of water ingested by the different groups. Because of variable amounts of unavoidable wastage, the total use varied from cage to cage as much as 14% in females and 25% in males from the means. Female mice took 3.86 and males 4.65 g/day. On a weight basis, mean use by males was 10.2 and by females 9.03 g/100 g/day, calculated during a representative year of adult life.[5] The fluid intake of male rats under identical conditions was 6.85 g and of females 7.51 g/100 g body weight/day.

As fluids contained 5 μg/g of metal, the intake including wastage could be roughly approximated at a maximum of 45 to 51 μg metal/100 g/day, or 16.48 to 18.62

[5] Use of water by single surviving males did not change in the several weeks or months before death; that by females usually doubled or tripled. Although the amount ingested could not be measured, there was no evidence that the intake of water and metal decreased in older animals.

TABLE 4

Organ concentrations of chromium and nickel in mice and man [1]

	No.	Kidney	Liver	Heart	Lung	Spleen	Mean [2]	Metal in food and water [3]
		μg/g wet wt	*μg/g wet wt*	*μg/g wet wt*	*μg/g wet wt*	*μg/g wet wt*		*ppm*
				Chromium				
Mice								5.1
Males	41	0.92	0.51	2.10	1.11	1.83	1.29	
Females	30	0.84	0.51	1.23	0.81	1.84	1.23	
% present [4]		100	100	92	91	81		
Control mice [5]								0.1
Males	33	0.38	0.09	1.04	0.39	0.40	0.46	
Females	30	0.34	0.15	0.31	0.17	0.42	0.28	
% present		75	65	74	70	40		
Adult man	145	0.01	0.007	0.015	0.13	0.005	< 0.09	0.04
% present		83	79	88	100	79		
				Nickel				
Mice								5.4
Males	33	0.93	0.78	1.05	1.13	2.76	1.33	
Females	27	1.07	0.75	0.72	0.53	4.16	1.45	
% present		71	78	75	70	91		
Control mice [5]								0.4
Males	69	0.52	0.62	0.43	0.32	0.42	0.46	
Females	30	0.46	0.20	0.0	0.61	0.33	0.32	
% present		100	87	67	67	75		
Adult man	145	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.14	0.2
% present		21	18	34	50	12		

[1] The levels for adult man are the means obtained by spectrographic methods from Tipton and Cook (10), subjects from the U.S. Mainland, and show general levels in tissues. The ranges in all cases were wide.
[2] The means of the averages for each organ are shown as indexes of relative tissue concentrations and are not to be construed as representing other than aids to the reader in comparing treated with control groups.
[3] Food and water values are measured levels for mice and approximations based on analyses for man (5–9).
[4] Percentage of organs analyzed in which the designated metal was detected.
[5] Controls represent all animals not given the metal indicated.
[6] No metal detected by analysis.

TABLE 5

Organ concentrations of titanium in mice and man [1]

	No.	Kidney	Liver	Heart	Lung	Spleen	Mean [2]	Metal in food and water [3]
		μg/g wet wt	*μg/g wet wt*	*μg/g wet wt*	*μg/g wet wt*	*μg/g wet wt*		*ppm*
				Titanium				
Mice								5.03
Males	41	2.86	1.81	8.80	4.81	6.83	5.02	
Females	37	2.89	2.05	4.10	1.66	3.70	2.88	
% present [4]		79	73	70	73	100		
Control mice [5]								0.03 [6]
Males	31	0.33	0.38	0.34	0.13	0.94	0.42	
Females	51	0.55	0.67	1.08	0.66	1.10	0.81	
% present		57	56	75	50	67		
Wild field mice	9	1.03	4.10	6.93	3.03	—	3.02	
Adult man	144	< 0.05	< 0.05	< 0.05	2.2	< 0.05	< 0.21	0.12
% present		15	23	17	99	22		

[1] The levels for adult man are the means obtained by spectrographic methods from Tipton and Cook (10), subjects from the U.S. Mainland, and show general levels in tissues. The ranges in all cases were wide.
[2] The means of the averages for each organ are shown as indexes of relative tissue concentrations and are not to be construed as representing other than aids to the reader in comparing treated with control groups.
[3] Food and water values are measured levels for mice and approximations based on analyses for man (5–9).
[4] Percentage of organs analyzed in which the designated metal was detected.
[5] Controls represent all animals not given the metal indicated.
[6] Bedding (softwood chips) contained 2.31 μg/g.

TABLE 6

Trace metals in tissues of mice in relation to age, both sexes: organs of highest mean concentrations [1]

Metal	Target organ	100–360 days	360–560 days	560–800 days
			μg/g wet weight	
Cadmium	kidney	5.19	2.82	1.68
	liver	1.82	0.57	0.07
Lead	kidney	1.32	1.43	0.68
	liver	1.00	1.38	0.25
	spleen	1.11	2.04	2.18
Chromium	spleen	2.38	1.10	1.47
	heart	1.02	0.69	1.77
Nickel	kidney	1.85	0.78	0.57
	spleen	3.60	1.27	3.57
Titanium	spleen	7.59	6.64	1.49
	heart	10.05	3.60	3.30

[1] 5 ppm metal were given in drinking water continuously.

mg/100 g/ year. Therefore, even if half were wasted, the largest part of each metal ingested was not absorbed by body tissues (tables 3–5). The order of absorption, assuming that organ concentrations are representative, was: titanium >> nickel > lead > chromium > cadmium, and of the incremental differences from controls: titanium >> nickel ≅ cadmium > lead ≅ chromium.

Metals in newborn. The whole carcasses of 5 stillborn mice were analyzed. The results: cadmium 0.0, lead 0.0, nickel 0.14 and chromium 0.31 μg/g wet weight, indicating that 2 metals crossed the placental barrier and 2 did not. The mothers' tissues contained the metals.

Metals in tumors. Two lung tumors in mice given cadmium and 7 in controls showed none. Lead was observed in 8 of 10 tumors of control females at 0.08 to 2.1 μg/g. Nickel appeared in 2 of 3 tumors in control animals, chromium in two and titanium in 1 of 2.

Reproduction. No experiments were made on the reproductive capabilities of mice on metals. Eight hundred mice, however, have been successfully bred and raised with the cadmium-free diet, and analyses of representative groups failed to show tissue cadmium.

DISCUSSION

This exploratory study was designed to reproduce the lifetime human experience by causing accumulations of trace metals in mice equal to those of man (10) and comparing effects with animals little exposed to the same metals. We were successful in that all values observed lay within human ranges. In the case of lead, mean values were similar, but we produced less than mean human concentrations of cadmium and caused greater than mean human concentrations of nickel, chromium and titanium in both control and metal-fed animals.

Several unexpected effects were observed. There were marked sex differences in the responses of mice, males often being affected as to mortality and females not. At the 99% level of confidence, longevity of males was decreased by cadmium and titanium and of females by lead. Nickel appeared to decrease the incidence of tumors in females, and cadmium and lead in males. No metal was carcinogenic. Cadmium, lead and titanium, which accumulate in man with age, did not so accumulate in mice at the doses used; in fact, younger animals often had the highest levels at death. Although higher organ concentrations were observed in animals given metals, in all cases a state of "balance" appeared to be achieved independent of the duration of exposures; this phenomenon was especially marked in the cases of lead, chromium and nickel. Despite careful control of intake, the ranges of analytical results were wide and there were overlapping values for all metals except cadmium. We have found the analytical methods dependable and sensitive (5–9) and do not believe that methodological errors were of this magnitude. Individual differences in mice towards ab-

sorption and accumulation, therefore, may explain the variations in organ content of metals, early mortality being associated with high tissue levels. Similar variations occur in the tissues of man (10), which have been presumed to result from differences in exposure.

Duplications in mice of human levels of hepatic and renal lead of 1.5 ± 1.10 (SD) and 1.2 ± 0.87 μg/g wet weight, respectively (10), were associated with increased male mortality compared with controls and with mice given chromium, decreased longevity and fewer tumors. Lead in these concentrations, therefore, exhibits adverse effects on male mice not balanced by partial inhibition of tumors.

One-fifth to one-tenth of human renal cadmium levels of 32 ± 15 (SD) μg/g (10), in the range of children and African natives (11), were accompanied by decreased male survival and longevity despite suppression of tumors of the lungs. Deficiency of cadmium caused no obvious adverse effects on any parameter. Therefore, cadmium is not an essential micro-nutrient for mice but exhibits an innate toxicity in males at relatively low concentrations. In rats the major manifestation is hypertension (12, 13).

Nickel is not common in human tissues, except skin (10). Mice had higher levels whether fed it or not, although within human ranges. Whereas manifestations other than on female tumors were not obvious, nickel appears to have innate toxicity of an undefined nature, expressed by lessened male survival.

Chromium occurs in all human lungs but is not ubiquitous in other adult tissues; when present, it is only in small amounts (10). All but one of the mouse tissues analyzed had levels within the adult human extremes and resembled tissues of infants and children who have considerably larger concentrations (8). The improved growth and survival of both mice (1) and rats (14) at these levels, and the absence of adverse effects, suggest that chromium may have an essential function. Other evidence for its physiological role has been obtained (15).

Titanium had not been given chronically to animals, to our knowledge. Human lung contains a considerable amount, with a usual range of 0.55 to 7.0 μg/g and extremes up to 20 μg/g. Other tissues have little; therefore our mice resembled the human experiment in respect to lung but not to other organs. The unexpectedly large accumulations of titanium in laboratory and field mice suggest that homeostatic mechanisms are not active. Although improving the rate of growth (1), titanium is probably not an essential trace element because of its adverse effects on longevity.

Changes such as these here reported might occur sometimes in mice raised with various commercial diets which contain 0.4 to 1.9 ppm lead, 0.4 to 3.0 ppm nickel, 0 to 11.7 ppm titanium, 0 to 4.5 ppm chromium and 0.09 to 0.2 ppm cadmium (1). Our basal diet was low or deficient in these metals. One or more of them might affect growth, survival, longevity or tumors. If so, studies on mice should include control of the metal content of the diets given.

Comparison of our control data with those of Lindop (16) on a much larger series of SAS/4 mice reveal differences. The survival curve of our females, their median age and longest lifespan were somewhat less than hers, whereas body weights were greater. The incidence of tumors (38.6%) was also less (53.9%). Our control males had considerably shorter survivals, median age and longest lifespan than Lindop's and the incidence of tumors, 25%, was also less than the 41.0% reported by her. Whereas differing strains and diets probably account for these differences, the reduction in tumors in mice given metals is unlikely to be the result of these factors.[6]

These experiments demonstrate innate toxicities of lead, titanium, nickel and cadmium in male mice at tissue levels within human ranges, and a favorable effect of chromium. Low tissue affinity for chromium suggests that barriers to absorption may be present, as they may be in

[6] Although the incidence of tumors in inbred mice varies markedly with the strain (17), randombred animals in large enough numbers from the same source are not likely to show such great differences. The chance that 50 animals will have an incidence of tumors of 24% and another 50 or more obtained at the same time from the same source have 2% is small (< 0.01).

man (8), whereas such barriers are not as active for cadmium, lead, nickel and especially titanium. These experiments also indicate that in the oral doses employed, none of these metals is carcinogenic for mice.

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ADDENDUM

Since this report was submitted for publication, evidence has been obtained that rats on an identical regimen are partly deficient in chromium in respect to glucose metabolism.

LITERATURE CITED

1. Schroeder, H. A., W. H. Vinton, Jr. and J. J. Balassa 1963 Effect of chromium, cadmium and other trace metals on the growth and survival of mice. J. Nutrition, *80:* 39.
2. Saltzman, B. E. 1953 Colorimetric microdetermination of cadmium with dithiozone. Analyt. Chem., *25:* 493.
3. Saltzman, B. E. 1952 Micro-determination of chromium with diphenylcarbazide by permanganate oxidation. Analyt. Chem., *34:* 1016.
4. Sandell, E. B. 1959 Colorimetric Determination of Traces of Metals. Interscience Publishers, New York.
5. Schroeder, H. A., and J. J. Balassa 1961 Abnormal trace metals in man: Cadmium. J. Chron. Dis., *14:* 236.
6. Schroeder, H. A., and J. J. Balassa 1961 Abnormal trace metals in man: Lead. J. Chron. Dis., *14:* 408.
7. Schroeder, H. A., J. J. Balassa and I. H. Tipton 1962 Abnormal trace metals in man: Nickel. J. Chron. Dis., *15:* 51.
8. Schroeder, H. A., J. J. Balassa and I. H. Tipton 1962 Abnormal trace metals in man: Chromium. J. Chron. Dis. *15:* 941.
9. Schroeder, H. A., J. J. Balassa and I. H. Tipton 1963 Abnormal trace metals in man: Titanium. J. Chron. Dis. *16:* 55.
10. Tipton, I. H., and M. J. Cook 1963 Trace elements in human tissue. II. Adult subjects from the United States. Health Physics, *9:* 103.
11. Tipton, I. H., H. A. Schroeder, H. M. Perry, Jr. and M. J. Cook 1963 Trace elements in human tissue. III. Subjects from Africa, the Near and Far East and Europe. Health Phys., in press.
12. Schroeder, H. A., and W. H. Vinton, Jr. 1962 Hypertension induced in rats by small doses of cadmium. Am. J. Physiol., 202: 515.
13. Schroeder, H. A. 1964 Cadmium hypertension in rats. Am. J. Physiol., in press.
14. Schroeder, H. A., W. H. Vinton, Jr. and J. J. Balassa 1963 Effect of chromium, cadmium and lead on the growth and survival of rats. J. Nutrition, *80:* 48.
15. Schwarz, K., and W. Mertz 1959 Chromium (III) and the glucose tolerance factor. Arch. Biochem. Biophys., *85:* 292.
16. Lindop, P. J. 1961 Growth rate, lifespan and causes of death SAS/4 mice. Gerontologia, 5: 193.
17. Hoag, W. G. 1963 Spontaneous cancer in mice. Ann. N. Y. Acad. Sci., *108:* 805.

Effects of Feeding Frequency on Metabolism, Rate and Efficiency of Gain and on Carcass Quality of Pigs [1]

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ABSTRACT The effect of feeding pigs a 2000-g ration and 5000 ml water in one or 5 meals daily was determined by metabolism, growth and carcass quality measurements. In the first experiment, with 50-kg pigs, differences in digestibility coefficients due to increased feeding frequency were negligible. The efficiency of calorie conversion to fat and protein gain, derived from nitrogen and carbon balances, was about 49% for both groups of pigs. The more frequently fed pigs eliminated more fecal but less urinary water than the pigs fed once daily. The same restricted feeding regimens used for the caged pigs in experiment 1 were applied to individually penned, 60-kg pigs in the second experiment. A higher rate of gain by the pigs fed once daily was attributed to the effect of "fill" as evidenced by lighter carcass weights and smaller carcass gains. No significant difference between the 2 groups of pigs could be shown by any of the carcass measurements taken which included specific density.

The metabolism of experimental animals may be influenced by the frequency of feed intake (1). Less body fat, but more body protein and water was stored by rats feeding at frequent intervals ad libitum than by rats force-fed twice daily the same ration. Greater urinary excretion of nonprotein nitrogen and urea nitrogen by force-fed rats has also been reported (2). It has been suggested that by increasing the frequency of feeding pigs, improvements may be obtained in efficiency of feed conversion and carcass quality (3). Few studies have been made with pigs on the effect of single or multiple feeding. Braude et al. (4) observed little or no effect on the performance and carcass quality of pigs fed once or twice daily. However, better weight gain and a tendency towards improved feed conversion was recorded for pigs of about 50- and 90-kg body weight, fed twice daily compared with others fed 4 times daily (5).

Pšeničnyj (6) reported on the effects of the number of daily feedings on the digestibility of feed and on metabolism in pigs. Pregnant and lactating sows were fed twice or 3 times daily in one test, and 8-month old fattening pigs fed either 2, 3 or 4 times daily in a second test. In both tests, the digestibility of protein and the retention of nitrogen appeared to be improved by less frequent feeding. A decrease in the number of feedings from 4 to 2, reduced the production of carbon dioxide and increased the daily gain in body weight of fattening pigs. The purpose of the present study was to determine the effects on the metabolism, growth rate, feed conversion and carcass quality of feeding pigs either once or 5 times daily.

EXPERIMENTAL

Experiment 1. Eight Yorkshire barrows averaging 60 kg body weight were put into individual, metal metabolism cages. During a 4-week preliminary period, pigs were paired according to body weight and fed twice daily the same quantities of a grower ration[2] and added water. A maximal voluntary intake of 2000 g ration and 5000 ml water was established. A schedule was then introduced whereby these quantities, consumed by each caged pig within 20 minutes, were given to one member of each pair in one feeding daily,[3] at 8 AM, and to its mate in 5 equal feedings daily, at 8 AM, 11 AM, 2 PM, 5 PM, and 8 PM. The pigs assigned these 2 regimens will be referred to as "single-feeders" and "multiple-feeders," respectively.

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[2] The ration consisted of the following: (in per cent) barley meal, 88.15; soybean meal (44% C.P.), 4.50; white fishmeal (66% C.P.), 5.50; limestone, 0.80; iodized salt, 0.50; vitamin A and D_3 mixture (1500 IU and 300 IU/g), 0.15; vitamin B_{12}, (9 mg/454 g), 0.05; antibiotic (10 g chlortetracycline/454 g), 0.05; $ZnSO_4$, 0.05; dicalcium phosphate, 0.25. The ration analyzed (as per cent of dry matter) crude protein, 19.1; crude fiber, 5.4.

[3] 4000 ml of water were given with the ration at 8 AM, and 1000 ml of water only at 5 PM.

A 6-week experimental period followed, during which carbon-nitrogen balances were obtained for each pig. A 24-hour determination of carbon dioxide production was made for each pig at intervals of 2 weeks, at a rate of 4 pigs/week. A respiration chamber (measuring 194 cm long, 169 cm high and 72 cm wide) was used for this purpose. A metabolism cage, allowing separate collection of feces and urine from the test pig in it, was wheeled into the chamber which became airtight on closing its hinged door. Transparent plastic for the roof and sides provided adequate visibility for the animal confined in the otherwise all-metal chamber.

An electric fan circulated the air in the chamber, with temperature and humidity being regulated by a thermostatically controlled refrigeration unit, connected to a large cooling plate within the chamber. Air flow was started 30 minutes after the pig was put in the chamber. A preliminary, standardization period of 75 minutes preceded the 24-hour test. The air from the chamber was passed through a respiration gas meter [4] taking continuous 0.1% samples. Barometric pressure and the air temperature at the meter were recorded regularly during the day. Test pigs were fed quickly by means of a porthole in the roof of the chamber; the same feeding motions were enacted 5 times daily for pigs fed only once daily.

