DIETARY PROTEIN AND THE DEVELOPMENT OF RAT LATHYRISM

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The literature dealing with lathyrism contains many references to the possible role of protein in the development of the syndrome. The earlier papers have been reviewed by Lewis and his group ('48) and deal largely with the question of the existence of toxic material in some species of *Lathyrus*, in contrast with the idea that protein insufficiency is the causative agent. Acceptance of the presence of an actual toxic agent has been followed by a shift in emphasis toward exploration of the question of whether proteins play a specific role in altering the course of the syndrome.

Of immediate interest among this latter group of publications are the papers of Vivanco, Diaz and Palacios ('50), Dasler ('54) and Bachhuber and Lalich ('54). Dasler found that both casein and gelatin exert a protective action against the development of the skeletal deformities characteristic of the syndrome and also promote growth; gelatin being less effective than casein. Bachhuber and Lalich found it difficult to produce aortic aneurism in the presence of dietary casein levels above 10%. Vivanco, Diaz and Palacios state there is a factor associated with the animal protein factor which is effective against rat lathyrism.

During the development in this laboratory of a procedure for the isolation of the toxic agent in the Singletary pea (*L. pusillus*), it was observed that two distinct toxicity patterns developed. One conformed to the classical picture of rat lathyrism in which skeletal changes are dominant; the

other involved the sudden onset of paralysis with only minor skeletal change, if any. These patterns were tentatively correlated with variation in toxin intake and the type of ration fed. This led to the investigation of factors which might be involved. The details with respect to the influence of proteins are presented in this paper.

METHODS

Weanling albino rats of a local strain served as test animals. With the one exception noted in the text, all comparisons were made on the basis of matched littermates. The weight, food consumption and condition of each rat were recorded daily. Each animal was rated on the basis of general appearance, abnormal gait, development of palpable exotoses and of spinal curvature. A scale of one plus to 4 plus was used to denote the progression from slight to very severe change. Definite muscular paralysis was separately noted.

The basal rations employed are given in table 1. All were fed ad libitum unless otherwise specified. Supplements were

TABLE 1

Composition of basal rations

CONSTITUENT	DIET A	DIET B	DIET C
	%	%	%
Singletary pea meal	70	70	60
Dextrin, white	25	20	..
Sucrose	..	..	29.5
Corn oil, fortified [1]	..	5	5
Dextrose, fortified [2]	..	3	3
Salt mix [3]	2	2	2
Brewers' yeast [4]	2	..	..
Delsterol	1	..	..
DL-methionine	..	..	0.5

[1] Add 1.0 gm alpha-tocopherol, 1.0 mg calciferol, 1.0 gm vitamin A ester concentrate to 498 gm Mazola brand oil.

[2] Add 20.0 gm choline chloride, 1.0 gm niacin, 0.2 gm calcium pantothenate, 50 mg riboflavin, 25 mg thiamine hydrochloride, 20 mg pyridoxine hydrochloride, 5 mg folic acid, 2 mg biotin, 2 mg vitamin B_{12} to 279 gm U.S.P. dextrose.

[3] Hubbell, Mendel and Wakeman ('37).

[4] Fleischmann.

introduced at the expense of dextrin or sucrose. Ration A was first employed and was devised to simulate the type of ration with which the clinical syndrome develops. When the paramount effect of protein supplementation became evident, it was abandoned in favor of the more desirable rations.

The detoxified form of ration B contained Singletary pea meal which had been thoroughly extracted with methanol. Prior results indicated such a meal to be nontoxic.

The experimental periods employed varied from 13 to 35 days. Data concerning weight and skeletal changes are presented on the 15-day basis. Study of records has shown this period to be predictive of the overall pattern of growth and skeletal change and such a presentation avoids the troublesome questions of the effect of inanition and variability in length of experimental period frequently caused after the 15th day by paralysis.

RESULTS

Effect of protein on growth and skeletal change

The results given in table 2 show that supplementation with casein (either technical or vitamin-test grades), an acid-hydrolized casein, a tryptic casein hydrolyzate or with lactalbumin consistently improved weight gain and postponed the onset of lathyritic skeletal changes. This effect was most marked when the casein-supplemented ration B and the unsupplemented ration B were pair-fed to equalize the intake of toxic material. Gelatin was not as effective as casein in promoting weight gain, but did slow the onset of skeletal change in a definite manner; with zein there was some weight increase, probably because of its methionine content, but there was very little delay in the onset of skeletal change.

The 10 amino acids regarded as essential for rat growth were then incorporated individually into ration A at levels approximating the amount supplied by 25% of casein in the ration. None of these 10 supplemented rations seemed to alter the pattern of skeletal change observed in the paired animals receiving the unsupplemented ration.

TABLE 2

The effects on growth and skeletal change produced by introducing protein into lathyrogenic rations

NO. OF PAIRS	SEX	AVERAGE GAIN [1]		LATHYRITIC SEVERITY [1]		SUPPLEMENT
		Basal only	Plus protein	Basal only	Plus protein	
		gm	*gm*			
			A. Basal ration A			
3	F	23	39	2 +	1 +	25% casein [2]
3	M	17	49	2 +	1 +	
5	F	21	27	2 +	1 +	25% gelatin [3]
1	M	13	28	3 +	1 +	
2	F	19	27	2 +	2 +	25% zein [4]
1	M	24	28	2 +	3 +	
			B. Basal ration B			
3	F	30	57	2 +	0 +	20% casein [4]
6	M	32	63	3 +	1 +	
2	F	26	42	2 +	1 +	20% gelatin [3]
5	M	32	38	3 +	1 +	
2	F	28	37	2 +	1 +	20% acid
3	M	33	50	2 +	1 +	casein hydrolyzate [5]
2	F	28	44	2 +	1 +	20% tryptic
3	M	32	57	2 +	1 +	casein hydrolyzate [6]
			C. Basal ration B; rats pair-fed			
2	F	29	43	3 +	0	20% casein [4]
3	M	24	40	2 +	0	
			D. Basal ration C			
2	F	41	52	2 +	0	25% casein [2]
3	M	24	40	2 +	0	
3	F	42	48	2 +	1 +	25% lactalbumin [5]
1	M	41	61	2 +	1 +	

[1] Over a 15-day period. All values rounded.
[2] Vitamin-test casein.
[3] U.S.P. gelatin.
[4] Technical grade.
[5] From Nutritional Biochemicals.
[6] From General Biochemicals.

