HISTOLOGICAL DIFFERENTIATION OF FATTY LIVERS PRODUCED BY THREONINE OR CHOLINE DEFICIENCY [1]

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THREE FIGURES

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INTRODUCTION

Fat deposition in the livers of young rats fed 9% casein-sucrose diets containing choline is reduced when such diets are suplemented with additional protein (Litwack et al., '52; Hawk and Elvehjem, '53; Harper et al., '53a), threonine (Singal et al., '53; Harper et al., '53a), or glycine (Harper et al., '54a) and when sucrose is replaced by either dextrin or cerelose (Harper et al., '53b). Although there have been a number of detailed histological studies on the livers of rats fed either choline-deficient (Follis, '48; Hartroft, '50), or low-protein diets (Wang et al., '49; Koch-Weser et al., '53), histological studies of the livers of rats fed each of the above dietary regimens have not been published. One report (Dick et al., '52) on structural changes in the livers of rats fed amino acid diets completely deficient in either threonine or lysine has been presented.

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[2] Rockefeller Foundation fellow, from the National University of Colombia, Bogota.

This paper includes a comparison of the structural changes observed in the livers of animals fed various low-fat, low-protein diets with particular reference to the effects of partial deficiency of threonine.

EXPERIMENTAL

Male weanling rats of the Sprague-Dawley strain weighing from 40 to 50 gm were used throughout these studies. The animals were divided into similar groups of 6 each on the basis of body weight and were maintained in individual cages with raised screen bottoms. They were fed ad libitum and each animal was weighed weekly during the experimental period of two weeks.

The per cent composition of the basal diet was a follows: sucrose, 81.4; casein, 9.0; corn oil, 5.0; salts IV (Hegsted, Mills, Elvehjem and Hart, '41), 4.0; methionine, 0.3; tryptophan, 0.1; choline chloride, 0.13. Vitamins were added to provide, in milligrams per kilogram of ration: thiamine, 5.0; riboflavin, 5.0; niacin, 10.0; calcium pantothenate, 20.0; pyridoxine, 2.5; folic acid, 0.2; biotin, 0.1; vitamin B_{12}, 0.02 and inositol, 100.0. Two drops of halibut liver oil fortified to provide vitamin A, 1,000 I.U.; vitamin D, 100 I.U.; 2-methyl-1-4-naphthoquinone, 0.04 mg and alpha tocopherol, 0.8 mg, were administered weekly. Supplements unless otherwise indicated replaced an equal weight of carbohydrate. In the choline-deficient diets methionine was replaced by cystine.

At the end of the experimental period each rat was anesthetized with di-ethyl ether and two samples were taken from the left lobe of the liver. They were immediately fixed, one with Bouin's and the other with Zenker's techniques. The prepared samples were embedded in paraffin and a series of sections 7 μ thick were cut from a continuous zone of about 500 μ. The slides were stained using Hematoxylin Eosin and Mallory's Collagen techniques. After the samples for histological studies were taken the remainder of the liver was removed for the determination of fat. Fat was determined

on the dried and ground liver by ether extraction using the method outlined by Hawk and Elvehjem ('53).

RESULTS

Liver fat

The results presented in table 1 include liver fat values and growth rates for the groups of animals from which the liver samples were taken and correspond to the photomicrographs presented in figures 1 to 3. These results are representative of a large number of trials. Liver fat values for animals fed choline-deficient diets containing either 9% or 18% casein (group 1, A and C) were 46.6 and 41.6% (dry weight) resectively. When these diets were supplemented with 0.13% choline and the cystine was replaced with methioinne (group 1, B and D) fat values of 27.3 and 10.0%, respectively, were obtained. The latter is taken to represent a normal level of liver fat. Although the inclusion of choline chloride in the 18% casein diet reduced liver fat to a normal level, it is evident, as has been pointed out previously (Harper et al., '53a; Singal et al., '53), that liver fat values for rats fed the 9% casein diet containing both choline and methionine are considerably above normal.

The results in table 1 (group 2) show that liver fat values for rats fed the basal diet supplemented with gelatin, threonine or a combination of threonine and glycine were appreciably lower than that of the control group. The level of threonine used, it should be noted, is double that provided by 6% gelatin. In contrast, when choline was omitted from the basal diet, supplementation with threonine caused no reduction in the level of fat in the liver (Harper et al., '53a; Singal et al., '53).

The last section of table 1 includes results obtained when different carbohydrates were used in the basal diet. Both fructose and sucrose induced higher fat deposition than did either dextrin or cerelose. These differences have been attributed to the effects of the different carbohydrates on the

utilization of low protein diets (Harper et al., '53b). That differences in the rates or mechanism of metabolism of the carbohydrates may be of significance cannot be dismissed,

TABLE 1

Growth and liver fat of rats used for histological studies[1]

		DIET	RATE OF GAIN	LIVER FAT	
			gm/wk.[2]	*% dry wt.*[2]	*% wet wt.*[2]
Group 1.	A	9% casein + 0.3% cystine. No choline	14.5 ± 0.7	46.6 ± 3.7	16.7 ± 2.1
	B	9% casein + 0.3% methionine + 0.15% choline chloride	14.2 ± 0.9	27.3 ± 2.9	8.7 ± 1.1
	C	18% casein + 0.3% cystine. No choline	29.8 ± 2.1	41.6 ± 3.2	15.7 ± 1.8
	D	18% casein + 0.3% methionine + 0.15% choline chloride	34.9 ± 2.2	10.0 ± 0.6	2.9 ± 0.2
Group 2.	A	9% casein + 0.3% methionine + 0.15% choline chloride (basal)	19.7 ± 1.4	37.0 ± 2.2	13.0 ± 1.3
	B	Basal + 6% gelatin	24.1 ± 1.0	21.8 ± 1.0	6.7 ± 0.3
	C	Basal + 0.36% DL threonine	20.8 ± 1.0	14.3 ± 0.6	4.0 ± 0.3
	D	Basal + 0.36% DL threonine + 1.5% glycine	24.3 ± 1.3	10.4 ± 0.5	2.9 ± 0.2
Group 3.	A	Basal	19.7 ± 1.9	34.1 ± 2.9	10.8 ± 1.2
	B	Basal, sucrose replaced by fructose	13.8 ± 1.2	29.6 ± 2.6	9.5 ± 1.0
	C	Basal, sucrose replaced by cerelose	28.0 ± 1.3	15.0 ± 0.4	4.1 ± 0.2
	D	Basal, sucrose replaced by dextrin	36.2 ± 0.6	15.4 ± 1.2	4.1 ± 0.4

[1] Some of these results have been reported previously (Harper et al., '53a, '53b). They are included here to permit ready comparison with the histological observations.

[2] Values represent mean ± standard error of the mean for a group of 6 animals.

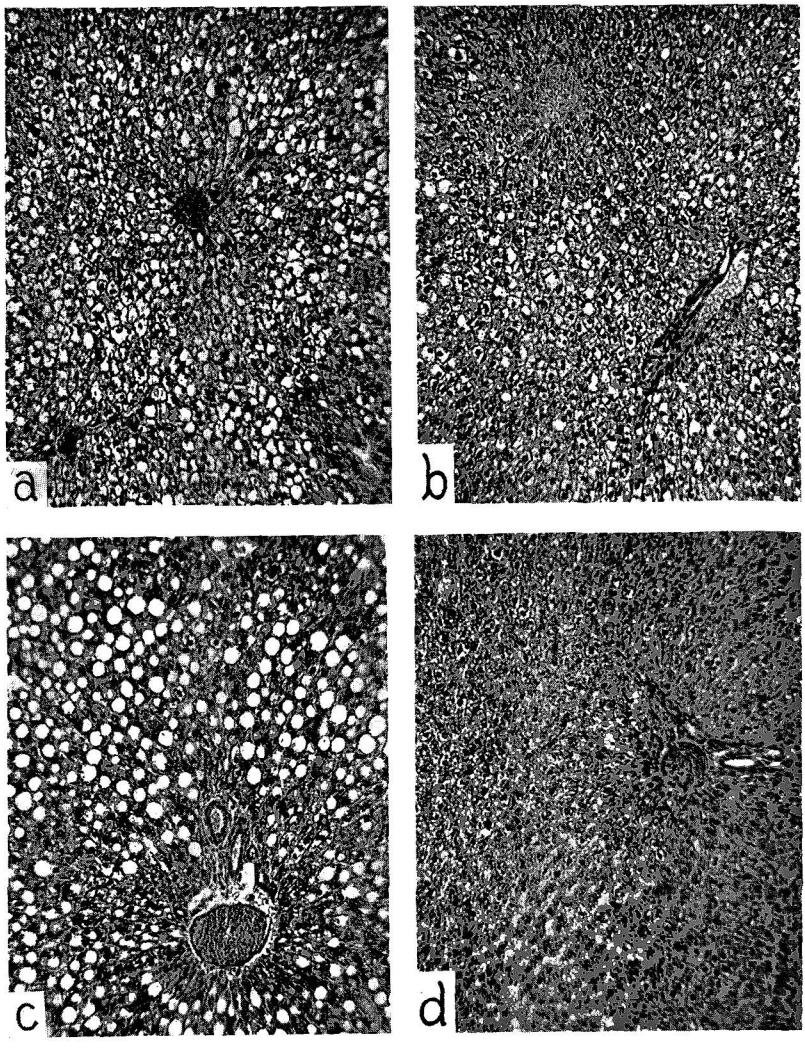

Fig. 1 Photomicrographs of representative livers from group I (see table 1). Bouin's fixative. H and E stain.

A Nine per cent casein, 0.3% cystine, choline-free diet. Diffuse fatty metamorphosis and hydropic-like degeneration of liver cells.

B Nine per cent casein, 0.3% cystine, condition similar to A but less severe.

C Eighteen per cent casein, 0.3% cystine, choline-deficient diet. Diffuse fatty metamorphosis of liver cells. Other cells show evidence of nuclear and protoplasmic hyperchromatosis.

D Eighteen per cent casein, 0.3% cystine. Mild nuclear hyperchromatosis in some liver cells. Otherwise normal in appearance.

but since fat accumulates to the same extent in the livers of rats fed 6% casein diets containing either dextrin or cerelose as it does in the livers of rats fed 6% casein diets containing sucrose (unpblished data, Harper, Benton, Winje and Elevhjem), it is suggested that protein utilization is the most significant factor.

Histology

Post-mortem examinations showed no indications of undernourishment, anemia or jaundice in any of the animals. The livers from rats in which fat accumulated were enlarged and pale, soft and friable. The edges were rounded and not well defined and usually the surface was mottled. These alterations were most prominent in animals fed choline-deficient diets.

Photomicrographs representative of each of the livers studied are presented in figures 1 to 3. No evidence of cirrhosis or fibrosis was observed in any of the animals. In rats fed choline-free diets containing 9% casein (fig. 1-a) diffuse fatty metamorphosis of the liver cells was observed. The protoplasm and nuclei of the non-fatty cells varied in their affinity for the stains used resulting in an image resembling the hydropic degeneration described by Engel and Phillips ('39). Sinusoids were not defined and no lobular delimitation was observed.

When the protein level of the choline deficient diets was increased to 18% (fig. 1-c) fewer cells were affected and these were enlarged and spherical. The remaining liver cells stained more intensely and the protoplasm appeared to be irregularly clumped.

In samples from the livers of animals fed the 9% casein ration containing choline (figs. 1-b, 2-a, 3-a) the fatty cells were arranged in zones resembling a network. The fatty cells were similar in appearance to those found in livers of animals fed the choline-free casein diets. The remaining cells showed increased affinity for protoplasmic and nuclear stains. Oc-

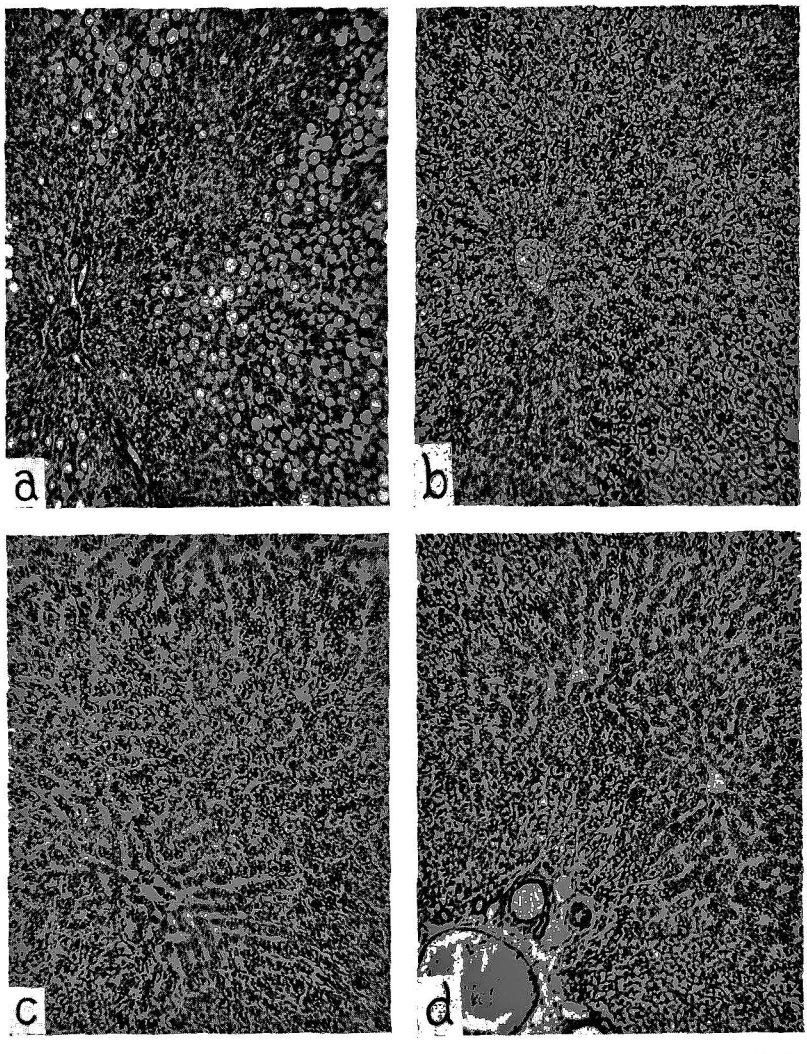

Fig. 2 Photomicrographs of representative livers of rats from group 2 (see table 1). Bouin's fixative. H and E stain.

A Nine per cent casein, 0.3% methionine. Fatty cells are distributed in a network pattern. Nuclear and protoplasmic hyperchromatosis in non-fatty cells.

B Nine per cent casein, 0.3% methionine, 6% gelatin. Mild hydropic-like degeneration of liver cells, nuclear hyperchromatosis. Typical fatty cells are absent.

C Nine per cent casein, 0.3% methionine, 0.36% threonine. Fatty cells are not observed. Mild and occasional nuclear hyperchromatosis. Sinusoids are well defined.

D Nine per cent casein, 0.3% methionine. 0.36% threonine, 1.5% glycine. A few cells show indication of nuclear hyperchromatosis. Otherwise normal.

casionally necrotic and replacement cells were seen. Some lobules, however, were not structurally modified.

The addition of 6% gelatin to the basal diet produced a considerable reduction in the number of fatty cells (fig. 2–b). Moderate hydropic-like changes and nuclear hyperchromatosis were the main features. No lobular delimitation was observed. Sections from animals that received a supplement of 0.36% threonine (fig. 2-c) showed no fatty metamorphosis of hepatic cells. The sinusoids maintained their normal structure but a slight protoplasmic clearing could be observed. Supplementation with 1.5% glycine and 0.36% threonine (fig. 2-d) produced an almost normal appearance of the liver and the only possible abnormality was a mild and occasional hyperchromatosis.

When sucrose in the diet was replaced by fructose the histological pattern was not altered. The zonification was more distinct and the groups of normal and fatty cells were more clearly differentiated. When dextrin or cerelose were used in place of sucrose the livers appeared structurally normal.

DISCUSSION

The histological observations on the livers of animals fed diets deficient in choline are in general agreement with the results of previously published reports (Follis, '48). The observations on the livers of animals fed low protein diets also agree in some respects with the results of other investigators. Wang et al. ('49) using protein--depleted rats, and Koch-Weser et al. ('53) using rats fed high-fat, low-protein diets, observed occasional necrosis, protoplasmic clearing, and variations in staining of non-fatty cells, findings similar to those reported in this paper. Dick et al. ('52) reported diffuse fatty infiltration analagous to that observed in choline deficiency, in the livers of rats fed diets completely deficient in threonine. In the present study, differences in the cellular appearance of livers from animals fed choline-deficient diets and those from animals fed low casein diets containing cho-

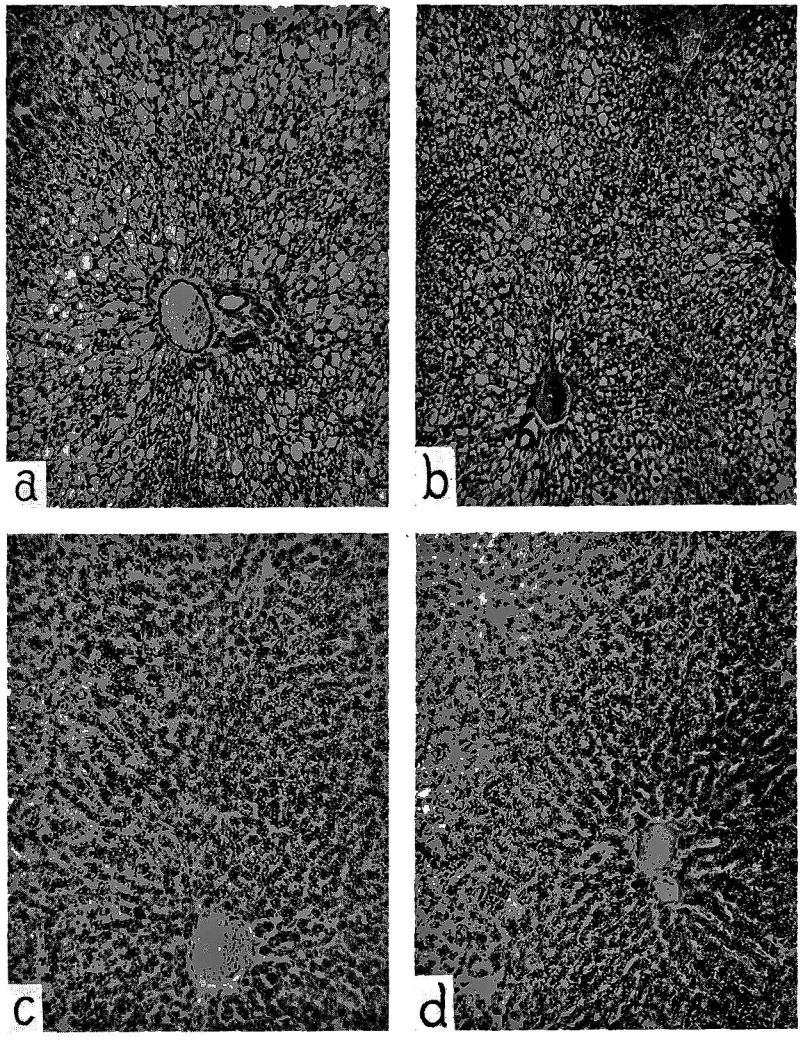

Fig. 3 Photomicrographs of representative livers of rats from group 3 (see table 1). Bouin's fixative. H and E stain.

A Sucrose. Condition similar to that described for figure 2–A. Fatty cells are arranged in zones. Hyperchromatosis in non-fatty cells and some focal necrosis are observed.

B Fructose. Zonification is more distinct. Groups of normal and nuclear hyperchromatic cells are observed among the zones of non-fatty cells.

C Dextrin. Liver maintains its normal structure.

D Cerelose. Liver maintains its normal structure, sinusoids and lobules are more clearly defined than in the previous photomicrograph.

line make it possible to distinguish between these two conditions. In the livers of choline deficient animals the fatty infiltration is diffuse and is most severe in the vicinity of the central vein. In the livers of animals receiving low-protein diets containing choline the distribution of fatty cells results in a network appearance in which zones of normal cells are interspersed among the zones of fatty cells and only occasionally is the fatty infiltration most severe around the central vein of the lobule. The magnitude of the fatty infiltration is much greater in rats fed a diet deficient in choline.

The increased affinity of the non-fatty cells, in the livers of rats fed the basal diet (9% casein plus choline), for the stains used, is suggestive of some modification of the metabolic function of these cells. The addition of threonine to this diet, besides reducing the number of fatty cells, also results in a normal appearance of the non-fatty cells.

There is a close corrleation between the extent of fat deposition as determined chemically and the severity of fatty infiltration as determined histologically in the livers of animals fed the various diets described. The results of the histological studies confirm the earlier observations that fat accumulates in the livers of animals fed 9% casein diets containing choline and methionine. Since fatty infiltration was prevented when the basal diet was supplemented with either threonine or threonine and glycine this is further evidence that both threonine and choline (and possibly other amino acids) are required to maintain a normal level of fat in the livers of young animals fed 9% casein diets.

The need for both choline and either additional protein or threonine to prevent fatty infiltration in the livers of animals fed the low-protein, choline-free diet indicates that the fatty infiltration in this case is the result of two distinct deficiencies (fig. 1-a). The dual nature of this deficiency is further illustrated by other comparisons. Choline alone was completely effective in preventing the fatty infiltration when the 18% casein diet was fed (fig. 1-c vs. fig. 1-d) and protein alone or threonine alone prevented the fatty infiltration when cho-

line was included in the 9% casein diet (fig. 1-b vs. fig. 1-d and fig. 2-a vs. fig. 2-c). The earlier failures to demonstrate this effect of threonine when choline-free diets were fed (Singal et al., '53 and Harper et al., '53a) has been explained as a result of the greater severity of the choline deficiency which masks the protein or threonine effect unless the diet contains close to the stated requirement of lipotropic factors (Harper et al., '54b). These conclusions from the histological observations support the earlier conclusions based on the chemical studies.

SUMMARY

Histological studies have been made on the livers of rats fed various low-casein diets in an attempt to correlate alterations in fat deposition with structural changes in the liver tissues.

The fatty infiltration and occasional necrosis observed in the livers of rats fed a 9% casein-sucrose diet containing choline were not apparent when diets containing additional protein, threonine, or threonine and glycine were fed. The livers of rats fed diets in which the sucrose was replaced by either glucose or dextrin also appeared structurally normal.

The fatty infiltration in the livers of rats fed the basal diet was less severe than that observed when choline was omitted from the diet. The occasional necrosis and the network-like distribution of fatty cells also make it possible to differentiate this condition from the diffuse fatty infiltration that occurs in choline-deficient rats.

ACKNOWLEDGMENTS

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EFFECT UPON GROWTH OF THE D ISOMERS IN SYNTHETIC MIXTURES OF THE ESSENTIAL AMINO ACIDS [1]

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THREE FIGURES

When an ample supply of the L forms of the essential amino acids is provided in the diet, but care is taken to avoid an excess of methionine, at least an equal quantity of the D isomers can be included without producing any apparent growth-retarding effect in the experimental rat (Van Pilsum and Berg, '50). Addition of glycine and ammonium citrate to such diets fails to increase the growth rate, but acceleration of the less rapid growth obtained when mixtures of the essential amino acids are fed at lower dietary levels has been reported (Rose, Smith, Womack and Shane, '49).

Preliminary tests made in this laboratory (Van Pilsum, '49) have indicated that rate of growth is retarded markedly when tryptophan, histidine, methionine and phenylalanine are all fed simultaneously in the D form only, despite the fact that the D isomers of these amino acids will each promote growth readily when tested singly (Berg and Potgieter,

[1] The data in this paper are taken from a dissertation submitted in February, 1953, by William A. Phillips in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate College of the State University of Iowa.

The work was aided by a grant from The Dow Chemical Company of Midland, Michigan. A summary of the study was presented at a symposium on amino acids at the 125th meeting of the American Chemical Society in Kansas City, on March 26, 1954.

'31–32; du Vigneaud, Sealock and Van Etten, '32; Berg, '34; Cox and Berg, '34; Jackson and Block, '37–38; and Rose and Womack, '46).

The purpose of this communication is to present data which show that growth on diets containing lower levels of the 10 essential amino acids than those used by Van Pilsum and Berg ('50) is indeed stimulated, not only through the addition of glycine and ammonium citrate, but also by including the extraneous D forms of the essential amino acids whose D isomers are inverted too slowly to promote growth in diets devoid of their L counterparts. In the latter instance the enhanced rate of growth obtained may reflect the catabolism of the poorly-invertible D components to provide extra nitrogen (and possibly also carbon residues) for the synthesis of the non-essential amino acids. In confirmation of the preliminary tests cited above, the simultaneous replacement by D isomers of all the L-amino acids known to be produceable by inversion failed to promote as rapid growth as that attained when these amino acids were fed in the L or the DL forms. The slower rate of growth apparently reflects a limited capacity to invert so many D-amino acids simultaneously.

EXPERIMENTAL

Most of the DL-amino acids used in these experiments were products of The Dow Chemical Company.[2] DL-Histidine monohydrochloride dihydrate, DL-isoleucine and DL-threonine were bought from Merck and Company. DL-Arginine monohydrochloride was prepared from gelatin by the method of Schein and Berg ('46) and isolated by the method of Cox ('28). All of the amino acids were recrystallized to give correct amino nitrogen or total nitrogen values, as determined by the Van Slyke and Kjeldahl methods.

Most of the optically active amino acids were prepared in this laboratory, chiefly by resolution. For some resolu-

[2] These were provided by The Dow Chemical Company through the courtesy of Dr. J. E. Johnson. We are happy to acknowledge our appreciation of this additional generosity.

TABLE 1

Specific rotations of the L- *and* D-*amino acids used in the experimental diets*

AMINO ACID	$[\alpha]_D$ [1]		SOLVENT USED	SOLUTE PER 100 ML OF SOLUTION	BIBLIOGRAPHIC REFERENCE [2]
	Found	Recorded			
	degrees	*degrees*		*gm*	
L-Valine [3]	+ 27.7	+ 27.4	6N HCl	2.0 [4]	Fischer, '06; (Price, Gilbert and Greenstein, '49)
L-Leucine	+ 15.7	+ 15.9	6N HCl	3.8	Price, Gilbert and Greenstein, '49
L-Isoleucine [3]	+ 40.6	+ 40.8	6N HCl	2.48	(Locquin, '07; Price, Gilbert and Greenstein, '49; Greenstein, Levintow, Baker and White, '51)
L-Threonine [3]	— 28.0	— 28.3	H_2O	2.0 [5]	(West and Carter, '37)
L-Lysine monohydrochloride [3]	+ 21.0	+ 20.5	6.08N HCl	3.0	Berg, '36; (Doherty and Popenoe, '51)
L-Tryptophan	— 31.0	— 32.1	H_2O	0.5	Cox and King, '30; (Berg, '33); Shabica and Tishler, '49
D-Tryptophan	+ 32.2	+ 32.45	H_2O	0.5	Shabica and Tishler, '49; (Berg, '33)
L-Methionine	+ 21.4	+ 21.6	0.2N HCl	0.88	Price, Gilbert and Greenstein, '49
D-Methionine	— 21.4	— 21.5	0.2N HCl	0.88	Price, Gilbert and Greenstein, '49; Windus and Marvel, '31
L-Phenylalanine	— 34.2	— 34.8 and — 35.2	H_2O	2.0	Gilbert, Price and Greenstein, '49; Wretlind, '50
D-Phenylalanine	+ 34.5	+ 35.2	H_2O	2.0	Wretlind, '50
L-Histidine monohydrochloride monohydrate	+ 9.4	+ 9.42 [6]	1N HCl	2.0 [6]	(Dunn, Frieden, Stoddard and Brown, '42)
D-Histidine monohydrochloride monohydrate	— 9.6 [7]		1N HCl	2.0	Pyman, '11
L-Arginine monohydrochloride [3]	+ 22.0	+ 22.8	5N HCl	2.0	(Birnbaum and Greenstein, '52)
D-Arginine monohydrochloride	— 21.8	— 23.0	5N HCl	2.0	(Birnbaum and Greenstein, '52)

[1] Except where otherwise indicated, conditions used in determining the optical rotation were the same as those cited in the reference given. Polarizing temperatures varied from 23° to 27°C. in the various tests.

[2] Reference numbers which are not enclosed in parentheses indicate method of preparation. When no enclosed reference is given, the citation also includes reference data on optical rotation. Enclosure in parentheses indicates that the reference cited served only to provide standards of optical purity.

[3] Small parts of the L-valine, the D-methionine and the L-isoleucine were prepared by Dr. John F. Van Pilsum. Most of the L-isoleucine was kindly provided by Dr. H. C. White of the Dow Chemical Company. The bulk of the L-threonine was generously supplied by the Sterling-Winthrop Research Institute, through the courtesy of Dr. M. L. Tainter, Director. A portion was from a supply previously made available by Dr. E. E. Howe of the Research Laboratory of Merck and Company. Part of the L-lysine monohydrochloride was purchased from the Nutritional Biochemicals Corporation. The L-histidine monohydrochloride monohydrate and the L-arginine monohydrochloride were purchased from Merck and Company.

[4] The concentration recorded in the reference was 1.0.

[5] The reference does not record the concentration used.

[6] Calculated for the monohydrochloride monohydrate from the $[\alpha]_D^{24.8} = + 12.72$ degrees given in the reference cited for free L-Histidine in 1.003N HCl, c = 1.6395. Recalculated c became 2.22. For c = 8.0 in water, plus HCl equivalent to the monohydrochloride, the $[\alpha]_D^{27}$ was + 8.13 degrees. Under these conditions, Cox and Berg ('34) record + 8.12 degrees.