Samples of expired air were analyzed for carbon dioxide content by gas-solid chromatography.[5] Carbon analysis of feed, feces and urine was by the method of Cunningham (7). Nitrogen balances from 7-day total collection periods were obtained for all pigs each week of the experimental period. Urine was preserved in hydrochloric acid and stored at 1°C. Apart from a single collection for a pig in the respiration chamber, feces were collected twice daily and stored frozen. Analyses for dry matter, nitrogen and crude fiber were by standard methods (8). Fecal crude protein (nitrogen × 6.25) and dry matter were determined with thawed, wet feces and the crude fiber with the air-dried feces.

Experiment 2. Twenty littermate pairs[6] of Yorkshire barrows, matched for initial body weight (averaging 50.4 kg) were used in this experiment to test on penned pigs the feeding regimens previously imposed on caged pigs in experiment 1. Each pig was kept in an individual pen (measuring 1.2 × 4.3 meters) in which neither bedding nor water was provided. Pigs were allowed a week to adjust from a system of ad libitum, dry-feeding in groups to one of individual, restricted, wet-feeding. All pigs were fed the same daily quantities of ration (2043 g) and of water (5000 ml) but after the end of the first week, one pig in each pair was fed once daily and its pairmate 5 times daily. The ration formulation and feeding regimen were the same as described for experiment 1.

Pigs were weighed weekly at about 10 AM each weighing day. Hence, compared with pigs fed 5 times daily that had received only one fifth of their daily ration when finally weighed, those pigs fed once daily had consumed by that time the whole of their daily ration before weighing. All pigs that had reached 85.1 kg were slaughtered 2 days later. The last feeding for the "single-feeders" was at 8 AM, and for the "multiple-feeders" at 8 PM on the day before slaughter. The carcass of each pig was weighed while warm and measured after chilling (1°C) for length, *longissimus dorsi* area and depth of shoulder and loin fat according to standard methods (9).

A specific density determination was made on each chilled carcass, as a sensitive measure of its composition, by a modification of a procedure described by Holme et al. (10). The left and the right side of a carcass, without its head and tail, were weighed in air and in water, about 24 hours after slaughter. The accuracy of weighing was to within 114 g and 28 g, respectively. A cylindrical, metal tank (2.44 m high and 1.83 m diameter) was filled to overflowing with water for the in-water weights. The temperature of the water was recorded regularly and corrections [7] were made for its variation (between 6.5 and 14°C) when calculating

[4] Zentralwerkstatt Göttingen G.m.b.H., Göttingen, West Germany.

[5] Beckman Model GC-2A, using a silica-gel column and helium as carrier gas. Carbon dioxide was calculated by peak height comparison against a reference sample containing 1.08% CO_2.

[6] The pigs in one pair were from different litters but suckled by the same sow.

[7] Handbook of Chemistry and Physics, ed. 41 1959 Chemical Rubber Publishing Company, Ohio, p. 2129.

specific density. A formula given by Meyer and Nelson (11) was used to calculate, from specific density values, the percentage fat in each carcass.

RESULTS AND DISCUSSION

Experiment 1. Differences in weight gain, efficiency of feed conversion and digestibility of the ration between the "single" and the "multiple" feeders were negligible (table 1). The efficiency of caloric conversion to fat and protein gain was close to 49% for both groups of pigs. A difference in feed conversion on this basis has been observed between rats fed ad libitum or force-fed (1). Cunningham et al. (12) reported that pigs of about 87-kg body weight may store over 50% of the digestible calories as fat when fed 2450 g daily of a 12% crude protein ration. At a level of 1600 g daily, the efficiency was reduced to 39%, maintenance requirements apparently taking a larger proportion of the caloric intake. The caloric conversion in the present experiment, expressed in comparable terms of fat gain only, was 41%, therefore agreeing closely with the value previously reported.

The apparent digestibility of crude protein and crude fiber was not changed by feeding pigs once or 5 times daily. However, Pšeničnyj (6) gives data showing a tendency for improved crude protein digestibility with fewer feedings daily.

Cohn et al. (2) working with rats, and Wu and Wu (13) with a human subject, observed that fewer feedings daily increased urinary nitrogen. In the present study with pigs, differences in urinary nitrogen and fecal nitrogen between "single" and "multiple" feeders were small, nonsignificant and resulted in almost identical nitrogen retention (table 2). Urinary nitrogen was slightly lower and fecal nitrogen higher for the "multiple-fed" than for the "single-fed" pigs. The experimentally restricted protein intake by the pigs, may have influenced the urinary nitrogen results obtained (13). Rakes et al. (14) noted no difference in fecal nitrogen (as nitrogen absorption) between sheep fed either once or 8 times daily. However, urinary nitrogen was lower in the more frequently fed young sheep but no different in more mature sheep. A significantly greater nitrogen retention was reported by Satter and Baumgardt (15) for cows fed 8 times daily compared with those fed twice daily. Fewer feedings daily, increased fecal and urinary nitrogen, but

TABLE 1

Effect of feeding frequency on weight gain, efficiency of feed conversion and coefficients of apparent digestibility of caged pigs (exp. 1)

	Frequency of feeding	
	Once daily	5 times daily
Feed/day, g	2000	2000
Water/day, ml	5000	5000
Final weight, kg	96.70	93.70
Initial weight, kg	74.42	71.20
Gain/day, kg	0.53	0.54
Feed conversion, kg feed/kg gain	3.73	3.77
Kilocalories retained/kcal consumed, %[1]	49.73	48.80
Digestibility coefficients, %		
Dry matter	80.84	80.78
Crude protein	85.00	84.36
Crude fiber	25.58	26.42

[1] Based on a caloric intake of 3.12 digestible kcal/g feed (previously determined) and on the protein and fat gains obtained by nutritional balances. Combustion values of 5.7 kcal/g protein gain and 9.5 kcal/g fat gain were used (16).

TABLE 2

Effect of feeding frequency on the daily nitrogen and carbon balances and on the fecal and urinary loss of water for caged pigs (exp. 1)

	Frequency of feeding	
	Once daily	5 times daily
Weight of feces, g	851	965[1]
Feces dry matter, %	39.69	34.92[1]
Feces water, g	516	629[1]
Feces nitrogen, g	8.03	8.38
Weight of urine, kg	3.63	3.47[1]
Urine dry matter, %	2.65	2.56
Urine water, kg	3.54	3.39[1]
Urine nitrogen, g	30.71	30.29
Feces and urine water, kg	4.06	4.02
Grams water loss/g water intake,[2] %	77.29	76.56
Nitrogen retention, g	14.81	14.89
Feces and urine carbon, g	167.99	166.42
Respiratory carbon, g	361.92	368.68
Carbon balance, g	249.90	254.83
Protein carbon,[3] g	47.41	47.37
Protein gain, g	92.56	93.06
Fat gain,[3] g	264.74	271.14

[1] $P < 0.01$.

[2] Determined from water lost in feces and urine, and from water taken with and in the feed (12.41% moisture content).

[3] A factor of 52.7% carbon in body protein and 76.5% carbon in carcass fat was used (16).

the digestibility of nitrogen by the cows was not significantly changed.

Results from the pig experiment (table 2), showed significantly ($P < 0.01$) more feces and less urine from the "multiple" than from the "single" feeders. Both groups of pigs consumed the same total daily quantity of water, but the feces from the "multiple-fed" pigs were lower ($P < 0.01$) in dry matter content. The difference in weight of urine appeared to be the result of greater ($P < 0.01$) elimination of urinary water by the "single" feeders since it could not be accounted for by a difference in dry matter content. Feeding 5 times daily was conducive to water elimination via the feces, whereas once-daily feeding resulted in greater urinary loss of water. The total water loss via the feces and urine was the same for both groups, but the feces of the more frequently fed pigs contained 15.7% and those of the other pigs 12.8% of the total water.

The difference of nearly 7 g respiratory carbon daily (table 2), measured as carbon dioxide, was not significant. Carbon dioxide production by pregnant and suckling sows was reported to be independent of the number of feedings daily; with fattening pigs however, the production was reduced by feeding twice instead of 4 times daily (6).

Less muscle activity, measured as a greater reserve alkalinity of the blood, was noted (6) with twice daily compared with 3 or 4 times daily feeding. Visual observations on this aspect of feeding frequency were made on the pigs in the present study. Those fed only once daily in the morning soon became accustomed and relatively indifferent to the anxiety and excitement shown by the "multiple" feeders at each of their 4 subsequent feeding times. The opposite effect was reported for caged sheep (14); but the daily expenditure of energy was not significantly affected.

Protein gain was the same for both the "single" and "multiple" feeders, and the slightly greater fat gain by the "multiple" feeders was not significant. Evidently there was no change in weight gain, feed conversion, digestibility and carbon-nitrogen balance of caged pigs fed the same restricted total quantity of ration in either one or 5 feeds daily.

Experiment 2. All animals were slaughtered at about the same body weight, and since initial body weight was not significantly different, weight gains by pigs of each group were, as expected, markedly similar (table 3). However, differences in "fill" due to the feeding regimens described previously were probably responsible for the significant ($P < 0.01$) increase in rate of gain by the "single" feeders. This is supported by the evidence of lighter carcass weights ($P < 0.01$) and smaller calculated carcass gains ($P < 0.01$) of this group of pigs. Since carcass gain was directly related to the different ($P < 0.01$) number of days on test, carcass gain per day was almost the same for both groups

TABLE 3

Effect of feeding frequency on weight gain, efficiency of feed conversion and carcass measurements of individually penned pigs (exp. 2)

	Frequency of feeding	
	Once daily	5 times daily
Feed/day, g	2043	2043
Water/day, ml	5000	5000
Age at slaughter, days	191	200
Days on test	85	94 [1]
Weight gain, kg	37.45	35.86
Gain/day, kg	0.44	0.38 [2]
Carcass weight, kg	67.00	70.18 [2]
Carcass gain, kg [3]	31.59	33.95 [2]
Carcass gain/day, kg	0.37	0.35
Feed consumption, kg	173.27	191.86 [1]
Kilograms feed/kg live gain	4.64	5.36 [2]
Kilograms feed/kg carcass gain	5.55	5.72
Carcass length, mm	783.59	774.45
Carcass specific density [4]	1.0566	1.0531
Percentage carcass fat [5]	30.03	31.81
Total fat thickness, mm [6]	51.82	56.89
Longissimus dorsi area, cm [2]	27.81	28.00

[1] $P < 0.05$.
[2] $P < 0.01$.
[3] Estimated as the final carcass weight minus 71% of the initial bodyweight.
[4] From averages of the right and left sides of each carcass.
[5] Calculated from a formula given by Meyer and Nelson (12), using specific density determinations.
[6] Sum of the shoulder and the loin fat thickness.

of pigs. Feed consumption was greater ($P < 0.05$) for the "multiple" feeders because they were on test longer. Their feed conversion was correspondingly lower ($P < 0.01$) on a live gain basis but no different on a carcass gain basis from that of the "single" feeders.

No significant difference between the 2 groups of pigs could be shown by covariance analysis with adjustment for final carcass weight, for any of the carcass quality measurements taken. The apparent but not significant difference in total fat thickness was due probably to a particularly lean "single-feeder" carcass measuring 12.70 mm of fat. The percentage carcass fat, calculated from specific density, averaged 31%, which was not unreasonably lower than the 38% reported for pigs of 68 kg body weight restricted in feed intake (12). An examination of the carcass density data showed that the weight in air and the specific density were each significantly[8] ($P < 0.01$) greater for the left side (31.19 kg and 1.0556, respectively) than for the right side of the carcass (30.54 kg and 1.0541, respectively). The differences probably arise from a tendency for carcasses to be sawed[9] to one side or other of the midline. Although it is likely that the percentage of bone may have only a small influence on carcass density (10), the production of sides (called "hard" and "soft" sides) with unequal proportions of bone was considered to be a possible cause for differences in weight and density.

At the conclusion of experiment 1, it was considered that the apparent similarity of performance by the pigs fed either once or 5 times daily, was the effect of their confinement in metabolism cages. The results from experiment 2, however, were in agreement with those from experiment 1. A measurable effect of increased feeding frequency might have been a greater energy expenditure and consequently less body fat storage by the more active "multiple-fed" pigs; but no such difference could be detected between the 2 groups of penned pigs. It could not be determined from the results of these experiments whether a larger, total intake of feed would have produced measurable carcass differences between pigs fed once or 5 times daily.

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LITERATURE CITED

1. Cohn, C., and D. Joseph 1959 Changes in body composition attendant on force-feeding. Am. J. Physiol., *196:* 965.
2. Cohn, C., D. Joseph, L. Bell and L. Oler 1963 Feeding frequency and protein metabolism. Am. J. Physiol., 205: 71.
3. Cohn, C., D. Joseph and M. D. Allweiss 1962 Nutritional effects of feeding frequency. Am. J. Clin. Nutrition, *11:* 356.
4. Braude, R., M. J. Townsend, G. Harrington and J. G. Rowell 1963 A comparison of feeding growing pigs once or twice daily. J. Agr. Sci., 60: 389.
5. Mel'nikov, S. V., and I. I. Struk 1956 Frequency of feeding pigs during intensive fattening. Svinovodstvo., no. 8: 25 (cited in Nutrition Abstr. Rev., 27: 591, 1957).
6. Pšeničnyj, P. D. 1958 Vlijanie kratnosti kormlenija no perevarimost i obmen veščestv u svinej. Vestnik Selskokhozyaystvennoy Nauki., 3: 96.
7. Cunningham, H. M. 1963 Note on a rapid method for determining the carbon content of feeds and excreta. Canad. J. Animal Sci., *43:* 212.
8. Association of Official Agricultural Chemists 1960 Official Methods of Analysis, ed. 9. Washington, D. C.
9. Canada Department of Agriculture 1959 Record of performance of swine. Production and Marketing Branch, Ottawa.
10. Holme, D. W., W. E. Coey and K. L. Robinson 1963 The prediction of pig carcass composition from measurements of carcass density. J. Agr. Sci., *61:* 9.
11. Meyer, J. H., and A. D. Nelson 1963 Efficiency of feed utilization by various animal species fed similar rations. J. Nutrition, *80:* 343.

[8] Paired comparison using the *t* test.
[9] Each carcass, suspended by the hind legs with the belly facing the operator, was divided into 2 sides with an electrically powered, circular saw.

12. Cunningham, H. M., D. W. Friend and J. W. G. Nicholson 1962 Efficiency of conversion of protein, energy and carbon in pigs restricted late in the fattening period. Canad. J. Animal Sci., 42: 176.
13. Wu, H., and D. Y. Wu 1950 Influence of feeding schedule on nitrogen utilization and excretion. Proc. Soc. Exp. Biol. Med., 74: 78.
14. Rakes, A. H., E. E. Lister and J. T. Reid 1961 Some effects of feeding frequency on the utilization of iso-caloric diets by young and adult sheep. J. Nutrition, 75: 86.
15. Satter, L. D., and B. R. Baumgardt 1962 Changes in digestive physiology of the bovine associated with various feeding frequencies. J. Animal Sci., 21: 897.
16. Armsby, H. P. 1928 The Nutrition of Farm Animals. The Macmillan Company, New York.

A Pepsin Pancreatin Digest Index of Protein Quality Evaluation [1]

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ABSTRACT A pepsin pancreatin digest index was devised for a rapid, accurate estimation of protein quality. The index was calculated from the amino acids released by an in vitro digestion with pepsin followed by pancreatin. The amino acids were determined by automatic amino acid analysis which allowed rapid protein quality evaluations with very small samples. Using whole egg as a standard, excellent correlation was observed between the pepsin pancreatin index values for 12 proteins and their biological values reported in the literature from feeding trials. The proteins tested were selected to cover a wide range of protein quality. The pepsin pancreatin digest index values showed better correlation with the biological values for the growing rat than did the essential amino acid index which tended to overestimate the value, or the chemical score which tended to underestimate it.

Although the nutritional quality of proteins must, in the final analysis, be established with feeding trials, in vitro methods of protein evaluation are useful in screening new protein foods and processing methods because of their rapidity. Recent reviews of laboratory methods of protein quality evaluation have been given by Grau and Carroll (1), Campbell (2), Pearson and Darby (3), Frost (4), and Mauron (5). Two procedures widely used for screening potential protein foodstuffs, the chemical score (6–8) and the essential amino acid index (9, 10) were based on the total amino acid composition. These procedures were rapid and in many cases accurate; however, no allowance was made for variations in the digestibility and availability of amino acids. Sheffner et al. (11) developed the "pepsin-digest-residue amino acid index" which combined the pattern of essential amino acids released by in vitro pepsin digestion with the amino acid pattern in the remainder of the protein. The work involved was considerable since 10 amino acids had to be determined in an acid hydrolysate as well as in a pepsin digest by the use of microbiological techniques. Therefore, this method did not appear suitable for evaluation of large numbers of food proteins. The procedure to be described in the present paper gives an amino acid index based on the release of amino acids by pepsin followed by pancreatin. The labor and sample size required for assay was reduced by the use of automatic amino acid analysis.

METHODS AND MATERIALS

Pepsin digests were prepared by incubating with shaking 500 mg dried protein (samples were dried to constant weight over P_2O_5 at room temperature) with 12.5 mg pepsin [2] in 15 ml of 0.1 N hydrochloric acid for 24 hours at 37° in a water bath. Pepsin followed by pancreatin digests were prepared by incubating 100 mg protein with 1.5 mg pepsin in 15 ml of 0.1 N hydrochloric acid at 37° for 3 hours. After neutralization with 7.5 ml of 0.2 N sodium hydroxide and addition of 4 mg pancreatin [3] in 7.5 ml of pH 8.0 phosphate buffer, the digestion mixtures were incubated for an additional 24 hours at 37°. Enzyme blanks were prepared by incubation under the described conditions with the protein sample omitted. Fifty parts per million merthiolate which were added to the digestion mixture to prevent growth of microorganisms did not interfere with the digestion and subsequent analysis. Ten milliliters of digestion mixture were added

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[2] B grade, California Corporation for Biochemical Research, Los Angeles.
[3] See footnote 2.

to 50 ml of one per cent picric acid solution and centrifuged for 30 minutes at 1000 × *g* to remove undigested protein and larger peptides.

Fifty milliliters of supernatant were passed through a column containing anion exchange resin [4] in chloride form into a 250 ml lyophilizing bottle. After rinsing the column with three 5-ml portions of 0.02 N hydrochloric acid, the samples were dried by lyophilization. The dried samples were dissolved and diluted to 10 ml with pH 2.2 buffer. Amino acid analysis of the samples was made by the ion exchange method of Moore et al. (12–14) with a Spinco model 120 amino acid analyzer. Basic amino acids were separated on a 10-cm column using pH 5.28 buffer. The acidic and neutral amino acids were separated on a 159-cm column using pH 3.25 buffer followed by pH 4.25 buffer after 8 hours and 30 minutes from zero time.