Supplements of DL-methionine did, however, improve growth slightly. Varying the level of methionine between the 0.6 and 1.6% levels did not alter the magnitude of the response.

This finding raised the question of whether this effect of methionine on weight gain was due to a methionine deficiency of pea protein or to a deficiency specifically induced by the lathyrogenic factor in the pea. To answer this, matched trios of littermates were fed separately the detoxified form of ration B, the same containing 0.6% of DL-methionine and the same containing 20% of casein. The results, given in table 3, part A, indicate that a methionine deficiency existed in the basal detoxified ration. They also seem to indicate that no other amino acid is critically deficient in the ration as the casein supplement was no better than the methionine supplement. This was not the case when the lathyrogenic form of ration B was used in the same manner. Here, casein supplementation was far more effective than the methionine addition (see table 3, part B). Further evidence of the same nature may be found in table 2, part D. Hence, casein must have supplied some factor other than methionine which acted to offset a growth-depressing effect of the lathyrogenic agent of the pea.

With the finding that β-aminopropionitrile and aminoacetonitrile can induce the typical lathyritic syndrome in the rat (Wawzonek et al., '55), the effectiveness of a casein supplement against these materials was tested using detoxified ration B as the basal ration. It was found that the incorporation of casein at the 20% level did not slow the onset of lathyritic symptoms when the concentration of β-aminopropionitrile was above 0.1% of the ration. The rations were simply too toxic. This is in agreement with the negative results of Bachhuber et al. ('55) obtained with rations containing 21.5% of casein and 0.3% of β-aminopropionitrile. At levels of 0.1 and 0.05% of added β-aminopropionitrile, animals fed the casein-supplemented ration had recorded lathyritic severities of 1, 1, and 1 +, and 1 and 1 + at day 15. In the

absence of dietary casein, the recorded lathyritic severities of the animals were 2 and 2 + and 2, 1, 1, 2 and 2 + at day 15 for these two levels of added β-aminopropionitrile. These animals were not matched littermates; still, the same pattern of protection seems to be evident.

The results with aminoacetonitrile were equivocal at levels of 0.03 and 0.06% and negative at higher levels.

TABLE 3

The effects of methionine and casein when fed with the detoxified and lathyrogenic forms of ration B

NO. OF TRIOS	SEX	AVERAGE [1] GAIN	LATHYRITIC [1] SEVERITY	SUPPLEMENT
		gm		
		A. Fed detoxified ration		
2	M	54	0	None
		86	0	0.6% DL-methionine
		85	0	0.6% DL-methionine + 0.2% L-tryptophan + 1.6% L-lysine HCl
3	F	50	0	None
		72	0	20% casein [2]
		70	0	0.6% DL-methionine
1	M	60	0	None
		85	0	20% casein [2]
		87	0	0.6% DL-methionine
		B. Fed lathyrogenic ration		
2	F	34	3	None
		43	3	0.6% DL-methionine
		58	1	20% casein [2]
1	M	27	2	None
		39	3	0.6% DL-methionine
		61	0	20% casein [2]

[1] Over a 15-day period. Values rounded.

[2] Technical grade.

Effect of protein on paralysis

The paralysis observed during the early phase of the study appeared only during the terminal stage when an animal was severely crippled. This paralysis was found to be largely irreversible (Lee, '50).

In this study, the paralysis obtained with ration A, containing casein, appeared much earlier both in point of time and degree of crippling skeletal change. Eight animals in a group of 34 (23.5%) became paralyzed between the 19th and 30th days. The recorded severities of lathyrism for these animals at the time of paralysis were 1, 1, 2, 1, 2, 2, 3 and 2 +. These animals recovered the use of their hind legs when they were returned to the stock laboratory ration and nursed carefully; however, the skeletal changes noted were still observable. None of the paired unsupplemented animals became paralyzed during the 35-day experimental period.

With ration B, containing casein, the onset of paralysis without severe skeletal change came still earlier and with greater frequency. Twelve animals out of 27 (44.4%) became afflicted; 4 before the 15th day and 6 more before the 20th day. Recorded lathyritic severities at time of paralysis were 1, 1, 1, 1, 1, 0, 1, 1, 0, 0, 1 and 1 +. Only three of the paired unsupplemented animals became paralyzed (11.1%); two before the 20th day and the third before the 25th day. Lathyritic severities at the onset of paralysis were recorded as 3 + in all three cases.

The same exacerbation of paralysis seemed to occur when rations supplemented with gelatin or lactalbumin were used. The numbers of animals fed these rations were small compared to those available for casein and, therefore, the data are not presented.

DISCUSSION

The finding that casein can retard the onset of the skeletal change when β-aminopropionitrile in known amount is the lathyritic agent indicates that this effect is definitely related to the metabolic effects of a lathyrogenic factor in the seed.

Inasmuch as with rations containing the detoxified seed, the addition of either methionine or casein results in equally good growth, and yet methionine alone cannot replace casein in the lathyrogenic diets, it would appear that a growth depression is induced by the lathyrogenic factor which can be

alleviated by some factor or factors in casein other than methionine. The same is true for the skeletal effects.

The relatively rapid onset of a reversible paralysis with little skeletal change when casein is fed indicates that paralysis is a specific effect of the lathyritic factor and not merely a secondary effect of vertebral collapse as has been suggested (Lewis et al., '48). This is probably the cause of a secondary paralysis where severe crippling has occurred; thus two types of paralysis may occur in experimental rat lathyrism.

It is suggested as a working hypothesis that the skeletal and growth effects of casein, gelatin and lactalbumin result from the partial reversal of an interference with cellular uptake or utilization of amino acids. The protein may overcome the block induced by the lathyrogenic material by providing either a single specific substance or by the mass provision of a select group of amino acids. Too high a level of toxin would have the reverse mass effect and negate the desirable effect of protein. The superior effect of casein on growth, in comparison with gelatin, is then a reflection of a more desirable pattern of amino acids. The alternative seems to be the questionable hypothesis that protein is supplying not one but two entities; one specific for promoting growth, the second for prevention of skeletal deformity.