[7] The conditions employed were the same as for the L isomer. At c = 8.0 in water, which contained HCl equivalent to the D monohydrochloride, the $[\alpha]_D^{28}$ was — 8.32. Cox and Berg ('34) found — 8.25 degrees under these conditions.

tions enzymatic procedures were found preferable, for others chemical methods seemed better. In the choice of procedure, the time required to prepare an enzyme system of sufficient purity and the difficulty of obtaining complete enzymolysis were sometimes determining factors.

Table 1 gives the specific rotations of the L- and D-amino acids used in the growth tests, references to the methods of resolution or isolation employed and to data for specific rotations which served as standards of comparison, and information concerning the sources of the L- and D-amino acids which were not prepared in this laboratory. The D-arginine monohydrochloride was isolated from the mixture obtained by the action of arginase on a buffered solution of DL-arginine monohydrochloride. The procedure was essentially a large scale adaptation of the method described by Hunter and Dauphinee ('29–30). At the completion of the incubation period the solution was adjusted to pH 4.0 with hydrochloric acid, boiled to coagulate the protein, and filtered. The monohydrochloride of D-arginine was isolated by the method of Cox ('28).

Weanling male rats of the Sprague-Dawley strain, weighing 30 to 40 gm each, served as the experimental animals. They were housed individually in false-bottomed wire cages at a room temperature of 80 $\pm$ 2°F. and were permitted to consume food and water ad libitum. Food consumption and weights were recorded every 4 days during a 36-day experimental period.

The chief variations in the diets were in the compositions of the amino acid mixtures which served as the sole source of protein nitrogen. For convenience, the amino acids were divided qualitatively into two groups, the first consisting of valine, leucine, isoleucine, lysine, and threonine, and designated group A, the second composed of tryptophan, methionine, phenylalanine, histidine, and arginine, and called group B. At the time these studies were begun, indications were that the D forms of the amino acids of group A were only poorly utilizable for growth, or not utilizable at all; but

that the D forms of the amino acids of group B (with the possible exception of D-arginine for which no data were found) were readily available. The various compositions of group A and group B are given in table 2. The several combinations

TABLE 2

Variations in composition of the essential amino acid mixtures of the diets [1]

GROUP A	L-1 or DL-1	L/2	DL-2	DL-4
Valine	0.70	0.35	1.40	2.80
Leucine	0.80	0.40	1.60	3.20
Isoleucine	0.50	0.25	1.00	2.00
Lysine [2]	1.00	0.50	2.00	4.00
Threonine	0.50	0.25	1.00	2.00
	3.50	1.75	7.00	14.00

GROUP B, UNCOMPENSATED	L-1, DL-1, or D-1	L/2	DL-2 or D-2
Tryptophan	0.20	0.10	0.40
Methionine	0.60	0.30	1.20
Phenylalanine	1.00	0.50	2.00
Histidine [2]	0.40	0.20	0.80
Arginine [2]	0.20	0.10	0.40
	2.40	1.20	4.80

GROUP B, COMPENSATED	DLc-1	Dc-1	DLc/2	Dc/2
Tryptophan	0.23	0.27	0.12	0.15
Methionine	0.66	0.73	0.36	0.45
Phenylalanine	1.10	1.22	0.60	0.75
Histidine [2]	0.48	0.60	0.24	0.30
Arginine [2]	0.23	0.27	0.12	0.15
	2.70	3.09	1.44	1.80

[1] The groups are designated by letters indicating the configuration of the component amino acids, followed by numbers indicating that the relative composition is the same (1) or half (/2), twice (2) or four times (4) that in the reference L-1 diet. The letter c indicates compensatory additions to allow for incomplete inversion of the D components.

[2] The figures for histidine, arginine and lysine represent the amounts of these amino acids provided in the monohydrochlorides fed. The DL-histidine monohydrochloride was a dihydrate, the L a monohydrate. To compensate for the HCl, an equivalent of $NaHCO_3$ was added. The $NaHCO_3$ and the derivative fed displaced an equal quantity of dextrin from the diet.

of groups A and B used in formulating the diets fed are indicated in the protocols of the experiments.

In addition to the amino acid mixture, each 100 gm of diet contained sucrose 15.0, Cellu flour 2.0, salt mixture (Jones and Foster, '42) 4.0, corn oil 2.0, vitamin A and D concentrate [3] 0.08, inositol 0.1, choline chloride 0.2, liver extract [4] 0.4, vitamin B_{12} concentrate [5] 0.007 gm, and dextrin to make 100 gm. The other vitamin supplements were provided by adding to each kilo of diet thiamine hydrochloride 5.0, riboflavin 10.0, pyridoxine hydrochloride 5.0, nicotinic acid 5.0, calcium d-pantothenate 25.0, p-aminobenzoic acid 300.0, α-tocopherol (acetate) 25.0, 2-methyl-1,4-napthoquinone 2.0, biotin 0.1 and folic acid 0.1 mg.

Three series of tests were conducted, the second and third simultaneously. In series I and II the physiologically active amino acid levels of the diets approximated 5.9%, and in series III 2.95%.

Data pertaining to series I are summarized in figure 1. Comparisons of the growth rates observed show that at this level provision of the L-amino acids of group A in the DL form (experiment 2) induced greater food consumption and better growth than when the amino acids of both groups were fed in the L form (experiment 1). The increase in growth rate was highly significant statistically ($P < 0.01$),[6] but was not as great as that obtained when supplements of glycine and ammonium citrate (experiment 3) were added to the L-amino acid mixture, despite the fact that these additions provided considerably less nitrogen than did the D-amino acid components. When the group B amino acids were pro-

[3] Oleum percomorphum, Mead Johnson and Company. Contains not less than 60,000 vitamin A units and 8,500 vitamin D units, U.S.P., per gram.

[4] Liver concentrate, N. F., kindly supplied by The Wilson Laboratories, through the courtesy of Dr. S. W. Hier.

[5] We are indebted to Dr. L. R. Hines and E. R. Squibb and Sons for a generous supply of preparation M-1499-1, which contained 2.4 μg of vitamin B_{12} per gram, with starch as the diluent.

[6] $P < 0.05$ is considered to be of doubtful significance, $P < 0.02$ to be significant and $P < 0.01$ highly significant.

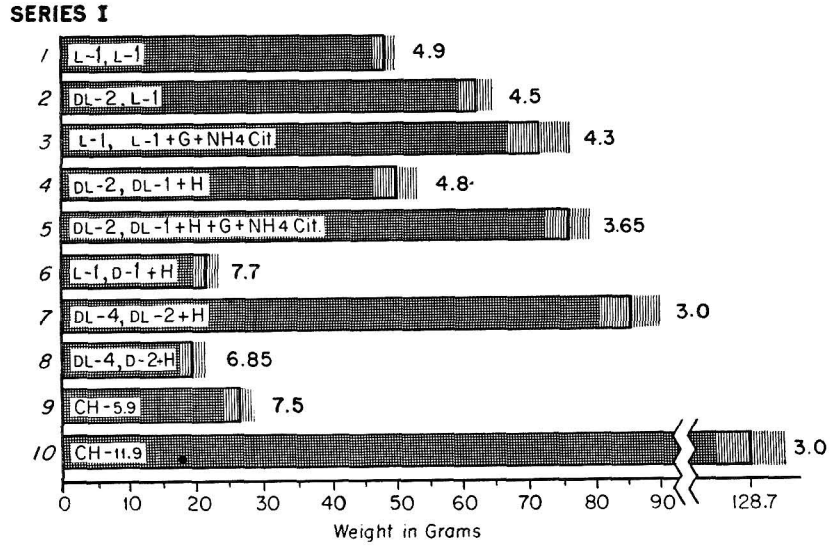

Fig. 1 *Gains in 36 days on essential amino acid mixtures fed primarily at* physiologically active levels approximating 5.9% of the diet. The influence of extra nitrogen.

Each bar represents the growth response of 6 male rats. The portion enclosed by the heavy line indicates median gain in weight. The vertical shading shows the standard error $\frac{s}{\sqrt{n}}$, where s is the standard deviation, n the number of animals. The experiment number appears at the left of the bar, the grams of food consumed per gram of gain at the right. The first letters in the first 8 bars refer to the group A amino acids, the second to the B group. The number following the letter indicates relative dietary level at which the group A or group B was fed. For details refer to table 2.

Designations CH-5.9 and CH-11.9 refer to diets containing 5.9 gm and 11.9 gm of a casein hydrolysate per 100 gm. The acid hydrolysate used was generously provided by the Upjohn Company, through the courtesy of Dr. John T. Correll and Dr. Curtis E. Meyer. It had been fortified before use with 0.6 gm of DL-methionine and 3.4 gm of DL-tryptophan per 100 gm; 5.9 gm was isonitrogenous with the amino acid mixture provided in experiment 1.

In experiment 3, G + NH_4Cit indicates addition of 0.34 gm of glycine and 0.27 gm of ammonium citrate per 100 gm of diet; in experiment 5, the additions of these were 0.95 and 0.74 gm, respectively, per 100 gm. H refers to the addition of 0.1 gm of extra DL-histidine per 100 gm of diet in experiments 4 and 5, 0.2 gm in experiment 7, 0.2 gm of extra D-histidine in experiment 6 and 0.4 gm extra in experiment 8.

vided in the DL form at the same level (except for histidine) as the L-amino acid components had been in experiment 2 (see experiment 4), the growth acceleration observed in experiment 2 did not occur. Further supplementation with glycine and ammonium citrate (see experiment 5) induced definite growth stimulation ($P < 0.01$). Growth on a casein hydrolysate, adequately fortified with tryptophan and methionine when fed at a level of 11.9% (experiment 10) was markedly

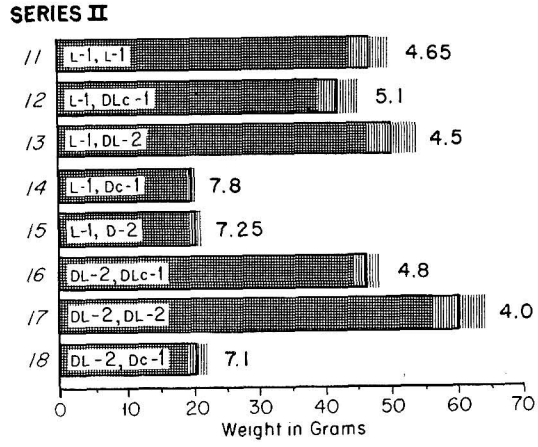

Fig. 2 Gains in 36 days on essential amino acid mixtures fed primarily at physiologically active levels approximating 5.9% of the diet. The influence of feeding the readily-invertible D-isomers.

For aid in the interpretation of this figure, see the legend to figure 1. The letter c indicates the inclusion of extra DL- or D-amino acids of group B, made in an attempt to compensate for incomplete inversion. For detailed dietary compositions see table 2.

lowered when the fortified hydrolysate was fed at half that level (experiment 9). In the latter case the fortified hydrolysate probably failed to provide an adequate supply of the essential amino acids, notably methionine. Mass substitution of D-amino acids for the L forms in group B (experiment 6) promoted a decidedly inferior weight gain in the 36-day period. The high growth rate observed when the diet contained 19.0% of DL-amino acids (experiment 7) was not attained when the DL-amino acids in group B were replaced

with D-amino acids (experiment 8). In experiments 6 to 8 the only compensation for possible incomplete inversion of the group B amino acids was in the use of more histidine.

Experiments 11 to 18 of series II, figure 2, represent further attempts to analyze the effects of supplying the group B amino acids in the DL or the D forms. The allotments of these forms in the group B mixtures used in experiments 12 and 16 (DLc-1) and experiments 14 and 18 (Dc-1) were increased in an attempt to compensate for the slow or incomplete inversion of the D components (Cox and Berg, '34; Oesterling and Rose, '52; Wretlind, '52a, '52b). The D-arginine content was increased by one-third. The growth response noted in the control experiment (11) of this series essentially duplicated the response in experiment 1. The apparent differences in mean rates of growth in experiments 12 and 13, in which the DL amino acids of group B were fed at different levels are not statistically significant ($P < 0.20$). In experiment 16, in which the poorly invertible group A amino acids were fed at twice the control L level, growth was not improved, as it had been in experiment 2, figure 1, which contained the same quantity of extraneous D amino acids as in group A, but only L-amino acids as components of group B. When the DL amino acids of both groups A and B were fed at double the control L level (see experiment 17) the growth rate increased significantly ($P < 0.02$) over that noted in experiment 16 and approximated that attained in experiment 2, figure 1. When the group B amino acids were supplied in the D form exclusively (experiments 14, 15 and 18), growth occurred at a considerably slower rate than in the control experiment (11). This was true even when the D-amino acids of group B were fed at twice the level (experiment 15) of the L-amino acids employed in the control experiment. Feeding the group A amino acids in the DL form at twice the L level did not improve the rate of growth significantly (cf. experiments 18 and 14).

The data obtained in the third series of tests (experiments 19–27) are recorded in figure 3. In the control experiment

(19) in which only the L amino acids were fed at half the level employed in experiment 11, growth was but one-fourth as rapid. Feeding the group B amino acids in the DL form at a compensated level 20% higher (experiment 22) afforded about the same rate of growth. Doubling the DL amino acids of group B (experiment 23) produced better growth ($P < 0.05$). Feeding the group B amino acids in the D form at compensated levels 50% higher (experiment 25) than the L

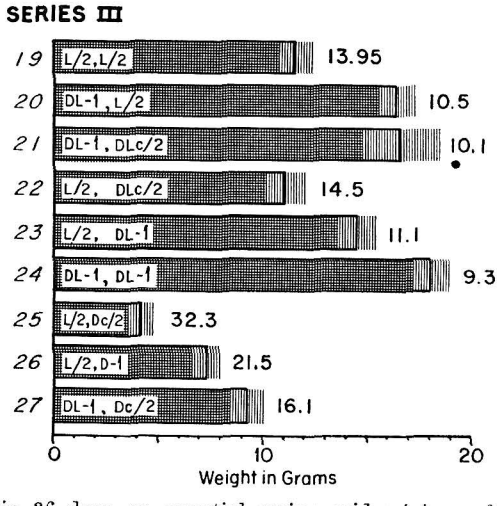

Fig. 3 Gains in 36 days on essential amino acid mixtures fed primarily at physiologically active levels approximating 2.95% of the diet. The influence of feeding the poorly-invertible and the readily-invertible D-isomers.

For aid in interpreting this figure, see the legends to figures 1 and 2.

level in experiment 19 caused a very significant drop in the rate of growth ($P < 0.01$). Doubling the D amino acids in this group promoted a highly significant ($P < 0.01$) acceleration in growth (see experiment 26). When the group A amino acids were all fed in the DL form at twice the L level, marked stimulation in growth occurred (for experiments 20 vs. 19, $P < 0.01$; 21 vs. 19, $P < 0.05$; 21 vs. 22, $P < 0.05$). This was still further enhanced when both groups were fed in the DL form (experiment 24 vs. 23, $P < 0.02$). Feeding the group A amino acids in the DL form also accelerated the

growth response to diets in series III which contained the group B amino acids in the D form (see experiments 27 and 25, $P < 0.01$).

In general, food consumption in the three series of tests reflected the capacity of the diet employed to promote growth. Almost invariably on the diets containing the purified amino acid mixtures, the amount of food required to induce unit gain in weight within each series of tests was less on the more rapid growth-promoting than on the poorer growth-promoting diets.

DISCUSSION

Comparisons in all three series of experiments seem to indicate clearly that the extraneous D-amino acids that were provided when the L-amino acids of the non-invertible or poorly invertible group (group A) were fed in the DL form stimulated growth beyond that observed when only the L-amino acids of this group were fed. The increment in growth attained, however, was less than that induced by the addition to the diets of glycine and ammonium citrate (fig. 1), despite the fact that only about one-fifth as much nitrogen was contributed by the latter.

The possibility that ammonia or other forms of nitrogen were produced metabolically from the D forms of the group A amino acids seems good. The D isomers of valine, leucine, and isoleucine are all readily attacked by D-amino acid oxidase (Klein and Handler, '41; Karrer and Frank, '40). In feeding tests, at least part of the nitrogen of D-lysine is converted to urea and ammonia (Ratner, Weissman and Schoenheimer, '43; Neuberger and Sanger, '44). Release of nitrogen as ammonia might also be effected by the flora of the intestinal tract. To what extent such release may occur in studies of this type is uncertain (Rose and Smith, '50).

In the studies recorded, the possibility has not been ruled out that some inversion of the D isomers of the group A amino acids could have occurred. Experiments with doubly labeled D-leucine have indeed shown that a limited stereonaturalization of this isomer can take place (Ratner, Schoenheimer and

Rittenberg, '40), but the inversion is too small in degree to permit the L-leucine thereby produced to meet even the needs for maintenance in the young animal (Rose, '38). After the present study was begun, White, Fones and Sober ('52) reported that, contrary to information previously available (Rose, '38), D-valine could be utilized for repletion in the rat which had previously subsisted on a diet devoid of valine. However, unless lack of the L enantiomorph were the specific limiting factor in the L-amino acid mixture provided in our control experiments, inversion of D-valine could not alone have been responsible for the growth acceleration. The amount of each amino acid fed in the L-amino acid mixture at the 5.9% level, with the single exception of the phenylalanine, which was 0.1% higher, was the tentative minimal level of Rose et al. ('49), i.e., the minimum amount needed to promote optimum growth when all of the non-essential amino acids are also provided. There is at present no known reason to assume that the omission of the non-essential amino acids would impose an extra demand specifically for valine or for leucine, or for that matter, for any specific other group A amino acid. On the other hand, specific relationships between certain of the non-essential amino acids and the group B amino acids are known. The sulfur of methionine is used in the synthesis of cystine (Tarver and Schmidt, '39) and phenylalanine is converted to tyrosine (Moss and Schoenheimer, '40). Histidine may also be a ready source of glutamic acid (Edlbacher and Kraus, '30), and arginine a ready source of glutamic acid, proline and hydroxproline (Stetten and Schoenhemier, '44; Womack and Rose, '47; Stetten, '51).

At the 5.9% L-amino acid level employed in the control tests in series I and II, replacement of the L-amino acids of the readily-invertible group B with the same, or approximately the same, quantities of the DL or D forms, limited the stimulating effect upon growth otherwise noted when the group A amino acids were fed in the DL form at twice or 4 times the level of the L form. Also in these series, doubling the allotment of D-amino acids of the readily-invertible group

B did not accelerate the growth response. These observations suggest that a ceiling is imposed on the overall capacity of the rat to provide L-amino acids by inversion when the readily-invertible amino acids (group B) are supplied *en masse* in the D form. On the other hand, the availability of the readily-invertible D-amino acids does not seem to be markedly impaired in the presence of relatively large amounts of the poorly-invertible D-amino acids, several of which (leucine, isoleucine, and valine) are known to be attacked readily by D-amino acid oxidase (Karrer and Frank, '40; Klein and Handler, '41), the enzyme usually assumed to be involved in the initial degradation of the amino acids and in the process of stereonaturalization. From these observations it seems fair to conclude that the D forms of the poorly-invertible group of amino acids do not inhibit competitively the inversion of the readily-invertible D-amino acids.

At the lower dietary level employed in the series III tests, feeding the group B amino acids in the DL or D form obviously necessitated less inversion. When the group A amino acids were fed in the DL form at double the L levels, the same degree of stimulation was noted whether the group B amino acids were supplied in the L form or fed in the DL form in quantities calculated to provide the L form in equal amount, partly as such and partly by inversion. Feeding the group B amino acids in the D form at an analogous level caused marked growth retardation, but feeding the group A amino acids in the DL form at double the L levels led to improvement, probably attributable to catabolism of the poorly-invertible D forms to provide nitrogen for the synthesis of the non-essential amino acids. In this series, feeding the group B amino acids in the D form at double the L levels produced an accelerated response, thus indicating that in this instance the capacity of the animal to effect inversion had not been reached.

In most of the previous tests recorded in the literature concerning the availability of the invertible D amino acids for growth, the diet employed provided optimum levels of the other essential amino acids, and the non-essential acids

as well. Under such conditions D-histidine was somewhat less efficient than L-histidine (Cox and Berg, '34). In diets which promoted maximum growth, 0.2% of D-tryptophan produced a slower response than did 0.2% of L-tryptophan (Oesterling and Rose, '52); in earlier diets which were higher in fat and promoted less rapid growth, no differences were discernible (du Vigneaud, Sealock and Van Etten, '32; Berg, '34). Direct comparisons of D- and L-methionine, in diets which contained 0.2% of L-cystine (Wretlind and Rose, '50) showed essentially equal utilization, even at the suboptimum level of 0.2%. Tests of D-phenylalanine in diets high in fat, but containing amino acid mixtures devoid of tyrosine (Rose and Womack, '46), showed almost, if not quite, as satisfactory growth promotion at levels of 0.5 and 1.0% as was attained with L-phenylalanine.

Wretlind ('52a) has recently reported that suboptimum amounts of D-methionine (0.25%) in dietary mixtures containing only the essential amino acids (no arginine) produced less rapid growth in 10 days than like amounts of L-methionine. When 5 of the other essential amino acids were fed in the L form, three in the DL (isoleucine, threonine, and valine), the divergence was small, but when the 8 other amino acids were all fed in the DL form, growth promotion by D-methionine was considerably slower. In an analogous study of phenylalanine in which the 8 other essential amino acids (no arginine) were all fed only in the DL form 0.5% of D-phenylalanine produced less rapid growth than 0.5% of L-phenylalanine (Wretlind, '52b). He attributes the differences observed between growth on the D and L isomers to retardation of stereonaturalization by the competitive inhibition, by the other D-amino acids, of the participating enzyme systems. Our data would seem to indicate that, if competitive inhibition is involved at all, it must be associated primarily with the D-amino acids of the readily-invertible group.

Increasing the quantity of group B amino acids fed exclusively in the D forms at the lower dietary level increased the

growth response; at the higher dietary level, neither an increase nor a decrease was noted. Failure of the growth rate to show a decrease would seem to indicate that toxicity was not involved, but that a limit had been reached in the capacity to retain the D-amino acids or to effect their inversion.

Energy is probably required in the inversion of the D-amino acids, as well as in the synthesis of the non-essential amino acids and in the fabrication of new tissue. It may well be that the demands imposed by the simultaneous inversion of so many D-amino acids divert energy which might otherwise be used in growth.

SUMMARY

Rats fed suboptimal percentages of the 10 essential amino acids as the chief source of dietary nitrogen grew somewhat better when the poorly invertible group of essential amino acids (valine, leucine, isoleucine, lysine and threonine) was supplied in the DL form at twice the L level. Presumably the extraneous D-amino acids provide nitrogen for limited synthesis of the non-essential amino acids, though much less readily than do even smaller amounts of glycine and ammonium citrate.

When the group of amino acids whose D modifications are readily invertible (tryptophan, methionine, phenylalanine, histidine and arginine) when each is fed singly were all fed simultaneously in the DL form in amounts calculated to provide adequate amounts of each L form after inversion somewhat slower growth occurred at the basic 5.9% level than when the diet contained these amino acids in the L form only. Less growth was attained when this group of amino acids was supplied only in the D form, and increasing the allotment of these D-amino acids as a group did not increase the rate of growth. In analogous tests at a basically lower (2.95%) dietary level, growth when this group of amino acids was supplied in the DL form was about the same as when the group was fed in the L form. When the readily-invertible group was provided only as D-amino acids, the growth response was poorer, but it improved with increase in allotment.

Failure of the D forms of the invertible group to promote as rapid growth as in the L controls can be attributed largely to incomplete or to slow inversion. Failure to increase the rate of growth at the higher dietary levels by increasing the allotment of the readily invertible D-amino acids suggests that a ceiling is imposed on the overall capacity of the animal to effect stereonaturalization *en masse* of the D-amino acids whose inversions singly occur readily. Reasons for making this assumption are discussed.

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NUTRITIONAL INTAKE OF CHILDREN

II. CALCIUM, PHOSPHORUS AND IRON [1]

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FOUR FIGURES

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INTRODUCTION

The pattern of the changing food intake of children during the years of growth presents many interesting facets which merit further investigation. During infancy and adolescence, when physical growth is most rapid, nutritional requirements are high and, in a child with good physical and psychological health, appetite increases and food intake becomes greater.

However, during the latter part of the first year and through the pre-school years, demands for growth are somewhat lessened. At the same time development and maturation are progressing rapidly. The child in this post-infancy period is exploring his world, adding many new accomplishments, becoming independent in eating and in general activity, learning bowel control, and exerting his will in making selections. There are intervals when appetite is decreased and the intake of some or all nutrients is lowered. The observation that the decrease in consumption is most marked in a few specific foods and therefore the intake levels of some nutrients are decreased while the levels of others remain stationary or increase is leading to further study of the factors involved.

The purpose of this series of papers is to present the findings on nutrient intake of a group of healthy children during

[1] This study was aided by a grant from the Nutrition Foundation.

the first 5 years of life. Other aspects of the growth and development of these children which are pertinent to the changes in food intake will be reported at a later date.

EXPERIMENTAL

The background and techniques of this study were reported in detail in a previous publication (Beal, '53) and will be only briefly summarized here. Nutrition studies were added in 1946 to the program of the Child Research Council, which for many years has been following the growth and development — physical, physiological and psychological — of a group of children from "upper middle class" families of the Denver area. Since the purpose of this organization is research rather than therapy, and since the children enrolled are under the care of pediatricians not on the Council staff, no effort is made by the staff to influence dietary intake.

Nutrition data are obtained by a series of interviews and 24-hour intakes, carefully recorded at monthly intervals during the first 6 months of life, and thereafter at intervals of three months. Nutrients are calculated from food value tables (Bowes and Church, '51; U. S. Department of Agriculture, '48, '50).

The data in this paper represent 795 histories on 58 children (26 boys and 32 girls) who now range in age from 4 months to 9 years. Only the first 5 years of life are included. Eliminated from these data are histories on breast-fed infants during the period of such feeding and two single histories on older children who had illnesses of sufficient severity and duration to decrease markedly their food intake during the three-month period. All others histories taken on these 58 children are included except as otherwise specified.

The first report in this series presented intake levels of calories, carbohydrate, fat and protein. The present report is concerned only with the intake of calcium, phosphorus and iron in food and does not include mineral concentrates which have been given to some of the children for varying

periods of time. Subsequent reports will deal with vitamin intakes.

RESULTS AND DISCUSSION

As previously reported, the intakes of calories, carbohydrate and fat rise rapidly during the first 12 to 18 months, then show only slight increases until three to 4 years, when the increase is accelerated. Protein intake, however, remains stationary during the period from 15 months until after three years of age. The pattern of these 4 nutrients is in sharp contrast to the pattern of the three minerals, each of which shows a distinct decrease during the post-infancy period. This contrast is obvious both in the group data and in the intakes of individual children.

The intakes of calcium and phosphorus of the children in this study from birth to 5 years of age are presented in table 1. Because of the skewness of the data, percentiles are used in preference to means and standard deviations. For each nutrient the table gives the values determined from visual smoothing of the 25th, 50th and 75th percentiles, with the lowest and highest intakes observed to date.

Intake of calcium rises rapidly in the first 6 months, with the median reaching 1.0 gm by 5 months. There is a slight further increase between 6 and 9 months, then the intake falls steadily to its lowest level between two and three years, when the median is 0.75 gm. This is followed by an acceleration so that by 5 years the median is again at the 1.0 gm level. An analysis of the data by sex shows relatively little difference between boys and girls during the first 5 months, but the levels for boys are higher between 6 and 15 months. It is of interest that the girls' intake of calcium begins to decrease after 9 months, while the boys reach a higher level of intake than the girls and maintain this higher level until after one year, when their intake also decreases. This is consistent with the relatively greater growth of the boys during the latter part of the first year (Boyd, '52). No observable sex difference in calcium intake is seen between 15

TABLE 1

Calcium and phosphorus intake of children from birth to 5 years of age

AGE	NO. OF CASES	CALCIUM, GM					PHOSPHORUS, GM				
		Percentile					Percentile				
		Lowest	25	50	75	Highest	Lowest	25	50	75	Highest
years months											
0–0 to 0–1	27	0.19	0.49	0.58	0.65	0.92	0.15	0.38	0.46	0.53	0.77
0–1 to 0–2	34	0.27	0.70	0.81	0.94	1.17	0.21	0.56	0.66	0.76	0.90
0–2 to 0–3	35	0.31	0.82	0.91	1.04	1.29	0.27	0.66	0.75	0.86	1.00
0–3 to 0–4	38	0.31	0.86	0.96	1.09	1.37	0.28	0.73	0.81	0.92	1.10
0–4 to 0–5	37	0.33	0.89	1.00	1.14	1.38	0.32	0.78	0.86	0.99	1.20
0–5 to 0–6	38	0.36	0.91	1.04	1.16	1.55	0.39	0.83	0.91	1.03	1.32
0–6 to 0–9	40	0.66	0.92	1.05	1.17	1.57	0.70	0.88	0.95	1.09	1.32
0–9 to 1–0	41	0.65	0.88	1.02	1.16	1.44	0.71	0.90	0.99	1.14	1.40
1–0 to 1–3	42	0.58	0.79	0.97	1.13	1.35	0.56	0.87	0.99	1.16	1.37
1–3 to 1–6	40	0.48	0.72	0.90	1.07	1.53	0.64	0.83	0.94	1.12	1.56
1–6 to 1–9	37	0.44	0.66	0.82	1.00	1.52	0.47	0.79	0.90	1.07	1.54
1–9 to 2–0	36	0.38	0.62	0.77	0.95	1.37	0.45	0.76	0.87	1.02	1.42
2–0 to 2–3	36	0.36	0.61	0.76	0.92	1.33	0.42	0.74	0.85	0.99	1.36
2–3 to 2–6	37	0.48	0.60	0.75	0.91	1.34	0.52	0.73	0.84	0.97	1.37
2–6 to 2–9	34	0.23	0.60	0.75	0.91	1.36	0.37	0.73	0.84	0.97	1.33
2–9 to 3–0	33	0.32	0.61	0.75	0.92	1.41	0.44	0.73	0.84	0.98	1.28
3–0 to 3–3	31	0.35	0.62	0.76	0.93	1.56	0.46	0.74	0.85	1.00	1.45
3–3 to 3–6	29	0.42	0.65	0.78	0.96	1.37	0.39	0.76	0.88	1.03	1.38
3–6 to 3–9	27	0.46	0.68	0.82	1.00	1.36	0.46	0.78	0.91	1.07	1.28
3–9 to 4–0	25	0.48	0.71	0.87	1.05	1.40	0.59	0.80	0.96	1.10	1.31
4–0 to 4–3	26	0.50	0.74	0.92	1.09	1.53	0.57	0.82	1.00	1.13	1.41
4–3 to 4–6	24	0.43	0.76	0.96	1.13	1.79	0.49	0.85	1.03	1.16	1.61
4–6 to 4–9	24	0.49	0.78	0.99	1.16	1.47	0.56	0.87	1.06	1.19	1.45
4–9 to 5–0	24	0.38	0.79	1.01	1.18	1.44	0.55	0.89	1.09	1.22	1.41

months and three years; thereafter the number of cases is as yet too small to permit adequate differentiation.