Chromatograms of pepsin and pepsin pancreatin hydrolysates of egg white protein are shown in figure 1. The pepsin hydrolysate represented 53 mg of protein and the pepsin pancreatin hydrolysate represented 5.3 mg of protein. Analyses of pepsin hydrolysates were unsatisfactory since large concentrations of peptides obscured peaks of some amino acids, many of which were present in small concentrations. Chromatograms of the pepsin followed by pancreatin hydrolysates gave excellent resolution of the amino acids. In those areas showing peptides, a slanting line was drawn as illustrated in figure 1 which served as a baseline for integrating the amino acid peaks. Preliminary studies showed this method of integration to be more accurate and reproducible than the usual method (14) when varying concentrations of the enzyme hydrolysate were analyzed.

The total amino acid content of the samples was determined on acid hydrolysates. Fifty-milligram samples were hydrolyzed with 2 ml of 6 N hydrochloric acid for 22 hours at 110° in a sealed tube containing a nitrogen atmosphere. After filtration and evaporation to dryness 3 times with vacuum distillation, the samples were dissolved in pH 2.2 citrate buffer and diluted to 25 ml. Tryptophan which was destroyed by acid hydrolysis was analyzed after hydrolysis with 5 N sodium hydroxide in a manner similar to that of Dreze (15).

The pepsin pancreatin digest index was calculated in essentially the same manner as the "pepsin-digest-residue amino acid index" described by Sheffner et al. (11) with slight modifications. The amino acid concentrations were expressed as grams

[4] AG 2-X 8, 200–400 mesh, California Corporation for Biochemical Research, Los Angeles.

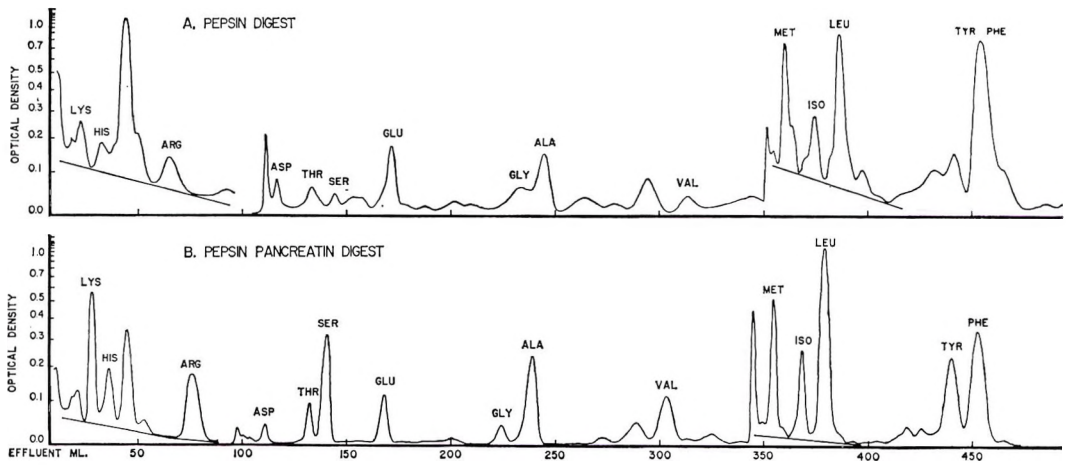

Fig. 1 Representative chromatograms from the Spinco Model 120 amino acid analyzer. A, a chromatogram of a pepsin hydrolysate of egg white; B.a chromatogram of a pepsin followed by pancreatin hydrolysate of egg white. The chromatogram of the pepsin hydrolysate shows peptides and amino acids from 10 times more protein as the chromatogram of the pepsin-pancreatin hydrolysate.

per 100 g total amino acid rather than as milligrams per gram of protein. Amino acids used in all calculations included lysine, phenylalanine plus tyrosine, methionine, threonine, valine, isoleucine, leucine, and histidine, the amino acids classified by Rose (16, 17) as essential for the growing rat. Tryptophan was destroyed during the picric acid procedure and not determined in the enzyme hydrolysates. A correction for the tryptophan content was therefore included in some calculations to see whether the tryptophan content of the proteins tested would influence the results. This correction which was the ratio of total tryptophan in the sample to total tryptophan in the reference protein was included in the geometric mean of each fraction.

The procedure was tested with 12 food proteins which covered the entire range of protein quality. Commercial preparations tested were egg white, lactalbumin, casein, soybean protein, gluten, zein,[5] wheat flour,[6] yeast[7] and gelatin.[8] Whole egg (raw), milk (pasteurized) and beef (roasted) samples were prepared by lyophilization followed by grinding and mixing with a mortar and pestle. Before lyophilization, the beef samples were homogenized with 50 ml of water with a Virtis homogenizer.

RESULTS AND DISCUSSION

The pepsin pancreatin amino acid index values calculated with and without the tryptophan correction are shown in table 1, columns 4 and 3, respectively. The biological values for growing rats as reported in the literature for the 12 proteins are shown in column 2. One or more reports of the biological values were found for

[5] All obtained from Nutritional Biochemicals Corporation, Cleveland.
[6] Pillsbury's Best, The Pillsbury Company, Minneapolis.
[7] Red Star Food Yeast, Red Star Yeast and Products Company, Milwaukee, Wisconsin.
[8] Bacto Gelatin, Difco Laboratories, Detroit, Michigan.

TABLE 1

Comparison of pepsin pancreatin digest index (calculated with and without the tryptophan correction) with biological values reported in the literature for the growing rat, the chemical score [1] and the essential amino acid index [2]

Food protein	Literature biological value for growing rats	Pepsin pancreatin digest index		Chemical score	Essential amino acid index
		Excluding tryptophan correction	Including tryptophan correction		
Whole egg	96,[3] 97 [4]	100	100	100	100
Egg white	83,[3] 82,[5] 97 [6]	89	90	100	97
Milk	90,[3,5] 84 [3] (dried), 84 [4]	87	86	79	89
Lactalbumin	85,[3] 84 [5]	85	87	79	88
Beef	76 [3,5]	80	78	81	83
Casein	73,[5] 69,[3] 78 [7]	78	79	66	89
Soybean	raw: 57,[3] 59 [7] heated: 75,[3,5] 74 [7]	68	67	51	82
Yeast	63,[3] 69 [5]	74	74	55	81
Wheat flour	52 [3,5]	54	52	42	61
Gluten	40,[6] 61 [7]	49	47	40	55
Zein	—	42	27	17	33
Gelatin	25,[5] 0 [8]	29	18	17	23
Correlation coefficient, r =		0.982 (0.995) [9]	0.990 (0.994) [9]	0.940	0.979
Regression equation,[10] Y =		1.097X − 11.00 (0.954X + 0.65) [9]	0.970X − 0.54 (0.940X + 1.98) [9]	0.831X + 14.48	1.006X − 9.46
SE of estimate =		1.45 (0.92) [9]	1.05 (1.03) [9]	2.58	1.65

[1] Bender (8).
[2] Oser (20).
[3] Block and Mitchell (7).
[4] Sommer (24).
[5] Mitchell and Block (6).
[6] Mitchell and Beadles (25).
[7] Rippon (26).
[8] Bender, Miller and Tannan (27).
[9] Values in parentheses exclude gelatin.
[10] Y = predicted biological value; X = pepsin pancreatin digest index.

each protein except zein which does not support growth or maintain body weight without supplementation (18).

Whole egg was selected as the standard and set equal to 100 since it had the highest biological value of the proteins tested. A better correlation was observed between the pepsin pancreatin digest index and the biological value when whole egg was used as the standard ($r = 0.990$) than when egg white was used as the standard ($r = 0.976$).

Arginine which has been classified as a semi-indispensable amino acid (19) and is required only in small amounts (16) was not included in the calculations of the pepsin pancreatin digest index shown in table 1. A better correlation was observed between the pepsin pancreatin digest index and the biological values when arginine was excluded ($r = 0.990$) than when it was included ($r = 0.972$) in the calculations.

Index values calculated with the tryptophan correction showed better correlation with biological values from the literature ($r = 0.990$) than did index values calculated without the tryptophan correction ($r = 0.982$). The index values were virtually the same with both methods of calculation for all proteins tested except for zein and gelatin which were devoid of tryptophan. When indexes for zein and gelatin were excluded from the correlation, the correlation coefficients were the same whether the tryptophan correction was included ($r = 0.994$) or excluded ($r = 0.995$). Rose (16) showed the minimal level of tryptophan required for growth was lower (0.2%) than for the other essential amino acids. Therefore, good estimations can be made of the biological value without including the tryptophan correction except for proteins low in or devoid of tryptophan. This would eliminate the need for a separate basic hydrolysis and short column run.

Table 1 shows a comparison of the pepsin pancreatin digest index values with the chemical score of Bender (8) and the essential amino acid index of Oser (20). The latter 2 methods were calculated from the total amino acid content of the food proteins tested. The pepsin pancreatin digest index showed better correlation with the literature biological values ($r = 0.990$) than did the chemical score ($r = 0.940$) or the essential amino acid index ($r = 0.979$). Similarly the standard error of estimate was smaller for the pepsin pancreatin digest index (1.05) than for the chemical score (2.58) and the essential amino acid index (1.65). In general, the chemical score underestimated the biological value of the protein, whereas the essential amino acid index overestimated the biological value. There were cases where the agreement between the essential amino acid index or the chemical score and the biological value was poor. For example, the essential amino acid indexes of casein, milk, and lactalbumin were equal, but the biological value of casein was actually lower than milk and lactalbumin.

Because a value of 100 rather than 96 or 97 was assigned to the egg standard, the pepsin pancreatin digest index slightly overestimated the biological values. More precise values may be obtained by multiplying the index by the correction factor (biological value of whole egg/100). This correction agreed closely with the regression line ($Y = 0.970X - 0.54$). This correction could not be accurately applied to the essential amino acid index or the chemical score because their corresponding regression lines ($Y = 1.006X - 9.46$ and $Y = 0.831X - 14.48$) did not correspond to the correction factor.

The essential amino acid index and the chemical score are useful in screening large numbers of potential foods. However, many potential food sources such as seed and leaf proteins require extensive processing which often may have a deleterious effect on the nutritive value of the protein (21). For example, carbohydrates may react with the side chain of some amino acids like lysine (22). Other processes may improve the nutritive value by inactivating digestive enzyme inhibitors. Procedures based on the amino acid composition using acid hydrolysis may not reveal these changes in the nutritive value induced by the processing. However, they would be detected by the pepsin pancreatin digestion. Hence, the pepsin pancreatin digest index would be useful not only in estimating the nutritive value of processed proteins but also in evaluating processing procedures.

A disadvantage of this procedure is the calculation required for the index. Furthermore, any evaluation based on amino acid analysis does not take into account the contribution to the welfare of the animal of factors other than amino acids. Mitchell (23) suggested that some protein foods may contain factors other than amino acids which add to their nutritive value. Although processing procedures and processed foodstuffs must, in the final analysis, be tested with feeding trials, a procedure such as the one described would be useful in preliminary work because of its rapidity and accuracy. The procedure's greatest advantage was the small sample size required. Only 150 mg of protein were required for estimation of the biological value. Thus many variations of a process could be readily tested on a small laboratory scale.

LITERATURE CITED

1. Grau, C. R., and R. W. Carroll 1958 Evaluation of protein quality. In Processed Plant Protein Foodstuffs, eds., A. M. Alschul. Academic Press, New York, p. 153.
2. Campbell, J. A. 1961 A critical appraisal of methods for evaluation of proteins in food. R. 1 Add. 37 WHO/FAO/UNICEF-PAG, United Nations, New York.
3. Pearson, W. N., and W. J. Darby 1961 Protein nutrition. In Annual Review of Biochemistry. Annual Reviews, Inc., Palo Alto, California, p. 325.
4. Frost, D. V. 1959 Measuring the nutritive value of proteins. In Protein and Amino Acid Nutrition, ed., A. A. Albanese. Academic Press, New York, p. 255.
5. Mauron, J. 1961 The concept of amino acid availability and its bearing on protein evaluation. In Meeting Protein Needs of Infants and Children, pub. 843. National Academy of Sciences — National Research Council, Washington, D. C., p. 424.
6. Mitchell, H. H., and R. J. Block 1946 Some relationships between the amino acid contents of proteins and their nutritive values for the rat. J. Biol. Chem., *163:* 599.
7. Block, R. J., and H. H. Mitchell 1946 The correlation of the amino acid composition of proteins with their nutritive value. Nutrition Abstr. Rev., *16:* 249.
8. Bender, A. E. 1961 Determination of nutritive value of protein by chemical analysis. In Meeting Protein Needs of Infants and Children, pub. 843. National Academy of Sciences — National Research Council, Washington, D. C., p. 407.
9. Oser, B. L. 1951 Method for integrating essential amino acid content in the nutritional evaluation of protein. J. Am. Dietet. A., *27:* 396.
10. Mitchell, H. H. 1954 Biological value of proteins and amino acid interaction. Quartermaster Food and Container Inst. Surveys, National Research Council, Washington, D. C.
11. Sheffner, A. L., G. A. Eckfeldt and H. Spector 1956 The pepsin-digest-residue (PDR) amino acid index of net protein utilization. J. Nutrition, *60:* 105.
12. Moore, S., D. H. Spackman and W. H. Stein 1958 Automatic recording apparatus for use in the chromatography of amino acids. Federation Proc., *17:* 1107.
13. Moore, S., D. H. Spackman and W. H. Stein 1958 Chromatography of amino acids on sulfonated polystyrene resin. An improved system. Anal. Chem., *30:* 1185.
14. Spackman, D. H., W. H. Stein and S. Moore 1958 Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem., *30:* 1190.
15. Dreze, A. 1956 Le dosage du tryptophane dans les milieux naturels. 1. Stabilité du tryptophane vis-a-vis des agents d'hydrolyse. Bul. Soc. Chim. Biol., *38:* 243.
16. Rose, W. C. 1937 The nutritive significance of the amino acids and certain related compounds. Science, *86:* 298.
17. Rose, W. C. 1938 Nutritive significance of amino acids. Physiol. Rev., *18:* 109.
18. Osborne, T. B., and L. B. Mendel 1915 The comparative nutritive value of certain proteins in growth, and the problem of the protein minimum. J. Biol. Chem., *20:* 351.
19. Block, R. J., and D. Bolling 1944 Nutritional opportunities with amino acids. J. Am. Dietet. A., *20:* 69.
20. Oser, B. L. 1959 An integrated essential amino acid index for predicting the biological value of proteins. In Protein and Amino Acid Nutrition, ed., A. A. Albanese. Academic Press, New York, p. 281.
21. Krehl, W. A., and J. J. Barboriak 1960 Factors affecting utilization of food nutrients by the normal organism. In Nutritional Evaluation of Food Processing, eds., R. S. Harris and H. V. Loesecke. John Wiley and Sons, Inc., New York, p. 521.
22. Carpenter, K. J. 1958 Chemical methods of evaluating protein quality. Proc. Nutrition Soc., *17:* 91.
23. Mitchell, H. H. 1952 The nutritional evaluation of protein: A half century of progress. Nutrition Rev., *10:* 33.
24. Sommer, E. E. 1938 The biological value of milk and egg protein in young and mature rats. J. Nutrition, *16:* 129.
25. Mitchell, H. H., and J. R. Beadles 1950 Biological values of six partially purified proteins for the adult albino rat. J. Nutrition, *40:* 25.
26. Rippon, W. P. 1959 A comparison of several methods for estimating the nutritive value of proteins. Brit. J. Nutrition, *13:* 243.
27. Bender, A. E., D. S. Miller and E. J. Tannah 1953 The biological value of gelatin. Chem. Ind. (London), *30:* 799.

A Study of the Hypocholesterolemic Activity of the Ethyl Esters of the Polyunsaturated Fatty Acids of Cod Liver Oil in the Rat

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ABSTRACT The ethyl esters of the polyunsaturated fatty acids of cod liver oil reduced the hypercholesterolemia produced in rats fed diets supplemented with a coconut oil, cholesterol, and sodium taurocholate. As little as 0.1% ethyl ester added to the diet caused a reduction of blood cholesterol concentration in the hypercholesterolemic rat. The hypocholesterolemic activity of the polyunsaturated fatty esters was sustained after withdrawal of the fatty esters from the ration. Long-term feeding of the polyunsaturated fatty esters resulted in a consistent lower blood cholesterol through the duration of a 154-day feeding experiment. Control animals fed the hypercholesterolemic diet attained high blood cholesterol values which declined after 7 weeks; however, their blood cholesterol level never reached the lower value of the polyunsaturated fatty ester supplemented rats. Subcutaneous administration of the polyunsaturated fatty esters also caused a reduction in the blood cholesterol of hypercholesterolemic rats. It is suggested that the polyunsaturated fatty acids function through different mechanisms in reducing blood cholesterol levels in the rat and chicken.

The feeding of marine oils has been reported (1–4)[1,2] to reduce blood cholesterol in the rat. Recently, Howe and Bosshardt (5) published results in which cod liver oil and a polyunsaturated fatty acid concentrate derived from cod liver oil depressed plasma cholesterol in the mouse. The hypocholesterolemic effect of the ethyl esters of the polyunsaturated fatty acids of cod liver oil (EECLO) in the chicken have previously been reported from this laboratory (6). The effect of the EECLO preparation on whole blood total cholesterol in the rat is reported in this paper.

EXPERIMENTAL

Animals were housed in individual cages and maintained at an environmental temperature of 22°C. Feed and water were fed ad libitum. Female rats (Sprague Dawley strain) were used in most of these experiments, because they develop greater hypercholesterolemia than male rats (7).

The experimental diet (M54) used had the following percentage composition: sucrose, 49.00; casein, 18.00; coconut oil,[3] 20.00; salt mix,[4] 4.50; diatomaceous earth,[5] 3.00; vitamin mix,[6] 2.50; cholesterol, 2.00; sodium taurocholate, 1.00; choline chloride, 0.40; butylated hydroxy toluene, 0.05. Coconut oil, a saturated fat, was used in the ration because it produces greater hypercholesterolemia than most other fats (8). Whole blood was collected for cholesterol assay by bleeding from the tail into heparinized capillary tubes. Whole blood cholesterol was determined using a modification[7] of the Albers and Lowry (9) fluorometric procedure. Whole blood cholesterol values are higher than reported serum and plasma cholesterol values. The preparation and analyses of the ethyl ester preparations of cod liver oil were previously outlined (6).