The exacerbation of the paralytic effect may be explained as a combination of the stress induced by rapid growth and the increased intake of toxic material, protein having no direct effect whatever. No paralysis occurred within the experimental period when pair-feeding was employed to equalize intake of toxic material and limit growth, although it was common without such limitation. The idea that the paralytic effect may be the result of cumulative toxic action must be included because of the experiences during isolation studies where fractions containing little activity frequently produced paralysis after long periods of feeding.

The question of specificity of protein effect then remains. It does not appear to involve singly any of the amino acids ordinarily listed as essential for the rat. The possibility that an unusual induced deficiency of one of the unessential acids is present is not thought to be too probable because of the performance of zein. If the protein is acting as a carrier of an active non-protein material, it must be very tightly bound because vitamin-test casein was apparently as effective as the technical grade. This is thought less probable than the remaining possibilities that the protein is effective either because of its supply of a select group of amino acids or of a specific peptide.

It is implicit in this pattern of thought that anything which affects the utilization of the amino acids might well have a definite effect on development of the lathyritic syndrome. This could explain the different results recorded in the literature as due to the divergent lathyrogenic rations employed.

SUMMARY

Casein, casein hydrolyzates, lactalbumin and gelatin are effective in minimizing the growth depression and skeletal change induced by feeding Singletary pea seed to albino rats. Zein is not effective. Casein is also effective where low levels of β-aminopropionitrile are used as lathyrogenic agent. Diets containing casein exacerbate the paralytic effect of the seed so that a reversible paralysis is produced which does not involve spinal compression or other severe skeletal change.

Methionine is the first limiting amino acid in the seed protein, but neither it nor any other of the essential amino acids can replace the effective protein supplements.

It is suggested that the skeletal and growth effects of the effective proteins result from a reversal of an interference with amino acid metabolism; protein providing either a select group of amino acids or a specific peptide. The exacerbation of the specific paralytic effect is then caused by the stress of more normal growth combined with larger intake of toxin.

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STUDIES OF PROTEIN ABSORPTION USING NITROGEN ^{15} AS A TAG

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Increasing interest is found in the literature concerning the assimilation and utilization of protein foods as one of the major metabolic deficits of the geriatric patient. However, a marked decrease in utilization capacity, as a function of age alone, is not reported in well controlled studies. The work of Horwitt ('53), with older human subjects, indicates a decreased protein need, proportional to decreased caloric requirements, while Hegsted et al. ('46) suggest that nitrogen equilibrium in man can be maintained on as little as 30 to 40 gm of protein per day.

Studies of nutrition in relation to specific diseases and to starvation are also reported. Bolker ('53) conducted a study of increased tumor sensitivity to roentgen rays among cancer patients who were in negative nitrogen balance prior to the start of forced protein therapy; and Brock ('55) has reviewed and summarized the dietary management of patients suffering from kwashiorkor and other forms of chronic protein malnutrition.

Continued interest is found also in the biologic value, that is, the repleteness and digestibility of protein foods (Allison and Anderson, '45; Bricker, Mitchell and Kinsman, '45; Benton et al., '55). Thomas ('09) has defined the biologic value of proteins in terms of the amount of nitrogen retained in an animal. Allison ('49) has reviewed the many methods

of determining the absorption and retention of protein. He comments: "Dietary proteins replete tissues differently than they support growth in young animals or maintain the nitrogen integrity of the adult."

Many methods for the evaluation of protein assimilation are applicable to humans as well as to animals. Of recognized methods, nitrogen balance, as developed by Mitchell ('44, '45) and Murlin ('46a, b, c) and their respective co-workers is the most readily applied to adults. This method involves the use of a nitrogen-free diet for a control period in which fecal and urinary nitrogen are determined, and employs a shift to a diet containing the test protein. Murlin et al. ('46a) found that the urinary nitrogen on a nitrogen-free diet varied in man with (a) the level of protein in the pre-experimental diet, (b) the position of the no-protein period, (c) the nature of the supporting protein, (d) conditions antecedent to the supporting proteins which could affect the accrued nitrogen deficit prior to the beginning of the no- protein period. These variables would make difficult the study of the absorption and retention during high constant protein intake, especially with comparison between young and old subjects.

The concept postulated by Folin ('05), which has been given strong support by Mitchell ('55) makes necessary a distinction between endogenous and exogenous nitrogen in protein metabolism. Starvation, or at least the use of a nitrogen-free diet, is the classical method of determining the endogenous nitrogen plateau, but it is a difficult dietary discipline to maintain among a group of human subjects.

Other workers (West, Wilson and Eyles, '46) have used the postalimentary rise in plasma acids as a measure of the absorption of amino acids. Tagged iodinated casein has been used by Lavik et al. ('52) to determine protein absorption by the measurement of plasma iodine values, as well as urinary and fecal excretion of radioactive iodine. Use of iodine as a protein tag has been critically evaluated (Anonymous, '53).

In our work, N^{15} was selected as a protein tag because it introduced none of the assumptions concerning protein metabolism which would have accompanied the use of C^{14} or I^{131}. The need for direct evaluation of endogenous nitrogen is avoided through the use of a single feeding of the tagged protein

Yeast was selected as the protein source because of the relative simplicity of incorporation of the tag into synthetic culture media; the large yield of tagged protein obtainable from a given amount of N^{15}, and the high N^{15} to N^{14} ratio which can be attained in a final yeast product (Roll et al., '49). The ability of yeast to grow well on ammonium salts as the sole nitrogen source indicates synthesis of all common amino acids, and, hence, general distribution of the tag among all of the amino acids. Yeast in moderate supplemental amounts, is well tolerated by patients and its nutritive values have been well established.[1] It was, therefore, possible to maintain the patients on a full diet, plus an identical unlabelled yeast source of protein, so that the experimental feeding conditions could be uniform throughout the study.

PROCEDURE

Preparation and analysis of yeast. Two gram of N^{15} were purchased in the form of ammonium radical (NH_4) enriched ammonium nitrate with an N^{15} content of 67% in the ammonium (NH_4) radical, and with no N^{15} enrichment in the nitrate radical.[2] The ammonium nitrate was converted to di-ammonium phosphate to eliminate the nitrate radical. Synthetic culture media were prepared [3] and, prior to N^{15} addition, were completely free of nitrogen, except for the minute amounts which the necessary vitamin fractions contained. Identical proportions of untagged di-ammonium phosphate were introduced into larger quantities of the same nitrogen-

[1] Yeast, 1: 3, Anheuser-Busch (1949) St. Louis.