Since milk is the major source of calcium in the diets of these children and since the pattern of milk intake of individual children is being subjected to further study, figure 1 is presented to show the contrast of the median intake of total calcium to the median intake of milk calcium; for ease of conversion, grams of calcium and equivalent values in ounces of milk are both indicated on the graph. The values for milk

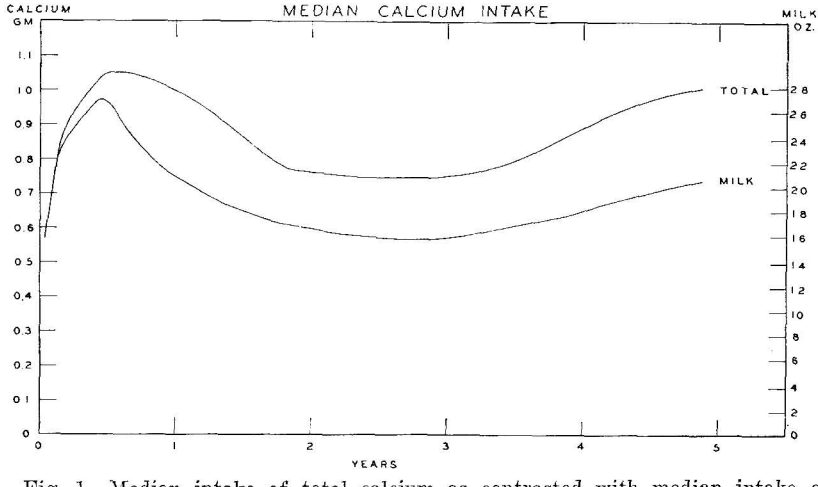

Fig. 1 Median intake of total calcium as contrasted with median intake of milk calcium of children in the first 5 years of life.

calcium include only the milk consumed as a beverage and on cereal; other sources of milk in the diet, such as pudding, soup and creamed dishes, have been excluded. Also excluded from the milk calcium median were 4 instances in which formulas simulating breast milk were used, since the low level of calcium in these formulas necessitates a different basis of conversion to ounces of milk.

As figure 1 indicates, milk is the essential source of calcium in these diets during the first three months, after which cereal and other solids contribute to the calcium content of the diet. The curves of the two medians thus become increasingly

separated throughout the first year, then from one through 5 years they are more nearly parallel. The consumption of milk as a beverage reaches a maximum at 6 months, when the median is about 27 ounces, then decreases to its lowest level of 16 ounces at two and one-half to three years before it starts to increase again. The range of intake is very wide.

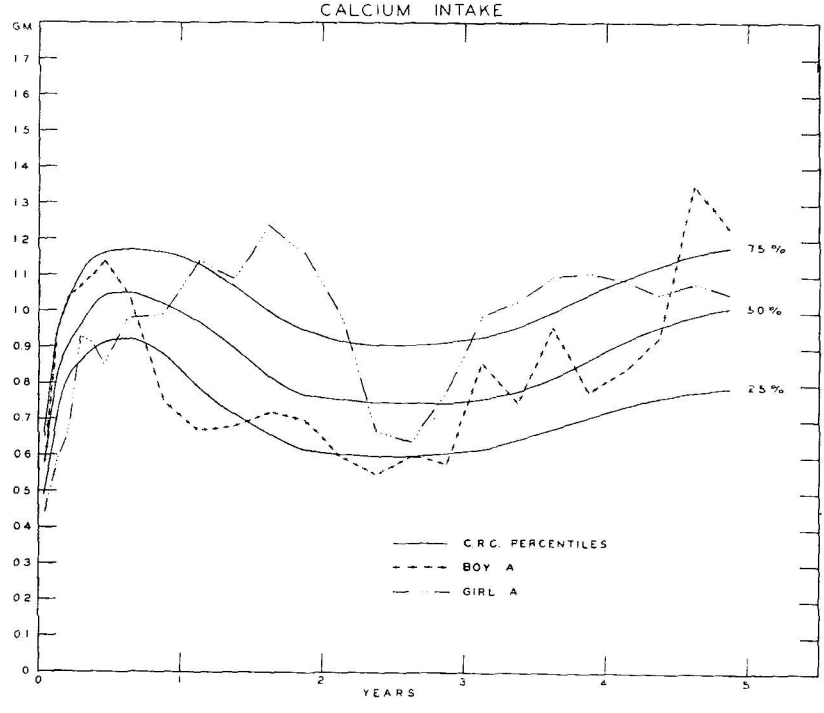

Fig. 2 Examples of individual variation in calcium intake from birth to 5 years of age.

For example, at two years and 9 months the lowest intake was 0.10 gm and the highest 1.15 gm, a range from approximately three to 32 ounces of milk. It should be noted that the children in whom this wide range is found are healthy children whose patterns of growth, with one exception, are satisfactory and who are not restricted in food intake by economic limitations.

Individual variations in calcium intake are shown in figures 2 and 3, in which 4 different patterns are indicated. Boy A had a relatively high intake during the first 6 to 9 months, after which his calcium intake decreased to below the 25th percentile by one year, remained low until nearly three years, and then rose to above the 75th percentile by

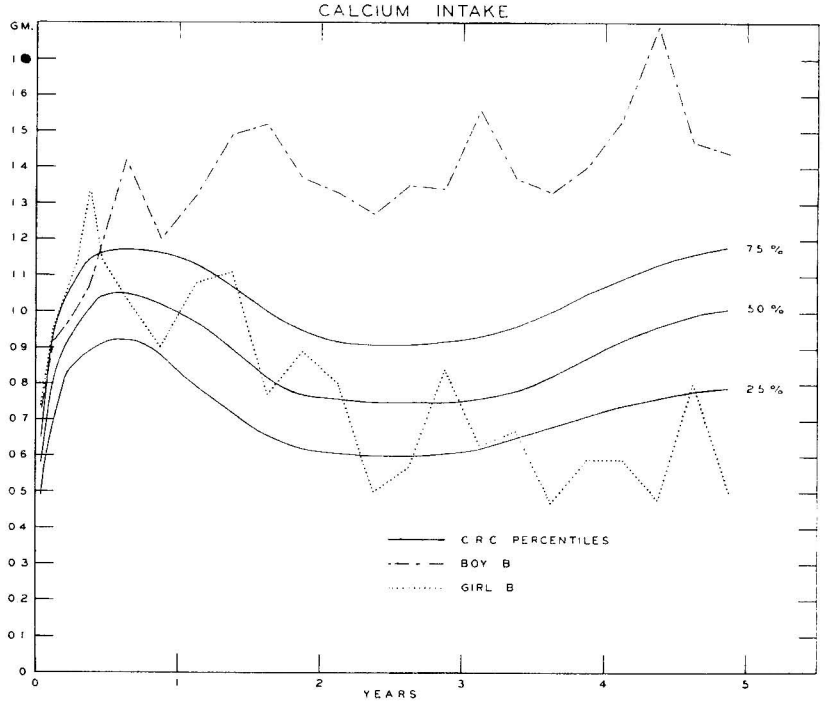

Fig. 3 Examples of individual variation in calcium intake from birth to 5 years of age.

$4\frac{3}{4}$ years. However, his calorie intake has been consistently in the highest quartile and his protein intake above the median, indicating that a higher consumption of other foods was compensating for the decrease in milk. In contrast, the calcium decrease in Girl A, which was restricted to the period between two and three years of age, was reflected in calorie and protein levels as well.

Boy B and Girl B (fig. 3) present patterns of intake which are somewhat more unusual in this group. Boy B is one of the two children to date who have not had marked decrease in milk intake during this age range. While his calorie intake showed a decrease between two and 4 years of age, when his appetite was poor and consumption of many foods dropped, his protein and calcium intakes in this age period were consistently above the 75th percentile. Girl B, on the other hand, has shown an erratic pattern of calcium intake which decreased markedly between 6 months and two and one-half years and maintained a low level with no real increase between two and one-half and 5 years. Her intake of calories and protein reflected the milk decrease during the last half of the first year, then rose to the median or higher until $3\frac{1}{2}$ years, when a general drop in food consumption lowered calorie and protein intake to levels comparable to that of calcium. The failure of this child to take an increasing amount of milk after three to 4 years of age, thus diverging from the pattern of the group, will make her an interesting subject of investigation of the physical and/or psychological factors involved.

While the patterns of intake vary from one child to another, there is a tendency in this group for the most common changes in intake to be similar to those of Boy A and Girl A. There is a period of marked decrease, occurring usually near the end of the first year and persisting for one to two years, with the lowest intake being shortly after two years of age, followed by an increase. These changes in level of intake parallel a theoretical calcium retention curve formulated by Stearns ('52) from calcium needs for growth during childhood. Since physical growth is relatively small in the period from two to 4 years of age, calcium needs are probably lower than at any other time until adulthood. However, the Recommended Allowance for calcium established by the Food and Nutrition Board of the National Research Council ('53) is maintained at a level of 1.0 gm from 10 months through 9 years of age.

Phosphorus intake shows a pattern similar to but less striking in change than that of calcium, since phosphorus reflects to a lesser extent the change in milk intake. The phosphorus

TABLE 2

Iron intake of children from birth to 5 years of age

AGE	NO. OF CASES	IRON, MG				
		Percentile				
		Lowest	25	50	75	Highest
years months						
0–0 to 0–1	23	0.2	0.3	0.4	0.9	1.4
0–1 to 0–2	31	0.4	0.6	1.0	2.1	6.4
0–2 to 0–3	34	0.5	1.0	2.2	4.2	5.9
0–3 to 0–4	37	0.6	1.8	4.0	5.8	10.4
0–4 to 0–5	36	0.9	3.5	5.6	7.6	15.1
0–5 to 0–6	37	3.4	5.0	6.9	9.5	20.6
0–6 to 0–9	40	3.5	7.0	9.8	12.3	17.8
0–9 to 1–0	41	2.8	8.4	10.8	14.7	24.0
1–0 to 1–3	42	3.4	7.0	9.6	13.0	19.0
1–3 to 1–6	40	2.4	5.9	8.2	11.5	19.7
1–6 to 1–9	37	2.3	5.1	7.3	10.3	20.5
1–9 to 2–0	36	3.4	4.7	6.7	9.3	15.9
2–0 to 2–3	36	3.4	4.5	6.2	8.4	16.1
2–3 to 2–6	37	3.3	4.5	5.9	7.8	12.0
2–6 to 2–9	34	2.1	4.6	5.8	7.3	11.9
2–9 to 3–0	33	3.0	4.6	5.7	6.9	9.5
3–0 to 3–3	31	3.6	4.7	5.7	6.8	13.1
3–3 to 3–6	29	3.3	4.9	5.7	6.7	11.2
3–6 to 3–9	27	3.3	5.0	5.8	6.8	8.9
3–9 to 4–0	25	3.6	5.1	5.9	6.9	8.2
4–0 to 4–3	26	3.5	5.2	6.1	7.1	10.8
4–3 to 4–6	24	2.8	5.3	6.2	7.3	9.6
4–6 to 4–9	24	3.6	5.4	6.4	7.4	11.1
4–9 to 5–0	24	4.0	5.5	6.6	7.6	9.2

percentiles rise in the first year. During the period when protein levels maintain a plateau and calcium levels decrease markedly, phosphorus follows an intermediate course, then increases as do the other two nutrients after three to 4 years of age.

While the intakes of the nutrients thus far presented are largely a reflection of voluntary consumption by the children, the iron levels during the first two and one-half years are affected by the large amount of iron added to the specially prepared baby foods which are commonly fed to the infants in this series. This high iron content, particularly of the cereals, results in an intake which rises sharply from the

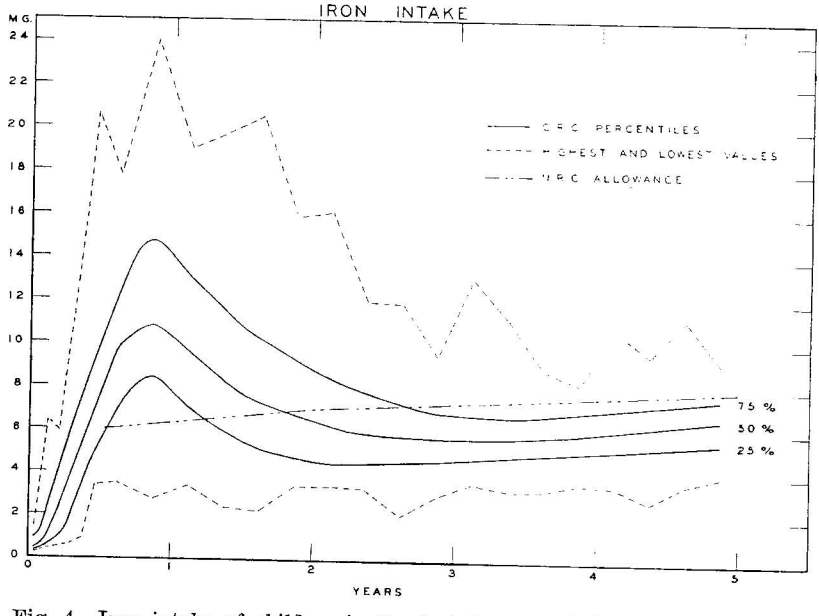

Fig. 4 Iron intake of children in the first 5 years of life, showing smoothed 25th, 50th and 75th percentiles and observed minimum and maximum values as contrasted with the Recommended Dietary Allowance.

time of introduction of solids into the diet to a peak at about one year of age, after which there is a decline as these foods are replaced by foods prepared at home. Thus, as may be seen in table 2, the median iron intake, which reaches a peak of more than 10 mg daily by one year, drops in the next 18 months to a level under 6 mg daily. These percentile values were determined with the exclusion of 4 instances in which high-iron formulas were given.

After two and one-half years of age, more than 75% of the children in this group consume an amount of iron which is less than the Recommended Allowance of the National Research Council (fig. 4). There is evidence from recent balance studies, some using radioactive iron, that absorption of iron from food is relatively small (Macy, '42; Moore and Dubach, '52) and that the dietary requirements of iron for children need review (Johnston, '53; Darby et al., '47). The fact that the children in this series have, on the whole, satisfactory levels of hemoglobin and erythrocytes (Meyers, unpublished data) leads one to believe that these levels of iron intake are adequate to meet their needs.

SUMMARY

Data have been presented from 795 nutrition histories on 58 children in the first 5 years of life. Calcium, phosphorus and iron intakes have been computed in terms of quartiles and maximum and minimum levels observed. In addition, some of the individual patterns of calcium intake have been shown.

Intake of calcium rises rapidly in the first 6 months, less rapidly between 6 and 9 months, then decreases to a lower level between two and three years, when the median calcium level is 0.75 gm and the median milk intake is 16 ounces. This is followed by an increase in milk and in total calcium. There is a sex difference in calcium intake between 6 and 15 months, with the boys reaching a higher level than the girls and maintaining that level for a longer period of time.

Phosphorus intake increases during the first year, then shows a pattern intermediate between the stationary intake of protein and the markedly decreased intake of calcium in the early pre-school years, increasing again between three and 4 years.

The sharp rise of iron intake during the first year, due primarily to the high iron content of commercially prepared infant cereals, is followed by a decrease as these foods are replaced in the diet. After three years, levels of iron intake

increase, but from two and one-half years to 5 years more than 75% of the intakes remain below the Recommended Allowance of the National Research Council.

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INFLUENCE OF ARSANILIC ACID ON DIETARY REQUIREMENT OF CHICKS FOR CERTAIN UNIDENTIFIED GROWTH FACTORS [1, 2]

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Previous reports clearly indicate that at least two different unidentified growth factors are required by poultry for rapid growth (Arscott and Combs, '50; Kohler and Graham, '51; Menge et al., '52; Couch et al., '52; Scott and Jensen, '52; McGinnis et al., '53; and Combs et al., '54). One of these factors is present in various liver and fish products while the other is supplied by dried whey products, dried brewers' yeast, distillers' solubles and certain fermentation products. The sparing effect of orally administered antibiotics on the dietary requirement for these unidentified growth factors also has been reported (Groschke, '50; Jones and Combs, '51; Heuser and Norris, '51; Scott and Jensen, '52; and Combs et al., '54).

Morehouse and Mayfield ('46), Morehouse ('49), Bird et al. ('49) and Combs and Laurent ('53) have shown that orally administered 3-nitro-4-hydrophenylarsonic acid stimulates growth of chicks. Arsanilic acid (p-aminophenylarsonic acid) also has been reported to improve chick growth (Elam et al., '53). It is believed that antibiotics exert their effect on chick growth, in part at least, through an alteration of the intestinal microflora. Limited observations indicate that

[1] Scientific paper no. A 455, Contribution no. 2527 of the Maryland Agricultural Experiment Station (Department of Poultry Husbandry).

[2] This investigation was supported in part by a research grant from Abbott Laboratories, North Chicago, Illinois.

arsanilic acid also influences, to a certain extent, the microbial population within the intestinal tract of chicks and poults (Anderson et al., '52; Elam et al., '53).

In view of the possible similarity in action of arsenicals and antibiotics, experiments were conducted to determine if orally administered arsanilic acid would influence the dietary requirement of the chick for the unidentified growth factors present in fish products and dried whey.

EXPERIMENTAL

This report includes the results of two separate chick experiments, each composed of 14 groups of sexed chicks maintained in floor pen units with litter. Each experimental group consisted of 100 male and 100 female New Hampshire chicks. At the start of each experiment, 25 males and 25 females in each group were wingbanded at random. Individual weights were obtained at the end of each experiment for the surviving banded chicks and total weights were taken en masse by sexes. Experiments I and II were terminated after 8 and 8.5 weeks, respectively. Since the average weights of all chicks obtained by mass weighing and the corresponding average weights of the prebanded chicks obtained from individual weights were in excellent agreement for the various groups in both experiments, the average individual weights only are shown in the tables. Accordingly, these data are based on the average individual weights of the first 20 prebanded males and the first 20 prebanded females per group in experiment I, and the first 22 prebanded males and the first 22 prebanded females per group in experiment II. The use of these individual weights made statistical analysis possible. The method of analysis of variance was applied to the individual weights in both experiments. In the calculation of the feed required per unit of gain, the total mass weights were used with appropriate corrections made for mortality.

Three basal rations (A, B and C) were used in these experiments. The composition of the basal rations A and B is shown in table 1. Ration C differed from ration B only in

that it contained 2.5% corn fermentation solubles in place of an equal amount of corn. These rations are adequate in all known nutrients required by the chick, based on calculated analysis. Furthermore, in previous studies involving similar basal rations, no growth response in chicks has been obtained

TABLE 1

Composition of basal rations

INGREDIENT	RATION A (Exp. 1)	RATION B (Exp. 2)
	%	%
Ground yellow corn	57.45	60.45
Soybean oil meal,, 44% protein	32.5	32.5
Dhy. alfalfa meal, 17% protein	2.5	2.5
Butyl molasses fermentation solubles (250 μg riboflavin/gram)	1.0	..
Dried whey, 65% lactose	2.0	..
Limestone	1.0	1.0
Bone meal, steamed	2.75	2.75
Salt, iodized	0.3	0.3
Manganese sulfate, 65% tech. grade	0.025	0.025
Vitamin A and D feeding oil (300 I.C.U. of vit. D and 2250 I.U. of vit. A/gm)	0.2	0.2
"D"-activated animal sterols (1500 I.C.U. of vita. D/gm)	0.025	0.025
Choline chloride, 25% mixture	0.05	0.05
Vitamin B_{12} and antibiotic supplement (3 mg of vit. B_{12} and 2 gm procaine penicillin/lb.)	0.1	0.1
DL-methionine	0.05	0.05
Nitrofurazone, 11.2% mixture	0.05	0.05
	mg/lb.	*mg/lb.*
Riboflavin	1.5	2.5
Calcium pantothenate	2.0	3.0
Niacin	15.0	15.0

from the inclusion of additional amounts of known required nutrients in their diet. When supplements were added, adjustments in the basal rations were made as required to maintain comparable levels of calcium, phosphorus and protein. Dried whey (65% lactose) or the arsanilic acid supplement (Pro-Gen, containing 20% arsanilic acid) replaced an

equal amount of corn. The 2% condensed fish solubles supplement replaced 1.5% soybean oil meal and 0.5% corn in the rations. In experiment I, when 5% fish meal was fed, 7.5% soybean oil meal and 1.25% steamed bone meal were removed from basal ration A, while 0.25% limestone and 3.5% additional corn were added. When 2.5% of the dried fish solubles product was added to basal ration A, 2.5% soybean oil meal, 0.5% limestone and 0.25% steamed bone meal were removed from the ration and 0.75% additional corn was added. The fish solubles used in the preparation of the dried fish solubles product was obtained from the same lot of the condensed fish solubles used in experiment I and was dried on crab meal. The resulting dried product contained approximately 40% fish solubles and 60% crab meal on a solids basis.

RESULTS

The results obtained in experiment I are shown in table 2. The addition of 5% fish meal, 2.5% dried fish solubles product, 2% condensed fish solubles or 5% fish meal plus 2% condensed fish solubles failed to stimulate significantly the growth of chicks in the absence of arsanilic acid. These supplements, however, when fed in the presence of arsanilic acid at either level used, significantly improved the average 8-week weights. Improvement in feed efficiency was consistently obtained when either of these supplements was added to the ration but the improvement was decidedly greater in the presence of arsanilic acid. The addition of arsanilic acid at levels of either 90 or 120 p.p.m. failed to improve the growth rate when supplied in the unsupplemented basal ration A. On the other hand, consistent, statistically significant responses were noted when arsanilic acid was added at either level to the rations containing a fish product. Likewise, improvement in feed efficiency was noted when arsanilic acid was added to diets containing a fish product, while little or no improvement was noted in the absence of a "fish factor" supplement. Basal ration A contained a source of the "whey factor." Accordingly, the results of this experiment indicate that the addition

TABLE 2

Effect of arsanilic acid and fish products on growth and feed efficiency of growing chickens (exp. 1)

GP. NO.	SUPPLEMENT TO RATION A	AV. 8-WK. WT., BOTH SEXES [1]	CHANGE IN WT. DUE TO		FEED/ UNIT GAIN	CHANGE IN FEED/ UNIT GAIN DUE TO	
			Fish product [2]	Arsanilic Acid [3]		Fish product	Arsanilic acid
		gm	*gm*	*gm*	*gm*	*gm*	
		No arsanilic acid series					
1	None	942	..	..	2.66	..	..
2	5% fish meal	936	— 6	..	2.51	—.15	..
3	2.5% dried fish sol. product	956	+ 14	..	2.37	—.29	..
4	2% condensed fish solubles	977	+ 35	..	2.45	—.21	..
5	as 2 + 4	988	+ 46	..	2.48	—.18	..
		90 p.p.m. arsanilic acid series					
6	None	928	..	— 14	2.72	..	+ .06
7	5% fish meal	1001	+ 73	+ 65	2.24	—.48	—.27
8	2.5% dried fish sol. product	989	+ 61	+ 33	2.34	—.38	—.03
9	2% condensed fish solubles	1042	+ 114	+ 65	2.38	—.34	—.07
		120 p.p.m. arsanilic acid series					
10	None	918	..	— 24	2.57	..	—.09
11	5% fish meal	977	+ 59	+ 41	2.24	—.33	—.27
12	2.5% dried fish sol. product	1004	+ 86	+ 48	2.30	—.27	—.07
13	2% condensed fish solubles	1057	+ 139	+ 80	2.11	—.47	—.34
14	as 11 + 13	1020	+ 102	+ 32	2.27	—.30	—.21

[1] Averages based on individual weights of 20 males and 20 females per treatment.

[2] Not statistically significant when no arsanilic acid was fed; in presence of arsanilic acid (both levels combined), the response from fish meal supplementation was statistically significant to the 5% level and that from condensed fish solubles or dried fish solubles product supplementation was statistically significant to the 1% level.

[3] Differences not statistically significant when diet contained no fish product; however, the average response obtained with either level of arsanilic acid was statistically significant to the 1% level when the fish products (all groups combined) were included in the diet.

of arsanilic acid increased the need for a supplemental source of the unidentified growth factor supplied by each of the fish products used. At the same time, the response obtained from the addition of arsanilic acid appeared to be dependent, to a large extent, on the adequacy of the ration with respect to this unidentified growth factor.

TABLE 3

Response of chicks to dried whey and arsanilic acid, fed as supplements alone and in combination (exp. 2)

SUPPLEMENT	RATION C SERIES	RATION C + 2% FISH SOL. SERIES	AV. OF BOTH SERIES	AV. INCREASE DUE TO TREATMENT
	Av. 8.5-wk. weight, both sexes [1]			
	gm	*gm*	*gm*	*gm*
None	1100	1132	1116	. .
90 p.p.m. arsanilic acid	1082	1149	1116	± 0.0
2.5% dried whey	1131	1167	1149	+ 33
2.5% dried whey + 90 p.p.m. arsanilic acid	1207	1256	1232 [2]	+ 116
Average	1130	1176		
	Feed required/unit gain			
	gm	*gm*	*gm*	*gm*
None	2.64	2.68	2.66	. .
90 p.p.m. arsanilic acid	2.68	2.59	2.64	— .02
2.5% dried whey	2.68	2.50	2.59	— .07
2.5% dried whey + 90 p.p.m. arsanilic acid	2.67	2.62	2.65	— .01
Average	2.67	2.60		

[1] Averages based on the individual weights of 22 males and 22 females per treatment.

[2] The difference between this value and each of the other corresponding values is statistically significant to the 1% level.

Part of the results obtained in experiment II are shown in tables 3 and 4. The influence of arsanilic acid on the growth response of chicks to dried whey is shown in table 3. When 2.5% dried whey was added to ration C or to ration C modified to contain 2% fish solubles, the improvement in

growth obtained was not statistically significant. However, the addition of dried whey to these rations in the presence of 90 p.p.m. arsanilic acid resulted in a highly significant growth response. Likewise, the addition of 90 p.p.m. of arsanilic acid to the rations containing no whey failed to improve the growth rate but significantly improved it when 2.5% dried whey was present in the ration. No appreciable influence on feed efficiency was noted. These results indicate that arsanilic acid supplementation of the ration also increases the chicks' requirement for the unidentified factor present in dried whey. The data also suggest that the ration should

TABLE 4

Response of chicks to the addition of condensed fish solubles and dried whey to their diet, in the presence of arsanilic acid (exp. 2)

SUPPLEMENT	RATION B [1] SERIES	RATION C [1] SERIES	AV. OF BOTH SERIES	AV. CHANGE DUE TO TREATMENT [2]
	Av. 8.5-wk. weight,[3] both sexes			
	gm	*gm*	*gm*	*gm*
None	1061	1082	1072	. .
2.5% dried whey	1158	1207	1183	+ 111
2% condensed fish sol.	1202	1149	1176	+ 104
2% cond. fish sol. + 2.5% dried whey	1254	1256	1255	+ 183
	Feed required/unit gain			
	gm	*gm*	*gm*	*gm*
None	2.68	2.68	2.68	. .
2.5% dried whey	2.60	2.60	2.60	— .08
2% condensed fish sol.	2.59	2.59	2.59	— .09
2% cond. fish sol. + 2.5% dried whey	2.51	2.62	2.56	— .12

[1] Modified to contain 90 p.p.m. arsanilic acid.

[2] Average changes in body weight due to the addition of either dried whey or condensed fish solubles to the diet are statistically significant to the 1% level. When both were added, the further increase in growth also is statistically significant to the 1% level.

[3] Averages based on individual weights of 22 males and 22 females per treatment.

be adequate in the "whey factor" in order to permit maximum growth response from orally administered arsanilic acid.

The responses obtained from the addition of dried whey and condensed fish solubles in the presence of 90 p.p.m. arsanilic acid (experiment II) are shown in table 4. Significant

TABLE 5

Response of chicks to condensed fish solubles and arsanilic acid as supplements, alone and in combination

SUPPLEMENT	RATION A SERIES (EXP. 1)	RATION B SERIES (EXP. 2)	RATION C SERIES (EXP. 2)	AV. OF THREE SERIES	AV. CHANGE DUE TO TREATMENT
		Av. weight, both sexes,[1] *gm*			
None	942	1153	1100	1065	..
90 p.p.m. arsanilic acid	928	1061	1082	1024[2]	— 41
2% condensed fish solubles	977	1170	1132	1093	+ 28
2% cond. fish sol. + 90 p.p.m. arsanilic acid	1042	1202	1149	1131[3]	+ 66
		Feed required/unit gain			
None	2.66	2.58	2.64	2.63	..
90 p.p.m. arsanilic acid	2.72	2.68	2.68	2.69	+ .06
2% condensed fish solubles	2.45	2.54	2.68	2.56	— .07
2% cond. fish sol. + 90 p.p.m. arsanilic acid	2.38	2.59	2.59	2.52	— .11

[1] For experiment 1, averages based on 8-week individual weights of 20 males and 20 females per treatment; for experiment 2, averages based on 8.5-week individual weights of 22 males and 22 females per treatment.