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[1] Peifer, J. J., and W. O. Lundberg 1957 Effects of unsaturated acids and fish oils on plasma and tissue lipids from hypercholesterolemic rats. Federation Proc., *18:* 300 (abstract).

[2] Peifer, J. J., and W. O. Lundberg 1961 Effect of dietary fats and oil fractions on cardiovascular tissues. Federation Proc., *20:* 93 (abstract).

[3] Neobee "O," E. F. Drew Co., New York.

[4] Salt mix: (g/kg diet) $CaHPO_4 \cdot 2H_2O$, 16.369; $K_3C_6H_5O_7 \cdot H_2O$, 10.632; $CaCO_3$, 7.367; NaCl, 4.868; K_2HPO_4, 3.841; $MgCO_3$, 1.841; $FeC_6H_5O_7 \cdot 3H_2O$, 0.720; $MnSO_4 \cdot H_2O$, 0.065; $CuSO_4$, 0.008; $AlKSO_4 \cdot 12H_2O$, 0.0004; $CoCl_2 \cdot 6H_2O$, 0.0004; KI, 0.0002; $ZnCO_3$, 0.0002; NaF, 0.00003.

[5] Micro-Cel, Johns-Manville Products Corporation, Manville, New Jersey.

[6] Vitamin mix: (mg/kg diet) inositol, 2000; *p*-aminobenzoic acid, 100; Ca pantothenate, 60; *d*-α-tocopheryl succinate, 50; nicotamide, 40; riboflavin, 12; thiamine·NO_3, 6; pyridoxine·HCl, 6; vitamin A acetate, 5.486; menadione, 4; folic acid, 0.5; biotin, 0.2; vitamin D_3, 0.041; cyanocobalamin, 0.030.

[7] Kahn, S. G., and H. Yacowitz 1958 Aspects of fluorometric analysis for total cholesterol in serum and tissues. Federation Proc., *17:* 991 (abstract).

RESULTS AND DISCUSSION

Table 1 presents results obtained from the feeding of different amounts of the EECLO preparation (iodine number, 320). The results represent a composite sample from 2 groups of female rats that were started on test at weaning age. The hypocholesterolemic effect of EECLO was not the effect of a reduction in cholesterol consumption, since feed consumption and mean body weight gain values were similar among the different test groups. Supplementation with 0.25% appeared to be the lowest level resulting in significant hypo-

TABLE 1

Effect of feeding the polyunsaturated ethyl esters of cod liver oil on total whole blood cholesterol in female rats

Treatment [1]	Blood cholesterol						
	Initial [2]	7-Day	14-Day	21-Day	28-Day	35-Day	42-Day
	mg/100 ml	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
M54	179 ± 5 [3,4]	299 ± 9	342 ± 11	376 ± 31	414 ± 33	564 ± 40	590 ± 28
M54 + 1% EECLO	185 ± 6 [3]	254 ± 12 [5]	267 ± 7 [5]	245 ± 19 [5]	284 ± 15 [5]	280 ± 20 [5]	261 ± 82 [5]
M54 + 0.5% EECLO	182 ± 4 [3]	290 ± 10	289 ± 16 [6]	315 ± 21	295 ± 11 [5]	329 ± 13 [5]	285 ± 21 [5]
M54 + 0.25% EECLO	176 ± 4 [3]	263 ± 12 [6]	294 ± 10 [5]	322 ± 20	358 ± 25	390 ± 21 [5]	313 ± 16 [5]
M54 + 0.1% EECLO	187 ± 6	—	354 ± 23	—	477 ± 43	—	390 ± 19 [5]
M54 + 0.05% EECLO	196 ± 5 [6]	—	330 ± 18	—	389 ± 26	—	458 ± 34 [5]
M54 + 0.01% EECLO	178 ± 3	276 ± 13		384 ± 50		518 ± 41	
M54 + 0.005% EECLO	171 ± 6	303 ± 25		378 ± 29		620 ± 51	

[1] EECLO indicates ethyl esters of the polyunsaturated fatty acids of cod liver oil with an iodine number of 320. An equal amount of coconut oil was removed from the diet when a test oil was included.
[2] Weanling rats were used, with 10/treatment except where indicated.
[3] These values represent 20 rats.
[4] Mean ± SE.
[5] Confidence limit of 99% or greater. Cholesterol values within a bleeding period were compared with their respective M54 cholesterol value.
[6] Confidence limits between 95% and 99%.

TABLE 2

Effect of feeding the polyunsaturated ethyl esters of cod liver oil on total whole blood cholesterol in female rats during treatment and after withdrawal

Treatment [1]	Blood cholesterol				
	Days on treatment			Days after withdrawal	
	Initial [2]	7	14	7	31
	mg/100 ml	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
M54	695 ± 38 [3]	784 ± 44	780 ± 66	714 ± 77	710 ± 134
M54 + 1% EECLO	666 ± 55	407 ± 42 [4]	337 ± 16 [4]	346 ± 26 [4]	354 ± 26 [5]
M54 ± 0.5% EECLO	664 ± 65	439 ± 28 [4]	347 ± 11 [4]	369 ± 34 [4]	496 ± 49
M54 + 0.25% EECLO	759 ± 61	572 ± 82 [4]	488 ± 46 [4]	426 ± 41 [4]	529 ± 58
M54 + 0.1% EECLO	704 ± 75	729 ± 60	659 ± 80	520 ± 66 [5]	485 ± 55
M54 + 0.05% EECLO	798 ± 55	730 ± 85 [5]	690 ± 79 [5]	685 ± 89	535 ± 45

[1] Fed pre-test diet (M54) from weaning to 7 weeks of age. EECLO indicates ethyl esters of the polyunsaturated fatty acids of cod liver oil with an iodine number of 320. An equal amount of coconut oil was removed from the diet when a test oil was included.
[2] Ten rats/treatment.
[3] Mean ± SE.
[4] Confidence limit of 99% or greater. The increment between the initial and subsequent cholesterol values within a treatment were compared with the respective change in blood cholesterol for the M54 control group.
[5] Confidence limits between 95% and 99%.

cholesterolemic activity, during the initial weeks of EECLO feeding. This dietary level of EECLO was equivalent to a daily intake of approximately 25 mg of the polyunsaturated fatty ester preparation. After 6 weeks of continued EECLO feeding, hypocholesterolemic activity was noted at levels as low as 0.05%. The hypocholesterolemic activity of EECLO was not lost but was sustained even after its withdrawal from the ration (table 2). This had not been observed in the chicken (6). The phenomenon of a significant reduction in blood cholesterol sustained after the withdrawal of EECLO from the diet suggests that the rat, unlike the chicken, may be able to store polyunsaturated fatty acids, and that these substances are released from body stores over a period of time. The results also suggest that the polyunsaturated fatty acids may exert their effect after absorption through the intestinal wall, and may not act by reducing cholesterol absorption from the intestinal tract, as was speculated for cholesterol-fed chicks (10). It is not illogical to suggest that the rat and chick differ in their mechanism for handling surplus amounts of blood cholesterol — particularly since feeding a cholesterol-supplemented diet to chicks contributes directly to the increase of serum cholesterol and to the development of atheromatous plaques in the aorta, whereas rats fed under similar dietary conditions do not develop plaques in their aorta and hypercholesterolemia is not as severe. Wissler et al. (11) showed that it is necessary to feed thiouracil if experimental atherosclerosis is to be produced in the rat.

A 0.5% supplement of EECLO did not reduce blood cholesterol in a 154-day study conducted with weanling male and female rats fed a modified M54 diet from which cholesterol and sodium taurocholate had been omitted. However, when the cholesterol-containing diet, M54, was fed for this length of time (table 3), the addition of 0.5% EECLO resulted in a significantly lower blood cholesterol in both sexes. The hypocholesterolemic effect of EECLO was also observed when it was added to diets of animals that had previously attained their maximal blood cholesterol level during 49 days of feeding diet M54. The blood

TABLE 3

Effect of long-term feeding of the polyunsaturated ethyl esters of cod liver oil on total whole blood cholesterol in rats

Treatment [1]	Sex	Blood cholesterol						
		Initial [2]	21-Day	49-Day	70-Day	106-Day	118-Day	140-Day	154 Day
		mg/100 ml	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
1	M	167 ± 4 [3]	690 ± 44	758 ± 73	587 ± 67	433 ± 31	433 ± 23	433 ± 27	425 ± 38
2	M	174 ± 7	378 ± 28 [4,6]	388 ± 22 [4,6]	276 ± 7 [4]	309 ± 9 [4]	278 ± 13 [4]	267 ± 12 [4]	258 ± 10 [4]
3	M	163 ± 3	543 ± 43 [5]	580 ± 39 [5]	271 ± 39 [5]	269 ± 9 [4]	289 ± 16 [4]	243 ± 7 [4]	235 ± 8 [4]
1	F	164 ± 5	746 ± 95	1111 ± 93	798 ± 79	883 ± 93	849 ± 92	756 ± 119	965 ± 113
2	F	160 ± 6	446 ± 33 [4,6]	319 ± 17 [4,6]	312 ± 16 [4]	301 ± 10 [4]	315 ± 13 [4]	344 ± 19 [4]	282 ± 16 [4]
3	F	152 ± 4	697 ± 69	944 ± 84	361 ± 47 [4]	309 ± 17 [4]	289 ± 16 [4]	292 ± 14 [4]	287 ± 25 [4]

[1] Treatments: 1 = M54; 2 = M54 + 0.5% EECLO; 3 = M54 for the initial 49 days after which the diet was supplemented with 0.5% EECLO. EECLO indicates ethyl esters of the polyunsaturated fatty acids of cod liver oil with an iodine number of 320.
[2] Ten weanling rats of each sex/treatment.
[3] Mean ± SE.
[4] Confidence limits of 99% or greater. Cholesterol values of treatments 2 and 3 within a bleeding period for a given sex were compared with their respective control (treatment 1).
[5] Confidence limits between 95% and 99%.
[6] Confidence limits of 98% or greater. Treatment 2 compared with treatment 3.

cholesterol level of these animals was reduced to a value similar to that of the rats receiving EECLO from weaning age. The difference in elevated blood cholesterol between males and females was eliminated with EECLO feeding. However, EECLO feeding never reduced blood cholesterol to the normal level observed in rats fed the modified M54 diet which lacked cholesterol and sodium taurocholate. A peak blood cholesterol value was reached in rats of both sexes between 21 and 49 days of feeding diet M54. A similar observation in rats fed a hypercholesterolemic diet has been described by Cuthbertson et al. (7). After this maximum was attained there was a decline to a lower but still much elevated blood cholesterol level. Similar observations have been reported in chickens by Rodbard (12) and by Kahn (6). Mean body weights did not differ between animals of the same sex, suggesting that comparable amounts of food were consumed, and that the lower blood cholesterol for the EECLO-supplemented groups was not caused by a reduction in cholesterol intake.

Two additional very unsaturated EECLO preparations were fed in various amounts to hypercholesterolemic female rats (table 4). The iodine numbers for these preparations (EECLO-7 and EECLO-10) were 347 and 376, respectively. An increase in the unsaturation of the ethyl ester preparations resulted in an increase in hypocholesterolemic activity. This was shown by the significant decrease in blood cholesterol of rats fed between 0.05 and 0.1% of these EECLO preparations. When an ethyl ester preparation with an iodine number of 320 was fed (table 1), a dose of 0.25% was the lowest level that gave a significant hypocholesterolemic activity.

Small quantities of EECLO were administered to test rats by subcutaneous injection, and control groups received an equal volume of coconut oil, thus all injections were of the same volume, 0.1 ml/rat/day. This small amount of oil appeared to be absorbed from the site of injection, since nodules of oil were not observed at the injection sites when the studies were completed. Table 5 shows that a hypocholesterolemic effect could be obtained from the unsaturated fatty esters when given in this manner to hypercholesterolemic female rats. This route of administering EECLO did not affect blood cholesterol in normocholesterolemic rats fed a commercial rat diet. Subcutaneous injections of

TABLE 4

A comparison of hypocholesterolemic effects of two ethyl ester fractions of cod liver oil fed to female rats

Treatment [1]		Blood cholesterol	
		Initial [2]	7-Day
		mg/100 ml	*mg/100 ml*
Group A			
	M54	473 ± 52 [3]	671 ± 54
	M54 + 0.5% EECLO-7	551 ± 57	336 ± 28 [4]
	M54 + 0.25% EECLO-7	489 ± 42	459 ± 62 [4]
	M54 + 0.10% EECLO-7	542 ± 61	608 ± 55
	M54 + 0.05% 2EECLO-7	548 ± 53	523 ± 55 [4]
Group B			
	M54	496 ± 72	582 ± 62
	M54 + 0.5% EECLO-10	609 ± 46	317 ± 17 [4]
	M54 + 0.25% EECLO-10	476 ± 47	385 ± 30 [5]
	M54 + 0.1% EECLO-10	558 ± 47	472 ± 79 [4]
	M54 + 0.05% EECLO-10	557 ± 52	600 ± 39
	M54 + 0.025% EECLO-10	598 ± 78	651 ± 97

[1] Group A was fed pre-test diet (M54) from weaning to 8 weeks age and group B to 9 weeks age. EECLO-7 and EECLO-10 are ethyl esters of the polyunsaturated fatty acids of cod liver oil with iodine numbers of 347 and 376, respectively. An equal amount of coconut oil was removed from the diet when a test oil was included.

[2] Ten rats/treatment.

[3] Mean ± SE.

[4] Confidence limit of 99% or greater. The increment between the initial and 7-day cholesterol value within a treatment was compared with the respective change in blood cholesterol for the M54 control group.

[5] Confidence limits between 95% and 99%.

TABLE 5

Effect of the polyunsaturated ethyl esters of cod liver oil on total whole blood cholesterol when administered subcutaneously to female rats

Treatment [1]	Blood cholesterol	
	Initial [2]	Final [3]
	mg/100 ml	*mg/100 ml*
Group A		
M54 + 0.1 ml CO/day	350 ± 23 [4]	474 ± 45
M54 + 0.1 ml EECLO/day	302 ± 19	260 ± 12 [5]
M54 + 0.05 ml CO + 0.05 ml EECLO/day	359 ± 31	400 ± 33
M54 + 0.075 ml CO + 0.025 ml EECLO/day	314 ± 40	421 ± 28
M54 + 0.09 ml CO + 0.01 ml EECLO/day	331 ± 29	444 ± 31
M54 + 0.1 CO/day + 0.5% EECLO (in diet)	344 ± 52	328 ± 11 [6]
Group B		
M54 + 0.1 ml CO/day	264 ± 21	291 ± 23
M54 + 0.1 ml EECLO/day	251 ± 31	219 ± 19 [6]
Commercial diet + 0.1 ml CO/day	90 ± 2	87 ± 2
Commercial diet + 0.1 ml EECLO/day	91 ± 3	87 ± 3

[1] Group A was fed pre-test diet M54 from weaning to 7 weeks age and group B to 5 months age. EECLO indicates ethyl esters of the polyunsaturated fatty acids of cod liver oil with an iodine number of 320. CO indicates coconut oil with an iodine number of 10.
[2] In group A there were 10 rats/treatment; in group B, 8 rats/treatment.
[3] Final blood was collected from group A after 7 days and group B after 5 days.
[4] Mean ± SE.
[5] Confidence limit of 99% or greater. The increment between the initial and final cholesterol value within a treatment was compared with the respective change in blood cholesterol for its respective M54 + 0.1 ml CO/day control group.
[6] Confidence limits between 95% and 99%.

EECLO into hypercholesterolemic chickens did not reduce serum cholesterol.[8] This absence of a hypercholesterolemic effect in chickens may be due to slower absorption of the EECLO from the site of injection, or the polyunsaturated fatty acids reduce blood cholesterol in the hypercholesterolemic chicken through a different mechanism than in the hypercholesterolemic rat.

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LITERATURE CITED

1. DeGroot, A. P., and S. A. Reed 1959 Influence of dietary cod liver oil and some fractions of cod liver oil on serum cholesterol level of rats. Nature, *183:* 1191.
2. Hauge, J. G., and R. Nicolaysen 1959 The serum cholesterol depressive effect of linolenic, linolenic acids and of cod liver oil in experimental hypercholesterolemic rats. Acta Physiol. Scand., *45:* 26.
3. Peifer, J. J., F. Janssen, P. Ahn, W. Cox and W. O. Lundberg 1960 Studies on the distribution of lipids in hypercholesteremic rats. I. The effect of feeding palmitate, oleate, trioleate, linoleate, menhaden and tuna oils. Arch. Biochem. Biophysics, *86:* 302.
4. Nicholaysen, R., and R. Ragard 1961 Effect of various oils and fats on serum cholesterol in experimental hypercholesterolemic rats. J. Nutrition, *73:* 299.
5. Howe, E. E., and D. K. Bosshardt 1962 A study of experimental hypercholesterolemia in the mouse. J. Nutrition, *76:* 242.
6. Kahn, S. G., J. Vandeputte, S. Wind and H. Yacowitz 1963 A study of the hypocholesterolemic activity of the ethyl ester of the polyunsaturated fatty acid of cod liver oil in the chicken. I. Effect on total serum cholesterol. J. Nutrition, *80:* 403.
7. Cuthbertson, W. F., P. W. Elcoate, D. M. Ireland, D. C. B. Mills and P. Shearley 1959 Serum-cholesterol levels and atherosclerosis: Effects of composition of diet and tri-iodothyronine on the rat. Brit. J. Nutrition, *13:* 227.
8. Hegsted, D. M., A. Gotsis and F. J. Stare 1957 The effect of various fats upon experimental hypercholesteremia in the rat. J. Nutrition, *63:* 377.
9. Albers, R. W., and O. H. Lowry 1955 Fluorometric determination of 0.1 to 10 micrograms of cholesterol. Anal. Chem., *27:* 1829.
10. Kahn, S. G., S. Wind, A. Slocum, D. Pfeffer and H. Yacowitz 1963 A study of the hypocholesterolemic activity of the ethyl esters of the polyunsaturated fatty acids and cod liver oil in the chicken. II. Effect on serum and tissue cholesterol and aortic and coronary atherosclerosis. J. Nutrition, *80:* 414.
11. Rodbard, S. C., C. Bolene, R. Pick, M. Lowenthal, G. Gros and L. N. Katz 1950 The age factor in cholesteremia and atherosclerosis in the chick. Circulation, *2:* 473.

[8] Unpublished data.