[2] Eastman Organic Chemicals Dept., Distillation Products Industries, Rochester, New York.

[3] We are indebted to Drs. H. J. Buehler and R. D. Seeley of Anheuser-Busch for preparation of culture media and control and tagged yeasts.

free culture medium for the preparation of the control yeast. The nitrogen contents of the tagged and control yeasts (*S. cerevisiae*) were 8.25 and 8.89%, respectively, corrected for moisture, ash, and molecular weights (table 1). The purine nitrogen content was 12 and 13%, respectively, of total nitrogen, and the non-purine nitrogen by difference was 88% in the tagged, and 87% in the control yeast.

Determination of the N^{15}, as well as total nitrogen of all samples, yeast, urine or feces was accomplished by converting them to ammonia — then to nitrogen — followed by mass

TABLE 1

Total purine and non-purine nitrogen content of yeast

ITEM	N^{15} YEAST	CONTROL YEAST
	%	%
Moisture	6.4	4.5
Ash	6.7	8.7
Nitrogen, total (corrected for moisture and ash)	7.97	8.89
Nitrogen corrected to an approx. molecular wt. of 29 [1]	8.25	
Purine nitrogen (corrected for moisture and ash)	0.93	1.2
Purine nitrogen	12	13
Non-purine nitrogen	88	87

[1] Made up of 58.28% N^{15} and 41.72% N^{14}.

spectrometry assay for N^{15}. Macro-Kjeldahl determinations were run for total nitrogen, employing metallic selenium and mercuric oxide as catalysts for an 8-hour digestion period to completely convert all nitrogenous compounds to ammonia (Rittenberg, '46). The ammonia was distilled into standardized dilute sulfuric acid, and the residual acid titrated with base for total nitrogen estimation. Subsequently, the entire sample was re-acidified and reserved for N^{15} determination by a modified Rittenberg procedure.

The method [4] reported by Rittenberg and associates (Rittenberg, '46; Rittenberg et al., '39a, b; Schoenheimer et al., '38, '39a, b, c) was modified to permit the processing of

[4] This portion of the analyses was performed by Dr. R. D. Finkle of the Atomic Research Laboratory, Los Angeles.

samples with as much as 50 mg of nitrogen and the storage of these samples as nitrogen gas without serious dilution by air leakage through stopcocks. Samples were stored from two to 20 days before assay for N^{15}/N^{14} ratio.[5]

Mass spectrometric examination. The mass spectrometer used was not designed for maximum accuracy at high concentrations of N^{15} It was, therefore, necessary to dilute the N^{15} in the analysis of the tagged yeast with untagged nitrogen supplied from reagent grade ammonium sulfate which contained a natural isotopic enrichment of 0.0048 atom % excess N^{15} above reference tank nitrogen (table 2). The N^{15} content of the yeast was 58.28 atom % excess above reference tank nitrogen, allowing for the N^{15} in diluent ammonium sulfate.

As a result of the high N^{15} content, small amounts of tagged yeast (2.0 gm containing 83.57 mg N^{15}) which constituted about 1% of the protein of the diet could be fed experimentally; and the level of N^{15} in urine and fecal samples was sufficiently high for determination of N^{15} for a period up to 6 days subsequent to a single tagged feeding. To establish the natural occurrence of N^{15} in biologic samples, urine and stool specimens were obtained from patients who were maintained on a controlled high-protein diet for a minimum of three days and a maximum of 9 days prior to feeding of the N^{15} enriched yeast.[6] During this preliminary control period 1.83 gm of unenriched control yeast were added to the noon meal, daily. The isotopic content of the urine and stool specimens reflects biologic occurrence of N^{15} in their total diet during this period. Table 2 indicates that the biologic N^{15}/N^{14} ratio of samples from all patients averaged approximately 0.0066% above the natural occurrence of N^{15} in the reference tank nitrogen. The apparent tendency of some biologic processes to produce a greater concentration of heavy isotopes is a phenomenon which has been reported previously

[5] Sincere appreciation is expressed for the cooperation and assistance of Mr. Philip S. Fogg, president; Mr. J. K. Walker, manager and Mr. C. V. Bailey, supervisor — Analytical Service, and Mr. H. F. Froch, associate engineer; Consolidated Electrodynamics Corporation, Pasadena, California.

[6] Hospital studies were generously supported by the Swanson Foundation.

by Brewer ('37). This moderate N^{15} elevation above tank nitrogen was observed in all unenriched biologic material analyzed in this study. Therefore, the average enrichment value from these untagged specimens was used as a baseline for studying the subsequent enriched specimens. The reference nitrogen tank gas was used as a baseline for observation of instrument stability throughout the study.

TABLE 2

Nitrogen^{15} occurrence in unenriched biological samples

(Mass spectrometric analysis)

SUBJECT AND SPECIMEN [1]	SAMPLE	TANK NITROGEN	EXCESS OF ATOM % N^{15} IN SAMPLE	% NITROGEN FROM AIR BASED ON 32 PEAK	ATOM % EXCESS CORRECTED FOR NITROGEN FROM AIR 32 PEAK
	Atom % N^{15}	*Atom % N^{15}*			
L.G.–S	0.3709	0.3642	0.0067	2.45	
L.G.–U	0.3744	0.3656	0.0088	None	
G.S.–S	0.3709	0.3642	0.0067	2.4	
G.S.–U	0.3699	0.3644	(0.0058)	36.00 [2]	0.0076 [2]
A.E.–S	0.3735	0.3674	0.0061	None	
A.E.–U	0.3715	0.3657	0.0058	1	
S.B.–S	0.3728	0.3675	0.0053	None	
S.B.–U	0.3734	0.3660	0.0074	1	
B.S.–S	0.3691	0.3626	0.0065	None	
B.S.–U	0.3694	0.3637	0.0057	1	
N.H.–S	0.3681	0.3624	0.0067	0.5	
N.H.–U	0.3682	0.3628	0.0054	2.2	

Atom % excess N^{15} in sample

Average unenriched stool: 0.0063 ± 0.00043
Average unenriched urine: 0.0068 ± 0.0012
Average unenriched combined: 0.0066 ± 0.00076
Standardized unenriched $(NH_4)_2 SO_4$: 0.0048 ± 0.00065

[1] Obtained from pooled samples of urine (U); or stools (S) collected over a three-day period for each subject while on controlled diet supplemented by untagged yeast prior to N^{15} feeding.