[2] The difference between this value and the corresponding value obtained for the unsupplemented groups is statistically significant to the 5% level.

[3] The difference between this value and the corresponding value obtained for the unsupplemented groups is statistically significant to the 1% level.

responses were obtained when either 2.5% dried whey or 2% condensed fish solubles was added to basal rations B and C. When sources of both the "whey factor" and the "fish factor" were added a further improvement in growth was obtained which is statistically significant. Similarly, improvements in feed efficiency were noted when these unidentified factor supplements were supplied.

Results obtained in both experiments pertaining to the supplemental effects of arsanilic acid and condensed fish solubles are presented in table 5. The combined data confirm the observations made in experiment I, in that the addition of arsanilic acid increased the requirement for the unidentified factor present in fish solubles. No growth response was obtained from the addition of 2% condensed fish solubles in the absence of arsanilic acid, while highly significant responses were obtained from the addition of this supplement in the presence of arsanilic acid. The addition of arsanilic acid to basal rations A, B and C containing no source of the "fish factor" not only failed to improve growth but actually reduced the growth rate. When all three series are considered together, this growth depression is statistically significant. On the other hand, the addition of arsanilic acid to rations containing an adequate source of the "fish factor" further improved the growth rate. The amount of feed required per unit of gain for these groups also revealed the mutually supplemental effects of arsanilic acid and condensed fish solubles.

DISCUSSION

The lack of significant growth responses from the addition of fish products or dried whey to the different basal rations used in the present study may well be attributed to the presence of the antibiotic (procaine penicillin). Other work, cited above, has demonstrated the sparing effect of certain antibiotics, including penicillin, on the dietary requirement of the chick for both the "fish factor" and the "whey factor." This effect presumably occurs primarily through increased microbial synthesis or decreased bacterial utilization or both, or by destruction of these growth factors when the antibiotics are fed.

It is of special significance, then, that the addition of arsanilic acid to these rations containing procaine penicillin significantly increased the chick growth response to the unidentified growth factor supplements. Since arsonic acid

compounds also seem to exert an effect on the microbial population of the intestinal tract, it appears that the increased dietary requirement for the unidentified growth factors present in fish products may have been due to a partial suppression of bacterial synthesis of the "fish factor" by arsanilic acid. This interpretation may not explain why arsanilic acid increased the chick's response to "whey factor" supplementation, since the addition of arsanilic acid to the basal ration containing no supplemental source of the "whey factor" did not decrease the growth rate.

Griminger et al. ('53) and Sweet et al. ('54) found that arsanilic acid supplementation tended to increase the dietary need of chicks for vitamin K, presumably through reduced bacterial synthesis. Moreover, Bird et al. (49) reported that 3-nitro-4-hydroxyphenylarsonic acid did not effectively spare the requirement for fish meal in a vitamin B_{12}-deficient chick ration, but rather these additions were mutually supplemental. Similarly, Lih and Baumann ('51) found 3-nitro-4-hydroxyphenylarsonic ineffective in sparing the dietary requirement of rats for either thiamin or riboflavin, while certain antibiotics were effective. The observations are similar to those made with the unidentified growth factors in this study.

The fact that the addition of arsanilic acid to the rations containing sources of both the whey and fish factors further improved the growth rate of chicks, shows that this compound may also exert a beneficial effect on chick growth, even in the presence of procaine penicillin. This is presumed to be due to its effect on the microbial population in the intestinal tract. It appears important, however, that rations which are to be supplemented with arsenicals should be entirely adequate, at least in respect to the unidentified growth factors studied, for best results.

SUMMARY

The results of two experiments show that orally administered arsanilic acid increased the dietary requirement of

chicks for the unidentified growth factor present in fish products and dried whey. The growth response of chicks to these unidentified factors was significantly greater in the presence of arsanilic acid.

Arsanilic acid supplementation also has been shown to increase significantly the growth rate of chicks fed complete practical type rations containing an antibiotic and sources of unidentified growth factors. No growth response was obtained from arsanilic acid supplementation when the rations contained no additional source of these unidentified factors.

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ABSORPTION OF CARBOHYDRATE AND PROTEIN AS AFFECTED BY FEEDING CORNSTARCH, BANANA, OR GLUCOSE [1,2]

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The protein-sparing effect of carbohydrate is well known and was recently reviewed by Munro ('51). The addition of extra energy, either as fat or carbohydrate, to submaintenance diets, providing adequate amounts of protein, causes proportional improvement in nitrogen balance (Munro and Naismith, '53). The maximum sparing effect is manifested when the protein and carbohydrate are fed at the same time (Larson and Chaikoff; '50 Cuthbertson et al., '40; Geiger et al., '50).

Siliciano and Nasset ('53) found that rats fed a diet in which the protein was derived from casein and dried banana utilized the absorbed nitrogen more efficiently than those on the control diet, containing only casein as a source of protein. Harper and Katayama ('53) reported that rats on a 9% casein ration grew significantly better when the dietary source of carbohydrate was cornstarch rather than sucrose. Monson et al. ('50) found that rations containing dextrin pass through the digestive tract of chicks more slowly than those containing sucrose or lactose. These investigations support the idea that the protein-sparing action of carbohydrates is not constant for all conditions.

[1] The data in this paper were taken from a thesis submitted by Miss Lorraine Dreisbach to the Graduate School of the University of Rochester in partial fulfillment of the requirements for the degree Master of Science, June 1953.

[2] Supported by a grant from Tropic Foods, Inc.

The purpose of the present investigation was to compare the simultaneous absorption of dietary protein with three different sources of dietary carbohydrate. Cornstarch was chosen to represent the carbohydrates which require hydrolysis prior to absorption, and glucose to represent those that are absorbed without hydrolysis. Banana powder represents a source of carbohydrate intermediate in this respect because it requires partial hydrolysis before absorption. Each of these carbohydrates was fed as part of a normal diet, and the simultaneous absorption of carbohydrate and protein was observed over a period of 5 hours.

METHODS

Adult female albino rats 175 to 200 gm in weight of the Wistar strain from the department colony were used in all of the experiments. They were fed the stock diet until 5 days before the experiment began. Three of the 4 diets used contained 58% carbohydrate, 18% protein, 20% fat, 4% salt mixture and a vitamin supplement (see table 1). Diet IV was devoid of protein (0.04% N) and contained approximately 78% of cornstarch. The composition of each diet is given in table 1. The important variant in diets I, II, and III was the type of carbohydrate.

For purposes of adaptation the experimental diets were fed for 5 days prior to an experiment. In order to eliminate all food from the gastrointestinal tract the animals were fasted 48 hours. At the end of this fast the animals received a weighed quantity of the diet (1.50 to 2.50 gm) most of which was promptly eaten. The actual amount ingested was determined by difference. At intervals of 2, 3, 4, and 5 hours after feeding, two rats were sacrificed. This procedure was repeated three times for each diet, thus providing the results from 6 rats for each time interval. Dial-urethane [3] anesthesia was used, the cardia and ileocecal junction were ligated, the section between these ligatures removed, slit open, and the

[3] Solution of Dial-urethane generously supplied by the Ciba Pharmaceutical Products, Inc., through the courtesy of Dr. E. Oppenheimer.

contents washed out with approximately 200 ml of distilled water. This material was homogenized and made to a volume of 250.0 ml. Aliquot portions were analyzed for reducing sugar and total nitrogen.

Reducing sugar was determined by Somogyi's method ('45a, '45b) before and after acid hydrolysis of the intestinal contents. Undigested carbohydrate was hydrolyzed by boiling a 25.0 ml portion with 5.0 ml of HCl, sp. gr. 1.125, at

TABLE 1

Composition of diets

	I	II	III	IV
	gm	*gm*	*gm*	*gm*
Cornstarch	1286	....	....	84
Banana powder [1]	....	1380	....	..
Glucose	....	....	1100	..
Wesson oil	400	400	400	20
Casein	389	291	389	..
Salt mixture	80	80	80	4
Vitamin supplement [2]				

[1] Meloripe Banana Powder, Food Concentrates Inc., N. Y.

[2] Composed of the following per kilogram of diet: thiamine hydrochloride, 10 mg; riboflavin, 20 mg; niacinamide, 50 mg; folic acid, 1 mg; calcium pantothenate, 50 mg; pyridoxine hydrochloride, 10 mg; inositol, 250 mg; choline chloride, 2.0 mg; vitamin A, 6,500 I.U.; vitamin D, 1,500 I.U.; alpha tocopherol, 50 mg; methylnaphthoquinone, 15 mg.

atmospheric pressure under a reflux condenser for two and one-half hours. Nitrogen determinations were made with the Scales and Harrison ('20) modification of the Kjeldahl method. Intestinal contents, recovered under these conditions, contain, in addition to the unabsorbed protein of the food, other nitrogenous substances released in the secretions in response to the stimulus of food. In an attempt to assess the magnitude of this source of nitrogen, a series of observations was made on a group of rats receiving the nitrogen-free test meal (diet IV). Two of these rats were sacrificed at each

of the prescribed time intervals, and the total nitrogen of the alimentary tract contents determined. Three rats sacrificed after a 48-hour fast contained no reducing substances in their gastro-intestinal tracts.

RESULTS

Certain experimental and computed data are summarized in table 2. Each figure in this table is the arithmetic mean of 6 individual experimental results, excepting the third hour, diet III, and the 4th hour, diet II in each of which 5 animals were used. Diet I was the first to be fed and as the result of trial and error the mean size of the test meal turned out to be larger than for diets II and III. On this account the results have been expressed on a percentage basis as well as in absolute terms.

The data dealing with carbohydrate are relatively uncomplicated because little or no reducing substance is produced in the gastrointestinal tract. The results show that the percentage absorption of carbohydrate from starch is always less than it is from either banana or glucose, which, on the same basis, are nearly identical in the first and last periods.

The absorption of ingested protein is not so readily ascertained. The results in table 2 demonstrate that, in every experiment but one (diet I, 4th hour), over a period of two to 5 hours after feeding, the quantity of protein recovered from the gastrointestinal tract is greater than the amount ingested. The quantity of protein recovered was "corrected" by substracting the amount of protein present after feeding a non-protein test meal (see methods). The mean values for these experiments were, 0.29, 0.29, 0.26, and 0.16 gm protein for hours 2, 3, 4, and 5, respectively. The protein "absorbed" is obtained by subtracting the "corrected" recovered protein from the ingested protein. These computations are made with the full realization that protein absorption is not likely to be adequately represented by such a simple procedure.

TABLE 2

Absorption of carbohydrate and protein from test meals differing in type of carbohydrate

DIET [1]	HOURS AFTER FEEDING											
	2			3			4			5		
	I	II	III	I	II	III	I	II	III	I	II	III
Carbohydrate fed, gm	1.76	0.81	0.71	1.76	0.83	0.67	1.77	1.02	0.71	1.57	1.01	0.70
Carbohydrate absorbed, gm	0.47	0.55	0.42	0.78	0.61	0.40	0.90	0.81	0.53	0.92	0.93	0.63
Carbohydrate absorbed, %	27	69	64	45	74	67	55	80	75	59	93	92
Protein:												
Fed, gm	0.54	0.25	0.23	0.54	0.28	0.22	0.54	0.31	0.24	0.46	0.31	0.23
Recovered, gm	0.71	0.44	0.38	0.57	0.36	0.33	0.46	0.37	0.30	0.52	0.36	0.28
Recovered, %	121	188	171	116	128	181	88	125	131	118	116	124
Recovered "corrected," [2] gm	0.43	0.15	0.07	0.28	0.10	0.06	0.20	0.11	0.04	0.37	0.21	0.12
"Absorbed," [3] gm	0.12	0.09	0.14	0.25	0.19	0.16	0.34	0.21	0.20	0.12	0.11	0.11
"Absorbed," %	23	36	64	45	65	77	67	54	85	22	39	49

[1] I Dietary carbohydrate was starch.
II Dietary carbohydrate was supplied by banana.
III Dietary carbohydrate was glucose.

[2] Corrected for amount of protein recovered after feeding non-protein test meal.

[3] Protein "absorbed" = Protein fed- "corrected" protein recovered.[2]

DISCUSSION

More carbohydrate was ingested on diet I (starch) than on either of the other two but the maximum rate of absorption, 260 mg per hour, was not attained until the third hour. This result is not inconsistent with the fact that absorption of starch is dependent on, first, the secretion of amylase, and second, the hydrolysis of starch by amylase. As expected the carbohydrates of diets II and III, which contain monosaccharides, were absorbed at maximum rate at two hours. In the third, 4th, and 5th hours the rates of absorption diminished by approximately 30%. The rate of starch absorption declined to 225 mg per hour in the 4th hour and in the 5th hour it dropped still further and equaled the rate for diet II (banana), i.e. 185 mg/hour. The percentage carbohydrate absorption from diet I (starch) was always significantly less ($P < 0.01$) than the absorption of glucose or banana carbohydrate.

The source of carbohydrate in diet II was dried banana, which contains a mixture of reducing sugars, sucrose, starch and other complex carbohydrates (Von Loesecke, '49). The maximum rate of absorption of banana carbohydrate was attained in the second hour and is the highest in the entire series of experiments. The percentage of carbohydrate absorbed from banana (diet II) was always significantly greater than from starch diet I ($P < 0.01$) but it was greater than the absorption of glucose only in the third and 4th hours ($P < 0.01$). The absolute amount of carbohydrate absorbed was greatest with diet I (starch) in hours three and 4, but this result is probably due to the fact that the greatest amount of carbohydrate was ingested in these experiments.

Glucose was absorbed at the greatest rate during the first two hours (210 mg/hr.). It declined by 32% in hours three and 4, and by 40% in the 5th hour, when absorption was virtually complete. In percentage absorption the values for glucose consistently lie between those for starch and banana.

The data concerning absorption of protein digestion products suggest some interesting ideas. The fact that slightly

more protein is recovered than is fed, even in the 5th hour, suggests that the gastrointestinal tract must "turn over" somewhat more endogenous protein than is ingested. The quantities of protein ingested represent approximately one-tenth to one-sixth of the rat's protein intake when eating a normal diet ad libitum. It seems as if the "corrected" recovery of protein from the gastrointestinal tract, as entered in table 2, does not truly represent the difference between protein ingested and protein absorbed. In view of the secretagogue action of dietary protein it is fair to assume that the secretion of nitrogenous substances into the gastrointestinal tract is greater, especially in the 4th and 5th hours, than is indicated by the "blank" experiments in which non-protein test meals were fed. Protein evidently begets protein in the intestinal lumen and one is led to speculate as to when this autocatalytic process stops, if it does, and why. At any rate it is evident that a relatively large amount of protein must be synthesized, secreted and presumably recovered by the gastrointestinal tract in the process of digesting and absorbing a mixed meal. The most likely sources of these proteins are the numerous hydrolytic enzymes, as well as the mucoproteins present in the digestive juices. Nasset et al. (unpublished) have shown that the degradation of these endogenous proteins tends to maintain an amino acid mixture of constant composition in the intestinal lumen regardless of the type of meal ingested.

The great capacity of the gut wall to synthesize protein was well demonstrated by the work of Schoenheimer et al. ('39). They fed rats a normal diet supplemented with N^{15}-labeled leucine. At the end of three days the plasma proteins contained the greatest concentration of N^{15} and the gut wall was next with 90% as much as plasma and 59% more than liver.

The data in table 2 indicate that total protein "absorbed" increases with time except in the 5th hour. The rate of protein absorption is at the maximum for diet III (glucose) in the second hour, for diet II (banana) in the third hour, and

for diet III (starch) in the 4th hour. If these maxima prevailed to the end, the ingested protein would have been completely absorbed in the third hour for diet III, in the 4th hour for diet II, and in the 5th hour for diet I. Actually of course the "absorbed" protein, as indicated in table 2, is no greater at the end of the 5th hour than at the end of the second.

The superior protein-sparing effect of starch may be attributed to the fact that the carbohydrate/protein ratio in the gastrointestinal contents is consistently higher for diet I (starch) than for either diet II (banana) or diet III (glucose). With starch this ratio is always more than twice as great as for the other carbohydrates. This indicates that in the presence of starch a much larger quantity of carbohydrate is available for absorption simultaneously with protein. The carbohydrate/protein ratios determined by chemical analyses of diets I, II, and III as mixed were 3.3, 3.2, and 3.0, respectively. In the chymes derived from feeding these diets the carbohydrats/protein ratios were: 1.8, 1.7, 1.9, and 1.3 for diet I; 0.6, 0.6, 0.6, and 0.2 for diet II; and 0.8, 0.8, 0.6, and 0.3 for diet III, in hours 2, 3, 4, and 5 respectively. All samples of chyme were relatively impoverished in carbohydrate as compared with the feed but those obtained with diet I were much less so than either of the others.

SUMMARY

Rats were fed test meals of three diets which differed only in the type of carbohydrate, namely, cornstarch, dried banana, or glucose. Animals were sacrificed 2, 3, 4, and 5 hours post cibum and the contents of the gastrointestinal tract analyzed for carbohydrate and protein.

The carbohydrates of banana and glucose were absorbed at maximum rates in the second hour but starch absorption did not reach maximum values until the third hour. In both the third and 4th hour the absolute amount of starch absorbed was greater than either banana carbohydrate or glucose, but, expressed in per cent, starch absorption was always signifi-

cantly less than that of the carbohydrate of banana or of glucose.

The amount of nitrogenous material recovered from the gastrointestinal tract was always equal to or greater than the amount of protein ingested. This "turn over" of protein indicates a high degree of activity in the synthesis, secretion, degradation, and recovery of digestive enzymes, mucoproteins and other nitrogenous compounds.

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PARTIAL STARVATION AND ALCOHOL METABOLISM

AN EXAMPLE OF ADAPTATION TO UNDERNUTRITION [1]

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THREE FIGURES

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Previous studies in this laboratory have demonstrated that fasting markedly decreased the rate of alcohol oxidation by the intact rat as well as the ability of liver homogenates to oxidize alcohol *in vitro* (Vitale et al., '53). This effect was interpreted as reflecting a loss of liver enzymes (protein) required for the oxidation of alcohol. In somewhat similar studies upon the respiration of the intestinal mucosa (Vitale et al., '53a), the oxygen uptake by this tissue was lowered by food restriction but could be raised to nearly normal levels by feeding a 60% protein diet despite maintenance of the same caloric restriction. This was also interpreted as evidence of decreased enzymatic activity due to protein loss and the restoration or prevention of this loss by high protein feeding.

It is rather well known that the liver is one of the organs which promptly loses protein as the result of caloric or protein restriction. Approximately 10 to 15% of the total liver nitrogen may be lost during the first day of fasting or when a nitrogen-free diet is fed (Addis, Poo and Lew, '36; Koster-

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litz, '47; Wang et al., '49). The results of Wang and his associates ('49) suggest, however, that even when a protein-free diet is fed there may be some improvement in liver function or adaptation if the regimen is continued. They found that after approximately 4 weeks the livers of animals receiving a nitrogen-free diet showed a gradual return toward normalcy as judged by liver composition, cytological structure and liver size. No functional or metabolic tests were done.

A continuation of the studies upon alcohol metabolism in this laboratory has given results which are apparently pertinent to the problem of "Adaptation to Undernutrition" which has been discussed by Mitchell ('44). The results are presented in this paper.

EXPERIMENTAL

Adult female rats which weighed approximately 250 gm were obtained from the Charles River Breeding Laboratories. A standard purified (20% protein) diet was used (Vitale et al., '53). The food intake of the diet when offered ad libitum was approximately 12 to 14 gm per rat per day. In one group of 6 animals, 10 gm of food was offered. When the weight loss had ceased (no significant weight loss over a 5-day period) the ration was decreased to 8 gm per day and finally to 6 gm at which level it was held with no further change. A second group was immediately changed from ad libitum feeding to 5 gm of food per day and was kept at this level throughout the experiment. For purposes of discussion the latter group is labelled as "severely restricted" while the first group will be called the "gradually restricted" group.

At various time intervals, animals from either group were injected with 20% ethanol containing ethanol-1-C^{14} at a level of 2.0 gm per kilogram body weight. They were immediately placed in a respirometer for the collection of respired CO_2 for a period of 6 hours. The methods for the collection and determination of the activity of the respired CO_2 have been described by Vitale et al. ('53).

RESULTS

The rate of alcohol oxidation decreased as the animals lost weight. In this laboratory normal rats given 2 gm of alcohol per kilogram body weight metabolize approximately 80% in a 6-hour period. In the gradually restricted group (fig. 1) there was a 12% decrease in the amount of alcohol metabolized

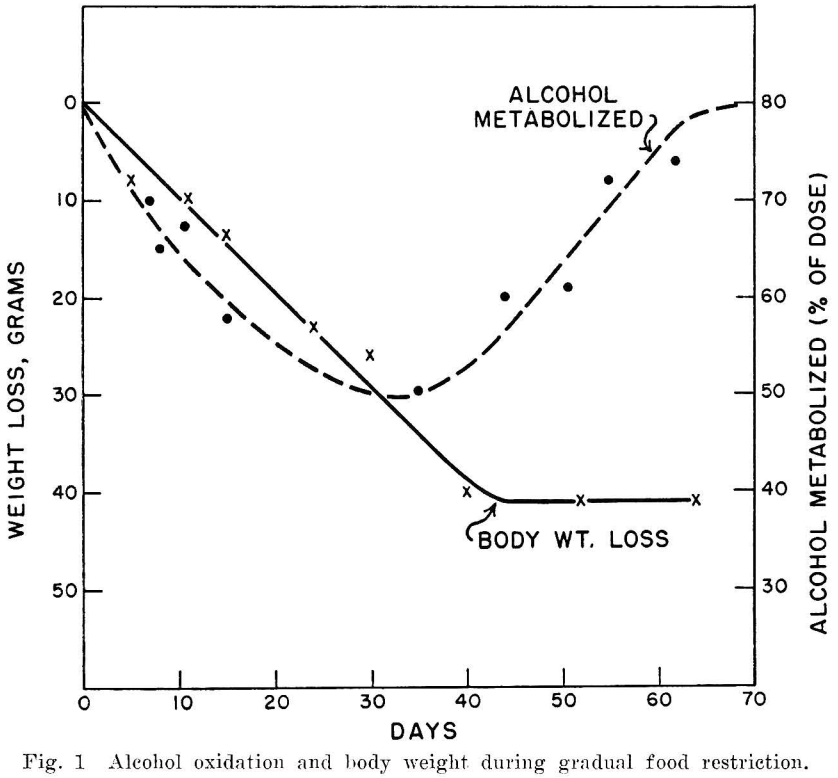

Fig. 1 Alcohol oxidation and body weight during gradual food restriction.

when the animals had lost 10 gm of body weight and a 40% decrease when they had lost 40 gm of body weight. With 6 gm of diet per day, their weight became stable at approximately 75 to 80% of their original body weight. The rate of alcohol metabolism returned to nearly normal. At the end of 50 days on restricted diet, approximately 72% of the administered alcohol was oxidized in the 6-hour period.

Increasing the rate of weight loss by more severe restriction accelerated the changes already observed (fig. 2). When the food intake was changed abruptly from ad libitum to 5 gm per day, the weight loss was more rapid, and stabilization at 70 to 80% of the original body weight occurred after

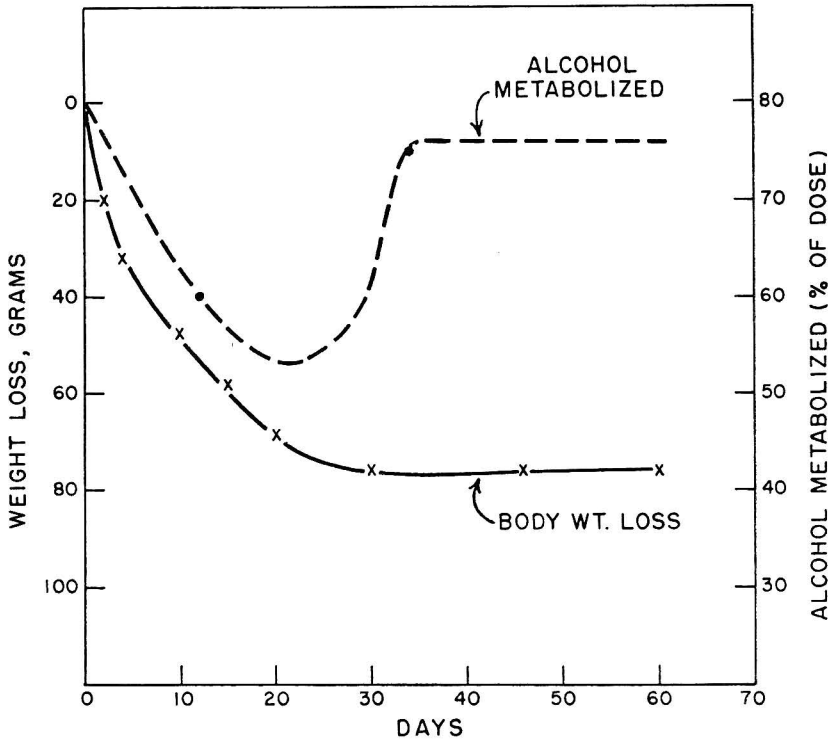

Fig. 2 Alcohol oxidation and body weight in animals fed 5 gm of food per day.

25 to 30 days. The amount of alcohol metabolized decreased as the animals lost weight but returned to nearly normal after body weight became stationary.

The metabolism of thiamine-deficient animals has been similarly studied. Figure 3 illustrates the effect of feeding a thiamine-deficient ration to adult animals. The amount of alcohol metabolized fell as the deficiency progressed and roughly paralleled the weight loss. However, in contrast to

the restricted groups, the weight of these animals never became stabilized and similarly the fall in alcohol oxidation continued until the death of the animals.

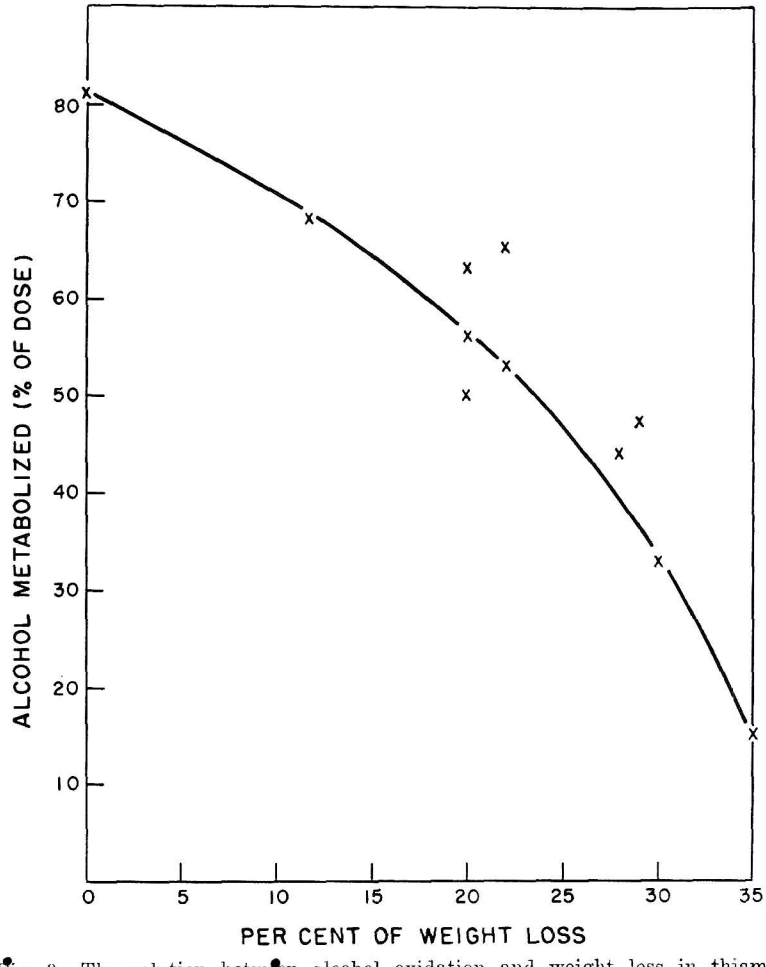

Fig. 3 The relation between alcohol oxidation and weight loss in thiamine-deficient animals.

DISCUSSION

When an organism is subjected to severe stress, it must either make certain adaptations or succumb to the stress. Adaptations to caloric restriction have been extensively reviewed by Keys et al. ('50). Undoubtedly the loss of weight

must be considered as one of the more effective adaptive mechanisms. The smaller body weight requires fewer calories for maintenance and the adaptation is made more or less in proportion to need; i.e., the more severely restricted animals lose weight most rapidly.

The liver responds rapidly to either protein or caloric restriction. Liver weight falls immediately after restriction is begun, but, as Wang et al. ('49) have shown in complete protein restrictions with ad libitum calorie feeding, the liver size reaches an apparent minimum. Further loss of body weight results in a return toward normal liver size in proportion to body weight. It is of considerable interest that the studies upon chemical composition and liver cytology showed that even though no protein was fed these animals, there appeared to be an improvement or a return toward normal as the deficiency was continued.

Studies upon the concentration of liver enzymes have shown that protein depletion causes marked losses in their activity (Miller, '50; Litwack et al., '50; Wanio et al., '53). The lability of various enzymes to protein depletion is not constant and some are more susceptible to depletion than others. Serial studies relating the losses to time have apparently not been done.