Availability of Amino Acids to Microorganisms

IV. COMPARISON OF HYDROLYSATES OF LACTALBUMIN, OATMEAL AND PEANUT BUTTER WITH SIMULATED AMINO ACID MIXTURES BY GROWTH RESPONSE OF MICROORGANISMS

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ABSTRACT A method described previously was used to compare hydrolysates of oatmeal, peanut butter and lactalbumin with their simulated mixtures of amino acids. Different growth responses of *Leuconostoc mesenteroides* P-60 were obtained with the hydrolysates of oatmeal and peanut butter and their simulated amino acid counterparts. The same growth responses were obtained with a hydrolysate of lactalbumin, a pure protein, and its amino acid counterpart. These results indicated that the oatmeal and peanut butter hydrolysates contained growth factors other than amino acids. To obtain equal growth with an amino acid mixture and an oatmeal hydrolysate, additional pyridoxal or pyridoxamine was required; to obtain equal growth with an amino acid mixture and a peanut butter hydrolysate, additional niacin was required. The effect of different forms of vitamin B_6 and niacin on growth response at constant nitrogen levels and the difficulty of evaluating amino acid patterns by the growth response of *L. mesenteroides* P-60 are discussed.

Study of the nutritional evaluation of food proteins by measuring availability of amino acids to microorganisms has been continued in this laboratory. In earlier work response of *Leuconostoc mesenteroides* P-60 to food hydrolysates and simulated mixtures of amino acids was different, even when adjustments were made for the effect of D-isomers, ammonium ions and carbohydrate content (1). A study of the factors, which when added to the amino acid mixtures, support growth responses equal to those obtained with hydrolysates of oatmeal, peanut butter and lactalbumin is the subject of the present paper.

METHODS

A quantity of food containing 127 mg[1] total nitrogen was hydrolyzed with 20% hydrochloric acid for 18 hours. The excess hydrochloric acid was removed, the hydrolysate filtered at pH 4 (3), and all the tryptophan and one-half the amount of cystine (4) calculated to be present in the food before hydrolysis were added to compensate for losses. After adjusting the pH to 6.8, the hydrolysate was made up to a volume of 500 ml.

The amino acid mixtures were prepared as outlined previously (1). In table 1 are shown the amino acid values and nitrogen distribution per 127 mg of total nitrogen in the amino acid mixtures for oatmeal, peanut butter and lactalbumin.

Using the method of Horn et al. (1), the growth response of *L. mesenteroides* P-60 was determined for the food hydrolysates and for the amino acid mixtures when each was added to a basal medium containing growth factors other than amino acids in amounts considered adequate to support maximal growth of the organism.

RESULTS

Comparison of microbial growth with oatmeal, peanut butter and lactalbumin hydrolysates and their corresponding amino acid mixtures. Different growth responses of the microorganism were obtained for the hydrolysates of oatmeal and peanut butter and their amino acid counterparts (table 2, lines 1 and 3; table 3, lines 1 and 5). However, the growth response for a hydrolysate of a pure protein, lactal-

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[1] In a previous study (2), 127 mg of total nitrogen of a good protein per 500 ml were found satisfactory.

TABLE 1

Free amino acids and nitrogen distribution [1] in amino acid mixtures simulating lactalbumin, oats and peanut butter hydrolysates

	Lactalbumin		Oats		Peanut butter	
	Amino acid value	Nitrogen	Amino acid value	Nitrogen	Amino acid value	Nitrogen
	mg	*mg*	*mg*	*mg*	*mg*	*mg*
Isoleucine	50.2	5.361	34.3	3.663	34.9	3.737
Leucine	101.3	10.819	59.2	6.322	50.0	5.340
Lysine	80.3	15.385	31.0	5.952	30.2	5.786
Methionine	17.6	1.653	12.8	1.202	8.8	0.826
Phenylalanine	26.9	2.281	45.3	3.841	41.3	3.502
Threonine	42.2	4.963	28.6	3.363	22.2	2.611
Tryptophan	14.7	2.017	10.3	1.413	8.0	1.097
Valine	46.6	5.573	47.6	5.693	34.9	4.174
Tyrosine	29.7	2.296	30.2	2.334	30.2	2.334
Cystine	32.6	3.801	23.9	2.787	15.1	1.761
Total EAAN [2]		54.149		36.570		31.158
Total EAA [3]	442.1		323.3		275.6	
Arginine	28.4	9.133	51.8	16.678	95.2	30.616
Histidine	14.5	3.928	17.0	4.591	18.3	4.955
Alanine	42.4	6.665	36.8	5.785	33.4	5.250
Aspartic acid	87.5	9.205	63.5	6.680	98.4	10.351
Glutamic acid	135.4	12.890	206.7	19.678	142.8	13.594
Glycine	22.0	4.105	38.1	7.109	47.6	8.882
Proline	43.0	5.233	42.8	5.209	49.3	6.000
Serine	41.3	5.505	44.4	5.918	35.7	4.759
Total NEAA N [4]		56.664		71.648		84.407
Total NEAA	414.5		501.1		520.7	
Total ammonium chloride N		16.187		18.782		11.435
Total N		127.000		127.000		127.000

[1] Per 127 mg total nitrogen.
[2] Essential amino acid nitrogen.
[3] Essential amino acids.
[4] Nonessential amino acid nitrogen.

bumin, was the same as for its amino acid counterpart at all levels of nitrogen (table 4, lines 1 and 3). The lactalbumin hydrolysate would be expected to contain only amino acids and ammonium ions. Therefore, the differences observed for oatmeal and peanut butter and their amino acid counterparts were postulated to be the result of factors other than amino acids and ammonium ions that affected the growth of the microorganism.

Factors responsible for growth difference with oatmeal hydrolysate and a simulated mixture of amino acids. For oatmeal, more growth was obtained with the amino acid mixtures than with the hydrolysate at the lower nitrogen levels and less growth at the higher nitrogen levels (table 2, lines 1 and 3). To determine whether these differences resulted from the presence of vitamins in the hydrolysate, the concentration of vitamins in the basal medium was doubled. With the additional vitamins, growth with the amino acid mixture decreased at the lower nitrogen levels and increased at the higher levels (lines 3 and 4). To determine which vitamins were present in the oatmeal hydrolysate in appreciable amounts, the hydrolysate was assayed using basal media lacking first one vitamin then another. We found that no vitamin except B_6 was present in amounts sufficient to change the response obtained with the regular basal medium. The hydrolysate contained enough vitamin B_6 to give the same response with or without pyridoxine in the basal medium (lines 1 and 2).

Lines 5 through 21 show the effect on growth response with the amino acid mixture when increasing amounts of pyridoxal and pyridoxamine were added to this mixture and when vitamin B_6 was omitted from the basal medium. As the amount of

TABLE 2

Growth response [1] *with oatmeal hydrolysate and a simulated mixture of amino acids at increasing nitrogen levels: the effect of B_6 vitamins*

	Food hydrolysate or synthetic mixture	Changes in basal medium		Added to amino acid mixture	Milligrams of total nitrogen				
		Vitamin B_6	Other vitamins		0.254	0.508	0.762	1.016	1.270
				μg/100 ml	*ml*	*ml*	*ml*	*ml*	*ml*
1	Hydrolysate	none	none		3.7	8.2	13.8	17.3	20.0
2		omitted	none		3.8	8.4	13.8	17.4	19.9
3	Mixture	none	none	0	4.5	10.5	14.8	17.0	18.4
4		doubled	doubled	0	4.2	9.9	15.4	19.1	21.2
5		omitted	none	0	3.8	4.8	7.3	9.0	11.1
6		omitted	none	0.1 al [2]	5.4	10.1	12.2	13.7	14.8
7		omitted	none	0.2 al	4.8	10.5	14.3	16.4	17.8
8		omitted	none	0.4 al	4.4	10.2	14.3	17.2	19.6
9		omitted	none	1.0 al	4.0	9.4	13.9	17.0	19.6
10		omitted	none	2.0 al	3.6	8.5	13.7	16.8	19.6
11		omitted	none	2.5 al	3.6	8.3	13.5	17.1	19.5
12		omitted	none	3.0 al	3.4	8.2	13.4	17.0	19.5
13		omitted	none	100 al	3.4	7.6	12.3	15.6	18.2
14		omitted	none	1.0 amine [3]	5.7	10.6	13.4	15.3	16.5
15		omitted	none	2.0 amine	5.2	10.8	14.1	15.9	17.4
16		omitted	none	4.0 amine	4.6	10.3	14.6	17.4	19.8
17		omitted	none	10.0 amine	4.1	9.1	14.1	17.3	19.8
18		omitted	none	16.0 amine	3.9	8.8	13.9	17.2	19.6
19		omitted	none	20.0 amine	3.9	8.9	13.9	17.3	19.9
20		omitted	none	25.0 amine	3.7	8.5	13.8	17.2	19.7
21		omitted	none	100 amine	3.6	7.9	12.6	15.4	18.6

[1] As measured by milliliters of 0.05 N NaOH.
[2] Pyridoxal.
[3] Pyridoxamine.

TABLE 3

Growth response [1] *with peanut butter hydrolysate and with a simulated amino acid mixture: effect of niacin*

	Food hydrolysate or amino acid mixture	Changes in basal medium		Added to amino acid mixture	Milligrams of total nitrogen				
		Vitamin B_6	Other vitamins		0.254	0.508	0.762	1.016	1.270
				μg/100 ml	*ml*	*ml*	*ml*	*ml*	*ml*
1	Hydrolysate	none	none		3.7	9.0	13.5	17.5	19.5
2		omitted	none		4.3	9.5	12.5	15.1	16.8
3		none	doubled		4.0	9.1	13.6	17.0	19.5
4		omitted	doubled		5.0	10.1	13.3	15.5	16.5
5	Mixture	none	none	0	4.1	9.0	13.8	16.6	17.5
6		omitted	none	0	4.6	5.3	5.9	6.7	8.0
7		omitted	none	25 ine [2]	4.3	9.7	12.4	14.1	15.1
8		omitted	none	50 ine [2]	4.3	9.9	13.9	16.1	17.1
9		none	doubled	0	4.4	9.4	14.5	18.0	19.0
10		none	none	80 niacin	4.0	9.0	14.0	17.7	19.3

[1] As measured by milliliters of 0.05 N NaOH.
[2] Pyridoxine.

pyridoxal or pyridoxamine was increased the response at all nitrogen levels increased and then decreased. However, larger amounts of the vitamins were required to produce a decrease in growth at the higher than at the lower nitrogen levels. At about 2.5 μg pyridoxal/100 ml of amino acid mixture the growth response equaled the response with oatmeal hydrolysate (lines 2 and 11). The same results were obtained with about 25 μg of pyridoxamine/100 ml of amino acid mixture (lines 2 and 20).

Factors responsible for growth difference with peanut butter hydrolysate and a simulated mixture of amino acids. For peanut butter, growth at the lower nitrogen levels

TABLE 4

Growth response [1] with a purified lactalbumin hydrolysate and a simulated amino acid mixture: effect of B_6 vitamins and niacin

	Food hydrolysate or amino acid mixture	Changes in basal medium		Added to amino acid mixture	Milligrams of total nitrogen				
		Vitamin B_6	Other vitamins		0.254	0.508	0.762	1.016	1.270
				μg/100 ml	*ml*	*ml*	*ml*	*ml*	*ml*
1	Hydrolysate	none	none		3.5	7.8	12.3	15.4	17.0
2		omitted	none		3.2	3.6	4.0	4.7	5.1
3	Mixture	none	none	0	3.6	7.8	12.6	15.5	17.1
4		omitted	none	0	3.0	3.2	3.6	4.1	4.7
5		omitted	none	4.0 amine [2]	3.5	7.3	11.9	15.1	17.8
6		omitted	none	0.4 al [3]	3.4	7.2	11.6	15.1	17.6
7		none	none	80.0 niacin	3.7	8.1	12.9	16.5	18.4
8		doubled	doubled	0	3.3	7.3	12.4	16.3	19.5

[1] As measured by milliliters of 0.05 N NaOH.
[2] Pyridoxamine.
[3] Pyridoxal.

was approximately the same for the hydrolysate and the amino acid mixture; at the higher nitrogen levels more growth was obtained with the hydrolysate than with the amino acid mixture (table 3, lines 1 and 5). When the hydrolysate was assayed using a basal medium containing no vitamin B_6, less growth was obtained at the higher nitrogen levels (lines 1 and 2) than with the regular basal medium. Thus, vitamin B_6 is not the factor responsible for the greater growth obtained with the hydrolysate than with the amino acid mixture, although the results obtained without vitamin B_6 show that the hydrolysate contains some vitamin B_6 (lines 2 and 6).

The amount of pyridoxine present in the peanut butter hydrolysate appears to be between 25 to 50 μg/100 ml as judged by growth responses (lines 2, 7 and 8). Using the regular amount of pyridoxine in the basal medium but doubling all the other vitamins suggested that the factors missing in the amino acid mixture were among the other 7 vitamins in the basal medium (lines 3 and 9). Of the 7 vitamins, only niacin had any effect on the response at higher levels of nitrogen. When graded amounts of niacin were added to the amino acid mixture, 80 μg/100 ml were required to produce a response equal to that with the peanut butter hydrolysate (lines 1 and 10).

Lactalbumin hydrolysate and its simulated amino acid mixture. It seemed desirable to study a pure protein hydrolysate which we were reasonably sure contained nothing but amino acids and ammonium ions. For these experiments a sample of lactalbumin was selected which had been purified in this laboratory and which contained 15.4% nitrogen (5). In table 4, lines 1 and 3, are shown the responses obtained with the unfiltered hydrolysate of lactalbumin and its simulated amino acid mixture. The agreement at all levels of the hydrolysate and the amino acid mixture indicates that no extra factors were present in the hydrolysate. However, the hydrolysate was tested for the presence of vitamins that had survived the purification and hydrolysis and none were noted. As an example, the absence of vitamin B_6 is shown by the limited growth with the lactalbumin hydrolysate when no vitamin B_6 was included in the basal medium (line 2).

Because of the results obtained for oats, the response to additions of pyridoxal and of pyridoxamine to the amino acid mixture simulating lactalbumin was determined (lines 5 and 6). Neither of these vitamins changed the response significantly. Addition of niacin increased the response at the last 2 levels of nitrogen (lines 3 and 7) as with the amino acid mixture simulating peanut butter. Doubling all the vitamins also increased the last 2 levels but lowered the first two (lines 3 and 8).

Reproducibility of results. When all phases of this method were rigidly controlled the reproducibility was excellent. A statistical analysis of each nitrogen level of results on oatmeal and peanut butter hydrolysates gave a coefficient of variation for oats of 4.4, 4.0, 1.3, 2.6, and 1.6%; average for all levels 2.8%; for peanut butter 3.2, 4.0, 2.0, 2.1, and 1.7%, average 2.6%.

DISCUSSION

A study of the nutritional value of the proteins of a food involves a determination of the amino acids of that food. This necessitates a hydrolysis of the food with strong acids for a considerable length of time. Other nutrients in the food having an effect on growth response of the microorganisms may be drastically changed by such treatment. However, some nutrients in these hydrolysates are still active and influence the growth response of the microorganism used. Among the active materials are some of the vitamins.

Generally an excess of a nutrient in the basal medium minimizes the effect of that nutrient in the food hydrolysate. Such a nutrient is niacin which, if present in excess in the basal medium, does not increase growth response even when large amounts are present in the hydrolysate. This is not true, however, when the food hydrolysate contains vitamin B_6. Various forms of vitamin B_6 undergo changes in activity when heated with mineral acid and when autoclaved with amino acids as shown by Snell et al. (6, 7) and Hochberg and associates (8). In the preparation of food hydrolysates with 20% acid, active vitamin B_6 compounds survived. The activity of these surving compounds cannot be compensated for by having an excess of vitamin B_6 in the basal medium, because an excess of pyridoxal or pyridoxamine will give low growth response at all levels of nitrogen (table 2).

The presence in food hydrolysates of nutrients other than amino acids which affect the growth of the microorganism makes difficult the evaluation of amino acid patterns in foods. For example, the same growth responses are obtained with oatmeal and peanut butter hydrolysates containing the same amount of total nitrogen but having entirely different amino acid patterns. For peanut butter hydrolysate the growth response is partially due to the presence of niacin, whereas for the oatmeal hydrolysate it is partially due to the presence of vitamin B_6. Thus in comparing 2 food hydrolysates, more growth might be obtained with one than with the other, not because of its amino acid pattern, but because of the presence of other nutrients.

The growth response with a hydrolysate of lactalbumin, a protein of relatively high biological value, was less, under standard conditions, than with oatmeal or peanut butter hydrolysates (tables 2 and 3). Even when the maximal amount of pyridoxal or pyridoxamine for the total nitrogen was added, the increased growth at the fifth level of nitrogen still was less than with oatmeal or peanut butter hydrolysates. Addition of more niacin resulted in increased growth at the high nitrogen levels, but at no level did it equal the response with oatmeal or peanut butter hydrolysates. Doubling all the vitamins in the basal medium, however, brought the fourth and fifth levels closer to the corresponding levels with oatmeal and peanut butter hydrolysates (table 4).

Purification in the preparation of lactalbumin may have removed unknown factors in milk which are necessary for the efficient use of this protein. Such factors also may have been removed in filtration during the preparation of other food hydrolysates. The effect of some of these factors is being investigated.

LITERATURE CITED

1. Horn, M. J., and H. W. Warren 1961 Availability of amino acids to micro-organisms. III. Development of a method for comparison of hydrolysates of foods with synthetic simulated mixtures. J. Nutrition, 74: 226.
2. Horn, M. J., A. E. Blum and M. Womack 1954 A rapid microbial method of determining proteins value. J. Nutrition, 52: 375.
3. Horn, M. J., A. E. Blum, C. E. F. Gersdorff and H. W. Warren 1953 Sources of error in microbiolgical determinations of amino acids on acid hydrolysates I. Effect of humin on amino acid values. J. Biol. Chem., 203: 907.
4. Horn, M. J., and A. E. Blum 1956 A microbiological method for determination of cystine in foods. Cereal Chem., 33: 18.
5. Jones, D. B., and C. O. Johns 1921 Determination of the monoaminoacids in the hy-

drolytic cleavage products of lactalbumin. J. Biol. Chem., *48:* 347.
6. Snell, E. E. 1944 The vitamin activities of "pyridoxal" and "pyridoxamine." J. Biol. Chem., *154:* 313.
7. Snell, E. E., B. M. Guivard and R. J. Williams 1942 Occurrence in natural products of a physiologically active metabolite of pyridoxine. J. Biol. Chem., *143:* 519.
8. Hochberg, M., D. Melnick and B. L. Oser 1944 Chemical determination of pyridoxine in biological materials and pharmaceutical products. The multiple nature of vitamin B_6. J. Biol. Chem., *155:* 119.