[2] Correspondence of 32 peak (oxygen) and 40 peak (argon) indicated sample contained air and the corrected (higher) value was used in this single case.

Selection of persons to be studied. Other studies by this group (Sharp and Hazlet, '54a; Sharp, Hazlet and Shankman, '54b) have dealt with testing of secretion levels of gastric hydrochloric acid, and with the utilization of hydrochloric acid [7] supplementation as therapy in achlorhydric and hypochlorhydric subjects. Published clinical findings and unpublished supplemental data indicate considerable improvement in utilization of protein foods for such patients. Therefore, one of the aims in this study was to assess with a precise analytical method the differences in protein absorption and retention between more aged achlorhydric or hypochlorhydric subjects and young euchlorhydric controls.

Six subjects were selected: two, each aged 24, a female and a male, were selected as controls on the basis of age and apparent normal gastric function; the remaining 4 were selected because impaired gastric function (decreased capacity to produce hydrochloric acid) could be expected — two, aged 57 and 66, females, had long-standing achlorhydria, and two, females, each aged 70, had well established hypochlorhydria. Each of these 4 subjects had been under observation of two of us for a minimum of 6 months. The primary complaint, referable to all 4, was irritable mucosal surfaces of the oral cavity. Hydrochloric acid supplementation and a high protein diet were prescribed as supporting therapy for the mouth complaints. Because of the marked improvement with this therapy, and because of the sustained high protein diet, these patients were felt to be suitable subjects, adequately preconditioned, for this study.

Selection of diet. The 1800 calorie menus selected provided a high content of animal protein, averaging 85 gm of a daily total of 110 gm, and were planned by the dietetic staff at the Huntington Memorial Hospital, Pasadena, California. A standard table of dietary values [8] was used in the calculation of protein content of all foods served. Three daily menus

[7] Normacid — The Stuart Company, Pasadena, California.

[8] Standard Table of Dietary Values — A Compilation of Diets 1953 The California Dietetic Association 7th ed. Los Angeles.

were prepared to provide variety and, with slight variation, the three were adhered to, in rotation, by all subjects in the study.

Administration of diet. For the 4 older, achlorhydric and hypochlorhydric, patients, the major (animal) protein components of the diet were furnished in weighed amounts for home preparation during the 10 days prior to hospitalization.

TABLE 3

Protein intake, fecal excretion and absorption by difference of nitrogen during 5-2/3 days

SUBJECT	TOTAL FOOD AND YEAST PROTEIN 17 MEALS [1]	AVERAGE PROTEIN PER MEAL	AVERAGE DAILY PROTEIN	AVERAGE DAILY NITROGEN	AVERAGE DAILY NITROGEN FECAL OUTPUT	% OF DAILY NITROGEN INTAKE ABSORBED BY DIFFERENCE [2]	% OF SINGLE NITROGEN FEEDING ABSORBED, BY DIFFERENCE [3]
	gm	*gm*	*gm*	*gm*	*gm*		
L.G.	623.84	36.68	110.04	17.62	1.61	90.86	87.3
G.S.	690.82	40.62	121.86	19.51	1.74	91.08	92.69
A.E.	617.65	36.32	108.96	17.44	1.57	90.10	90.93
S.B.	630.45	37.07	111.21	17.80	2.53	85.56	89.96
B.S.	612.45	36.01	108.03	17.30	1.56	90.98	90.22
N.H.	586.91	34.51	103.53	16.58	1.23	92.58	90.83
						Mean	Mean
						90.19 ± 1.576	90.34 ± 1.193

[1] Two meals prior to, and 14 meals subsequent to meal containing tagged yeast. All meals measured and served in hospital.

[2] Not corrected for endogenous nitrogen content of specimens or variables introduced by day to day fluctuations in fecal volume and excretion rate.

[3] Because of single feeding of N^{15} tag, endogenous nitrogen and rate variation correction not required.

Commencing 5 days before the tagged feeding, these patients received 1.83 gm of untagged yeast with their noon meal, daily. The young euchlorhydric control subjects received hospital-prepared meals and the daily 1.83 gm of untagged yeast during a three-day preconditioning interval. The yeast was supplied in a dry form in individual dose containers, in weighed amounts, and was usually stirred into milk; however, patients were permitted to mix the yeast with any food desired. No loss or rejection of yeast was reported through-

out the study. The daily noon feeding of the yeast continued throughout the study, except on the experimental day when 2.0 gm of N^{15} enriched yeast, comparable in total nitrogen content was substituted. Table 3 indicates the individual protein and nitrogen values of yeast and food consumed during 17 consecutive hospital-served meals. All foods were weighed, and rejected foods weighed for calculation of food values.

Collection of biological specimens. Commencing three days prior to tagged N^{15} feeding, all urine and fecal samples from all patients were collected. Wide-mouth Mason jars were provided each patient for direct collection of fecal and urine samples. HCl was used as a preservative to prevent loss of nitrogenous substances from both feces and urine. Upon receipt, the volume of each urine sample and the weight of each fecal sample were determined, with allowance for added measured preservative. Homogenization of fecal samples was necessary prior to aliquot taking.

RESULTS

All fecal specimens collected subsequent to the tagged feeding had a significant elevation of N^{15} concentration, with the single exception of one specimen from one subject (L. G.) collected three hours after tagged feeding in which an elevation of 0.0021 atom % (table 4) excess above the biological baseline was noted. Table 4 shows also a significant rise in N^{15} concentration in all other first specimens, and in a single second specimen (L. G.), on the morning following the noon tagged feedings ranging from 0.5 to 100 times the baseline. For two of the subjects these 20- and 21-hour specimens represented the peak concentrations found, while for the 4 remaining subjects the time of excretion peaks varied from 29 to 56 hours. In 5 of the 6 cases, the morning specimens of the 6th experimental day ($5\frac{2}{3}$ elapsed days) showed a return of the excretion rate to less than twice the baseline level. In the remaining case the level approximated a magnitude of 6 times the baseline. In other intermediate speci-

mens (not reported here) a consistent delay was also noted in the appearance of sizable early N^{15} concentrations, as well as delay in appearance of maximum peaks, indicating a much slower excretion rate for this subject than for the others studied.