It is generally agreed that the principal site of alcohol oxidation is the liver (Jacobsen, '52). It is, therefore, likely that the liver is the limiting factor in these studies upon alcohol oxidation. The decrease in oxidation during weight loss could be related to both the enzyme concentration in the liver and the total liver size. It may be noted that the block in alcohol oxidation is somehow related to both the total weight loss and the rate of weight loss. In the gradually restricted animals the rate of weight loss was about uniform during the 40-day period, yet the ability to oxidize alcohol became progressively worse. In the severely restricted group the fall in weight occurred more rapidly but not to a much greater extent. In both cases, however, as soon as the animals were able to stabilize their weight, the ability to oxidize alcohol

returned rapidly toward normal. When weight is maintained, the animals are obviously in "balance" with respect to both calories and protein. Under such conditions the adaptive mechanisms are apparently such that the body proteins are redistributed to provide relatively normal proportions and enzyme levels.

The studies with the thiamine-deficient animals provide a demonstration of the difficulties encountered in attempting to determine the "primary" lesion responsible for a metabolic abnormality. While one may be inclined on the basis of the present data to conclude that it is the weight loss which is related to the defect in alcohol oxidation, it will be extremely difficult to prove this point when weight loss and thiamine deficiency are apparently inseparable. Studies on chronic deficiency may be useful but will probably not be definitive.

The over-all problem of calorie-protein interrelationships has important implications in many kinds of nutrition studies. We have indicated (Vitale et al., '53a) that the paired feeding technique may not be an adequate or satisfactory method of separating the metabolic effects of a nutritional deficiency from the associated inanition which sometimes accompanies it. Clinically there is interest in nitrogen utilization during caloric insufficiency. The studies of Benditt et al. ('48) and of Cox et al. ('53) have demonstrated that within certain limits of supply of both calories and protein the deposition of nitrogen may be increased by feeding more of either protein or calories. The quantitative relations have not yet been defined although the latter group believes that at least 25% of the calorie requirement is needed before nitrogen utilization becomes significant. The work of Swanson ('51) also suggests a significant metabolic change in nitrogen conservation at intakes of calories near 25% of normal.

The parameters which must be considered in evaluating the effects of nutrition become increasingly more complex. It may be noted for example, that the animals were undoubtedly in nitrogen and caloric balance when their weight became constant. The functional test (alcohol metabolism) became

normal at the same time. By many criteria commonly used the animals would apparently be considered normal. The similarity in the state of these animals to various "underfed" human populations is obvious. The effects of short term and acute dietary restrictions clearly do not reflect the status of the animal in the "adapted state." How adequate the adaptation is in terms of health requires a great deal more study, but the long term studies of McCay and associates (McCay et al., '41; McCay, '52) indicate that such adaptation may not be detrimental.

SUMMARY

Adult rats were made to lose weight either rapidly or slowly by the restriction of their food intake. The ability to oxidize ethanol as measured by the excretion of $C^{14}O_2$ after giving ethanol-1-C^{14} decreased markedly while the animals were losing weight. However, as soon as the weight of the animals became stationary, the ability to oxidize alcohol returned to normal. The results are interpreted in the light of previous studies upon liver changes during starvation and protein deficiency. The study appears to provide a clear-cut example of *adaptation to undernutrition.*

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THE EFFECT ON THE GROWTH PERFORMANCE OF YOUNG PIGS OF ADDING COBALT, VITAMIN B_{12} AND ANTIBIOTICS TO SEMIPURIFIED RATIONS [1,2]

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TWO FIGURES

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The literature reveals very little conclusive evidence concerning the influence of trace minerals, particularly cobalt, on the growth performance of non-ruminant animals. The cobalt needs of ruminants have been widely investigated and in areas of cobalt deficiency the beneficial effects of supplemental dietary cobalt have been clearly demonstrated.

The discovery by Rickes et al. ('48) and Smith ('48), that vitamin B_{12} contains cobalt, and the observation by Abelson and Darby ('49) that rumen micro-organisms incorporated cobalt into vitamin B_{12} led to the hypothesis that the cobalt deficiency syndrome is caused by a lack of vitamin B_{12}.

It has not been established that any appreciable amount of inorganic cobalt is incorporated into vitamin B_{12} by micro-organisms in the intestinal tract of the pig. Braude et al. ('49) were unable to demonstrate the biosynthesis of radioactive vitamin B_{12} by feeding physiological doses of radioactive cobalt to two pigs over a six-week period.

[1] Journal Paper no. J-2463 of the Iowa Agricultural Experiment Station, Ames. Project. no. 2930.

[2] This work was partially supported by grants from Calcium Carbonate Company, Quincy, Illinois.

On the other hand improved growth response in pigs, attributable to cobalt supplementation, has been reported by a number of workers. William and Noland ('49) reported increased weight gains of swine fed corn-soybean oil meal rations supplemented with cobalt, copper, iron and manganese. Dinusson et al. ('50) observed improved gains in the growth and fattening of swine when the basal ration was supplemented with approximately 2 p.p.m. of cobalt. Robison ('50) observed that weight gains of young pigs were improved by the addition of cobalt. These gains were further improved when an APF concentrate obtained from aureomycin fermentation was added with cobalt. Swine rations supplemented with a mixture of trace minerals were found superior to non-supplemented rations by Speer et al. ('52). Richardson et al. ('51) observed that young pigs on corn-soybean oil meal rations fortified with trace minerals, including cobalt, and with vitamins except vitamin B_{12}, grew poorly when a combination of antibiotics were added to the ration.

The two experiments reported herein were designed to investigate the effect on the growth of swine of the supplementation of purified rations with cobalt or vitamin B_{12} or both. In formulating the basal rations for these experiments, attempts were made to produce a diet low in both cobalt and vitamin B_{12}. For these reasons, C. P. chemicals to supply the mineral elements and purified carbohydrates and proteins were used. The primary objective was to determine if cobalt supplementation influenced the vitamin B_{12} requirement of the pig. Although the growth-promoting effects of antibiotics have been demonstrated, antibiotics were fed to one half of the experimental animals in order to determine the influence they may have on the "intestinal synthesis" of vitamin B_{12}.

EXPERIMENTAL

Weanling pigs of known genetic and nutritional history were selected as the experimental animals. Their dams were maintained throughout lactation on corn-soybean oil meal

rations fortified only with vitamins A and D_2 and with minerals containing no added cobalt.

In the experiment in which no antibiotic was used, three litters of 4 pigs each were used. The 4 pigs from each litter were randomly alloted, one to each of 4 ration treatments, basal, basal + vitamin B_{12}, basal + cobalt, and basal + vitamin B_{12} + cobalt. In the experiment which included the antibiotic supplement two litters of 8 and 4 pigs each were used. In this case, two pigs from the one litter and one pig from the other litter were randomly assigned to each of the ration treatments.

The pigs were confined in individual wire-floored crates which were supplied with individual self feeders and watering pans. The crates were thoroughly washed 4 times daily as a precaution in preventing coprophagy.

A purified basal ration, consisting of protein, dextrinized corn starch, corn starch, corn oil, methionine, a complex vitamin mixture containing no vitamin B_{12}, C. P. minerals and trace minerals containing no cobalt is shown in table 1. This basal ration was deficient in vitamin B_{12} but not deficient in cobalt. The basal ration contained 0.10 p.p.m., the ration ingredients contributed 0.049 p.p.m. and the C. P. minerals 0.051 p.p.m. of cobalt. Vitamin B_{12} suplementation provided 10 μg of vitamin B_{12} and cobalt supplementation provided 3 mg of cobalt per pound of the respective rations.

The antibiotic-supplemented rations contained a mixture of 4 antibiotics added to provide 100 mg each of aureomycin hydrochloride, streptomycin (as the free base), terramycin hydrochloride, and procaine penicillin G per pound of total ration. Also, sulfathalidine was included at the rate of 100 mg per pound of ration. In a preliminary trial it was found that the tissues from pigs fed these high levels of antibiotics contained a sufficient quantity of antibiotics to inhibit the growth of certain test organisms. A depletion period of three to 5 days was found ample to deplete the tissue of any antibiotic carry over. The antibiotics and the sulfa drug were removed from the rations three days before the animals were taken off

the experiment for slaughter, as a precaution against subsequent interference of the antibiotics with the microbiological assay for tissue vitamins.

TABLE 1

Composition of the basal rations

INGREDIENTS	PROPORTIONS
	%
Drackett Ortho 220 protein [1]	18.00
Dextrinized corn starch	18.91
Corn starch (pearled)	51.74
Corn oil (crude)	4.00
Methionine Premix [2]	1.00
Vitamin Premix no. 12 [3] (without B_{12})	1.00
Trace mineral mixture [4] (without Co)	0.10
Mineral mixture no. 4 [5]	5.00
Iodized salt	0.25

[1] Ortho 220 manufactured by the Drackett Products Co., Cincinnati, Ohio. Analysis: N × 6.25, 83–84%; H_2O, 10%; Ash, 1.5–2.0%. When fed to provide a level of 18% in the final ration, contributed the following amounts of amino acids in per cent: histidine, 0.43; lysine, 1.16; methionine, 0.18; phenylalanine, 0.86; threonine, 0.65; tryptophan, 0.17; valine, 0.91; serine, 1.15; tyrosine, 0.56; alanine, 0.61; cystine, 0.10; isoleucine, 1.10 leucine, 1.26; and arginine, 1.41.

[2] Contributed 0.64% methionine to the ration.

[3] Vitamin Premix no. 12 supplied the following amounts of vitamin per pound of ration:

Alpha-tocopherol acetate	1.5 mg	Para amino benzoic acid	0.5 mg
Ascorbic acid	25.0 mg	Pyracin	1.0 mg
Biotin	0.2 mg	Pyridoxine	1.0 mg
Calcium pantothenate	6.0 mg	Riboflavin	1.5 mg
Choline chloride	450.0 mg	Thiamine	1.5 mg
Folic acid	0.5 mg	Vitamin K	1.0 mg
Inositol	200.0 mg	Vitamin A acetate	2000.0 I.U.
Niacin	15.0 mg	Vitamin D_2	400.0 U.S.P. units

[4] Contributed to the final ration: Fe, 70; Mn, 60; Zn, 20; Cu, 8; and F, 1 p.p.m.

[5] Mixture of C.P. grade salts: KH_2PO_4, 25.8; $CaHPO_4$, 26.2; $CaCO_3$, 30.0; and $MgSO_4 \cdot 7H_2O$, 18%.

An individual record of feed consumption and gain in weight was made for each pig at weekly intervals. The animals were slaughtered when they reached 100 pounds live weight.

RESULTS

Summaries of average values for gains, feed consumption and feed required per 100 pounds of gain are shown in tables

TABLE 2

Summary and analyses of variance of average daily gains, feed consumption and feed per 100 pounds gain, no-antibiotic pigs [1]

	AV. DAILY GAIN, LB.			AV. DAILY FEED, LB.			AV. FEED PER 100 LB. GAIN		
	No B_{12}	B_{12}	Av.	No B_{12}	B_{12}	Av.	No B_{12}	B_{12}	Av.
No Cobalt	1.12	1.41	1.27	2.73	3.15	2.94	244	225	235
Cobalt	1.18	1.23	1.21	2.85	2.83	2.84	244	230	237
Av.	1.15	1.32		2.79	2.99		244	228	

Analyses of variance

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARES		
		Av. daily gain	Av. daily feed	Av. feed per 100 lb. gain
Litters	2	0.10940	0.2169	763
Treatments	3	0.04693	0.1092	285
Vitamin B_{12}	1	0.08500 [2]	0.1323	817
Cobalt	1	0.01140	0.0320	14
$B_{12} \times$ Cobalt	1	0.04440 [2]	0.1633	24
Exp. error	6	0.00307	0.0647	384

[1] Average initial weight of pigs was 36.25 pounds.

[2] Significant at P = 0.05 or less.

TABLE 3

Summary and analyses of variance of average daily gains, feed consumption and feed per 100 pounds gain, antibiotic fed pigs [1]

	AV. DAILY GAIN, LB.			AV. DAILY FEED, LB.			AV. FEED PER 100 LB. GAIN		
	No B_{12}	B_{12}	Av.	No B_{12}	B_{12}	Av.	No B_{12}	B_{12}	Av.
No Cobalt	1.36	1.39	1.38	3.04	3.06	3.05	223	224	224
Cobalt	1.40	1.47	1.44	2.72	3.03	2.88	213	207	210
Av.	1.38	1.43		2.88	3.05		218	216	

Analyses of variance

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARES		
		Av. daily gain	Av. daily feed	Av. feed per 100 lb. gain
Litters	2	0.0480	0.0584	180
Treatments	3	0.0062	0.0860	195
Vitamin B_{12}	1	0.0080	0.0936	12
Cobalt	1	0.0090	0.1083	533
$B_{12} \times$ Cobalt	1	0.0014	0.0560	40
Exp. error	6	0.0174	0.0439	705

[1] Average initial weight of pigs was 22.93 pounds.

2 and 3. Figures 1 and 2 show the average live weights of the pigs receiving the various ration treatments.

The average effect of cobalt supplementation on average daily gain was not significant in the antibiotic-free group.

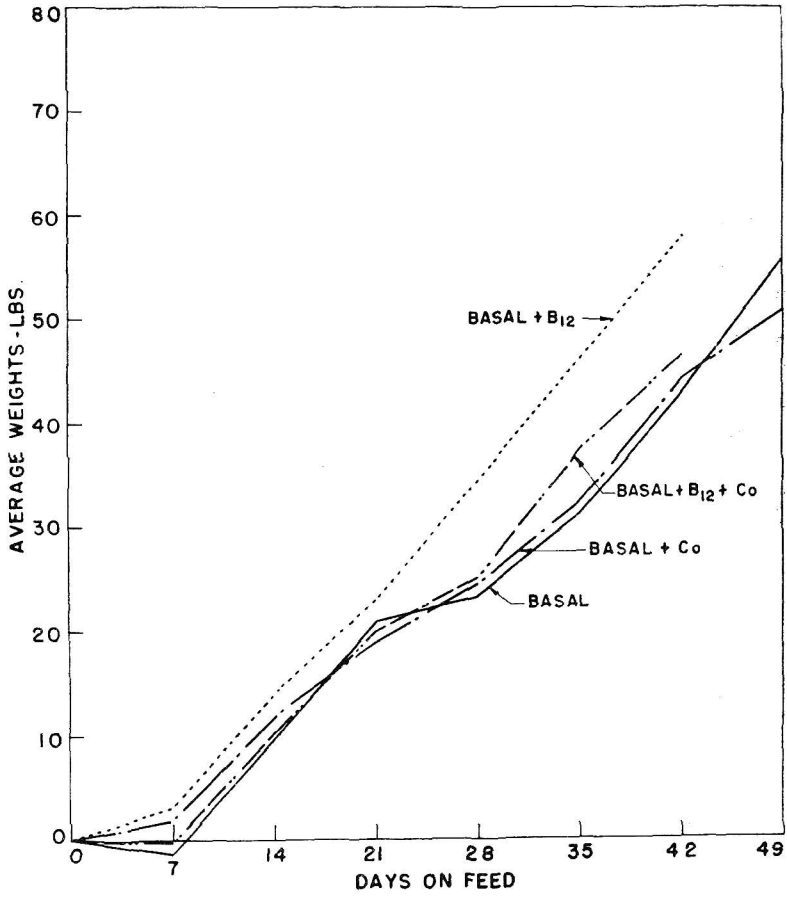

Fig. 1 Average live weights of pigs receiving the rations with no added antibiotic.

The average effect of vitamin B_{12} supplementation was to increase significantly the rate of gain. This was due almost entirely to its effect in the absence of cobalt. The daily gains of the pigs receiving both vitamin B_{12}- and cobalt-supple-

mented rations were not significantly greater than the gains of those pigs receiving cobalt supplementation only. In the case of the pigs without antibiotic in the ration, there were no significant effects of vitamin B_{12} or cobalt on daily feed intake or feed efficiency.

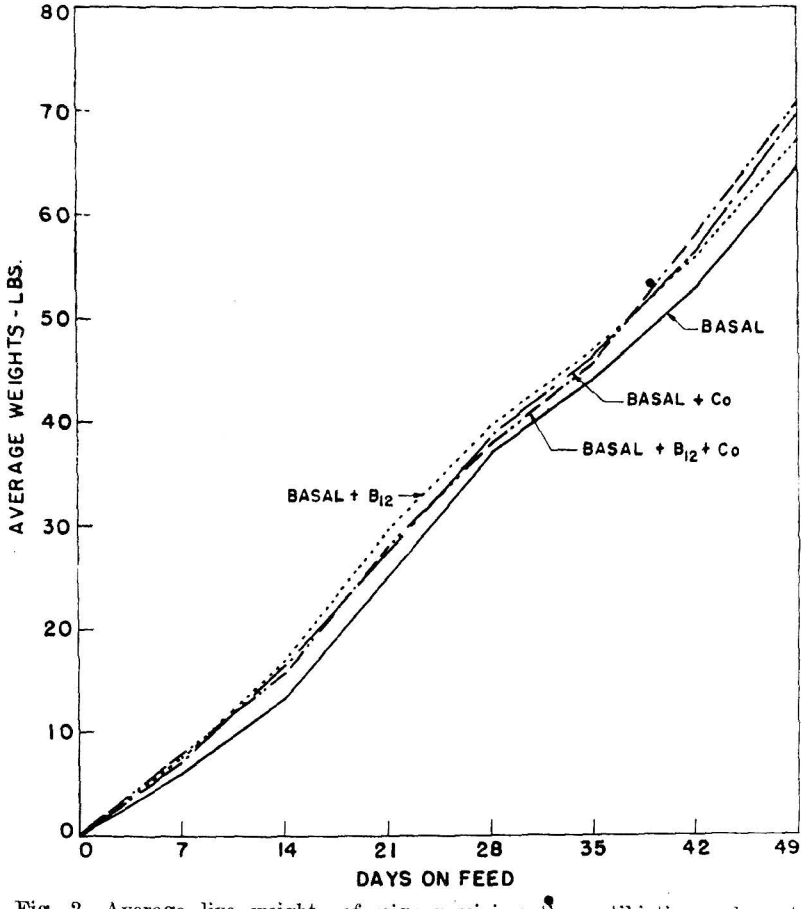

Fig. 2 Average live weights of pigs receiving the antibiotic-supplemented rations.

The inclusion of antibiotics in the ration resulted in the elimination of those differences found significant in the absence of the antibiotics. There was an apparent general increase in rate of gain, a result of improved feed utilization

TABLE 4

Microbiological plate counts[1] *made on pig fecal samples collected near the end of the feeding trials*

No-antibiotic and antibiotic groups (Values $\times 10^6$)

RATION	TYPE OF MICROORGANISMS													
	Acid former		NH_3 former		Strepto-cocci		Coliforms		*Fungi*		Tryptone glucose agar Total count		Proteo-lytic	
No-antibiotic														
Basal	200	11	195	10	1.3	14.6	285	7	.4	8.8	23	58	3.5	6
+ B_{12}	850	205	160	597	145 + Gas	5 + Gas	15	3	2.3	2.1	91	95	0.5	40
+ Co	130	30	150	205	52.5 + Gas	49.5 + Gas	121	6	12.1	2.0	156	59.5	1.0	30
+ B_{12} + Co	40	10	10	11	9.6 + Gas	4.9 No Gas	53	3	1.4	0.5	58	26	0.0	0.0
Antibiotics														
Basal	200	34	95	30	29 + Gas	00.0	93	1	57	17.5	110	16	0.0	2.0
+ B_{12}	30	155	215	26.5	30.0 + Gas	39 + Gas	42	25	65	21	48.0	340	0.0	2.0
+ Co	165	310	20	250	52 No Gas	45 No Gas	43	31	58	360	113	690	6.5	130
+ B_{12} + Co	20	45	230	119	30 + Gas	43 + Gas	40	11	58	60	112	202	0.5	11.0

[1] Counts made on two dates 10 days apart.

but little change in daily feed intake values. The observation that antibiotics improved the performance of these animals merely confirmed other work (Braude et al., '49).

In table 4 is shown the result of microbiological plate counts made on fecal samples collected from the various lots as indicated. Near the end of the feeding period, the largest number of glucose fermenters, proteolytic organisms and greatest total count were in the samples collected from the pigs on the antibiotic-free rations supplemented with vitamin B_{12}. The pigs on this ration, (basal + vitamin B_{12}) also had the best gains. There were fewer streptococci, coliforms, and fungi in the vitamin B_{12}, and vitamin B_{12} and cobalt lots than in the basal group. The cobalt lot with essentially the same and increased numbers of coliforms, and streptococci, respectively, showed somewhat slower gains than the pigs of the basal group toward the end of the feeding trial.

When antibiotics were fed, bacterial populations in pigs receiving the vitamin B_{12}, cobalt, and vitamin B_{12} and cobalt rations were generally increased over that found in pigs on the basal plus antibiotic ration.

DISCUSSION

The basal ration used in these studies was designed to provide a vitamin B_{12}-deficient diet low in cobalt. Chemical analysis for cobalt proved that the basal ration cannot be considered coblat-deficient. While the cobalt requirement of the pig has not been established, it has been reported by Askew and Dixon ('37) and Filmer and Underwood ('37) that 0.1 mg of cobalt per day would prevent or cure a deficiency in a sheep. Since the pigs on the basal ration consumed an average of three pounds of feed per day during the experimental trials, a cobalt intake of 1.4 mg per pig per day was assured. This amount of cobalt is far in excess of the quantity apparently required by the much more cobalt sensitive animal, the sheep.

The differences in the digestive processes of ruminant and non-ruminant animals offer a logical explanation of why the

pigs in this experiment did not respond to cobalt feeding and why vitamin B_{12} supplementation in the absence of antibiotics improved growth performance. This conclusion, however, offers no explanation for the improved growth performance of pigs receiving the high levels of antibiotics (approximately 100 times the recommended practical feeding levels) and no vitamin B_{12}. The hypothesis that high levels of antibiotics permit microbial synthesis of vitamin B_{12} in the intestinal tract because certain micro-organisms can flourish in the presence of antibiotics is attractive but it has not been proved. Another suggestion is that high levels of antibiotics inhibit the synthetic activity of micro-organisms and that nutritional deficiencies could develop as a consequence. The present results offer no support for this hypothesis nor do they provide any evidence against it.

These investigations indicate that increasing the cobalt content of the ration from 0.1 p.p.m. to 2.88 p.p.m. did not improve the growth performance of the pigs. An explanation for the difference between the weight gains of animals fed the basal ration supplemented with vitamin B_{12} alone and the gains of their litter mates which received the combination of vitamin B_{12} and cobalt (antibiotic-free group) cannot be given. The 2.88 p.p.m. of cobalt in the supplemented rations should not have provided an excessive intake of cobalt since Robison ('50) was able to demonstrate improved weight gains in pigs fed rations supplemented with about 7 p.p.m. of cobalt.

On the other hand, Speer et al. ('52) observed that pigs on rations supplemented with trace minerals grew better when fed rations containing less cobalt than 2.88 p.p.m. This observation indicates that the optimum level of cobalt in the pig rations is less than 3 p.p.m. However, definite proof for this is not available from these studies.

The excellent growth performance observed in animals on the rations deficient in vitamin B_{12} but containing a large quantity of antibiotics suggests that antibiotics modify the need for dietary vitamin B_{12}.

This explanation is not in harmony with the suggestion offered by Richardson et al. ('51) for explaining the poor performance of young pigs on all-plant rations, deficient in vitamin B_{12}, but supplemented with antibiotics, vitamin and minerals. The present data offer no evidence based upon growth performance which would suggest that antibiotics inhibited intestinal synthesis of vitamin B_{12}. These variations in results may indicate that the effectiveness of antibiotics in promoting growth of swine may be influenced by the type of ration used. In these instances, at least, widely different types of basal rations were used.

SUMMARY

The effect on the growth performance of young pigs of adding cobalt, vitamin B_{12} and antibiotics to semi-purified rations was studied.

In the absence of antibiotics, the average effect of vitamin B_{12} supplementation was to increase the rate of gain significantly. The addition of 2.88 p.p.m. of cobalt to a ration with or without antibiotics and containing 0.1 part per million of cobalt did not improve the growth performance of the pigs. The average effect of cobalt on the rate of gain was not significant.

Pigs receiving vitamin B_{12} and cobalt made gains similar to those made by animals receiving cobalt only. Daily feed intakes or feed efficiency were not affected by vitamin B_{12} or cobalt supplementation.

When antibiotics were added to these rations the differences found significant in the absence of antibiotics disappeared. Antibiotics produced an apparent general increase in rate of gain as a result of improved feed utilization. There was little change in the daily feed intake.

Bacterial studies on fecal samples showed a marked increase in the fungi counts of the pigs fed antibiotic and a decrease in fungi counts of pigs receiving vitamin B_{12} additions in both experiments. The counts were quite variable for other organisms.

Evidence that antibiotics inhibited intestinal synthesis of vitamin B_{12} was not obtained. Animals receiving the basal ration and rations to which extra cobalt and high levels of antibiotics were added made better gains than did animals on similar rations which did not contain antibiotics.

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INTERRELATIONSHIPS OF PANTOTHENIC ACID AND METHIONINE IN LYMPHOCYTE PRODUCTION BY RATS[1]

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INTRODUCTION

Several reports in the literature suggest a relationship between pantothenic acid and methionine in the nutrition of the rat. Nelson et al. ('47) reported that growth and survival of rats fed a pantothenic acid-deficient diet were improved when the casein content of the diet was increased from 24 to 64%. Subsequent work by this group (Nelson and Evans, '49) indicated that the same effect could be obtained by giving extra methionine to the pantothenic acid-deficient animals. In the course of studies of the effect of diet on antibody production, Lucovici et al. ('51) noted that pantothenic acid deficiency in rats greatly reduced the extent of antibody formation in response to an injected antigen. This reduction was in part overcome by the inclusion of extra amounts of methionine in the diet.

Since a considerable body of data suggest a relationship between antibodies and lymphocytes (Daugherty et al., '44; Ehrich and Harris, '45) the relationship of pantothenic acid to peripheral lymphocytes becomes of interest. Daft et al. ('45) reported that a high percentage of pantothenic acid-deficient rats exhibited granulocytopenia and although lym-

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phocyte counts were not included it is evident from the values observed for total leucocytes that peripheral lymphocytes were also greatly reduced in these animals. In a report from this laboratory (Dinning et al., '51) it was shown that rats fed a methionine-deficient diet developed leucopenia which was characterized by a reduction in peripheral lymphocytes. The present experiments were designed to investigate the possible interrelationships of pantothenic acid and methionine in leucocyte production by rats.

EXPERIMENTAL

Weanling littermate Sprague-Dawley rats of both sexes were housed in individual cages. The basal diet consisted of isolated soy bean protein,[2] 18 gm; hydrogenated vegetable shortening,[3] 10 gm; cod liver oil, 3 gm; salt mix,[4] 2 gm; sucrose, 66.3 gm; inositol, 0.1 gm; choline chloride, 0.1 gm; thiamine chloride, 0.5 mg; riboflavin, 0.5 mg; nicotinic acid, 2 mg; pyridoxine hydrochloride, 0.5 mg; biotin, 5 µg; and 2-methyl-1,4-naphthoquinone, 25 µg. This basal diet contains 0.27% methionine (Dinning et al., '51). This diet was fed to various groups of rats without supplement and with supplements of 0.1, 0.43, and 1.73% DL-methionine. These same 4 diets supplemented with 20 mg of calcium pantothenate per kilo were also fed to other groups of rats. The diets were fed for 55 days and records of growth, food intake, and mortality were kept. Total and differential leucocyte counts were made on tail blood drawn from rats after 48 to 52 days of feeding.

RESULTS AND DISCUSSION

Two separate experiments were conducted and the results are recorded in table 1 as series 1 and 2. When the diet contained no pantothenic acid the growth of the rats responded to increasing percentages of dietary methionine through the highest level tested. In contrast, when pantothenic acid was

[2] Alpha Protein, obtained from the Glidden Company, Chicago, Illinois.
[3] Crisco.
[4] Hubbell, Mendel and Wakeman, J. Nutrition, *14:* 273, 1937.

present in the diet the supplement of 0.43% methionine produced maximum growth. It thus appeared that in the absence of dietary pantothenic acid the methionine requirement for growth was increased. The mortality data also indicate an interrelationship between pantothenic acid and methionine. Deaths occurred during the 55-day observation

TABLE 1

The influence of dietary pantothenic acid and methionine on food intake, growth and survival of rats during a 55-day period

SUPPLEMENT	SERIES	NO. OF RATS	AV. DAILY FOOD INTAKE	AV. DAILY WT. GAIN	MORTALITY
			gm	*gm*	%
None	1	4	3.4	0	50
	2	6	3.9	—0.1	67
0.1% methionine	1	4	4.2	0.4	25
	2	4	6.0	0.3	50
0.43% methionine	1	4	5.2	0.9	0
	2	4	7.4	0.9	0
1.73% methionine	1	4	4.8	1.1	0
	2	4	7.5	1.3	0
Calcium pantothenate	1	4	4.9	0.2	0
	2	6	6.2	0.4	0
Calcium pantothenate	1	4	5.0	0.9	0
+ 0.1% methionine	2	4	7.9	1.4	0
Calcium pantothenate	1	4	9.1	2.3	0
+ 0.43% methionine	2	4	9.7	2.3	0
Calcium pantothenate	1	4	6.1	1.6	0
+ 1.73% methionine	2	4	8.9	2.3	0

period only in groups receiving the basal diet and the basal diet supplemented with 0.1% methionine. Survival was permitted either by increasing the methionine supplement or by the addition of pantothenic acid to the diet.