Effect of Dietary Carbohydrate on Glucokinase and Mannokinase Activities of Various Rat Tissues [1]

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ABSTRACT Glucokinase activities were determined in 9 rat tissues — liver, brain, muscle, kidney, small intestine, heart, spleen, adipose tissue and mammary gland. Significant amounts of glucokinase activity were particle-bound in brain (40 to 55%), in heart (50 to 60%) and in mammary gland (40%). In the other tissues, almost all of the activity appeared in the particle-free supernatant fraction. Except for a minor response by small intestine, liver was the only tissue in which glucokinase activity responded significantly to nutritional treatment of the rat. Evidence for the existence of 2 distinct enzymes capable of phosphorylating glucose was obtained from a study of the K_m (glucose) values for the enzymes in the particle-free supernatant fraction of rat liver. The hepatic enzyme with a high K_m value for glucose was shown to adapt to dietary treatment, whereas the hepatic glucokinase with a low K_m for glucose was non-adaptable. Mannokinase activities were determined in brain, liver and mammary gland. The rates at which brain, liver and mammary gland phosphorylated mannose were 33, 27 and 26%, respectively, of those observed for glucose.

The level of hepatic glucokinase activity[2] in the rat is influenced by the previous nutritional state of the animal. DiPietro and Weinhouse (1) have shown that glucokinase activity is lower in the liver of the fasted rat than in the fed rat. Rats fed diets high in carbohydrate for 20 and 40 days respond with increased activities (2). Blumenthal and associates (3) studied the effects of feeding a high carbohydrate diet (60% glucose) on hepatic glucokinase[3] of fasted rats and observed an increase in enzyme activity in 4 hours. These workers also demonstrated that the enzyme responds principally to carbohydrate. Long (4) compared the hexokinase[4] activity in the liver of the fasted rat with that in other tissues of the fed rat, and concluded that liver had the lowest activity. Since hepatic glucokinase activity responds to diet, his comparison is open to question.

In the present report we have investigated the effects of diet on glucokinase activities of rat liver, skeletal muscle, brain, kidney, heart, spleen, small intestine and epididymal adipose tissue, by means of an assay system that involved a coupled reaction with glucose-6-phosphate dehydrogenase. In addition, the method was modified to permit the study of mannokinase activities.[5]

EXPERIMENTAL

Animals and their treatment. Female rats (150 to 210 g) of the Long-Evans strain, raised and maintained with an adequate stock diet,[6] were used throughout, except in the experiments with epididymal fat pads.

The high carbohydrate diet consisted of 65% glucose, 22% vitamin-free casein,[7] 10% cellulose,[8] 2% salt mixture (5) and 1% liver.[9] The high protein diet contained 78% vitamin-free casein, 19% cellulose, 2% salt mixture (5) and 1% liver.[10]

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[1] This work was supported by a contract from the United States Atomic Energy Commission.
[2] Hexokinase (ATP: D-hexose 6- phosphotransferase) is used as a general designation for enzymes capable of transferring a phosphate group from ATP to the 6 position of any hexose. Glucokinase (ATP: D-glucose 6-phosphotransferase) is used to designate the enzyme that catalyzes the transfer of a phosphate group from ATP to the 6 position of glucose; and mannokinase (ATP: D-mannose 6-phosphotransferase) that which catalyzes the transfer of a phosphate from ATP and results in formation of mannose-6-phosphate. Glucokinase activity therefore refers to the rate of glucose phosphorylation and mannokinase activity to the rate of mannose phosphorylation.
[3] See footnote 2.
[4] See footnote 2.
[5] See footnote 2.
[6] Diablo Labration, Diablo Laboratories, Berkeley, California.
[7] "Vitamin-Test" Casein, Nutritional Biochemicals Corporation, Cleveland.
[8] Cellu Flour, Chicago Dietetic Supply House, Chicago.
[9] A raw liver substance, Vio-Bin Corporation, Monticello, Illinois.
[10] See footnote 9.

The rats were fed these specially prepared diets, ad libitum, for 3 days before they were killed. The rats ate 7 to 11 g/day regardless of which diet was fed.

Tissue preparations. The rats were stunned by cervical fracture, and bled. Liver, brain, kidney, spleen and epididymal adipose tissue were rapidly excised, and each tissue was blotted on filter paper and weighed. The heart was cut in half, and excess blood was removed. The small intestine was opened along its entire length, washed 3 times with 10 volumes of the homogenizing buffer, blotted dry, weighed and minced. Muscle was taken from the lateral thigh of the hind limb. This tissue was blotted dry, weighed and minced, with sharp scissors, in 1 ml of the homogenizing buffer. Mammary glands were excised from lactating rats that had suckled at least 6 pups for the entire lactational period (17 to 18 days). The glands were sliced (6), washed 4 to 5 times with 10 volumes of the homogenizing buffer, blotted dry, weighed and minced.

Unless otherwise stated, all tissues were homogenized for about 1 minute, with 3 ml of buffer/g, in a motor-driven, Potter-Elvehjem-type grinder provided with a loose-fitting Teflon pestle (0.5-mm tolerance). The buffer (pH 7.0) consisted of 0.15 M KCl, 0.005 M EDTA and 0.005 M $MgCl_2$. To each liter of buffer, 0.65 ml of 2-mercaptoethanol was added immediately before use (3). The resulting homogenates were centrifuged in a Model PR-2 International Refrigerated Centrifuge and the Model L Spinco Ultracentrifuge. All preparative procedures were carried out in a cold room, at 2 to 4°.

Glucokinase assay. A modification (3) of the method of DiPietro and Weinhouse (1) was used. The amount of glucose-6-phosphate formed by the enzyme was determined by following the appearance of TPNH in the presence of purified glucose-6-phosphate dehydrogenase and TPN. Each cuvette was made to contain 1.0 ml of a buffer (pH 8.0) mixture (consisting of 0.1 M histidine·HCl, 0.1 M Tris buffer, 0.01 M EDTA and 0.01 M $MgCl_2$), 0.025 ml of 0.55 M neutralized ATP, 0.02 ml of 0.06 M TPN, 0.002 ml of a suspension of purified glucose-6-phosphate dehydrogenase,[11] and 0.01 to 0.1 ml of the tissue preparations. The reaction was started by addition of 0.1 ml of 1 M glucose. The final volume of each mixture was adjusted with water to 2.0 ml,[12] and the reaction was carried out at 30°C. Blank cuvettes contained no added glucose.[13] Changes in optical density were measured spectrophotometrically at 340 mμ with a Beckman DU spectrophotometer equipped with a Gilford model 210 automatic cuvette positioner, a model 220 optical density converter, and a Leeds-Northrup 10-mv recorder. The absorbancies of 4 samples were recorded sequentially in automatic rotation at 10-second intervals. Measurements were taken continuously for the first 10 minutes after zero-order kinetics was observed. The extinction coefficient of 6.22×10^6 cm^2/mole for TPNH (7) was used to convert changes in optical density to amounts of TPNH formed. Glucokinase activities, measured in this manner, are expressed as (a) millimicromoles of TPNH formed per milligram protein per minute (specific activity), and (b) millimicromoles of TPNH formed per gram wet weight of tissue (total activity).

Purification of phosphoglucose isomerase and phosphomannose isomerase. The protein of the particle-free supernatant fraction, obtained from lactating rat mammary glands that precipitated in the presence of 55 to 75% saturation with ammonium sulfate, was used as the source for both isomerases. This preparation yielded the highest specific activities of both enzymes and contained very little kinase activity (table 1). Since the preparation of these enzymes from lactating rat mammary glands differed radically from that reported by Slein (8) for rabbit muscle, a description of our method is presented here: Ten grams of mammary glands ex-

[11] This amount of glucose-6-phosphate dehydrogenase is capable of producing 0.28 μmole of TPNH/minute when 0.1 ml of 0.5 M glucose-6-phosphate is used to initiate the reaction. The formation of TPNH in the coupled glucokinase assay was always kept at about 0.025 μmole/minute to insure at least a tenfold excess of glucose-6-phosphate dehydrogenase.

[12] This assay system therefore comprised 5×10^{-2} M glucose, 7×10^{-3} M ATP, 6×10^{-4} M TPN, 5×10^{-3} M $MgCl_2$ and 5×10^{-3} M EDTA.

[13] With the homogenate fractions obtained from liver, kidney and skeletal muscle, there was a slow linear increase in optical density without addition of glucose. Endogenous glucose, although accounting for a large proportion of this production of TPNH, was not the sole precursor of glucose-6-phosphate. The presence of glycogen in these fractions, together with substantial amounts of phosphorylase and phosphoglucomutase activity, could have accounted for the additional increase in the optical density observed with blank cuvettes.

TABLE 1

Purification of phosphohexose isomerases from lactating rat mammary gland[1]

Ammonium sulfate fractions	Enzyme activity			
	Phosphomannose isomerase	Phosphoglucose isomerase	Glucokinase	Mannokinase
% saturation	*mμmoles TPNH/mg protein/min*			
0 to 45	2.1		3.1	
45 to 55	9.5	45.1	8.4	1.7
55 to 75	39.5	484	2.1	0.6

[1] For description of ammonium sulfate fractions and enzyme assays see text. The averages, each of two closely agreeing values obtained with 2 rats, are given.

cised from a lactating, 17-day postpartum rat were sliced (6), washed 4 times with 15 ml of 0.25 M sucrose to remove as much of the contained milk as possible, minced, and finally homogenized with 35 ml of 0.25 M sucrose solution. The homogenate was centrifuged at 100,000 × *g* [14] for 45 minutes, and the clear supernatant fraction between the fatty layer and the pellet was separated. To 30 ml of this particle-free fraction enough solid ammonium sulfate was added to provide 45% saturation (9). After 15 minutes, the precipitate was separated by centrifugation, and the supernatant fraction was adjusted to 55% saturation with solid ammonium sulfate. The precipitate formed was separated by centrifugation for 15 minutes. Enough ammonium sulfate was then added to the supernatant fraction to yield a final concentration of 75%. The mixture was centrifuged, and the pellet separated. All procedures were carried out at 2°.

The precipitates obtained were dissolved in 0.25 M sucrose, and tested for enzymatic activity. Glucokinase activity was estimated as described above. Activities of phosphomannose isomerase (which converts mannose-6-phosphate to fructose-6-phosphate) and phosphoglucose isomerase (which converts fructose-6-phosphate to glucose-6-phosphate) were assayed in the same mixture without the additions of either glucose or ATP, but 0.05 ml of 0.07 M mannose-6-phosphate or 0.05 ml of 0.13 M fructose-6-phosphate was added to start the reaction.

Mannokinase assay.[15] The method used measured the conversion of mannose to mannose-6-phosphate. In the presence of excess phosphomannose isomerase, phosphoglucose isomerase and glucose-6-phosphate dehydrogenase, mannose-6-phosphate is converted to gluconic acid-6-phosphate, TPNH being produced by the dehydrogenase reaction.

Mannokinase activity was assayed in the same mixture as described above for glucokinase, except that purified phosphoglucose isomerase and phosphomannose isomerase were added in excess. The reaction was started with 0.1 ml of 1 M mannose, and the linear increase in optical density was followed for 10 minutes after an initial 20-minute lag period (10). The units used to express mannokinase activity were the same as those for glucokinase activity.

Preparation of brain mitochondria. Whole rat brains were homogenized with 7 ml/g of the buffer (pH 7.4 and composed of 0.075 M sucrose, 0.21 M mannitol, 0.005 M EDTA and 0.01 M Tris) described by Sacktor and Packer (11). The homogenate was first contrifuged at 800 × *g* for 10 minutes to remove cellular debris and nuclei. The pellet obtained by centrifugation at 10,000 × *g* for 10 minutes was resuspended in the same buffer by hand homogenization. Some heavy particles were removed by centrifugation at 800 × *g* for 10 minutes. The mitochondrial pellet was washed with the buffer and resedimented centrifugally. Washed mitochondria obtained in this way were suspended in the buffer and analyzed for enzymatic activity immediately after their preparation. All procedures were carried out at 2°C.

Chemical analyses. Protein was determined by the biuret method of Gornall et al. (12). Glucose was estimated enzymatically with purified yeast hexokinase

[14] Centrifugal force measured as the average in the center of the tube.

[15] See footnote 2.

and the glucokinase assay system described above. The anthrone procedure (13) was used for determination of glycogen.

Materials. The disodium salt of ATP [16] and triphosphopyridine nucleotide[17] were neutralized with KOH before use. Disodium glucose-6-phosphate and glucose-6-phosphate dehydrogenase (yeast) were obtained commercially [18] as was the purified hexokinase (yeast).[19] Mannose-6-phosphate was prepared according to the method of Slein (14), and isolated as the barium salt. It was converted to the potassium salt by the use of K_2SO_4 and Dowex-50-K^+. The barium salt of fructose-6-phosphate[20] was converted to the potassium salt. It was found to be contaminated with about 6% of glucose-6-phosphate when tested spectrophotometrically by the dehydrogenase assay system (15). It was purified so that it contained not more than 0.4% glucose-6-phosphate.[21] EDTA was obtained commercially.[22]

RESULTS

Intracellular distribution of glucokinase

The cellular distribution of glucokinase in various rat tissues is shown in table 2. Since the levels of glucokinase activity in the supernatant fractions prepared from homogenates of liver, muscle, kidney, spleen and adipose tissue were not diminished by centrifugation at speeds that sedimented cytoplasmic particles (15 minutes at 10,000 × *g* for mitochondria followed by 100,000 × *g* for 45 minutes, for microsomes), we concluded that almost all of the enzyme was in the particle-free fraction, which represents cellular cytoplasm (see table 2, columns 8 and 9). It is therefore not unexpected that the specific activities of glucokinase increased with removal of cytoplasmic particles, which do not contain this enzyme. In the case of liver, not only did the specific activity increase, but a measurable increase in total activity also occurred.

About 41% of the total glucokinase activity in brain was associated with the particles; the remainder was in the high-speed supernatant fraction (table 2). A similar pattern was observed with heart and lactating mammary gland homogenates. A small amount of glucokinase activity in the small intestine is probably bound to particles, but by far the major portion of the enzyme in this tissue is in the soluble fraction.

Particle-bound glucokinase of brain was further studied by isolation of its mitochondria. Attempts to solubilize the enzyme by freeze-thawing of these particles was not successful (table 3). Although treating them with 0.25% sodium desoxycholate in a 0.1 M sodium phosphate buffer (pH 7.2) solubilized about 50% of the total protein, this released very little glucokinase activity into the soluble fraction. In confirmation of the observations of Crane and Sols (16), the data in table 3 show that use of desoxycholate, by solubilizing some of the nonglucokinase-containing mitochondrial protein, resulted in a twofold increase in glucokinase activity which was, however, still bound to the insoluble portion.

Effect of previous nutritional state on glucokinase activity of various tissues

Table 4 shows that hepatic glucokinase is profoundly affected by dietary treatment. The specific activities of this enzyme were greater in rats fed a diet high in glucose than in those that were either fasted or fed a diet devoid of carbohydrate (but high in protein). Since total protein in the high-speed supernatant fractions from all 3 groups was about the same, it is not unexpected that total hepatic activity was lower in the fasted rats and in those fed the protein diet than in those fed the high-carbohydrate diet.

Glucokinase activity of small intestine (expressed as specific or total activity) also responded to the nutritional state of the rat (table 4), but the response was not so great as that observed in liver.

The specific activity of the enzyme in adipose tissue was lower in rats fed the high protein diet than in those fed the

[16] Pabst Laboratories, Milwaukee, Wisconsin.
[17] See footnote 16.
[18] California Corporation for Biochemical Research, Los Angeles.
[19] Sigma Chemical Company, St. Louis.
[20] Swartz Laboratories, Inc., Mt. Vernon, New York.
[21] Unpublished method.
[22] Fisher Scientific Company, Fairlawn, New Jersey.

TABLE 2

Glucokinase activities in homogenate fractions of various tissues [1]

Tissue	Total activity in supernatant fraction from centrifugation			Specific activity in supernatant fraction from centrifugation			Distribution of total glucokinase	
	5 min at 350 × *g*	15 min at 10,000 × *g*	45 min at 100,000 × *g*	5 min at 350 × *g*	15 min at 10,000 × *g*	45 min at 100,000 × *g*	Particles	Cytoplasm
							%	%
Liver	4.00 ± 0.44 [2]	5.20 ± 0.21	5.25 ± 0.19	22.7 ± 4.1	40.3 ± 4.0	57.0 ± 3.6		(100) [3]
Brain	4.30 ± 0.21	2.86 ± 0.12	2.53 ± 0.15	85.7 ± 2.3	58.8 ± 6.8	60.2 ± 5.2	41	59
Muscle	0.55 ± 0.06	0.58 ± 0.07	0.55 ± 0.08	6.4 ± 0.25	9.1 ± 0.31	12.1 ± 0.57	0	100
Kidney	1.65 ± 0.23	1.65 ± 0.18	1.65 ± 0.18	14.5 ± 1.0	26.4 ± 1.2	29.3 ± 1.3	0	100
Small intestine	1.41 ± 0.19	1.39 ± 0.15	1.22 ± 0.18	18.0 ± 1.1	25.0 ± 3.1	32.2 ± 3.1	13	87
Spleen	1.30 ± 0.19	1.25 ± 0.32	1.26 ± 0.33	5.5 ± 0.20	6.3 ± 0.24	10.2 ± 0.31	3	97
Heart	2.41 ± 0.13	1.76 ± 0.38	1.37 ± 0.08	16.5 ± 4.0	20.0 ± 3.4	19.5 ± 3.5	43	57
Adipose	0.35 ± 0.13	0.29 ± 0.09	0.32 ± 0.13	48.2 ± 3.4	51.6 ± 1.7	53.0 ± 4.1	8	92
Lactating mammary gland [4]	1.38 ± 0.18	0.84 ± 0.09	0.85 ± 0.09		13.8 ± 0.42	15.8 ± 0.51	38	62

[1] All rats were fed the high carbohydrate diet for 3 days before excision of tissues. Supernatant fractions obtained from tissue homogenates after the centrifugal speeds listed above were tested for glucokinase activity as described in text. Total glucokinase activity is expressed as micromoles of TPNH formed per minute per gram of wet tissue, and specific glucokinase activity as millimicromoles of TPNH formed per minute per milligram protein of homogenate fraction.

[2] Mean ± SD.

[3] The glucokinase activity expressed as total activity was higher in the particle-free supernatant fraction than in the fractions that contained particles.

[4] These lactating rats (18 days postpartum) were fed an adequate stock diet (36).