Calculations of atom % excess N^{15} over the baseline times milligrams of nitrogen derived from Kjeldahl determinations

TABLE 4

The rise and fall of N^{15} concentration in fecal specimens following tagged feeding

SUBJECT	FIRST SPECIMEN		SPECIMEN WITH PEAK N^{15} CONCENTRATION		FINAL SPECIMEN	
	Hrs. after tagged feeding	Concentration of N^{15} above biologic baseline [1]	Hrs. after tagged feeding	Atom % excess above baseline	Hrs. after tagged feeding	Atom % excess above baseline
L.G.	3	0.0021	..	.....	...	.....
L.G. (2nd spec.)	19	0.6576	29	0.7307	115	0.0074
G.S.	21	0.0764	45	0.3169	117	0.0105
G.S.	..	.....	..	.....	(141)	(0.0063)[2]
A.E.	20	0.2520	(1st spec. contained peak)		118	0.0083
S.B.	21	0.1453	(1st spec. contained peak)		117	0.0062
B.S.	21	0.2800	29	0.3187	118	0.0107
N.H.	20	0.0035	56	0.2467	117	0.0380

[1] Baseline equals 0.0066 atom % excess (see table 2).

[2] Supplemental specimens omitted from comparison calculations.

were made for all specimens. Table 5 shows that the final specimens collected contained 0.223% or less of the test dose, or approximately 2% of the average fecal excretion of N^{15}. Therefore, significantly large recovery of N^{15} would not be expected in subsequent specimens. The test period was prolonged by one day for one subject (G. S.), and a further decline of N^{15} recovery was noted from 0.22% to 0.15% of the test dose.

An average of 9.7% of the test dose was recovered in fecal specimens for all subjects during the 5⅔-day test interval. The individual and mean fecal excretion value would be slightly increased had collections been more extended, but it must be assumed that excretion at this point is largely re-excreted nitrogen. Therefore, by difference, 90.3% of the test dose is taken as the mean absorption of N^{15} by all subjects. This mean value and the calculated individual absorp-

TABLE 5

Quantitative recovery of tracer dose of N^{15} from feces during test period

SUBJECT	HOURS AFTER N^{15} TAGGED FEEDING	% OF TEST DOSE RECOVERED IN FINAL SPECIMEN	TOTAL N^{15} RECOVERED DURING 5⅔ DAYS AFTER TAGGED FEEDING	TOTAL % OF TRACER DOSE RECOVERED	TOTAL % TRACER ABSORBED BY DIFFERENCE
			mg		
L.G.	115	0.014	10.65	12.74	87.26
G.S.	117	0.221	5.98	7.16	92.84
G.S.[1]	(141)	(0.152)	(6.11)	(7.31)	(92.69)
A.E.	118	0.132	7.58	9.07	90.93
S.B.	117	0.169	8.39	10.04	89.96
B.S.	118	0.212	8.17	9.78	90.22
N.H.	117	0.223	7.66	9.17	90.83
Means		0.162 ± 0.061	8.072 ± 0.998	9.66 ± 1.193	90.34 ± 1.193

[1] Test period 6⅔ days.

tion values reveal an unexpectedly high absorption of the nitrogenous compounds in tagged yeast.

Inasmuch as the yeast cells were probably the most difficult structures to digest among the major sources of protein in the diet, the close agreement between the 90.3% average absorption of yeast protein and the 90.2% absorption (uncorrected for endogeneous nitrogen) of the daily protein intake of 110 gm is interesting (table 3). It should be noted that endogenous correction is not needed for yeast protein but is obligatory on untagged protein. The lowest, (87.26% — L. G.) and highest, (92.84% — G. S.) N^{15} absorption rates

were noted inothe y ung euclllorhydric control subjects — a variation of 6.35%. Subject A. E. absorbed 2.07% less than the maximum; N. H., 2.18% less than maximum; B. S. 2.84% below maximum and S. B. 3.11% below maximum. While these data alone do not show a decreased absorption of N^{15} in the achlorhydric and hypochlordric subjects, a subsequent publication dealing with the excretion and retention of the tag will present further support for the authors' published views. The discrepancies between individual percentages of dietary nitrogen absorption and individual percentages of N^{15} absorption represent the variable which cannot be smoothed out in a short-term nitrogen balance study by the classic techniques, as well as an elevation effect from re-excreted (endogenous) nitrogen. In a short-term study such as this the N^{15} data are considerably more reliable.

DISCUSSION

The use of a uniformly distributed N^{15} tag presents many advantages in the study of absorption of proteins in human subjects, including a markedly shortened experimental interval, a lessened need for large numbers of subjects, elimination of preconditioning low protein periods, and a simple method of minimizing the endogenous nitrogen problem. The so-called endogenous nitrogen level (Folin, '05), when determined in human subjects, is the sum of both the nitrogen excretion on a practical low protein (nitrogen) diet and the true endogenous nitrogen. In the theoretical case of a zero nitrogen intake, the excretion in the feces would represent the true endogenous nitrogen. The use of a single dose of N^{15} fulfills this condition, namely, no N^{15} intake (above biologic baseline) prior to the test dose. The level of N^{15} excreted at the end of the test period represents the sum of endogenous N^{15} and unabsorbed N^{15}. The minute amount of the test dose (0.223% maximum) found for both endogenous and unabsorbed N^{15} during the 5th day after the test dose indicates an upper limit for both endogenous or unabsorbed N^{15}. An

endogenous nitrogen of less than 0.22% may be disregarded in calculation of absorption of protein under these conditions.

SUMMARY

Methods for the study of protein absorption in man have been discussed. A yeast protein tag with an N^{15} content of 58.3 atom % excess was prepared. Evidence for the general distribution of the tag throughout the protein fraction of the yeast has been reviewed. Comparative absorption studies in young and old subjects have been presented. A mean yeast protein absorption of 90.3% among 6 subjects has been determined. Achlorhydria, hypochlorhydria, and age do not appear to produce depressing effects on the capacity to absorb a protein tracer during continued high-level protein intake. The role of gastric acidity in protein utilization will be further discussed in a subsequent communication. The single-dose tracer method of determining protein absorption has been shown to obviate the need for evaluation of endogenous nitrogen.