Peripheral leucocyte counts for rats receiving the various diets are given in table 2. In contrast to the report of Daft et al. ('45) the pantothenic acid-deficient rats in these experiments did not exhibit granulocytopenia. The number of

peripheral lymphocytes was greatly reduced in rats deficient in both methionine and pantothenic acid. In the absence of dietary pantothenic acid, a supplement of 1.73% methionine was required for the maintenance of normal levels of blood lymphocytes. When the diet contained pantothenic acid a supplement of 0.1% methionine resulted in normal levels of lymphocytes. The data were analyzed by the analysis of variance method (Snedecor, '46) and the effects of methionine and pantothenic acid on blood lymphocytes were significant ($P < 0.01$). A significant value was obtained for interaction

TABLE 2

The influence of dietary pantothenic acid and methionine on peripheral leucocytes of rats

(Cells reported as thousands per microliter)[1]

METHIONINE SUPPLEMENT %	WITH PANTOTHENIC ACID			WITHOUT PANTOTHENIC ACID		
	No. of rats	Gran.	Lymph.	No. of rats	Gran.	Lymph.
0	9	3.3	5.4	9	2.3	8.7
0.1	7	4.0	8.3	8	2.4	14.2
0.43	8	3.4	8.6	8	2.2	13.2
1.73	7	2.6	12.0	8	1.7	12.6

Peripheral leucocytes for chow-fed rats (Mean ± standard error) Gran. 2.1 ± 0.3; Lymph. 13.6 ± 1.6

[1] Counts were made between the 48th and 52nd experimental days.

($P < 0.05$), which indicates that the blood lymphocyte response to one of these dietary items was conditioned by the other.

The results of these experiments demonstrate the interrelationship of pantothenic acid and methionine by several criteria. The biochemical basis is not known but at least two possible explanations are suggested. It has been demonstrated (Olsen and Dinning, '54) that a deficiency of the sulfur-containing amino acids may result in a reduced coenzyme A content in tissues of rats fed adequate quantities of pantothenic acid. It is possible that in the present experiments, even though no pantothenic acid was supplied in the

diet, small amounts of the vitamin were made available to the animals by intestinal synthesis. Under such conditions the inclusion of extra amounts of methionine in the diet may have resulted in more efficient conversion of this small amount of pantothenic acid into coenzyme A. Alternatively, pantothenic acid may be concerned in the utilization of methionine for lymphocyte production. Since the methionine requirement for leucocyte formation seems to be related to its methyl-donating ability (Dinning et al., '51) it is possible that pantothenic acid is concerned in transmethylation or in methyl synthesis reactions.

SUMMARY

Sprague-Dawley rats were fed diets with graded levels of methionine with and without pantothenic acid. The methionine requirement for growth, survival, and lymphocyte production was higher in the absence of dietary pantothenic acid than in the presence of this vitamin. The results indicate that pantothenic acid and methionine are interrelated in the nutrition of the rat.

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THE EFFECT OF LEVEL OF DIETARY PROTEIN ON THE GROWTH OF CHICKS FED PURIFIED DIETS CONTAINING SUCROSE OR DEXTRIN [1]

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INTRODUCTION

Monson, Dietrich and Elvehjem ('50) reported that chicks raised on a complete purified diet containing dextrin (autoclaved cornstarch) grew more rapidly than those fed a similar diet with sucrose as the carbohydrate. The growth of chicks receiving the sucrose diet was improved when certain natural materials, such as 4% of primary yeast, were included in the diet (Dietrich et al., '52) but no improvement was observed when the levels of the known vitamins were increased (Monson et al., '52).

Since additional casein had been shown to improve the growth of chicks fed a diet containing 18% casein, 10% gelatin and cerelose (Lepp et al., '49), and since Harper and Katayama ('53) had observed that a diet containing 9% casein with cornstarch as the carbohydrate supported better growth of rats than did a sucrose diet containing the same level of protein, it was considered important to determine the effect

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of additional dietary protein on the growth of chicks fed the sucrose diet of Monson et al. ('50).

The effects of protein and amino acid supplements and of antibiotics on the growth of chicks fed a purified diet containing sucrose or dextrin as the carbohydrate are reported in this paper.

EXPERIMENTAL

Straight run (New Hampshire ♂ ♂ × Single Comb White Leghorn ♀ ♀) cross-bred chicks, which were the progeny of hens fed diet B-1 described previously (Robblee et al., '48) were used in all studies. The chicks were wing-banded and weighed at one day of age and were separated into similar groups of 15 birds each on the basis of their initial weights. They were housed in electrically heated brooder-type batteries with raised screen floors and were fed ad libitum. Individual weights were recorded weekly during the experimental periods of 4 weeks.

The purified basal ration was composed of carbohydrate, 60.2 gm; alcohol extracted casein, 18 gm; gelatin, 10 gm; salts V (Briggs et al., '43), 6 gm; soybean oil, 5 gm; L-cystine, 0.5 gm; thiamine hydrochloride, 0.6 mg; riboflavin, 1.2 mg; nicotinic acid, 10 mg; pyridoxine hydrochloride, 0.8 mg; calcium pantothenate, 4.0 mg; choline chloride, 170 mg; biotin, 0.06 mg; inositol, 100 mg; folic acid, 0.5 mg; vitamin B_{12}, 0.005 mg; para-aminobenzoic acid, 10 mg; 2-methyl-1,4-naphthoquinone, 0.05 mg; and α-tocopherol acetate, 1 mg. Fortified halibut liver oil (60,000 U.S.P units of vitamin A, 6,000 U.S.P. units of vitamin D_3 per gram) was given by dropper (two drops per bird per week). All birds also received as a supplement two drops of freshly prepared thiamine hydrochloride (10 mg/ml) at the end of the first and second weeks. All supplements were provided at the expense of the carbohydrate.

RESULTS

The results of 4 representative experiments, of the total of 9 experiments conducted in the first series, are presented

in table 1. These show the effects of different levels of casein and different supplements on the growth of chicks fed diets containing sucrose or dextrin as the source of carbohydrate. The 4-week weights of the chicks are tabulated separately for each experiment in order that each group may be compared with its own control. This is considered necessary because considerable variation is observed from experiment to experiment among groups receiving similar diets.

In experiment 1 various levels of casein were fed to groups of chicks receiving either sucrose or dextrin diets. When the level of casein in the diets was 18% or less, each group that received dextrin as the carbohydrate grew more rapidly than its sucrose control. The greatest absolute difference in growth was observed between the groups fed 18% casein diets. When the casein content of the diets was increased to 22% no appreciable difference in growth occurred. A similar growth response was obtained when the basal (18% casein-sucrose) diet was supplemented with 2% beef extract. In experiments 4 and 7 and in one experiment not reported in detail, in which sucrose and dextrin diets containing 18 and 25% casein were compared, similar results were obtained.

In experiment 1, and in a similar experiment not included in the table, groups that were fed 18% casein-sucrose diets supplemented with a mixture of 6 essential amino acids (see table 1) grew as well as the best of the groups fed dextrin diets. In experiment 2, although the amino acids improved growth somewhat, none of the sucrose groups grew as well as the dextrin control. In one experiment which is not reported, a much larger supplement of animo acids caused a growth depression. Similar cases have been reported (Almquist, '52), suggesting that a large excess of amino acids may exert deleterious effects.

Experiments 2 and 3 represent other situations that have been encountered during the course of this investigation. In experiment 2, although the group fed the 22% casein-sucrose diet grew better than that receiving the 18% casein-sucrose basal diet, it did not grow as well as the groups fed the dextrin

TABLE 1

Effect of level of dietary protein and type of dietary carbohydrate on chick growth

TYPE OF DIET		EXPERIMENT							
		1		2		3		4	
Casein	Supplement	Sucrose	Dextrin	Sucrose	Dextrin	Sucrose	Dextrin	Dextrin	Sucrose
%		gm [1]	gm [1]	gm [1]	gm [1]	gm [1]	gm [1]	gm [1]	gm [1]
13		199 ± 15	217 ± 16						
15		306 ± 17	335 ± 18						
18		342 ± 20	374 ± 18	299 ± 12	366 ± 13	350 ± 12	355 ± 9	300 ± 13	350 ± 14
22		369 ± 10	377 ± 10	327 ± 13	365 ± 11				
25						354 ± 12		364 ± 10	357 ± 14
18	2% Beef extract	376 ± 11		329 ± 17	364 ± 9	369 ± 16			
18	4% Beef extract					362 ± 14		354 ± 8	
18	Amino acid mix [2]	373 ± 11		317 ± 14					
18	Antibiotics [3]							378 ± 9	364 ± 9
25	Antibiotics [3]							384 ± 13	

[1] Values represent mean ± standard error of the mean of the 4-week weights for a group of 12 to 15 chicks.

[2] L-lysine·HCl, 0.1%; DL-threonine, 0.1%; DL-tryptophan, 0.15%; DL-valine, 0.1%; L-histidine·HCl·H_2O, 0.1%; and DL-methionine, 0.15% of the diet.

[3] Bacitracin, 2.5 mg + penicillin, 10 mg per 100 gram of ration.

diets. In this experiment the growth of the group receiving the sucrose basal was less than that of the comparable groups in experiments 1 and 3. This situation also occurred in two of the unreported experiments in which groups fed 22% or 25% casein-sucrose diets were included. In experiment 3 and in one unreported case in which the chicks receiving the sucrose basal diet grew extremely well, no response to either protein or dextrin occurred.

TABLE 2

Effect of supplements of different proteins on growth of chicks

DIET			EXPERIMENT 5
Casein	Carbohydrate	Supplement	Wt. at 4 weeks [1]
%			*gm*
18	Sucrose		293 ± 11
25	Sucrose		317 ± 12
30	Sucrose		336 ± 16
18	Sucrose	10% Fibrin	327 ± 13
18	Sucrose	10% Wheat gluten	339 ± 13
18	Dextrin		332 ± 16

[1] Mean ± standard error of mean for a group of 15 chicks.

Some growth response was observed (exp. 4) when an antibiotic mixture was fed with the dextrin basal diet and with the 25% casein-sucrose diet but the increase in growth was much smaller than that observed in groups receiving only 18% casein in sucrose diets.

The results presented in table 2 show that the growth of chicks fed 18% casein-sucrose diets was increased to that of groups fed 18% casein-dextrin diets when the protein content of the sucrose diet was increased by the addition of other proteins. In this experiment, as in experiments 1 and 4 (table 1), growth of the chicks receiving sucrose diets with higher levels of protein was as good as that of those fed the 18% casein-dextrin diet.

The results of three experiments in which chicks were fed sucrose diets containing different levels of casein are reported in table 3. The average 4-week weights for the males

and the females are presented as well as the combined averages. In each experiment growth (based on the group averages) was appreciably improved when the level of casein was increased from 18% to 25%. The results with the intermediate level of protein are less consistent but appear to follow this trend. When, however, the separate averages for males and females are compared, in only one experiment (exp. 7), in which the growth of the basal group was very poor, did the male birds show a response to the higher levels of casein.

TABLE 3

Effect of level of dietary casein and sex on growth of chicks fed sucrose diets

EXP. NO.	CASEIN LEVEL	WEIGHT AT 4 WEEKS [1]		
		Males	Females	Group
	%	*gm*	*gm*	*gm*
6	18	368 ± 22 (7)[2]	334 ± 28 (7)	351 ± 18
	22	388 ± 29 (7)	301 ± 23 (6)	347 ± 22
	25	366 ± 21 (9)	389 ± 23 (5)	374 ± 16
7	18	272 ± 6 (23)	260 ± 7 (20)	266 ± 5
	22	290 ± 14 (6)	277 ± 10 (8)	283 ± 8
	25	307 ± 16 (7)	293 ± 10 (5)	301 ± 10
8 [3]	18	318 ± 8 (7)	275 ± 6 (23)	286 ± 6
	22	328 ± 14 (11)	314 ± 8 (19)	319 ± 7
	25	309 ± 11 (13)	307 ± 9 (17)	308 ± 6

[1] Mean ± standard error of the mean.

[2] Figures in parentheses indicate the number of chicks in the group.

[3] Experiment 8 was of 25 days' duration.

In each experiment when the level of casein in the diet was increased from 18% to 25% the growth of the females was improved, the minimum increase in growth being 32 gm. A similar trend may be noted with the intermediate level of casein except in experiment 6.

DISCUSSION

The observation that chicks grew more rapidly when sucrose in purified diets containing 13%, 15% or 18% casein was replaced with dextrin parallels results obtained with rats

fed similar diets of lower protein content (Harper and Katayama, '53; Harper et al., '53). Since more protein was required in the sucrose than in the dextrin diets to support equivalent growth of chicks it would seem that the protein of purified diets containing up to 18% casein is better utilized, particularly by female chicks of the breed used, when the carbohydrate is provided as dextrin.

Although the results in this study were not as consistent as those reported previously with rats, in 10 of the 12 experiments a growth response was obtained when the casein content of the sucrose diets was increased above 18%. No growth response was obtained when the casein content of the comparable dextrin diets was increased. In three experiments the effects of different levels of casein were compared only in sucrose diets (table 3) so for these no direct comparison with groups receiving dextrin is possible. In 4 of the remaining experiments the rate of growth of chicks fed sucrose diets containing additional casein equalled that of those fed 18% casein and dextrin, and in three others, although an increase was noted it was not as great as that obtained when sucrose was replaced with dextrin. Of the two other experiments it should be pointed out that in one case growth was not improved either by additional protein or by dextrin.

Certain factors that may contribute to the greater variation in the chick experiments have been noted. Differences in the protein content of different lots of casein, for example, could account for some of the variation between experiments. This could not affect the results within an experiment because the same lot of casein was used for all diets in a single experiment. It might, however, account for the extremely good growth of all of the groups in occasional experiments, e.g., experiment 3.

Some experimental variation may be caused by differences in the response of different lots of chicks. Since males responded to additional protein only when the overall growth rate was very low (table 3), differences in the sex ratios of groups in the earlier experiments (exps. 1 to 5), in which

no sex differentiation was attempted, could reduce the magnitude of the average growth differences. In no instance, however, could this cause an apparent growth difference where none existed.

One point should be mentioned with regard to the standard errors reported in the tables. In many of the dextrin groups and better sucrose groups the variance is magnified by the presence of one or two excessively heavy birds while in the negative control groups (18% casein-sucrose) the variance is frequently exaggerated by the presence of a few birds considerably below the average. This situation which cannot be compensated for in the calculations tends to make the differences appear less significant.

The variability in the average final weights of the 18% casein-sucrose groups (273–354 gm) from experiment to experiment compared to the more consistent growth of the 18% casein-dextrin groups (332–387 gm), together with the failure from time to time of amino acid supplements and additional casein to induce a rate of gain of the sucrose groups equal to that of the dextrin controls, suggests that other factors, either physiological or nutritional, may be of importance. Monson et al. ('52) have reported that the growth of chicks fed an 18% casein-sucrose diet was improved by a supplement of 4% of primary yeast. Since the growth responses obtained with the amino acid mixture and with beef extract in the present study as well as that with yeast in the earlier study were similar in magnitude and variability to that obtained when the casein content of the sucrose diet was increased above 18%, and since each of these supplements provided additional amino acids (either free or as protein), the growth responses obtained with them appear to be due, in part at least, to the amino acids they contain. The results of experiment 5 (table 2), in which the growth of chicks fed the 18% casein-sucrose diet was improved when supplements of fibrin and wheat gluten were provided, also support this conclusion.

The results obtained with antibiotic supplements provide further support for this interpretation. Although a growth

response was obtained in each case when antibiotics were provided, the response to antibiotics was of doubtful significance in groups fed either the 25% casein-sucrose diet or the 18% casein-dextrin diet. Since, in groups that received only 18% casein with sucrose, a highly significant growth response was obtained, it would appear that both dextrin and antibiotics exert a protein-sparing action. Evidence for a protein-sparing action of antibiotics has been presented by Machlin et al. ('52) but Biely et al. ('52) and Scott et al. ('52) were unable to demonstrate such an action under their experimental conditions.

Both Monson et al. ('50) and Stokstad et al. ('53) have shown that sucrose diets pass more rapidly than dextrin diets through the gastro-intestinal tract of chicks. In work with rats Geiger ('51) has suggested that carbohydrate fed with protein reduces the rate of deamination of amino acids in the liver, and Harper and Katayama ('53) have suggested that the improvement in the growth of rats fed low protein diets in which sucrose was replaced with dextrin may have a similar basis. Womack et al. ('53) and Marshall and Womack ('54) have demonstrated increased nitrogen retention in rats fed low protein diets in which sucrose was replaced with dextrin. These factors might well contribute to improvement in the utilization of dietary protein in chicks fed dextrin diets.

Whether all of the improvement in growth due to dextrin can be accounted for when these interrelationships have been studied in greater detail or whether certain unknown growth factors may also be required, remains to be established. Despite the fact that recent developments in nutrition have made it possible to improve greatly the growth of chicks fed highly purified diets, there are still many reports showing that growth can be further improved by including certain crude materials in such diets (see Menge et al., '52, for references). When the growth rate of chicks is increased by providing the most complete diet at present possible, the balance of the

major dietary constituents assumes greater significance. Supplements of natural materials may, under such conditions, increase the rate of growth in two ways other than by providing unknown growth factors. Such supplements may provide additional amino acids and thus improve the entire amino acid balance of the diet (a point emphasized by the work of Grau and Kamei, '50; and Almquist, '52) or they may permit improved utilization of dietary protein in a manner similar to that postulated for dextrin.

SUMMARY

A previous report that chicks fed purified diets containing dextrin as the carbohydrate grow at a faster rate than those fed similar diets containing sucrose has been extended. The effects of different levels of dietary casein, of certain protein-containing supplements and of an antibiotic mixture included in such diets have been studied.

With the exception of groups that received antibiotics the best rate of growth occurred consistently in groups fed a diet containing 18% casein and dextrin. Chicks receiving dextrin grew more rapidly than those receiving sucrose when the level of casein was 18% or less. However, when the casein content of the sucrose diets was increased above 18% or when amino acids or supplements of other proteins were provided with 18% casein in sucrose diets, growth approached that of chicks receiving 18% casein with dextrin. The rate of growth of chicks receiving either sucrose or dextrin with 18% to 25% casein was increased when a mixture of penicillin and bacitracin was included in the diets, the effect of the antibiotics being much less in the presence of dextrin and higher levels of casein.

The effect of dextrin in improving growth was most evident in female chicks and it is suggested that dextrin exerts this effect largely by permitting better utilization of dietary protein.

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NUTRITIONAL REQUIREMENTS IN COLD CLIMATES

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Although it is generally recognized that proper feeding is an essential factor in maintaining normal health in a cold environment, the actual nutritional requirements under the great variety of arctic conditions are by no means firmly established. Reliable data pertaining to the energy requirements on arctic journeys are of considerable practical consequences, for the rations constitute a significant proportion of the load. On arctic journeys every pound of weight is important because weight is nearly always a critical factor.

It has been suggested by several authors that the arctic calorie requirements would be of the order of 5,500 to 6.000 calories per man per day both for men engaged in field operations, as well as for troops on garrison duties in the arctic or subarctic (Johnson et al., '49; Swain et al., '49). Marked increase in calorie intake by men in the arctic has also been reported in the papers by Johnson and Kark ('47) and Kark et al. ('48). On the other hand, a dietary survey carried out among European trappers in Greenland, 1939–40, showed that the white trappers maintained their body weight on an average gross consumption per man of 3,000 calories per day (Rodahl, '49). The miners in Spitsbergen who worked $8\frac{3}{4}$ to $9\frac{1}{2}$ hours a day, gained weight on an average consumption of 4,500 calories per man per day (Abs, '29). During the crossing of the inland ice of Greenland on skis in 1931, Höygaard and Mehren ('31) consumed 4,000 calories daily and found this

amount to be ample, even under the most strenuous conditions encountered during the expedition. A number of trail rations used on various other arctic or antarctic expeditions have varied between 4,000 and 6,000 calories per man per day (Lockhart, '45), but in most cases no data are available regarding the amounts of calories actually consumed and whether or not the rations were capable of maintaining the body weight. The energy requirements of adult Eskimos in Greenland with a body weight of 65 kg have been assessed to be 2,800 to 2,900 calories per day on the basis of data obtained by Krogh and by Höygaard (Krogh and Krogh, '13; Höygaard, '41).

In the case of United States soldiers in the arctic environment, it has been pointed out that the percentages of calories furnished by protein, fat and carbohydrate are not significantly different from those reported for United States troops eating garrison rations in temperate climates (Johnson and Kark, '46; Swain et al., '49). The primitive Eskimos apparently prefer a diet which covers almost half of the energy requirements by fat and half by protein (Höygaard, '41). They maintain that fat is necessary to keep themselves warm on cold journeys, and that they cannot stand cold when living on lean meat.

It has been suggested by several authors (DuBois, '28, Mitchell et al., '46; Keeton et al., '46, Keys, '49–'50, and others) that dietary modifications may exert a considerable and favorable effect upon the ability of man to withstand exposure to intense cold. Dugal, Leblond and Thérien ('45) have found that a diet rich in fats is decidedly superior to one rich in carbohydrates for adaptation and resistance to cold in animals. Available data indicate that requirements for a number of nutrients are markedly increased under conditions of low environmental temperature. Thus, Dugal and Thérian ('47) have found an increased requirement for ascorbic acid in animals exposed to cold. According to Ershoff and Greenberg ('50) an increased requirement for thiamine has also been demonstrated in animals following prolonged

exposure to low environmental temperatures. Some animal experiments have even indicated increased requirements for vitamin A (Ershoff, '52). In man, on the other hand, available experimental data have as yet failed to demonstrate clearly an increased vitamin requirement at low environmental temperatures (Glickman et al., '46), nor has a convincing beneficial effect of increased vitamin dosage in man against environmental stress been produced (Dahlberg, et al., '42).

During the two years 1950 to 1952 we had an unusual opportunity of carrying out detailed nutritional surveys for weekly periods during the 4 seasons of the year among a group of Infantry soldiers and a group of airmen on garrison duties at Ladd Air Force Base, Alaska, as well as in Eskimos from 4 different locations in Alaska. The purpose of this paper is to report briefly the main results of these studies. Further details regarding the Eskimo studies will be presented in a later publication.

MATERIAL AND METHODS

The white subjects totaled 36 young men (average age 20½ years, weight 70 kg and height 175 cm) representing two different groups. The Infantry group consisted of regular army soldiers engaged in the usual Infantry activities. The Air Force group included regular airmen on ordinary Air Force duties in Alaska. The men had lived in Alaska approximately a year at the time of the beginning of the study. Seven of the original subjects were surveyed at all 4 time periods. A total of 16 adult male Eskimos were used for comparison.

The temperature in the living and sleeping quarters, during the 4 periods of the study, was maintained at the same level as was customary for the subjects. The mean temperature in the sleeping quarters was about 75°F. The highest outdoor temperature recorded during this study was 90°F. in July and the coldest was —57°F. in January.

Careful clinical examinations and some limited laboratory tests were made in order to detect possible deficiency symp-

toms. The medical examinations included detailed medical histories, x-ray examination of the chest and long bones, oral temperature, pulse rate, blood pressure, night vision, capillary fragility tests, examination of the teeth and the gingiva, in addition to blood and urine examinations as well as determination of the basal metabolic rates and the determination of urinary nitrogen elimination.

During all examination periods a complete nutritional survey was carried out with individual food weighings at each meal. The subjects were allowed to select the food as usual in unlimited quantities. Each food item was collected on separate plates and in paper cups and accurately weighed. At the end of the meal each plate or cup together with the remaining unconsumed food was again weighed and the food consumption recorded. In-between-meals consumption was recorded on separate sheets by the subjects. These records were checked by the observer and added to the food consumption for that day. The calorie expenditure was estimated on the basis of time activity data. As an indication of calorie balance the body weight at the beginning and end of each period was used. For the computation of the results the values were taken from the standard tables, or calculated on the basis of the recipes. In the case of some unusual Eskimo foods, representative samples were analyzed.

RESULTS AND DISCUSSION

There was very little variation between the food served in the different periods of the study, and the food in the Infantry mess was very similar to that served in the Air Force mess-hall. The average consumption calculated by food groups is given in table 1.

In the Infantry group the gross consumption of calories varied between 3,100 and 3,400 per man per day, mean 3,200 (table 2). An average calorie expenditure for all 4 periods was estimated to be about 2,800 calories on the basis of the time activity data. Under these conditions no appreciable weight

TABLE 1

Average food consumption in grams per man per day of soldiers at Ladd Air Force Base during the 4 seasons of the year, calculated by food groups

FOOD GROUPS	PERIOD I FALL		PERIOE II WINTER		PERIOD III SPRING		PERIOD IV SUMMER	
	Air-men	Inf. men	Air-men	Inf. men	Air-men	Inf. men	Air-men	Inf. men
Breakfast, lunch, dinner:								
Meat, fish and poultry	219	241	206	213	198	163	197	230
Eggs	41	30	19	41	26	74	21	51
Milk and milk products	324	156	291	343	208	361	468	305
Butter	25	18	37	18	20	13	31	4
Cheese	11	..		..	9	6	1	18
Bread	186	204	170	235	121	221	152	172
Cereals and grain products	258	203	134	196	188	237	145	93
Potatoes	124	244	93	166	86	254	229	194
Other roots and tubers	5	12	5	24	4	10	5	3
Leaf and stem vegetables	20	9	6	24	9	32	6	43
Flower, fruit and seed vegetables	52	99	67	63	63	78	98	137
Salads	27	15	42	14	42	12	12	..
Fruit, fresh	14	82	16	13	15	2	116	155
Fruit, canned	76	68	74	40	25	53	63	39
Juices	..	90	41	80	86	88	260	228
Sugar, syrup, honey, jams & jellies	156	112	51	107	49	60	39	33
Dressings	17	15	21	16	34	12	8	8
Gravy	64	26	53	9	25	13	17	9
Sauces	2	12	2	4	38	8	1	20
Soups	110	9	52	8	..	37	..	..
Consumption between meals:								
Beverages (coca-cola, beer, etc.)	232	112	133	55	90	168	191	293
Milk shakes	12	10	..	15	..	5	..	23
Chocolate milk	20	4	31	16	..	11	8	12
Ice cream and sundaes	4	3	..	32	..	26	..	3
Sandwiches	24	6	11	1	..	..	2	7
Snacks (hamburgers, eggs, etc.)	13	..	9	10	2	..	..	5
Candy	15	15	24	19	27	9	1	2
Sugar	4	..	8	4	7	..	8	.
Fruit	56	14	20	33	..	7	..	..
Pies, cookies, bread, cereal	9	4	7	19	2	..	17	5
Greens	2	..	.	..	..	..	4	..
Nuts, popcorn	..	..	9	..	2	..	7	1

change occurred; there was a mean weight gain of 0.4 lbs. throughout the whole year.

In the Air Force group the average gross consumption varied between 2,000 and 3,000 calories per man per day, mean 2,950. The estimated mean calorie expenditure was 2,700. On this regime the mean body weight of all Air Force subjects for all 4 periods of the year remained unaltered.

In the adult male Eskimos an average daily gross consumption of approximately 3,100 calories was sufficient to maintain the body weight with an estimated daily energy expenditure of roughly 2,700 calories throughout the year.

These findings are in agreement with studies among Eskimos and trappers in Greenland. In the male adult Greenland Eskimo the calorie intake is, according to Höygaard ('41), in the order of 2,800. In the trappers the average gross consumption per man varied from 3,300 in the summer to 2,100 in the middle of the winter (Rodahl, '49).

The results for the arctic trappers in Greenland may appear surprisingly low. However, real hard work is only done occasionally by these trappers, for instance, during travelling on foot under difficult conditions. During the dark period of the year, when the food intake and calorie consumption is at a very low level, the weather conditions usually prevent any exercise taking place. At times, the trappers are confined indoors for several weeks in well-heated cabins. In the fall and early spring they travel by sledge and dogs along the fjord ice, and as the going is usually good, they are able to sit on the sledge during the journey, well-protected by warm fur clothing. During the late spring when the fjord ice is breaking up and the snow is melting on the land, the trappers are again confined to the cabin. In the summer, they usually travel by motor boat, which does not entail hard work. Similarly, the moderate calorie intakes in the Eskimos may be explained by the fact that the Eskimo does not normally go out in his kayak when the weather is bad, and he avoids travelling when the snow conditions are such that he must walk on foot instead of sitting comfortably on the sledge being

TABLE 2

Daily intake of essential nutrients by infantrymen and airmen in Alaska

	CALORIES	WATER	PROTEIN	FAT	TOTAL CARBO-HYDRATE	CALCIUM	PHOSPHORUS	IRON	COPPER	VIT. A	VIT. B_1	RIBO-FLAVIN	NICOTINIC ACID	VIT. C
		gm	*gm*	*gm*	*gm*	*mg*	*mg*	*mg*	*mg*	*I.U.*	*mg*	*mg*	*mg*	*mg*
Air Force group														
I. October	3500	1309	111	147	432	1115	1374	17.6	2.3	6748	1.8	2.3	20.0	32
II. January	3000	1066	90	149	325	809	1330	13.8	3.3	5958	1.4	1.7	19.5	70
III. April	2400	874	76	101	299	570	1135	14.0	3.1	7069	2.7	2.5	18.2	80
IV. July	2900	1491	91	107	342	1191	1626	15.0	1.7	5530	2.8	2.6	19.6	113
Mean	2950	1185	92	126	350	921	1366	15.1	2.6	6326	2.2	2.3	19.3	74
Infantry group														
I. October	3300	1070	102	129	442	789	1416	18.6	2.5	4296	2.0	1.8	19.3	99
II. January	3400	1123	103	139	432	1156	1751	19.5	3.3	9280	2.1	3.0	20.7	88
III. May	3200	1309	122	126	412	1246	1735	16.8	2.3	4937	2.4	2.6	18.4	91
IV. August	3100	1365	108	118	345	1004	1598	18.0	1.7	6270	2.0	2.2	21.4	128
Mean	3200	1217	109	128	408	1048	1625	18.2	2.5	6195	2.2	2.4	20.0	102

pulled by his dogs. This is especially true in Greenland. Although enormous amounts of work may occasionally be carried out during a short period, at times when the possibilities of hunting are exceptionally good, most of the time is spent in waiting for the game to appear or for the fish to bite. Furthermore, the Eskimos' clothing offers an excellent protection against loss of heat.