TABLE 3

Glucokinase activity in various brain homogenate fractions [1]

Homogenate fraction	Centrifugation		Glucokinase activity as % of activity in supernatant + mitochondria + microsomes		Total protein as % of protein in supernatant + mitochondria + microsomes
	Speed	Time	Specific activity	Total activity	
	× *g*	*min*			
Supernatant + mitochondria + microsomes	350	10	100 [2]	100	100
Supernatant + microsomes	10,000	10	80	46	57
Washed mitochondria	10,000	10	104	53	52
Pellet from mitochondria after freeze-thaw treatment	20,000	30	92	48	53
Supernatant from mitochondria after freeze-thaw treatment	20,000	30	70	3.8	5.6
Pellet from mitochondria after desoxycholate treatment	100,000	25	182	54	29
Supernatant from mitochondria after desoxycholate treatment	100,000	25	24	5.6	23

[1] Two whole rat brains (3.1 g) were homogenized with 21 ml of the sucrose-mannitol buffer mixture, and the homogenate was centrifugally fractionated as described in text. The supernatant fraction obtained by centrifugation at 350 × *g* for 10 minutes (supernatant plus mitochondria plus microsomes) contained 220 mg of protein. The individual fractions were tested for glucokinase activity. Values reported are the means of two separate, closely agreeing determinations obtained with 2 groups of rats.

[2] The specific activity of this fraction was 80 mμmoles of TPNH formed/mg protein/minute.

high glucose diet, but total enzyme activity did not change appreciably. This change in specific activity in the case of adipose tissue is related to the fact that the protein content of the particle-free supernatant fractions was higher in the experiments with the rats fed the high protein diet than in those with rats fed the high glucose diet.

Glucokinase activity in all of the other tissues examined failed to respond to dietary treatments.

Michaelis constants of the various tissue glucokinases

The Michaelis constants (K_m) were determined by the method of Lineweaver and Burk (17) and are recorded in column 12 of table 4. The values were of the order of 10^{-5} M glucose for all tissues except for liver of rats fed the high glucose diet. In the latter case, glucokinase had an apparent K_m of 7–9 × 10^{-3} M glucose.

To determine true K_m values it is important to assess carefully the significance of the endogenous substrates present in tissue homogenates so that the exact concentration of the enzyme's substrate is known. In the case of liver, endogenous glucose would certainly interfere with the determinations of the true K_m value for glucokinase. When the glucose concentration in the assay system was varied from 5 × 10^{-3} M to 2 × 10^{-1} M, the apparent K_m was of the order of 7–9 × 10^{-3}. This value agrees well with values of 1 × 10^{-2} to 4 × 10^{-2} reported by DiPietro et al. (18) who measured the K_m over the same range of glucose concentration. However, when the glucose concentration was lowered step by step to 1 × 10^{-4} M, we no longer obtained good linearity in the reciprocal Lineweaver-Burk plots as the curves sloped off. These observations suggest the possible existence of 2 enzymes, with different K_m values, as already reported by Walker (19) and Viñuela et al. (20).

An experiment to test whether the presence of a kinase with a low K_m for glucose could be detected by this assay procedure was performed. Purified yeast hexokinase (K_m of 1 × 10^{-4} M) was added to a particle-free supernatant fraction prepared from the homogenate of the liver of a rat that had been fed the high carbohydrate diet — the amount added being such that

TABLE 4

Effect of previous nutritional state on glucokinase activities of various rat tissues [1]

Tissue	Proportion of homogenizing buffer volume to wet tissue wt	Nutritional state of rat: Fed high carbohydrate diet for 72 hours			Fed high protein diet for 72 hours			Fasted for 48 hours			K_m[2] (M glucose)
		Protein	Specific activity	Total activity	Protein	Specific activity	Total activity	Protein	Specific activity	Total activity	
	ml/g	*mg/ml*			*mg/ml*			*mg/ml*			
Liver	3:1	28.5 ± 2.5 [3]	55.4 ± 3.6	5.13 ± 0.41	33.0 ± 2.5	6.6 ± 2.0	0.72 ± 0.11	36.8 ± 2.5	7.5 ± 1.2	0.82 ± 0.10	7–9 × 10^{-3}
Brain	3:1	16.0 ± 2.0	83.0 ± 9.3	4.40 ± 0.36	18.5 ± 2.0	84.5 ± 2.3	4.44 ± 0.50	17.5 ± 1.5	83.8 ± 6.5	4.35 ± 0.30	4–6 × 10^{-5}
Muscle	3:1	14.5 ± 2.0	11.9 ± 2.0	0.49 ± 0.07	16.0 ± 2.5	12.1 ± 3.2	0.56 ± 0.05	15.0 ± 1.5	9.5 ± 1.5	0.41 ± 0.07	7–9 × 10^{-5}
Kidney	3:1	18.5 ± 2.5	28.6 ± 3.1	1.53 ± 0.13	17.5 ± 2.5	24.8 ± 2.8 [4]	1.47 ± 0.22	17.5 ± 2.0	25.1 ± 1.0 [4]	1.41 ± 0.15	1–2 × 10^{-5}
Small intestine	3:1	12.5 ± 1.5	29.0 ± 4.3	1.07 ± 0.6	12.5 ± 0.5	21.2 ± 1.6 [5]	0.78 ± 0.09 [6]	10.5 ± 2.0	19.8 ± 1.2 [5]	0.73 ± 0.05 [6]	6–8 × 10^{-5}
Spleen	3:1			1.32 ± 0.06			1.26 ± 0.12				
Heart	10:1	8.0 ± 0.5	21.8 ± 1.7	1.80 ± 0.17	8.0 ± 0.5	19.5 ± 4.1	1.73 ± 0.26	9.0 ± 2.5	21.1 ± 0.7	1.93 ± 0.10	5–7 × 10^{-5}
Adipose	6:1	2.5 ± 0.5	53.0 ± 4.1	0.32 ± 0.13	4.0 ± 0.5	38.4 ± 7.6	0.41 ± 0.07				8–10 × 10^{-5}

[1] Glucokinase activity was assayed as described in text in supernatant fractions obtained by centrifugation of tissue homogenates (except brain) at 100,000 × *g* for 45 minutes. The brain fraction studied was the supernatant fraction obtained by centrifugation at 350 × *g* for 5 minutes. Specific activity is expressed as millimicromoles of TPNH formed per milligram protein per minute, and total activity as micromoles of TPNH formed per gram wet weight of tissue per minute.

[2] All K_m values were determined with tissue preparations prepared from rats fed the high carbohydrate diet in a glucose concentration range of 1×10^{-3} to 5×10^{-2} M.

[3] Each value reported is the mean ± SD of the results of 5 separate experiments.

[4] According to Student's *t* test: Differs from high carbohydrate diet values by $P > 0.1$ (considered not significant).

[5] Differs from high carbohydrate diet values by $P < 0.01$.

[6] Differs from high carbohydrate diet values by $P < 0.001$.

the maximal activity of the yeast hexokinase was 25% of that of the liver enzyme. When the K_m for glucose was determined in this mixture at high glucose concentrations (2.5×10^{-3} to 5×10^{-2} M), the apparent K_m was 2.5×10^{-3}, whereas at low glucose concentrations (5×10^{-4} to 1×10^{-3} M), the apparent K_m was 3×10^{-4}. We may conclude from the results of this experiment that if a kinase with a low K_m value were present along with the kinase with a high K_m value, it would not escape detection by this assay procedure.

The possible existence of 2 distinct glucokinases with different K_m values for the phosphorylation of glucose in liver (19, 20) was examined further. The glucokinase assay procedure used here was modified as described by Viñuela et al. (20) for the determination of the activity of the 2 glucokinases. Thus the activities were measured in the following way: (a) by the use of N-acetyl glucosamine, a potent inhibitor of the glucokinase, with a high K_m value; and (b) at a high glucose concentration (5×10^{-2} M), which yielded values for the sum of the activities of the 2 glucokinases, and at a low glucose concentration (8×10^{-4} M), which yielded values for the activity of the low K_m glucokinase. The results of experiments performed with dialyzed and undialyzed ammonium sulfate fractions of the particle-free supernatant fractions of homogenates prepared from the livers of rats fed high carbohydrate and of those fasted are presented in table 5. In the particle-free supernatant preparations, before ammonium sulfate fractionation, total glucokinase activity was about 7 times higher in the liver of the rat fed high carbohydrate (column 5, table 5) than in that of the fasted rat. The data in columns 7 and 8 clearly show that this variation in glucose phosphorylation induced by the dietary treatment is due to a change in the activity of only one of the two glucokinases. For this reason we have designated these enzymes as *adaptable* and *non-adaptable*. A distinct difference in the K_m values for glucose with these 2 enzymes could be demonstrated only in dialyzed ammonium sulfate fractions. For example, with the non-dialyzed ammonium sulfate fractions from both tissue preparations, we could detect only the high K_m value over a glucose concentration range of 1×10^{-3} to 5×10^{-2} M. Under these conditions, when the glucose concentration was decreased, the Lineweaver-Burk plots were no longer linear — as had been the case for the unfractionated particle-free supernatant preparations. This was apparently due to these 2 enzymes not having been adequately separated by the ammonium sulfate fractionation (columns 7 and 8).

Linear Lineweaver-Burk plots (17) over the whole glucose concentration range of 1×10^{-4} to 5×10^{-2} M were obtained only in the experiments with the dialyzed 20 to 50 and 60 to 75% ammonium sulfate fractions from the fasted rat liver preparation and with the dialyzed 60 to 75% ammonium sulfate fraction liver preparation from the rats fed high carbohydrate. In these dialyzed fractions we detected little or no cross contamination of one enzyme with the other, and the difference in the K_m values was therefore very clear. The K_m value observed with the non-adaptable glucokinase was 5×10^{-5} M glucose and that for the adaptable glucokinase was $7\text{–}9 \times 10^{-3}$ M glucose (table 5).

Apparently a major difficulty in the demonstration of these 2 liver enzymes with different kinetic properties is related to the degree of purity of the isolated fractions rather than to the presence of endogenous glucose (table 5). The data in table 5 also demonstrate that the adaptable glucokinase was unstable to prolonged dialysis, whereas the non-adaptable enzyme withstood such treatment.

Estimation of the actual amounts of glucose phosphorylated by various tissue homogenates

To relate the amount of TPNH generated in the glucokinase assay system to the amount of glucose phosphorylated, we determined the moles of TPNH generated per mole of glucose-6-phosphate formed from glucose at a specified time (10 minutes) for each tissue. In this connection the effect of 2 other enzymes must be considered: gluconic acid-6-phosphate dehydrogenase, an enzyme which reduces TPN, and phosphoglucose isomerase, which converts glucose - 6 - phosphate to

TABLE 5

Ammonium sulfate fractionation and dialysis of hepatic glucokinase [1]

Nutritional state of rat	Ammonium sulfate fraction	Before dialysis						After dialysis					
		Total protein	Total glucose	Glucokinase		Adaptable glucokinase activity	Non-adaptable glucokinase activity	Total protein	Total glucose	Glucokinase		Adaptable glucokinase activity	Non-adaptable glucokinase activity
				Activity	K_m [2]					Activity	K_m [3]		
	% saturation	*mg*	*mg*		*M glucose*			*mg*	*mg*		*M glucose*		
Fed high carbohydrate diet for 72 hours	unfractionated	752	226	40	8×10^{-3}	36	4.3	295	1.3	4.2	$0.5–5 \times 10^{-3}$	1.8	2.4
	20–50	334	4.3	12	8×10^{-3}	9.1	2.9	126		1.2	8×10^{-3}	1.0	0.20
	50–60	152		8.1	7×10^{-3}	7.9	0.20	113	0.7	0.53	9×10^{-3}	0.53	0
	60–75	125	2.6	4.2	9×10^{-3}	4.2	0						
	75–100	88		0									
Fasted for 72 hours	unfractionated	695	151	6.8	7×10^{-3}	2.2	4.6						
	20–50	300	2.9	2.9	9×10^{-3}	1.3	1.6	276	1.1	1.7	5×10^{-3}	0.20	1.5
	50–60	115		2.0	8×10^{-3}	1.7	0.30	98		0.51	$0.8–6 \times 10^{-3}$	0.22	0.28
	60–75	99	1.4	0.73	8×10^{-3}	0.73	0	88	0.8	0.10	7×10^{-3}	0.10	0
	75–100	95		0									

[1] The livers of 2 rats (8 g) in each group were pooled and homogenized with 24 ml of buffer, and the particle-free supernatant fractions were isolated by centrifugation as described in text. The fractions were then treated with solid ammonium sulfate (9), and the precipitates were dissolved in 2 to 5 ml of the same buffer that was used for the glucokinase assay system. In addition, 1-ml aliquots of the ammonium sulfate fractions were dialyzed against one liter of dissolving buffer for 16 hours at 2 to 4°. Glucose, in these fractions, was determined enzymatically as described in text. The activity of glucokinase, measured as given in text, is expressed as micromoles of TPNH produced per minute per total fraction. Calculations for determination of adaptable glucokinase activity are referred to in text.

[2] Linear Lineweaver-Burk plots (17) obtained only over the glucose concentration range of 1×10^{-3} to 5×10^{-2} M.

[3] Lineweaver-Burk plots (17) were made over the glucose concentration range of 1×10^{-4} to 5×10^{-2} M. When a K_m range is given above, the plots were not linear over the entire glucose concentration used.

TABLE 6

Amounts of TPNH formed from glucose-6-phosphate and fructose-6-phosphate in the glucokinase assay system [1]

Tissue	Dietary treatment	Centrifugation		Protein in assay	Substrate for TPNH production				Correction factor [4]
					Fructose-6-phosphate [2]		Glucose-6-phosphate [3]		
		Speed	Time		TPNH formed/ 10 min	TPNH formed / F-6-P added	TPNH formed/ 10 min	TPNH formed / G-6-P added	
		× g	*min*	*mg*	*mμmoles*	*per 10 min*	*mμmoles*	*per 10 min*	
Liver	high carbohydrate	100,000	45	0.29	248	1.44	301	1.46	1.45
Liver	high protein	100,000	45	1.65	307	1.78	354	1.73	1.76
Brain	high carbohydrate	350	5	0.32	179	1.03	216	1.05	1.04
Brain	high carbohydrate	100,000	45	0.20	166	0.96	210	1.02	0.99
Muscle	high carbohydrate	100,000	45	1.45	160	0.93	200	0.98	0.96
Kidney	high carbohydrate	100,000	45	0.55	192	1.11	276	1.35	1.23
Heart	high carbohydrate	100,000	45	0.24	141	0.82	201	0.98	0.95
Small intestine	high carbohydrate	100,000	45	0.37	230	1.33	256	1.25	1.29
Spleen	high carbohydrate	100,000	45	0.20	217	1.25	295	1.46	1.36
Adipose	high carbohydrate	100,000	45	0.50	260	1.50	336	1.64	1.57

[1] TPNH formation was followed continuously for 10 minutes upon addition of either 173 mμmoles of fructose-6-phosphate (F-6-P) or 205 mμmoles of glucose-6-phosphate (G-6-P) to the glucokinase assay system described in text, except that ATP and glucose were omitted. The amount of supernatant fraction assayed for each tissue was the same as that used in the glucokinase assays recorded in tables 2 and 4.

[2] The exact amount of fructose-6-phosphate added was determined by measuring TPNH production spectrophotometrically with purified phosphoglucose isomerase, purified glucose-6-phosphate dehydrogenase and TPN.

[3] The exact amount of glucose-6-phosphate added was determined by measuring TPNH production spectrophotometrically with purified glucose-6-phosphate dehydrogenase and TPN.

[4] These values are the averages of the numbers in columns 7 and 9.

fructose-6-phosphate. The effects of the activities of these 2 enzymes upon the glucokinase assay system are presented in table 6. In brain, muscle and heart, the activity of endogenous gluconic acid-6-phosphate dehydrogenase was very low compared with the large excess of glucose-6-phosphate dehydrogenase added to the assay system; hence the amounts of TPNH generated and the glucose-6-phosphate formed from the added glucose were almost identical within the 10-minute assay period. Apparently, in this assay system any fructose-6-phosphate formed by phosphoglucose isomerase is reconverted to glucose-6-phosphate — in preference to its conversion to hexose diphosphate — because the values for TPNH generation from added glucose-6-phosphate and fructose-6-phosphate in these tissue homogenate fractions (brain muscle and heart) are almost the same. With liver, kidney, small intestine, spleen and adipose tissue, however, the activity of the endogenous gluconic acid-6-phosphate dehydrogenase was such that it also produced TPNH during the 10-minute glucokinase assay. Thus, with some tissues, a correction factor, to take into account the extra TPNH formed by the second dehydrogenase, must be considered in calculating the actual amounts of glucose phosphorylated. The correction factors for these tissues are presented in column 10 of table 6.

In the experiments with liver homogenate fractions, as studied with both hexose-6-phosphates as substrates in the glucokinase assay system, about 1.6 μmoles of TPNH were generated per μmole of added glucose-6-phosphate or of added fructose-6-phosphate in the 10-minute assay period (column 10, table 6). When this generation of TPNH was allowed to proceed to completion (about 30 minutes), 2 μmoles of TPNH were produced per μmole of hexose phosphate added. These results affirm the suggestion that phosphofructokinase activity does not interfere with the glucokinase assay.

In the experiments with the liver fractions shown in table 2, total activity of hepatic glucokinase was unchanged when microsomes (the particles which contain glucose-6-phosphatase (21) were present in the assay system. This suggests that glucose-6-phosphatase did not interfere with our assay of hepatic glucokinase. The results of another type of experiment with liver homogenate fractions demonstrated directly that hepatic glucose-6-phosphatase did not influence our values for hepatic glucokinase activity. When glucose-6-phosphate or fructose-6-phosphate was added to the assay system along with either (a) the particle-free supernatant fraction, or (b) the particle-free supernatant plus microsomes, or (c) the particle-free supernatant fraction plus microsomes plus mitochondria, a production of 2 μmoles of TPNH per μmole of added hexosephosphate was observed after about 30 minutes. If any glucose-6-phosphate had been converted to glucose by any of these 3 liver fractions, the amount of TPNH formed would have been lower at equilibrium since no ATP was added.

Since none of the glucose-6-phosphate formed from glucose is diverted to pathways other than the one involving the glucose-6-phosphate dehydrogenase reaction, the correction factor determined for each tissue enabled us to calculate the actual amounts of glucose phosphorylated by the homogenate fractions of the various tissues studied. These amounts were obtained (table 7) by correcting the values for TPNH generation (table 4) by means of the correction factors determined for each tissue homogenate (table 6).