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GROWTH AND CREATINE BIOSYNTHESIS IN THE CHICK AS AFFECTED BY THE AMINO ACID DEFICIENCIES OF CASEIN[1,2]

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THREE FIGURES

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The continued use of casein as a primary protein source in purified chick diets emphasizes the necessity for further clarification of the effect of the amino acid shortcomings of this protein (arginine, glycine and the sulfur amino acids) on growth and protein utilization. Monson et al. ('55) reported increased growth when arginine and glycine replaced a gelatin supplement to purified diets containing 18 to 30% of casein. Wietlake et al. ('54) employed a 35% casein ration and reported a growth response from supplementation with arginine, glycine and creatine.

The work of Almquist ('41) generally supports the view that glycine and arginine are the biological precursors of creatine in the chick. They combine to form guanidoacetic acid (Bloch and Schoenheimer, '40) after which the methyl group of methionine is transferred to complete the formation of creatine (du Vigneaud et al., '41). Mellanby ('08), using an inadequate non-purified diet, reported an increase in chick

[1] Partial reports on this work have been presented at the Poultry Science Association and the American Chemical Society Meetings, 1955.

[2] Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers University, the State University of New Jersey, Departments of Poultry Husbandry and Agricultural Biochemistry, New Brunswick.

muscle creatine to age two weeks that could be influenced by dietary creatine. Almquist et al. ('43) reported a slight increase in chick muscle creatine when choline or methionine were added to a 25% casein ration deficient in the two methyl group donors. Fisher et al. ('55) reported a higher level of muscle creatine at the age of 24 days than at 17 days, muscle creatine was significantly increased by supplementing a 30% isolated soybean protein [3] diet with glycine.

In view of the importance of muscle creatine in energy metabolism, this experiment was designed to study growth and creatine formation in the growing chick as related to age and amino acid precursors by factorially supplementing a 25% casein ration with arginine, glycine and methionine. The basal ration supplemented with creatine as well as a practical chick starter were included for comparative purposes.

EXPERIMENTAL

Male and female chicks of a White Rock ♂ × New Hampshire ♀ cross were used in this study. Two lots of 20 birds each, separated according to sex, were assigned to each of 10 treatments, making a total of 400 birds. The chicks were weighed, wing-banded and placed on experiment in electrically heated brooders at one day of age; water and feed were supplied ad libitum. The composition of the basal ration is given in table 1. The basal ration was supplemented according to the experimental design shown in table 2. The levels of supplementation for arginine, glycine and methionine had been found satisfactory in previous work with similar diets (Fisher et al., '55), while creatine was added in excess of the molar equivalent of arginine. Experimental feeding was continued for 4 weeks. All chicks were individually weighed every 4 days, at which time two birds from each group were sacrificed for analyses of creatine in leg muscle. Creatine was determined as total creatinine by the method of Rose et al ('27).

[3] The Drakett Company, Cincinnati, Ohio.

TABLE 1

Composition of rations

BASAL DIET				CHICK STARTER	
Ingredients	Amount	Vitamins added	Amount	Ingredients	Amount
	%		*mg/kg*		*Per 100 lbs.*
Casein (crude)	25.00 [1]	Thiamine HCl	25	Corn meal	53.6 lbs.
Corn oil	3.00	Riboflavin	16	Soybean meal (50%)	34.0 lbs.
Non-nutritive fiber	3.00	Calcium pantothenate	20	Alfalfa	3.0 lbs.
Salt mixture [2]	5.34	Niacin	15	Corn distillers solubles	4.0 lbs.
Choline chloride	0.20	Pyridoxine HCl	6	Butyl fermentation	1.0 lbs
Vitamins A and D		Biotin	0.6	B_{12}-antibiotic supplement	0.5 lbs.
(10,000 A; 600 D_3)	0.10	Folic acid	4	Dicalcium phosphate	2.2 lbs.
Cerelose	63.36	Inositol	100	Mineral concentrate	1.0 lbs.
Vitamins	+	Para-amino benzoic acid	2	Salt	0.5 lbs.
	100.00	Menadione	5	Choline chloride (25%)	96 gm
		α-tocopherol acetate	20	Niacin	1 gm
		Ascorbic acid	250	Vitamins A and D	
		Vitamin B_{12}	.02	(1500 A; 300 D_3)	0.2 lbs.

[1] N × 6.25 = 20.4%.

[2] For composition see Fisher et al. ('54).

RESULTS

The growth curves for all treatments are presented in figure 1. Each point on the individual curves represents the average weight of 5 individual birds chosen at random. By this method the weight of any one bird is used only once and the bias of the rapid- or slow-growing bird within each group is eliminated from the growth curve. The curves can be considered as made up of independent variables along the Y axis.[4] Among the growth curves for the single supplements to the basal

TABLE 2

Experimental design

LOT NO.		SUPPLEMENT TO BASAL AT EXPENSE OF CERELOSE
Males	Females	
1	11	None
2	12	1% arginine [1]
3	13	2% glycine
4	14	0.3% methionine [2]
5	15	1% arginine + 2% glycine
6	16	1% arginine + 0.3% methionine
7	17	2% glycine + 0.3% methionine
8	18	1% arginine + 2% glycine + 0.3% methionine
9	19	0.8% creatine hydrate
10	20	Practical starting ration

[1] Arginine was supplied as L-arginine HCl.

[2] DL-Methionine.

ration, only arginine gave a highly significant response over the basal ($P < 0.001$). Chicks receiving the arginine-containing double supplements to the basal also grew significantly better than those on the basal. The double supplement of arginine + glycine gave significantly better growth than did arginine alone ($P < 0.05$), whereas the double supplement of arginine + methionine did not. The triple supplement, arginine + glycine + methionine was not significantly more effective than the arginine + glycine supplement. Thus,

[4] We wish to thank Mr. D. R. Embody, Squibb Institute for Therapeutic Research, for advice concerning the construction of these growth curves.