If the requirements for troops on arctic duty were in the order of 5,000 to 6,000 calories per man per day, any young soldier stationed in the Arctic should be calorically undernourished if his mean intake were considerably less than 5,000 calories daily over a prolonged period. Our results indicate clearly that this is not the case. The subjects had spent about a year in Alaska prior to the start of the study, and their diet had been roughly the same during the year prior to the study as during the period of the actual survey. They remained in excellent health and good physical condition. Since the body weights remained almost constant throughout the entire period of the study, the calorie intake during this period (2,950 to 3,200) may fairly closely represent the requirements necessary to maintain calorie balance. It would, therefore, seem justifiable to conclude that the calorie requirements under these conditions would be in the order of approximately 3,000 to 3,500 or slightly higher.

It appears probable that, in the past, figures for calorie expenditure in troops under arctic or subarctic conditions are somewhat overestimated. This depends, particularly in the case of energy required for surface travel, not only on the nature of the terrain, snow cover and the condition of the ground, but to a great extent on the training and experience of the individual soldier, for an experienced arctic traveller may cover the same ground with much less effort than a person who does not possess the know-how and the technique of arctic travel. In our material, the Infantry soldiers were all well-trained and thoroughly indoctrinated in arctic living and operations. In the case of the airmen, their exposure to environmental stress was probably not very much more than

that of men in similar occupations in the more temperate zone.

In animals the level of metabolism has been found to be inversely related to the environmental temperature. In man studies on the influence of climate on metabolism are conflicting, and the problem is greatly complicated by the possible effect of race and diet. In a recent study it was shown (Rodahl, '52) that about 9% of the higher metabolism in Eskimos is caused by apprehensiveness, and about 15% is caused by the

TABLE 3

Percentage of calories supplied from protein, fat and carbohydrate

	PERIOD	PROTEIN	FAT	CARBOHYDRATE
		%	%	%
Infantry group				
	Fall	12.2	34.8	53.0
	Winter	12.1	37.0	50.9
	Spring	14.9	34.7	50.4
	Summer	15.0	37.0	48.0
Air Force group				
	Fall	12.7	37.9	49.4
	Winter	12.0	44.7	43.3
	Spring	12.7	37.9	49.8
	Summer	13.5	35.7	50.7

specific dynamic action of the high meat diet of the Eskimo. When these two factors were eliminated, the basal metabolism of the Eskimo was almost exactly the same as in white controls. Thus the difference is not caused by racial factors. In whites living in Alaska, the basal metabolism was 6 to 8% below the DuBois standard, which is the same as the basal metabolism observed in white men living in temperate climates (Rodahl, '52). It thus appears that, under the conditions considered here, there would be no reason to expect an increased calorie requirement because of increase of basal metabolism due to climate.

Of the total calories consumed by our white subjects an average of 13% was derived from protein, 38% from fat and 49% from carbohydrate (table 3). Similar figures have been reported for United States troops eating a garrison ration in temperate or tropic climates (Johnson and Kark, '46; Swain et al., '49). Europeans living in the arctic environment, however, have a greater consumption of fat (Rodahl, '49), as is also the case with the Eskimos. In the Infantry group the percentage of calories supplied from fat was the same both in the winter and summer, while in the Air Force group 44.7% of the calories come from fat in the winter as against only 35.7% in the summer (table 3).

The average daily vitamin consumption in our white subjects was in all cases higher than the figures recommended by the National Research Council as standard allowances in temperate climates (table 2). Clinical examinations at each of the 4 periods revealed no significant pathological findings, and no evidence of deficiency symptoms was detected.

Recent studies have indicated that the requirement of vitaman A for adult men in temperate climates is approximately 2,500 I.U. vit. A (Hume and Krebs, '59). In Greenland the white trappers subsisted on 3,000 I.U. daily without showing clinical evidence of vitamin A deficiency (Rodahl, '49). The Greenland Eskimos' consumption of vitamin A is more than 10 times higher than that of the white trapper (Höygaard, '41). The Alaskan Eskimos consumed about 12,000 I.U. vit. A daily.

The National Research Council recommends 1.5 mg thiamine daily for physically active men. In our subjects the thiamine intake was slightly more than 2 mg. In Greenland less than 0.9 mg thiamine daily gave rise to vitamin B_1 deficiency, and one trapper developed beri-beri (Rodahl, '49).

The consumption of vitamin C by the European trappers in Greenland throughout the whole year was less than the figures considered as minimum human requirements (Rodahl, '49). Thus, in the middle of the winter the intake was less

than 10 mg vit. C. per day per individual although no distinct symptoms of scurvy were observed among these trappers. It has previously been found that Europeans in Greenland during sledging journeys of long duration subsisted on less than 15 mg vitamin C daily without any ill effect. In the Greenland Eskimo the daily average intake of vitamin C has been found to be 36 mg per individual (Höygarrd, '41). In the Alaskan Eskimos the daily intake of vitamin C was 28 mg, varying from 3 to 54 mg. No clinical evidence of vitamin C deficiency was detected.

It is well known that man may prevent, and even cure, scurvy on a diet of nothing but meat (Stefansson, '18). This is a remarkable fact, for it would be quite impossible for a white man to consume the amounts of vitamin C, normally considered as minimum requirements, from meat alone. In order to obtain 30 mg ascorbic acid, it would be necessary to consume 3 to 6 lbs. of meat per day, if the ascorbic acid content is 1 to 2 mg per 100 gm of meat. The explanation may be that the actual vitamin C requirement in reality is less than 30 mg per man per day in arctic conditions, or that the content of antiscorbutic substance in meat is higher than the figures obtained by the standard methods of assay. It is also possible that such a diet of meat causes changes in the intestinal flora, resulting in a decreased bacterial destruction of ascorbic acid. Finally, the possibility of synthesis of vitamin C in man has to be considered.

SUMMARY AND CONCLUSIONS

A series of nutritional surveys has been carried out among two groups of Whites (airmen and Infantry soldiers) in Alaska during the 4 seasons of the year from 1950 to 1952. Simultaneously, similar studies were made among 4 groups of Eskimos for comparison.

Individual food weighings showed an average daily calorie consumption per man of 3,000 in the Air Force group and 3,200 in the Infantry group. The average calorie expenditure

for the 4 seasons was estimated to be about 2,800 calories per man per day on the basis of time activity data. Under these conditions no appreciable weight change occurred, and the subjects remained in excellent health throughout the period of the study. It is concluded that the calorie requirements of the average man engaged in activities of similar magnitude and under similar climatic conditions as those of the subjects studied, would be in the order of approximately 3,000 to 3,500 calories per man per day at any season of the year. In adult male Eskimos at 4 different locations in Alaska, an average daily consumption of approximately 3,100 calories was sufficient to maintain the body weight with an estimated daily energy expenditure of roughly 2,700 calories throughout the year. These findings are in agreement with previous findings among Eskimos and trappers in Greenland (Höygaard, '41; Rodahl, '49).

The percentage of calories furnished by protein, fat and carbohydrate in the United States troops living in Alaska is not significantly different from that reported for United States troops eating a garrison ration in temperate or tropic climates. In the Air Force group, however, there was an increased consumption of fat in the winter.

The consumption of minerals was higher than the recommended allowances for temperate climates.

The data presented do not indicate that the human vitamin requirements are significantly higher in arctic or subarctic environments than in temperate climates. In the case of vitamin C, arctic travellers, trappers and Eskimos may subsist on ascorbic acid intakes of less than 15 mg daily without showing clinical evidence of vitamin C deficiency.

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MICROBIOLOGICAL EVALUATION OF PROTEIN QUALITY

II. STUDIES OF THE RESPONSES OF TETRAHYMENA PYRIFORMIS W TO INTACT PROTEINS [1]

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ONE FIGURE

(Received for publication March 15, 1954)

The development of a technique using a colorimetric procedure for the estimation of the growth of *Tetrahymena pyriformis* W was reported previously (Anderson and Williams, '51). Growth responses to the amino acids "essential" to this organism were measured by determination of the red triphenylformazan (TPF) formed by the enzymatic reduction of colorless 2,3,5-triphenyltetrazolium chloride (TPTZ).[3] These workers suggested that the nutritive value of a protein for the organism could be assessed with this method.

In the present study a modification in the method is given and the method is applied in evaluating the growth promoting values of partially purified proteins.

EXPERIMENTAL

Organism. The organism used for this work was described previously (Anderson and Williams, '51). Stock cultures

[1] This study was supported in part by a grant to Cornell University by the Herman Frasch Foundation.

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[3] Schwarz Laboratories, Inc., 230 Washington Street, Mount Vernon, New York.

carried in this laboratory under the same conditions for 4 years were used.

Preparation of stock suspensions of proteins. Finely ground samples of protein were weighed quantitatively into beakers; sodium hydroxide solution was added,[4] and the resulting suspensions were stirred slowly overnight at room

TABLE 1

Basal medium

COMPONENT	μG/ML OF SINGLE-STRENGTH MEDIUM	COMPONENT	μG/ML OF SINGLE-STRENGTH MEDIUM
Dextrose	1000.00	Guanylic acid	30.00
Sodium acetate	1000.00	Uracil	10.00
Ca pantothenate	0.60	Cytidylic acid	25.00
Riboflavin	0.60	Adenylic acid	20.00
Thiamine·HCl	6.00	$MgSO_4 \cdot 7H_2O$	100.00
Nicotinamide	0.60	K_2HPO_4	100.00
Pyridoxine·HCl	6.00	$CaCl_2 \cdot 2H_2O$	50.00
Pyridoxamine·HCl	0.60	$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	25.00
Pyridoxal·HCl	0.60	$CuCl_2 \cdot 2H_2O$	5.00
Biotin (free acid)	0.003	$FeCl_3 \cdot 6H_2O$	1.25
Folic acid	0.06	$MnCl_2 \cdot 4H_2O$	0.50
Choline chloride	6.00	$ZnCl_2$	0.05
Na-DL-6-thioctate [1]	0.0011		
or Protogen [1]	1 [2]		

[1] Kindly supplied by Dr. E. L. R. Stokstad, Lederle Laboratories, Pearl River, N. Y.

[2] Expressed as units per milliliter single strength medium.

temperature. The mixtures were brought to pH 7.0 and made to volume with distilled water. From these stock suspensions a series of dilutions containing twice the desired amount of nitrogen were made using distilled water.

Basal medium and growth conditions used. Double-strength basal medium (table 1), adjusted to pH 7.0, was pipetted in 2 ml amounts into 15-ml centrifuge tubes. Two milliliters of the double-strength protein suspensions were added into 4

[4] Twenty-five one-hundredths per cent NaOH was added so that an eventual ratio of 20 ml per 100 ml of stock suspension of protein existed.

replicate tubes for each nitrogen level of variable being assayed. The tubes were covered with aluminum caps and sterilized by autoclaving for 15 minutes at 121°C. The cooled tubes were inoculated, each with one loopful of 4- to 6-day stock culture, and were incubated for varying periods of time at 25°. The position of the tubes within the incubator was changed daily. At this time each tube was tapped gently to stir up the protein material.

Colorimetric procedure for estimating growth. At the end of the incubation period, one tube from each level of protein being assayed was inactivated by autoclaving (until the internal temperature of the autoclave reached 115°C.). These tubes served as blanks.

One milliliter of 2.5% TPTZ solution made up in 0.1 M phosphate buffer at pH 7.7 was added to each tube. The contents were stirred gently with a glass rod; the tubes were stoppered and placed immediately into a thermostatically controlled water bath set at 37°C. At the end of 15 minutes, 0.4 ml of 0.1 M acidic $HgCl_2$ [5] were added to each tube. The tubes were centrifuged; the supernatant solution was discarded; and the inverted tubes were allowed to drain for 5 minutes. Ten milliliters of acetone were added and the precipitated triphenylformazan (TPF) was dissolved with stirring. After centrifugation, the clear acetone extract was transferred to a cuvette and the transmittance was determined with a Model 14 Coleman Spectrophotometer, at a wave length of 485 mμ. Dilutions of the extracted TPF were made with acetone when necessary and all readings were calculated to a 10 ml volume.

MICROBIOLOGICAL ASSAYS

Comparison of growth response to casein when measured by the colorimetric procedure and by turbidimetric reading. Since protein efficiency with other species is often compared with a reference standard, casein was chosen as the standard for this study. Casein was selected because it is a protein

[5] Prepared by using 20 ml of concentrated HCl per liter.

(a) which can be obtained in a relatively purified form, (b) for which the amino acid composition has been well established, (c) which is readily hydrated and thus easily sampled, and (d) to which *Tetrahymena* shows differences in growth with varying levels of nitrogen.

Using the method outlined above, *Tetrahymena* cultures were grown for three days on unhydrolyzed casein. The growth response was measured by the colorimetric method and by turbidimetric measurement of the suspended *Tetrahymena* cells. An uninoculated tube served as the blank for each level of variable in the turbidimetric measurements. Measurements of turbidity are not always valid when protein is the source of nitrogen since some protein suspensions show decreased turbidity as the proteins are hydrolyzed. Casein solutions, however, are relatively clear at all stages and a fair estimate of growth can be determined by this method. The pattern of growth as measured by the turbidimetric method is reflected by the dye reduction method (fig. 1) which was used in all the subsequent assays.

Growth response of Tetrahymena to two sources of casein. During the time these investigations were being made two sources of casein were used as the reference standard. One, referred to as Rutgers casein, had been obtained from Rutgers University[6] and had been studied extensively in the cooperative project sponsored by the Bureau of Biological Research, Rutgers University (Rutgers University Report, '46–'50). The other, referred to as vitamin-test casein,[7] was a commercially available product.

In table 2 are presented the data for the three-day incubation response curves for which these two caseins were used. It would appear that the growth promoting value of the vitamin-test casein was inferior to that of the Rutgers sample. Also evident is the fact that the greatest variation between assays occurs at the lower levels of the protein. In the latter instance, either one or both of two factors may be responsible.

[6] Kindly supplied by Dr. James B. Allison.

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

The level of protein nitrogen may be below physiological limits for the organism, or the colorimetric method is less sensitive at very low levels of growth, or both, may be true.

Methods of quantitatively expressing the relationship between proteins. The pattern of the growth response curves for casein suggested the following methods for quantitatively

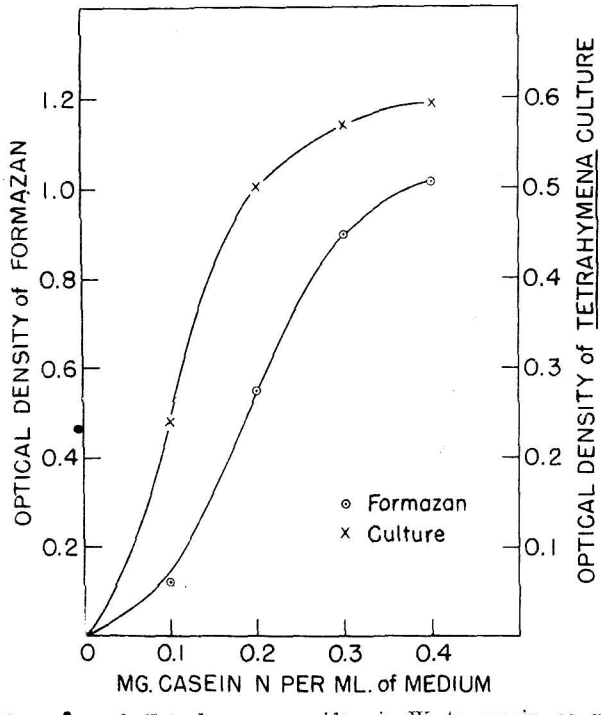

Fig. 1 Response of *Tetrahymena pyriformis* W to casein as measured by turbidimetric reading (560 mμ) and by TPTZ reduction (485 mμ).

expressing the relationships between the growth promoting abilities of a test protein and the reference protein, casein, to which the value of 100 would be assigned arbitrarily:

(a) Optical density per unit of nitrogen, or the relationship between the optical density found with the test protein versus the optical density found with casein when the levels of nitrogen are the same. An average of the values calcu-

lated from the portions of the curves where growth was increasing was used.

(b) Half-maximal growth responses; the relationship between the amounts of protein nitrogen necessary to give an optical density of 0.5 of the TPF formed (approximately half-maximal growth for casein).

(c) Units of nitrogen per increase in optical density; the relationship between the amounts of protein nitrogen required to cause an increase in optical density over a certain range, i.e., from 0.4 to 0.6.

TABLE 2

Comparison between assays of response of Tetrahymena to Rutgers casein and to vitamin-test casein after a three-day incubation period

PROTEIN NITROGEN	VITAMIN-TEST CASEIN				RUTGERS CASEIN			
	No. of assays	Mean O.D.[1]	S.D.[2]	C[3]	No. of assays	Mean O.D.[1]	S.D.[2]	C[3]
mg/ml								
0.10	6	0.09	± 0.06	66	9	0.17	± 0.06	35
0.20	6	0.42	± 0.09	21	9	0.62	± 0.08	13
0.30	11	0.79	± 0.13	16	9	0.91	± 0.12	13
0.40	6	0.86	± 0.13	15	9	1.02	± 0.08	8
0.50	6	0.92	± 0.16	10	3	1.10	± 0.03	3
0.60	4	1.02	± 0.08	8	3	1.08	± 0.07	3

[1] Mean optical density.

[2] Standard deviation of the mean optical density.

[3] Coefficient of variability $= \frac{(\text{Standard deviation} \times 100)}{\text{Mean}}$.

Growth response of Tetrahymena pyriformis W to partially purified proteins. A comparison of the nutritive values of the partially purified proteins [8] used in the cooperative study conducted by the Bureau of Biological Research of Rutgers University was undertaken using *Tetrahymena* as the test organism. The proteins used in this study represent ones which cover a wide range in nutritive values. In table 3 are assembled the data for the growth-promoting values of

[8] See footnote 6, page 592.

TABLE 3

The growth promoting values of defatted whole egg, wheat gluten meal, and peanut flour for Tetrahymena (Expressed as per cent of the value obtained for vitamin-test casein)

PROTEIN	DAYS OF INCUBATION	NO. OF LEVELS	METHODS OF EVALUATING PROTEIN [1] Method *a* Range	Method *a* Average	Method *b*	Method *c*	VALUES FOR OTHER SPECIES [2]	AVERAGE	RANGE
Defatted whole egg	3	4	72–144	120	113	118	Rat:	111	100–133
	5	5	85–102	96	90	100	Mouse:	97	80–115
							Dog:	108	
Peanut flour meal	3	4	24– 72	52	49	44	Rat:	51	38– 66
	5	4	44–115	82	71	100	Mouse:	64	59– 70
							Dog:	58	
Wheat gluten meal	4	1		11	17 [3]	[4]	Rat:	16	6– 23
	5	4	16– 19	16	18	21	Mouse:	36	32– 41
							Dog:	39	
Wheat gluten meal + L-lysine [5]	5	5	42– 73	54	47	22			

[1] See text for details.

[2] Rutgers University Report ('46–'50).

[3] An optical density of 0.4 instead of 0.5 was used.

[4] Unable to be calculated.

[5] L-lysine added so that total amount added plus that amount occurring in the wheat gluten, *per se*, had the same proportional value to tryptophan as it occurs in the vitamin-test casein standard.

three of the partially purified proteins and of wheat gluten meal supplemented with lysine. All three methods described above for evaluating the quality of the protein in relationship to casein were used where possible. For comparison, the protein efficiency ratios, expressed as per cent of the value obtained for casein are listed for the rat, mouse, and dog. The data indicate that any of the three methods of evaluation give values which fall within the range found for the growing rat, if assessment of quality by estimation of growth of *Tetrahymena* is made at a time when the organism is in an early phase of response to the protein. If the organism were allowed to incubate with defatted whole egg or peanut flour for 5 days, the three methods of evaluation did not give values which were within the range found for the rat (table 3).

The three methods always ranked the proteins in the same order, and method *b* cited above gave values which agreed within 5% of the average values found for the growing rat when the shorter incubation period was used.

As added proof that the *Tetrahymena* were capable of different growth responses to proteins of different quality, wheat gluten meal was supplemented with L-lysine. Growth increased over three-fold from that found with wheat gluten alone when methods *a* and *b* of growth evaluation were used. Method *c* did not indicate an increased response; this method of evaluation does not depict the growth-promoting value of a protein unless growth occurs within a nitrogen range comparable to the reference protein. Therefore, this method is invalid unless both proteins are of comparable nutritive value.

Preliminary experiments using defatted beef muscle and dried egg albumin indicate that these proteins can be evaluated with *Tetrahymena*. Quantitative assessment of protein quality has not been made.

Amino acid composition of Tetrahymena pyriformis W. Dried, washed cells of the protozoan, grown for 5 days on 0.4% yeast extract, were analyzed by microbiological methods

for 11 of the amino acids (Williams, '54) (table 4). The occurrence of the amino acids in descending order of magnitude was lysine, leucine, isoleucine, valine, threonine, phenylalanine, arginine, tyrosine, methionine, histidine, and tryptophan. This is a similar order to that found by Wu and Hogg ('52) except for lysine and leucine which are reversed. These authors have reported a similarity between the order of abundance of amino acids in this protozoa and in vertebrates.

TABLE 4

Content of eleven amino acids in Tetrahymena pyriformis W

AMINO ACID	CONTENT IN TETRAHYMENA [1]	AMINO ACID	CONTENT IN TETRAHYMENA [1]
	mg/100 mg N		*mg/100 mg N*
Tryptophan	7.2	Methionine	15.6
Arginine	31.7	Phenylalanine	32.1
Histidine	13.9	Threonine	34.9
Isoleucine	38.3	Valine	37.1
Leucine	47.6	Tyrosine	21.1
Lysine	54.4		

[1] All amino acids except tyrosine and tryptophan determined in acid hydrolysates.

The amino acid composition of *Tetrahymena* was compared with the amino acid composition of the proteins used in this study by means of product-moment correlation as suggested by Mitchell ('50). The following positive coefficients were found: defatted whole egg, 0.88, vitamin-test-casein, 0.87, wheat gluten meal, 0.62, peanut flour, 0.50. According to the findings of Beach et al. ('43) such coefficients would indicate that defatted whole egg and casein would promote better growth of the *Tetrahymena* than would wheat gluten meal or peanut flour. This was found to be so experimentally.

DISCUSSION

While the method does not yield absolute values which are always identical with those found in other laboratories

for the rat, it nevertheless, differentiates between good and poor growth-promoting proteins and ranks them in the same order as found with the rat. The organism also responded with additional growth when the poorer protein, wheat gluten, was supplemented with a limiting amino acid, lysine. Such findings indicate the ability of the organism to differentiate between proteins and of the present colorimetric method to detect this differentiation. This appeared to be true when a short-time incubation period was used and the organism was presumably dependent upon the protein, *per se,* for its supply of specific amino acids. With longer periods of incubation, increased growth and higher values relative to casein were noted for the "poor" proteins. It is possible that the longer time of contact with the protein enabled the protozoa to alter the structures of the supplied amino acids and perhaps use these products to better advantage than the original amino acids split off from the protein. *Tetrahymena* are also cannibalistic, digesting non-living *Tetrahymena* cells, and these amino acids thus would become available for growth.

Partially purified proteins were used in the present study and an extension of this work to so-called crude proteins is indicated. If erratic responses to the crude protein are noted, the amount of work necessary for fat-extraction and other methods of partial purification is still small in relation to the usual time-consuming methods of animal assay.

SUMMARY

A modification of the method for measuring the growth response of *Tetrahymena pyriformis* W by means of the enzymatic reduction of triphenyltetrazolium chloride (TP-TZ) is given.

The method was used to determine the growth promoting ability of the following proteins: casein, defatted whole egg, peanut flour meal, wheat gluten meal, and wheat gluten meal supplemented with L-lysine. The response of the organism reflects the quality of the protein and can be evaluated nu-

merically to give a value similar to the protein efficiency value of the protein for the growing rat.

The amino acid composition of *Tetrahymena* was determined and compared by means of product-moment correlation to that of the proteins used in the study. The coefficients indicated the growth promoting ability of the proteins as determined experimentally.

ACKNOWLEDGMENTS

The authors wish to thank Mrs. Jean Abraham and Mrs. Elizabeth Sprague for determining the amino acid content of the proteins and of the *Tetrahymena;* Dr. E. L. R. Stokstad, Lederle Laboratories, Pearl River, New York, for the protogen and the Na-DL-6-thioctate, and Dr. James B. Allison, Bureau of Biological Research, Rutgers University, New Brunswick, New Jersey, for the Rutgers Protein samples.

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DIETARY CONSTITUENTS WHICH MAY INFLUENCE THE USE OF FOOD CHOLESTEROL

II. PROTEIN, L-CYSTINE AND DL-METHIONINE INTAKE IN ADOLESCENT RATS [1]

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ONE FIGURE

(Received for publication February 23, 1954)

INTRODUCTION

Protein of high quality accompanies cholesterol in most of the natural foods which furnish it in any considerable concentration. Conversely, it is difficult to obtain an adequate diet supplying sufficient amounts of the sulfur-containing amino acids without using eggs, milk, or more than the small portion of muscle meat allowed in the typical low-fat, low-cholesterol diet. That protein intake may influence food cholesterol retention, and possibly even the rate of cholesterol metabolism, is suggested by previous work in this laboratory (Okey and Turner, '51) as well as by numerous papers dealing primarily with the lipotropic effect of protein toward glyceride. DL-methionine feeding has been reported to result in a slight decrease in liver cholesterol (Channon et al., '38). Very recently, cebus monkeys given cholesterol-rich diets low in the sulfur-containing amino acids have been found to develop atherosclerosis (Mann et al., '53). The same laboratory has, since this paper was prepared, reported the effect of supplementation of soybean protein with methionine (Fillios and Mann, '54).

[1] This work was supported in part by a grant-in-aid from the National Heart Institute USPHS, Department of Health, Education and Welfare.

Reported differences in the effect of protein intake on liver cholesterol, and even on liver fat, are large. Some of them are obviously due to differences in the adequacy or balance of nutrients furnished by the experimental diets. It is also not unreasonable to expect that there may be species differences in use of protein and cholesterol, that a weanling animal may not react as an adult, or that duration of feeding cholesterol and protein may be important.

A systematic investigation of the consequences of variations in protein intake within the ranges to be expected in human dietaries chosen at random has therefore been undertaken. The present paper reports a short-time feeding experiment in which the effects on liver cholesterol of a moderate supplement (15%) of DL-methionine-rich protein were compared with those of equivalent amounts of DL-methionine, L-cystine, and a DL-methionine-L-cystine mixture.

EXPERIMENTAL

Rats of the Long-Evans strain from the Home Economics colony were used. They were placed, at weaning, on the stock diet,[2] and transferred to the experimental diets when the males attained weights of 150 gm and the females, 135 gm. Groups receiving each diet consisted of 10 males and 11 females.[3]

[2] The stock diet had the following composition: Ground whole wheat, 32 parts; raw casein, 16.6; skim milk powder, 8.3; whole milk powder, 8.3; ground alfalfa, 12.5; wheat germ, 12.5; dried brewers yeast, 5; $CaCO_3$, 1; iodized salt, 1; A and D vitamin mix, 2.8. Protein content, approximately 26%.

[3] In the interest of having groups of maximum size compatible with available facilities for caging in the laboratory at the same time, no animals on the corresponding cholesterol-free diets were included in this series. Such groups have, however, been studied. Variations in total liver cholesterol and in liver lipid have been in the same direction as those reported for cholesterol-fed rats, but of much smaller magnitude. Ranges of means were 20.8 to 24.9 mg total cholesterol per liver for males, and 11.5 to 16.9 mg for females. Corresponding total liver lipid values were 322 to 357 mg for males, and 263 to 316 mg for females. The last were taken as evidence that the choline content of diet B was adequate. Evaluation of these data is to be reported in detail with the growth study of which they constitute a part.

The composition of the diets is given in table 1. The methionine and cystine supplements in diets BM (basal plus methionine), BC (basal plus cystine), and BCM (basal plus cystine plus methionine) were calculated to be equal to those furnished by the extra 15% of egg white in diet HP (high protein).

TABLE 1

Composition of diet

INGREDIENTS	BASAL "B"	HIGH PROTEIN "HP"	BASAL + METHIONINE "BM"	BASAL + CYSTINE "BC"	BASAL + CYSTINE + METHIONINE "BCM"
	%	%	%	%	%
Casein (vitamin-free)	5.0	5.0	5.0	5.0	5.0
Egg albumin (dry powder)	10.0	25.0	10.0	10.0	10.0
Fat (Primex)	13.5	13.5	13.5	13.5	13.5
Salt mixture [1]	4.0	4.0	4.0	4.0	4.0
Cholesterol	1.0	1.0	1.0	1.0	1.0
Sucrose	64.5	49.5	63.6	64.2	63.3
ADEK mixture [2]	1.0	1.0	1.0	1.0	1.0
B mixture [3]	1.0	1.0	1.0	1.0	1.0
DL-methionine	0.0	0.0	0.9	0.0	0.9
L-cystine	0.0	0.0	0.0	0.3	0.3

[1] Hubbell, R. B., L. B. Mendel and A. J. Wakeman, J. Nutrition, *14*: 273, 1937.