TABLE 7

Actual amount of glucose phosphorylated by various rat tissues [1]

Tissue	Nutritional state of rat	
	Fed high carbohydrate diet	Fed high protein diet
Liver	38.2	3.8
Brain	80.0	81.1
Muscle	12.4	12.6
Kidney	23.2	20.1
Small intestine	22.5	16.4
Heart	23.0	20.5
Adipose	33.8	24.5

[1] The specific activities of glucokinase were calculated by dividing the values for the amounts of TPNH formed in the glucokinase assay system for the various rat tissue homogenate fractions (see table 4) by the correction factors recorded in table 6. The values are presented as millimicromoles of glucose phosphorylated per milligram protein per minute.

Mannokinase activity of liver, brain and lactating mammary gland

In liver, mannokinase activity was highest in the particle-free supernatant fraction. This observation resembles that with hepatic glucokinase. On the other hand—as was also true for brain glucokinase—a large portion of the brain mannokinase activity was associated with the mitochondria (table 8).

As for glucokinase, hepatic mannokinase activity responded to the previous nutritional state of the rat, but that of brain mannokinase did not. The specific activities for hepatic mannokinase observed in rats fed a diet containing 65% glucose were about 7 times higher than those in rats fed a diet low in available glucose and containing 78% protein. This difference in the specific activities of hepatic mannokinase closely resembles that observed for hepatic glucokinase. Glucokinase and mannokinase activities of the particle-free, supernatant fraction obtained from lactating rat mammary gland homogenates were also compared. This study revealed that, whereas the specific activity of glucokinase was 16.3, that for mannokinase was 4.2. When total activity toward each of these substrates was compared, glucokinase activity was 1.04 and mannokinase activity, 0.19. Thus, the ratio of glucokinase to mannokinase in this tissue was about 5, a value corresponding well with values noted for liver and brain.

TABLE 8

Mannokinase activities in liver and brain homogenate fractions

Tissue	Dietary treatment	Centrifugation		Mannokinase activity [1]		
		Speed	Time	Specific	Total	K_m
		× *g*	*min*			M *mannose*
Liver	high carbohydrate	100,000	45	13.3 ± 0.67 [2]	1.24 ± 0.07	8×10^{-3}
Liver	high protein	100,000	45	1.9 ± 0.3	0.21 ± 0.04	
Brain	high carbohydrate	350	5	28.0 ± 1.7	1.54 ± 0.09	6×10^{-5}
Brain	high carbohydrate	100,000	45	19.8 ± 1.3	0.84 ± 0.06	6×10^{-5}

[1] Mannokinase activity was measured as described in text and is expressed as millimicromoles TPNH formed per milligram protein per minute (specific activity), and micromoles TPNH formed per gram wet weight tissue per minute (total activity).

[2] The values are means ± SD of results of 3 separate experiments with supernatant fractions obtained by centrifugal treatments given above.

TABLE 9

Inhibition of glucokinase activity by mannose

Exp.	Tissue	Substrate added		TPNH		A/B
		Glucose	Mannose	Observed (A)	Calculated if glucokinase and mannokinase activities were additive (B)	
		μmoles	*μmoles*	*mμmoles formed/min/mg protein*		
1 [1]	Liver	100	0	55		
	Liver	0	100	2 [3]		
	Liver	100	100	25	57	0.44
2 [2]	Brain	100	0	63		
	Brain	0	100	19		
	Brain	100	100	47	82	0.57

[1] Expermient 1 was performed with the 100,000 × *g* supernatant fraction obtained from homogenates prepared from the liver of a rat fed a high carbohydrate diet. The conditions for the measurements of enzymatic activity are given in the text, under glucokinase assay, except as noted above. The measurements were made for 10 minutes immediately after addition of substrate.

[2] Experiment 2 was performed with the 100,000 × *g* supernatant fraction obtained from brain homogenates prepared from a rat fed a high carbohydrate diet. The conditions for the measurement of enzymatic activity are given in the text, under mannokinase assay, except as noted above. The measurements were taken after a 20-minute lag period and were followed for 10 additional minutes.

[3] This value cannot be compared with the values for hepatic mannokinase activity as the assay system did not contain a large excess of the phosphohexose isomerases.

The apparent K_m values for hepatic mannokinase (table 8) and hepatic glucokinase (table 4) were of the same order, namely, $7–9 \times 10^{-3}$ M; those for brain mannokinase (table 7) and glucokinase (table 4) were both about $4–6 \times 10^{-5}$ M.

Hepatic glucokinase was inhibited by mannose (table 9) to about 50% at equimolar concentrations of the 2 hexoses. In brain homogenate fractions, a similar observation was made.

DISCUSSION

In 1953 Crane and Sols (16) studied the cellular location of hepatic glucokinase by using a glucose disappearance method for assay of the enzyme, and observed that as much as 35% of the total glucokinase was particle-bound. Subsequently, Lange and Kohn (22), using a microtitrimetric procedure (23), reported that 22% of the total glucokinase in liver was associated with the particles. According to Thielmann and Blume (24), 70 to 90% of the hexokinase activity of normal rat liver is localized in the particle-free supernatant fraction. DiPietro and Weinhouse (1) observed, however, that soluble glucokinase of liver can account adequately for the total phosphorylation of glucose by liver slices. The results of the present study are in accord with their observation (1), for we found all of the hepatic glucokinase activity in the cytoplasmic fraction of the cell.

In contrast with this situation in liver, a large portion of brain glucokinase is particle-bound, a result that confirms the observations of Crane and Sols (16,25). In the present study, in which a spectrophotometric assay method was used, 2 types of experiments revealed that 40 to 55% of the glucokinase activity in brain was particle-bound. In the first, a brain homogenate fraction composed of mitochondria plus microsomes plus cytoplasmic fluid had about 40% more activity than a brain fraction composed of microsomes plus cytoplasmic fluid. This suggests that mitochondria contained 40% of the total brain glucokinase. In the second experiment, isolated, washed mitochondria accounted for about 55% of the brain glucokinase activity. Crane and Sols (16, 25), in their study of the cellular location of brain glucokinase, observed that about 70 to 90% of the total activity was in the mitochondrial fraction. It is unlikely that the homogenization procedure used in this study solubilized appreciable amounts of brain glucokinase; even our attempts to solubilize the enzyme by a freeze-thawing technique and desoxycholate treatment of the mitochondria resulted in little release of the enzyme.

In homogenates of spleen, skeletal muscle and adipose tissue, almost all of the glucokinase activity was observed in the particle-free supernatant fraction. Similar results were reported by Jervell et al. (26) for rat diaphragm and by Walaas and Walaas[23] for rat skeletal muscle. (To our knowledge, no study of the cellular location of glucokinase in adipose tissue or spleen has appeared.) The reports of Crane and Sols (16) that 73% of the glucokinase in intestinal mucosa can be sedimented at $18,000 \times g$ for 60 minutes, and of Lange and Kohn (22) that almost 90% of this enzyme in rat intestine is present in the pellet obtained after centrifugation at $20,000 \times g$ for 30 minutes, have not been confirmed in the present study.

The increase in total hepatic glucokinase activity observed when the particles sedimented at $10,000 \times g$ are removed from the liver homogenates is worthy of comment. This particulate fraction contains mitochondria, and its ability to utilize TPNH is well known. The possibility that these particles reduce the amount of measured TPNH generated in the glucokinase assay system, thereby lowering the apparent glucokinase activity, must be considered. We tested this possibility experimentally, using the $350 \times g$ supernatant fraction obtained from liver homogenates as the enzyme source in the glucokinase assay system with only 0.2 μmole of glucose as substrate. Optical density increased slowly for about 30 minutes, by which time the reaction produced 0.4 μmole of TPNH. When this reaction mixture was followed for at least 30 minutes more, no decline in the level of TPNH occurred. The same result was observed when 0.4 μmole of TPNH was added as substrate instead of glucose. These results show that no oxidation of TPNH occurred in our assay system. Similar results

[23] Private communication.

were obtained with the 350 × *g* supernatant fraction of brain homogenates. It is not unexpected that no TPNH oxidation by liver or brain cytoplasmic particles occurs in this system, as adequate substrates for such an oxidation are not present.

Curiously enough, removal of the particle fraction obtained with 100,000 × *g* failed to affect total hepatic glucokinase activity. This fraction contains microsomes and, according to de Duve et al. (21), all of the glucose-6-phosphatase is located here. This hydrolytic enzyme dephosphorylates glucose-6-phosphate, and thus the presence of microsomes would be expected to decrease the apparent total glucokinase activity. That no such decrease was observed in the present study might be explained by the observations of Beaufay et al. (27) who showed that (a) the optimal pH for glucose-6-phosphatase is 6.0; (b) the enzyme is inhibited by glucose; and (c) the enzyme needs a certain concentration of its substrate, glucose-6-phosphate, to be active. Considering their results the conditions in our experiments were unfavorable for the activity of this enzyme for the following reasons: (a) our glucokinase system contained a high glucose concentration; (b) glucose-6-phosphate was rapidly removed by the large excess of added dehydrogenase; and (c) the pH of the medium was 8.0. Thus, our results demonstrate that it is necessary to clear the liver homogenate of the heavy particles to obtain maximal glucokinase activity. However, the presence of microsomes, which contain glucose-6-phosphatase, does not interfere with the assay.

The nutritional state of the rat is of extreme importance in the study of activities of certain enzymes, particularly those of liver. Many workers (1–3, 20, 28–33) have emphasized that the levels of activity of many enzymes concerned with carbohydrate metabolism respond to dietary treatment. In a comparison of glucokinase activity of various rat tissues, Long (4) concluded that the activity of this enzyme was lowest in liver. Unfortunately, he compared the activity of this enzyme in *fasted* rats with those of other tissues in *fed* rats. Our results demonstrate that the nutritional state of the rat has a marked effect on hepatic glucokinase. Although the feeding of diets low in available carbohydrate decreased liver glucose phosphorylating activity, this activity in other tissue was unaffected. When glucose phosphorylation in various tissues of the rat fed the high glucose diet was compared, the liver had an activity almost as high as that of brain, but much higher than that in kidney, small intestine, spleen, heart, skeletal muscle and adipose tissue.

The affinity of glucokinase for glucose can be expressed in terms of K_m values. Both DiPietro et al. (18) and Viñuela et al. (20) reported values of 1×10^{-2} M glucose for the K_m when they used the spectrophotometric assay for hepatic glucokinase with crude enzyme preparations. Walker (19), who used the same assay method, observed that the value for this constant was $3–4 \times 10^{-3}$ M glucose. However, Lange and Kohn (22) reported a value of 4×10^{-5} M glucose for the hepatic enzyme, which was assayed by a microtitrimetric procedure. Our value with the crude particle-free supernatant fraction of liver ($7–9 \times 10^{-3}$ M glucose) agrees well with the higher figures reported.

In a recent report, Viñuela et al. (20) suggested that the liver of the well-fed rat contains 2 enzymes capable of phosphorylating glucose. On the basis of inhibition experiments and ammonium sulfate fractions, they postulated the existence of one kinase with a high K_m (1×10^{-2} M glucose), which is adaptable to diet or hormonal environment, and another with a low K_m (1×10^{-5} M glucose), which is not adaptable. The results presented in this report confirm the observations of these workers (20). Thus the adaptable glucokinase of liver, as compared with the nonadaptable glucokinases of other tissues, has a remarkably low affinity for glucose.

This low affinity, combined with the pronounced response to dietary carbohydrate, makes the glucokinase step an excellent regulatory and rate-limiting mechanism for glucose metabolism in the liver. The liver cell, according to Cahill et al. (34), is freely permeable to glucose, so that transport of glucose from extracellular to intracellular compartments cannot be rate limiting. This is in contrast with other cells such as those of skeletal muscle, heart, brain, and adipose tissue, in which

the transport process is presumed to be rate-limiting, and glucose reaching the intracellular compartment is rapidly phosphorylated by a non-adaptable glucokinase with a high affinity for this substrate.

Sols and Crane (35) reported that brain glucokinase also catalyzes phosphorylation of mannose and other hexoses. (The ratio of relative maximal rates of glucose and mannose for the brain kinase observed by these workers was two to one.) We have confirmed this observation. Our observations indicate that glucose and mannose are phosphorylated in liver by the same enzymes (adaptable and non-adaptable), as previously reported by Viñuela et al. (20), and that the affinities for the 2 substrates are approximately the same. The rates of dissociation of the enzyme-substrate complex into the enzyme and products, however, must differ, because differences in the maximal activities with glucose and mannose were observed. In the case of liver, the ratio of relative maximal rates of glucose and mannose phosphorylation was about three to one.

LITERATURE CITED

1. DiPietro, D. L., and S. Weinhouse 1960 Hepatic glucokinase in the fed, fasted, and alloxan-diabetic rat. J. Biol. Chem., 235: 2542.
2. Vaughan, D. A., J. P. Hannon and L. N. Vaughan 1960 Effect of diet on selected glycolytic enzymes of the rat. Am. J. Physiol., 199: 1041.
3. Blumenthal, M. D., S. Abraham and I. L. Chaikoff 1964 Dietary control of liver glucokinase activity in the normal rat. Arch. Biochem. Biophys., 104: 215.
4. Long, C. 1952 Studies involving enzymic phosphorylation. I. The hexokinase activity of rat tissues. Biochem. J., 50: 407.
5. Hawk, P. B., B. L. Oser and W. H. Summerson 1947 Practical Physiological Chemistry, ed. 12. Blakiston, Philadelphia, p. 1273.
6. McIlwain, H., and H. L. Buddle 1953 Techniques in tissue metabolism. I. A mechanical chopper. Biochem. J., 53: 412.
7. Horecker, B. L., and A. Kornberg 1948 The extinction coefficients of the reduced band of pyridine nucleotides. J. Biol. Chem., 175: 385.
8. Slein, M. W. 1955 In Methods in Enzymology, eds., S. P. Colowick, and N. O. Kaplan, vol. 1. Academic Press, Inc., New York, p. 299.
9. Taylor, J. F. 1953 In The Proteins, eds., H. Neurath and K. Bailey. Academic Press, Inc., New York, p. 1.
10. Abraham, S., W. M. Fitch and I. L. Chaikoff 1961 Mannose metabolism and the demonstration of mannokinase and phosphomannoisomerase activities in the lactating rat mammary gland. Arch. Biochem. Biophys., 93: 278.
11. Sacktor, B., and L. Packer 1962 Reactions of the respiratory chain in brain mitochondrial preparations. J. Neurochem., 9: 371.
12. Gornall, A. G., C. J. Bardawill and M. M. David 1949 Determination of serum proteins by means of the biuret reaction. J. Biol. Chem., 177: 751.
13. Hassid, W. Z., and S. Abraham 1957 In Methods in Enzymology, eds. S. P. Colowick, and N. O. Kaplan, vol. 3. Academic Press, Inc., New York, p. 34.
14. Slein, M. W. 1957 In Methods in Enzymology, eds. S. P. Colowick and N. O. Kaplan, vol. 3. Academic Press, Inc., New York, p. 154.
15. Kornberg, A., and B. L. Horecker 1955 In Methods in Enzymology, eds., S. P. Colowick, and N. O. Kaplan, vol. 1. Academic Press, Inc., New York, p. 323.
16. Crane, R. K., and A. Sols 1953 The association of hexokinase with particulate fractions of brain and other tissue homogenates. J. Biol. Chem., 203: 273.
17. Lineweaver, H., and D. Burk 1934 The determination of enzyme dissociation constants. J. Am. Chem. Soc., 56: 658.
18. DiPietro, D. L., C. Sharma and S. Weinhouse 1962 Studies on glucose phosphorylation in rat liver. Biochemistry, 1: 455.
19. Walker, D. G. 1962 The development of hepatic hexokinases after birth. Biochem. J., 84: 118P.
20. Viñuela, E., M. Salas and A. Sols 1963 Glucokinase and hexokinase in liver in relation to glycogen synthesis. J. Biol. Chem., 238: PC1175.
21. de Duve, C., B. C. Pressman, R. Gianetto, R. Wattiaux and E. Appelmans 1955 Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. Biochem. J., 60: 604.
22. Lange, C. F., Jr., and P. Kohn 1961 Substrate specificity of hexokinase. J. Biol. Chem., 236: 1.
23. Schwartz, M., and T. C. Myers 1958 Simple microtitrimetric constant-pH method for accurate enzyme assays. Anal. Chem., 30: 1150.
24. Thielmann, K., and E. Blume 1962 Intrazelluläre Verteilung von Hexokinase und ATP in der Leber Alloxandiabetischer Mäuse. Ztschr. Physiol. Chem., 328: 164.
25. Sols, A., and R. K. Crane 1955 In Methods in Enzymology, eds., S. P. Colowick and N. O. Kaplan, vol. 1. Academic Press, Inc., New York, p. 277.
26. Jervell, K. F., E. Walaas and O. Walaas 1956 The inhibition of hexokinase reaction in the rat diaphragm by 2, 4-dinitrophenol. Acta Physiol. Scand., 35: 321.
27. Beaufay, H., H. G. Hers, J. Berthet and C. de Duve 1954 Le système hexose-phosphatasi-

que. V. Influence de divers agents sur l'activité et la stabilité de la glucose-6-phosphatase. Bull. Soc. Chim. Biol., 36: 1539.
28. Tepperman, J., and H. M. Tepperman 1957 Effects of antecedent food intake pattern on hepatic lipogenesis. Am. J. Physiol., *193:* 55.
29. Fitch, W. M., and I. L. Chaikoff 1960 Extent and pattern of adaptation of enzyme activities in livers of normal rats fed diets high in glucose and fructose. J. Biol. Chem., 235: 554.
30. Fitch, W. M., R. Hill and I. L. Chaikoff 1959 The effect of fructose feeding on glycolytic enzyme activities of the normal rat liver. J. Biol. Chem., 234: 1048.
31. Freedland, R. A., and A. E. Harper 1959 Metabolic adaptations in higher animals. V. The study of metabolic pathways by means of metabolic adaptations. J. Biol. Chem., 234: 1350.
32. Weber, G., G. Banerjee and S. B. Bronstein 1961 Selective induction and prevention of enzyme synthesis in mammalian liver. Biochem. Biophys. Res. Comm., *4:* 332.
33. Willmer, J. S., and T. S. Foster 1962 Enzymatic and metabolic adaptations. I. Changes in intact rats subjected to dietary modifications. Canad. J. Biochem. Physiol., *40:* 953.
34. Cahill, G. F., Jr., J. Ashmore, A. S. Earle and S. Zottu 1958 Glucose penetration into liver. Am. J. Physiol., *192:* 491.
35. Sols, A., and R. K. Crane 1954 Substrate specificity of brain hexokinase. J. Biol. Chem., *210:* 581.
36. Abraham, S., P. F. Hirsch and I. L. Chaikoff 1954 The quantitative significance of glycolysis and non-glycolysis in glucose utilization by rat mammary gland. J. Biol. Chem., *211:* 31.