[2] The B vitamin mixture furnished, per kilo of diet, 2.5 mg thiamine, 4 mg riboflavin, 2 mg pyridoxine, 1.5 mg biotin, 2 mg folacin, 10 mg calcium pantothenate, 10 mg niacin, 10 mg paraminobenzoic acid, 100 mg ascorbic acid, 500 mg inositol, and 500 mg choline.

[3] The ADEK mix furnished 10,000 I.U. vitamin A (as distillate), 1,000 I.U. vitamin D, 100 mg mixed tocopherol, and 5 mg menadione, per kilogram diet. It was diluted with cottonseed oil. Weekly food intakes varied from 90 (BM) to 97 gm (BC) for males and from 74 (BM) to 85 gm (BC) for females.

After 21 days on the experimental diet, each rat was killed by decapitation, subjected to careful autopsy, and the liver tissue was prepared for analysis.

Moisture determinations were made by drying weighed samples of liver tissue, in vacuo at 60°C., to constant weight. For lipid analysis, liver samples were weighed and immediately homogenized with redistilled 95% ethanol. They were stored under this ethanol until analyzed. Extraction was

completed by one two-hour heating under the ethanol at 65°C., decantation through a filter, and reextraction for one hour with a second portion of ethanol. The undissolved residue was then extracted, with freshly distilled ethyl ether, in a Soxhlet for 24 hours. The extracts were combined and made to volume.

Aliquot portions were saponified by heating on a steam bath with fresh normal sodium ethylate according to the method of Bloor ('28). The acidified residue was extracted with petroleum ether extract. A second aliquot was measured into a centrifuge tube, the petroleum ether was evaporated off, and the residue was taken up with acetone-alcohol. Analyses for total cholesterol were completed by the procedure of Sperry and Webb ('50). Free cholesterols were determined by evaporating an aliquot of the original alcohol-ether extract and completing the determinations according to the Sperry and Webb method. Careful checking showed that the modified procedure was at least as accurate as the published methods.

The advantages were: (1) the stability of the ethanol suspension, and (2) the elimination of the need for separate extraction and saponification for the determination of total lipids.

RESULTS AND DISCUSSION

Data for weight gains, liver size, and liver lipids are given in figure 1. For the sake of comparison, liver cholesterol figures were computed in three ways. Expression as percentage of the fat-free dry weight seemed to make no great difference in the pattern of significance as suggested by Ridout et al. ('52). It is recognized that this may not hold good under all experimental conditions. Because of the difficulty in securing, in rats, exactly uniform time intervals between the last ingestion of food and autopsy and because of the variability in glycogen content of livers of rats fed similar diets (unpublished work of Alta Garrison in this laboratory), computation of cholesterol as milligrams per liver seems the method of choice.

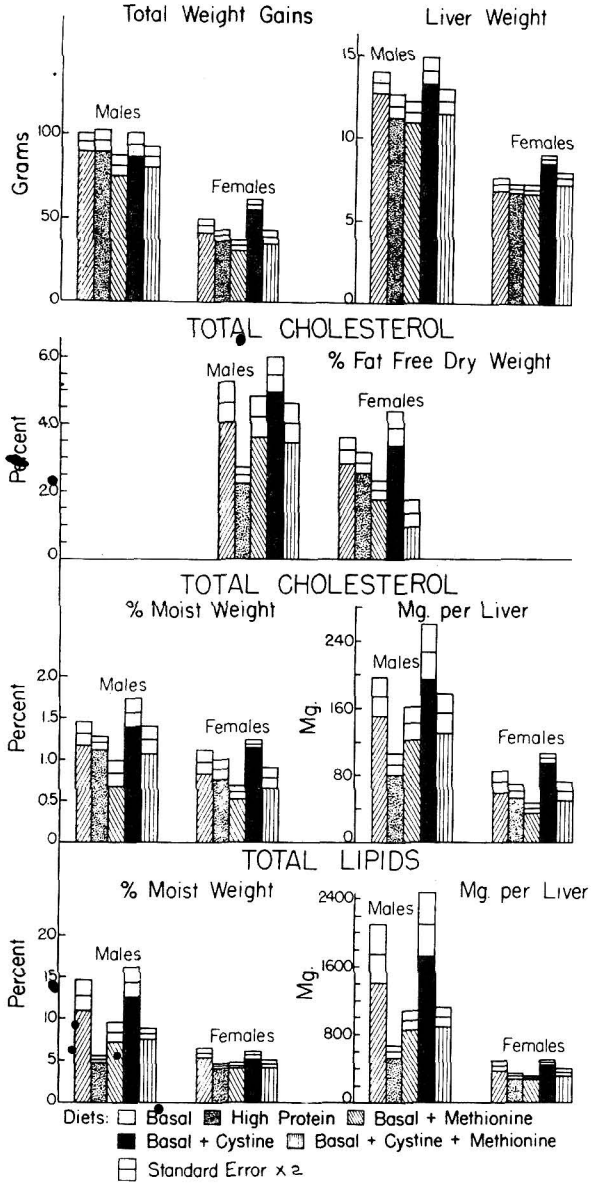

Fig. 1 Weight gains, liver weights, and liver lipids of rats fed the various experimental diets.

The total height of a column represents the mean value plus one standard error, the height to the line in the center of the unshaded area the mean value for each group.

Differences in liver cholesterol were confined almost entirely to the ester fraction. Free liver cholesterol for males varied between 0.223 and 0.242%, and between 28.1 and 35.7 mg per liver. For females, figures were 0.242 to 0.270%, and 16.8 to 21.8 mg.

Mean weekly food intakes for the last two weeks of the experiment were: males, B (basal) diet, 93 gm; HP diet, 95 gm; BM diet, 90 gm; BC diet, 97 gm; BCM diet, 82 gm. Food intakes and weight gains for individual animals showed close correlation. (Mean intake data for the entire period are not given because of a mistake made during the first few days of the experiment in records for part of the animals.)

The addition of an extra 15% of egg albumin to the basal diet resulted, in three weeks, in a significant reduction in total liver cholesterol as well as in liver fat in male rats ($p < .01$). The reduction in total liver lipids was probably significant in female rats ($p < .05$), but there was no significant reduction in liver cholesterol ($p < 0.5$). Free liver cholesterols were low for both males and females. Relatively low levels of liver fat and cholesterol have also been observed in female rats in other cholesterol feeding experiments with diets of moderate protein content. It is to be noted that food consumption and weight gains were fully as high for the males on the high-protein diet as for those on the basal diet. Females fed the HP diet gained somewhat less than did females fed diet B, a fact which may be related to fat storage.

The addition of methionine to the basal diet significantly lowered liver cholesterol storage ($p < .05$) in female but not in male rats ($p < 0.3$). Free cholesterols, as milligrams per liver, were higher than for the HP groups. In this case, however, lowered liver cholesterol storage was associated with lowered food intakes and significantly depressed weight gains ($p < .05$). On the basis of data for the B and BM groups alone, one might be tempted to conclude that the lipotropic effect of methionine was due primarily to reduction in food intake.

Comparison of the figures for the HP and BM groups, however, hardly supports such a conclusion. Significant differences in percentage, but not in amounts, of liver cholesterol were found between the HP males with relatively high food intakes and weight gains, and the BM males with lowered food intakes and weight gains and slightly smaller livers.

Significant lowering of cholesterol storage ($p < .05$) also appeared when BM females were compared with those on the HP diet, and in this case, food intakes and weight gains of the two groups were almost equal. The data do not, therefore, justify the conclusion that depression of cholesterol storage is solely a result of the depression of growth produced by methionine. Treadwell's concept ('44) of the preferential use of methionine for growth, rather than for lipotropic activity, is also difficult to reconcile with the data. It is interesting, in this connection, that other amino acids reported to have lipotropic activity, including threonine, have also been reported to depress growth.

The addition to the basal diet of L-*cystine,* in the amount furnished by 15% egg albumin, stimulated appetite and mean growth rate consistently in females, but not in males. Liver size was increased in both sexes. Liver lipid and cholesterol were not significantly increased except when the cholesterol data for females were computed as milligrams per liver ($p < .05$). The cholesterol content of the livers of the L-cystine-fed rats was consistently higher than that of either the HP or the BM groups, and was somewhat higher than that of the BCM group. In females, especially, L-cystine-induced increases in liver cholesterol storage were proportionately greater than increases in liver fat. An occasional L-cystine-fed male had a liver which was very fatty but not very rich in cholesterol. But when the cholesterol content of this liver was recalculated to percentage fat-free dry weight, or even to milligrams per liver, it did not differ markedly from that of other individuals in the group. However, it is hard to escape the conclusion that the lack of statistical significance

in the differences between cholesterol storage of the B and the BC males was due to the high individual variability.

The addition of both L-*cystine and* DL-*methionine* to the basal diet B did not have so great a lipotropic effect as did the high-protein diet, in the case of males. In the females there was no real difference between the HP and BCM groups. Growth differences between the rats on the HP and BCM diets were not significant.

Liver lipid storage showed positive correlation with liver cholesterol storage (on the basis of "r" values) for all groups except males on diet B and females fed diets BC and BM. How important these exceptions may be can only be determined by further study. It is hardly to be expected that groups of 10 animals each would give "r" values indicative of significant correlation except where few variances existed.

In view of problems of weight gain and weight reduction in relation to cholesterol retention, tests of possible correlation between weight gain and liver cholesterol storage also seemed indicated. Calculation of correlation coefficients ("r" values) gave significant figures for the BC males and the HP females only. However, scatter diagrams and arrangement of data for individual groups in order of magnitude gave some further information. Rats with the smallest amount of liver cholesterol were usually in the lowest quartile when the groups were arranged in the order of increasing weight gains. High values for liver cholesterol were usually associated with the largest weight gains. However, in each group there were some animals which failed to show this relationship. Moreover, it was observed that the rats from the same litters, even when they were fed different diets, tended to be found in the same quartiles when distribution was based on cholesterol storage.

Sex differences in the responses of the rats to cholesterol-rich diets containing different percentages of protein have been striking and consistent in studies of long as well as short duration. The weight gains and liver weights in this experiment were, as might be expected, significantly smaller

for females than for males. Differences between the HP groups (male and female) were less marked ($p < .05$) than were comparable differences in the other diet groups ($p < .01$).

Cholesterol feeding consistently resulted in less liver cholesterol storage in females than in males, even when figures were recomputed to storage per 100 gm body weight. Because of the greater tendency of the male rats to accumulate fat in the liver, sex differences in liver cholesterol are somewhat smaller when computed to a dry fat-free basis. So computed, however, the BC, BM, and BCM females still had significantly lower percentages of liver cholesterol than did the males fed the same diets.

Failure to recognize the extent of the sex differences in response to cholesterol feeding may well account for some of the contradictory findings reported in the literature. The high incidence of conditions due to abnormal cholesterol deposition in tissue in the young human male is well recognized. The animals in this study were just reaching maturity, and it seems fair to assume that their needs, both for protein and for cholesterol (as a precursor of sex hormones), were very high. Two possible explanations for the findings in this study are: (1) the female's increased tolerance for cholesterol and her failure to respond to protein feeding *per se* may be related to a lower rate of accumulation of body protein (i.e., growth) and a proportionately greater demand for cholesterol as a precursor of steroid hormones; (2) accumulation of the notably higher percentage of total body fat in the female than in the male may be linked with some mechanism which involves a more rapid turnover of liver lipid. Some evidence for the first possibility has been reported from this laboratory (Okey et al., '50); but neither possibility explains why the females showed the more significant responses to the feeding of free methionine or free cystine.

Attention should be drawn to the fact that the foregoing is a report of a three-week cholesterol feeding experiment with adolescent rats, carried out under set conditions so that

resulting data could be compared with those from other laboratories as well as evaluated in relation to studies of different duration in our own. Diets were purified, adequate insofar as we could determine, and furnished a fairly high percentage of fat. The time of feeding was, admittedly, short. Results are presented with no implication that the lipotropic effect of protein and methionine may not vary with differences in species, age, or range of growth of the test animal, duration of feeding, or with alteration of any one of a number constituents of the test diet. It seems significant, however, that liver cholesterol storage in male rats is, in a short time, as sensitive to protein intake as these data indicate.

SUMMARY

A three-week cholesterol feeding experiment with adolescent rats is reported. It was planned to measure the effect, on liver cholesterol storage, of adding 15% egg albumin to a diet already adequate for good growth. Five groups of rats were used. One group was given only the basal diet (B). Each of the other groups received, respectively, the basal diet plus 15% extra egg albumin (HP); DL-methionine (BM) L-cystine (BC); DL-methionine and L-cystine (BCM). The amounts of the amino acids were equivalent to those furnished by the extra egg albumin. Each group consisted of 10 males and 11 females.

Males given the extra egg albumin had significantly smaller amounts of liver cholesterol than did those fed the basal diet. Females had consistently lower liver cholesterol values than did males, but showed no significant decrease with increased protein intake. DL-methionine tended to decrease liver cholesterol storage, and L-cystine, to increase it. Differences were significant for females only, and were related to weight gain.

Sex differences in response to both protein and cholesterol were so marked as to make separate evaluation of data for males and females imperative.

ACKNOWLEDGMENT

The writers are indebted to Marcia McLean Stone for assistance in the autopsies and for the determinations of free cholesterol.

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THE USE OF CHROMIC OXIDE IN DIGESTIBILITY AND BALANCE STUDIES WITH DOGS[1]

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An important problem associated with the techniques of both digestion trials and balance studies employing either "time" or "marker" collection of feces is that of accurately identifying the fecal residue of the test diet. This difficulty may be overcome by calculating digestibility by a ratio method. This method involves the inclusion in the diet of a physiologically inert substance, and providing it is quantitatively and evenly eliminated in the feces, accurate digestion coefficients may be obtained.

Chromic oxide (Cr_2O_3) exemplifies such an inert substance, and its usefulness in digestion trials has been widely demonstrated. This index material has been successfully used in this respect with humans (Edin, Kihlen and Nordfeldt, '44; Kreula, '47, '50; and Irwin and Crampton, '51); with rats (Schürch, Lloyd and Crampton, '50); with pigs (Schürch et al., '52); with ruminants (Edin, Kihlen and Nordfeldt, '44; Kane, Jacobson and Moore, '50; Chanda et al., '51; and Crampton and Lloyd, '51); with horses (Skulmowski et al., '43; and Olsson, Kihlen and Cagell, '49); with rabbits, foxes and mink (Edin, Kihlen and Nordfeldt, '44); and with poultry (Edin, Kihlen and Nordfeldt, '44; Olsson, '50; and Dansky and Hill, '52).

In spite of the many species studied, no information is available as to the applicability of the Cr_2O_3 method to digesti-

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bility or balance studies with dogs. In addition to avoiding a quantitative determination of fecal output, the successful use of this ratio method makes possible a shorter collection period than that conventionally used for both types of study. Furthermore, quantitative records of feed intake are unnecessary if digestion coefficients only are required. Therefore, the primary objective of the work reported herein was to test the application of the Cr_2O_3 ratio method to digestibility or balance trials with dogs.

EXPERIMENTAL PROCEDURE

In order to establish the usefulness and accuracy of the Cr_2O_3 method of determining the apparent digestibility of ration nutrients by dogs, certain facts had first to be elucidated. For example, the evenness of the distribution of Cr_2O_3 in the feces, the length of time required for Cr_2O_3 excretion to become optimum, and the distribution in the feces of nutrients considered to be potentially the most variably excreted (nitrogen, calcium) were determined. An attempt was made to obtain these results under the most extreme circumstances of digestive activity. Therefore, relatively old dogs were employed in trial 1, since the gastro-intestinal motility of old animals would be expected to be more erratic than that of animals in younger age groups. Trial 2 consisted of an application to young dogs of certain results obtained in trial 1.

Trial 1. Four female dogs (a Fox Terrier, Cairn Terrier, Dachshund and a Beagle) were maintained in individual metabolism cages throughout the trial. The animals varied in age from 96 to 180 months. A standard commercial dog meal, containing Cr_2O_3 to the extent of 1% of its dry matter content, was fed each morning. A constant daily consumption of the complete ration was determined for each dog prior to the inclusion of the Cr_2O_3. The ration plus Cr_2O_3 was fed for a period of 20 days to represent the conventional 10-day preliminary and 10-day collection periods.

On the day following the initial administration of Cr_2O_3 with the ration, and subsequently for each of the 20 days, all fecal samples were collected twice daily (morning and evening) from each dog. These samples were oven-dried for 24 hours at 100°C. and ground for analysis.

Each fecal sample collected from each dog during the 20-day period was analyzed for Cr_2O_3, nitrogen and calcium. Ultimately, the fecal samples excreted from the 4th to the 7th day inclusive, following the initial administration of Cr_2O_3, were composited for each dog. On these composite samples, ash, crude protein and ether extract were determined by standard procedures.

Apparent digestion coefficients for dry matter were determined for each dog on each fecal sample by the Cr_2O_3 method, and for each dog by the conventional "time collection" method. Apparent digestion coefficients for crude protein, ether extract, total carbohydrate and ash were also determined for each dog by both methods.

Trial 2. Four 7-month-old Beagles were maintained in individual metabolism cages throughout the trial. The same commercial dog meal used in trial 1 was fed each morning. After a constant, daily consumption of the complete ration had been determined for each dog, Cr_2O_3 was included as 1% of its dry matter content. The ration plus Cr_2O_3 was fed for a preliminary period of three days followed by a collection period of 10 days.

Following the preliminary period, a quantitative record of fecal output was determined for 10 days. A representative sample of feces was retained daily from each animal for the first 4 days after the preliminary period. These daily fecal samples were oven-dried for 24 hours at 100°C., composited for each dog and ground for analysis.

Each composite fecal sample was analyzed for Cr_2O_3, ash, crude protein and ether extract. Apparent digestion coefficients for dry matter, ash, crude protein, ether extract and total carbohydrate were calculated by the Cr_2O_3 method and by the conventional "time collection" method.

RESULTS

Trial 1. The space required to illustrate the distribution of Cr_2O_3, nitrogen and calcium in individual samples of feces voided by the dogs at different times of the day is prohibitive. Therefore it must be sufficient to state that, in spite of the once a day feeding, the average distribution of Cr_2O_3, nitrogen and calcium was the same, whether the excrement was collected in the morning or in the evening.

The daily mean apparent dry matter digestion coefficients, as well as the concentration of nitrogen and calcium in the feces, weighted for morning and evening collections, are given in table 1 for each dog for the 20-day period. These coefficients indicate that on the third day following the administration of Cr_2O_3 in the feed, the index material was being excreted at its optimum level. In addition, after a preliminary period of three days, Cr_2O_3 was being fairly evenly distributed in the feces. Except for isolated extreme values, the distribution of both nitrogen and calcium in the feces excreted by the old dogs was also found to be relatively even from day to day.

It was shown by Schürch et al. ('52) that for pigs, the average of the Cr_2O_3 apparent dry matter digestion coefficients obtained for three days following an appropriate preliminary period was in agreement with the conventionally determined coefficient. However, after the preliminary period of three days, the averaging of the values for a 4-day period was considered more desirable in the case of dogs. The average apparent dry matter digestion coefficients for successive 4-day periods, as determined by the Cr_2O_3 method, are given in table 2. These, in effect, give average values for preliminary periods ranging from three to 17 days. The average values for nitrogen and calcium distribution in the feces for the corresponding 4-day periods are also presented in table 2.

In the majority of cases, the averaging of individual values over a 4-day period served to eliminate almost entirely the day-to-day variability in apparent dry matter digestibility,

TABLE 1

Weighted means of morning and evening values for Cr_2O_3 apparent dry matter digestion coefficients, and for nitrogen and calcium distribution in the feces

DAYS AFTER INITIAL Cr_2O_3 ADMINISTRATION	Cr_2O_3 DRY MATTER DIGESTION COEFFICIENTS				NITROGEN DISTRIBUTION IN FECES				CALCIUM DISTRIBUTION IN FECES			
	Dog no. 12	Dog no. 11	Dog no. 8	Dog no. 73	Dog 12	Dog 11	Dog 8	Dog 73	Dog no. 12	Dog no. 11	Dog no. 8	Dog no. 73
	%	%	%	%	*mg/gm*	*mg/gm*	*mg/gm*	*mg/gm*	*mg/gm*	*mg/gm*	*mg/gm*	*mg/gm*
1	67	60	55	62	41	43	42	43	58	61	67	66
2	74	72	73	74	40	39	39	44	66	63	63	61
3	73	73	75	75	38	42	40	42	64	57	65	63
4	71	71	75	75	41	41	39	42	61	57	65	66
5	75	70	76	76	41	46	39	43	62	56	63	67
6	75	71	76	75	39	45	41	40	65	55	67	66
7	77	72	74	75	39	45	43	44	67	57	66	63
8	75	70	76	76	38	42	47	43	61	57	69	67
9	75	71	75	76	42	42	40	40	63	51	63	68
10	76	71	76	75	40	40	41	41	64	55	63	65
11	74	73	74	74	40	40	42	42	58	57	63	63
12	75	73	76	76	40	41	38	41	66	54	67	65
13	75	71	76	75	40	41	39	42	61	53	64	71
14	76	72	75	74	39	40	40	42	63	58	62	64
15	77	65	76	74	42	41	41	40	63	52	66	63
16	75	74	76	74	41	42	38	42	63	59	65	66
17	75	70	76	74	42	43	40	42	59	52	62	63
18	75	74	75	74	42	39	41	42	65	58	63	62
19	74	72	77	74	40	40	37	42	61	57	63	68
20	77	71	76	74	41	42	42	44	67	53	64	63

and in nitrogen and calcium distribution in the feces. In addition, the values obtained after a preliminary period of three days corresponded as closely with the "time collection" value as did any of those obtained with preliminary periods of greater duration.

TABLE 2

A comparison of successive 4-day averages of weighted mean apparent dry matter digestion coefficients determined by the Cr_2O_3 method with those determined conventionally

PRELIMINARY FEEDING PERIOD	APPARENT DRY MATTER DIGESTION COEFFICIENTS DETERMINED BY THE Cr_2O_3 METHOD			
	Dog no. 12	Dog no. 11	Dog no. 8	Dog no. 73
days	%	%	%	%
3	73	71	75	75
4	74	71	75	75
5	75	71	75	75
6	75	71	75	75
7	76	71	75	75
8	75	72	75	75
9	75	72	75	75
10	75	72	76	75
11	75	72	75	75
12	76	70	76	75
13	76	70	76	75
14	76	70	76	74
15	76	71	76	74
16	75	72	76	74
17	75	72	76	74
Apparent dry matter digestion coefficients determined by "Time Collection" method	75	73	75	75

As a result of these findings, the feces excreted from the 4th to the 7th day were composited for each dog, and the composite samples were analyzed for ash, crude protein and ether extract. Total carbohydrate was determined by difference. The apparent digestion coefficients determined both by the conventional and Cr_2O_3 methods for each of these nutrients, as well as for dry matter, are shown in table 3.

TABLE 3

The apparent digestibility by old and young dogs of the nutrients of a commercial dog meal, as determined by two methods

AGE GROUP	DOG NO.	APPARENT DIGESTION COEFFICIENTS									
		Dry matter		Ash		Crude protein		Ether extract		Total carbohydrate	
		Collection	Cr_2O_3	Collection	Cr_2O_3	Collection	Cr_2O_3	Collection	Cr_2O_3	Collection	Cr_2O_3
Old dogs	12	75	73	32	27	77	75	92	92	78	77
	11	73	71	31	27	72	71	91	91	77	75
	8	75	75	30	30	77	77	90	90	79	79
	73	75	75	30	29	75	75	90	90	80	80
	Mean	74	74	31	28	75	74	91	91	79	78
Young dogs	B-84	72	73	34	36	68	68	86	87	78	79
	B-86	72	72	29	30	69	69	89	89	77	78
	C-13	74	74	32	30	73	72	85	85	80	80
	C-14	72	72	29	28	69	69	83	83	79	87
	Mean	73	73	31	31	70	70	86	86	79	79

The apparent digestion coefficients of the principal nutrients of the feed were in good agreement, whether determined by the conventional "time collection" method, or by the Cr_2O_3 method. Only in the case of ash were there appreciable discrepancies between methods in calculating apparent digestion coefficients. However, these differences, as well as those for other nutrients, were found by statistical analysis to be insignificant.

Trial 2. The apparent digestion coefficients for dry matter, ash, crude protein, ether extract and total carbohydrate, determined by both the conventional "time collection" method and by the Cr_2O_3 method using young dogs are presented in table 3.

The coefficients of all nutrients calculated by the index method agreed remarkably well with those determined conventionally. The slight discrepancy obtained between methods for the apparent digestibility of ash by the old dogs was not observed in the young animals.

DISCUSSION

The results obtained with both old and young dogs illustrate that the Cr_2O_3 ratio technique may be applied to digestibility and balance studies with this species. With its use, the quantitative determination of fecal output is eliminated, the actual collection period is shortened, and the necessity of retaining contaminated feces is avoided.

It was shown that the following steps are necessary for the successful application of this method to dogs:

1. At the conclusion of the ration adjustment period, Cr_2O_3 should be included in the ration for at least three days before the collection period commences. A convenient level of Cr_2O_3 was found to be 1% of the dry matter of the ration.

2. A collection period of 4 days sufficed to compensate for the day-to-day variations in Cr_2O_3 and nutrient excretion. Therefore, representative fecal samples should be retained daily for 4 successive days, each sample being dried for 24 hours at 100°C., and the composited sample for the 4-day period finely ground for analysis.

3. In the case of balance studies, total urine excretion should be recorded for the 4-day collection period, and suitable aliquots retained for analysis. The total excretion of a specific nutrient in the feces may be determined with the knowledge of its total intake during the period, and its digestion coefficient determined by the Cr_2O_3 method.

Aside from the original objective of this experiment, the results presented in table 3 illustrated an interesting situation with respect to the effect of age on nutrient utilization. It was noted that, regardless of method of determination, the apparent digestibility of the dry matter of the ration was greater for the old than for the young dogs. This enhancement was wholly a reflection of increases in the apparent digestibility of both crude protein and ether extract in the case of the old animals. The magnitude of these differences suggested that they were not due to chance variation, and indicated the need for further research into the effect of age on nutrient utilization.

SUMMARY

The Cr_2O_3-ratio method was found to be applicable to digestibility and balance studies with dogs. The advantages of this method indicated its replacement of the conventional "time collection" method.

A working procedure for the application of Cr_2O_3-ratio method to this type of study was given.

The apparent digestibility of both crude protein and ether extract was observed to be significantly greater for old than for young dogs. This observation indicated the need for further research into the effect of age nutrient utilization.

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FOR THE FOOD AND NUTRITION BOARD, NATIONAL RESEARCH COUNCIL

Editorial Note

The Journal of Nutrition is pleased to grant the request of the Food and Nutrition Board of the National Research Council to print from time to time official statements deemed important and of significant interest to professional workers in the field of nutrition. This is the first article to be published in accordance with such a plan.

A STATEMENT OF GENERAL POLICY CONCERNING THE ADDITION OF SPECIFIC NUTRIENTS TO FOODS

During the 1930's certain nutritional deficiencies were prevalent in the population of the United States and newly developed synthetic vitamins were being used in foods with little or no scientific guidance. In 1939 and again in 1945, the Council on Foods and Nutrition of the American Medical Association adopted policies on the proper addition of vitamins and minerals to foods.[1] In 1941 the Food and Nutrition Board (originally the Committee on Foods and Nutrition) of the National Research Council likewise adopted a policy on the addition of specific nutrients to foods. These statements of policy have now been reconsidered jointly by the Food and Nutrition Board and the Council on Foods and Nutrition in the light of experience and of new developments. There is good evidence to indicate that the policies have been beneficial to the public and have encouraged sound nutritional practices. The policies are therefore reaffirmed in principle and, with revision of wording, are embodied in the following statements:

1. With carefully defined limitations, the principle of the addition of specific nutrients to certain staple foods is

[1] Annual Meeting of the Council on Foods, 1939, J.A.M.A. *113:* 681 (Aug. 19). Also, Policies of the Council with Respect to the Nutritive Quality of Foods, 1945, ibid., *129:* 348–349 (Sept. 29).

endorsed for the purpose of maintaining good nutrition as well as for correcting deficiencies in the diets of the general population or of significant segments of the population. The requirements for endorsement of the addition of a particular nutrient to a particular food include (a) clear indications of probable advantage from increased intake of the nutrient, (b) assurance that the food item concerned would be an effective vehicle of distribution for the nutrient to be added, and (c) evidence that such addition would not be prejudicial to the achievement of a diet good in other respects. These requirements have been met in the specific cases indicated in paragraph 6 below.

2. The desirability of meeting the nutritional needs of the people by the use of natural foods as far as practicable is emphasized, and to that end education in the proper choice and preparation of foods and the betterment of food production, processing, storage, and distribution so as to provide more fully the essential nutrients native thereto are to be encouraged.

3. In order to avoid undue artificiality of food supply, foods chosen as vehicles for the distribution of additional nutrients should be, whenever practicable, those foods which have suffered loss in refining or other processing, and the nutrients added to such foods should preferably be the kinds and quantities native to the class of foods involved.

4. The addition of other than natural levels of nutrients to foods which are suitable vehicles of distribution may be favored when properly qualified judgement indicates that the addition will be advantageous to the public health and when other methods for effecting the desired purpose appear to be less feasible.

5. Whenever technological and economic developments lead to extensive reduction in the consumption of a staple food, with a consequent nutritionally significant reduction in the intake of an essential nutrient or nutrients, consideration by qualified bodies should be given to the desirability of restoring such nutrient or nutrients to the dietary.

6. The endorsement of the following is reaffirmed: the enrichment of flour, bread, degerminated corn meal, and corn grits; the nutritive improvement of whole grain corn meal and of white rice; the retention or restoration of thiamine, niacin and iron in processed food cereals; and the addition of vitamin D to milk, of vitamin A to table fats, and of iodine to table salt.

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