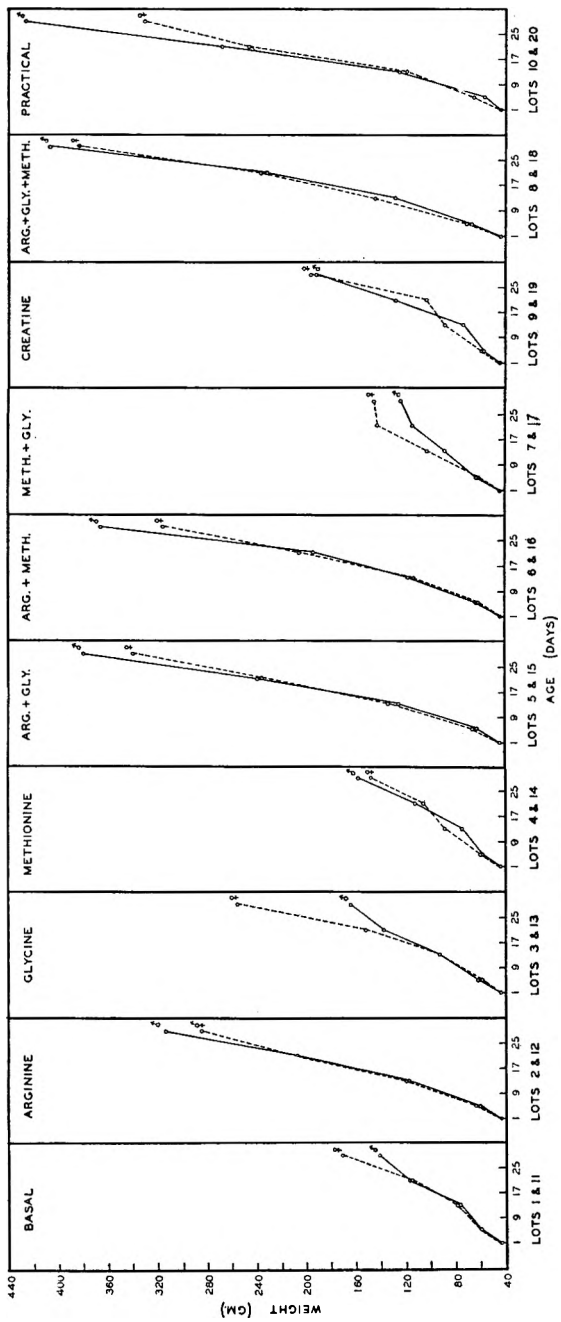

Fig. 1 Growth curves of chicks fed a casein diet supplemented with amino acids and creatine.

methionine showed a slight but insignificant improvement in growth over arginine alone and over arginine + glycine. Creatine supplementation of the basal had little effect upon growth. The excellent growth obtained from the arginine + glycine combination equalled that obtained with the prac tical chick starter.

As was to be expected, in all cases where good or "normal" growth occurred the males were significantly heavier than the females at 4 weeks of age. In the slower-growing lots — basal, glycine and glycine + methionine — it is interesting to note

TABLE 3

Coefficients of variation for experimental treatments

DIET	RATE OF GROWTH	
	Rapid	Slow
	%	%
Basal		19
Arginine	10	
Glycine		32
Methionine		20
Arginine + glycine	14	
Arginine + methionine	12	
Glycine + methionine		40
Arginine + glycine + methionine	11	
Creatine		28
Practical ration	13	

that the females grew better than the males, although the differences are not significant.

Table 3 shows the coefficients of variation of the chicks on the 10 diets. It can be seen that the arginine deficiency effected a very high degree of individual variability, as has been reported previously (Wietlake et al., '54; Fisher et al., '55).

The curves for muscle creatine vs. age are shown in figure 2. Since there were no apparent sex differences the sexes were pooled and each point on the curve represents the average of 4 individually determined values, two males and two females. Significant differences were determined as follows:

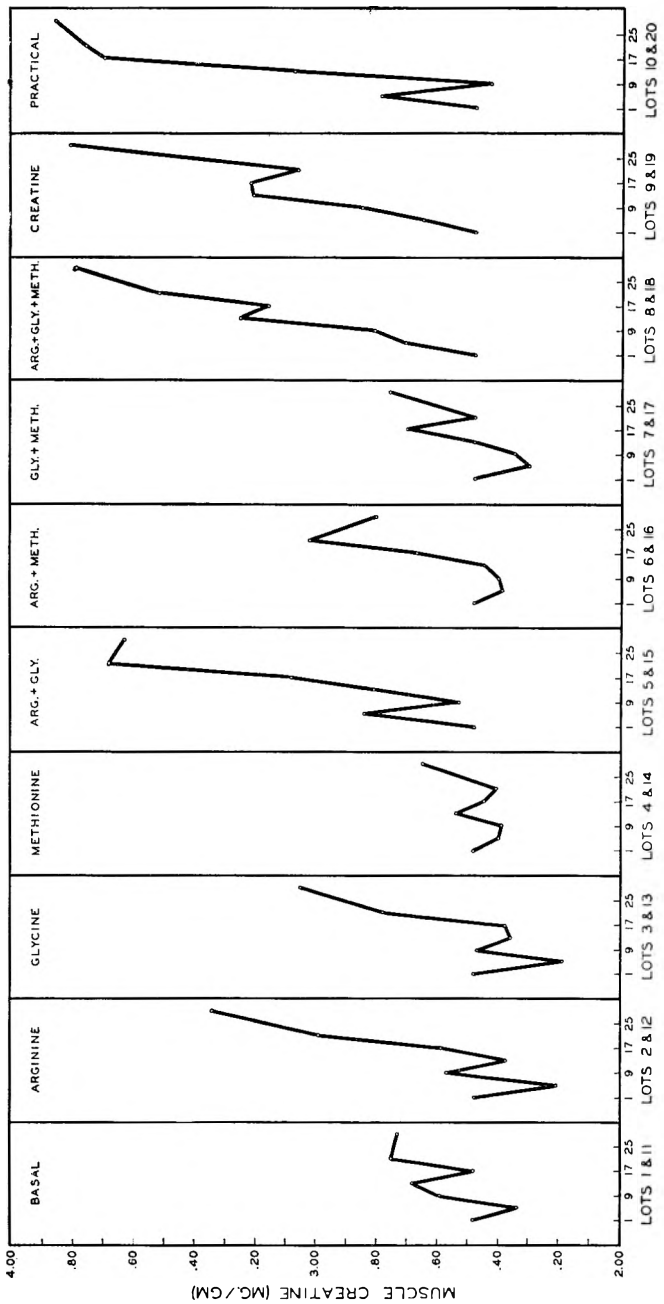

Fig. 2 Muscle creatine-age curves as influenced by dietary supplementation of amino acids and creatine to a casein basal ration.

the difference between each pair of creatine values determined for each separate sex, period and diet was expressed as a percentage of the sum of the pair of values. A standard deviation was calculated for all such differences in per-

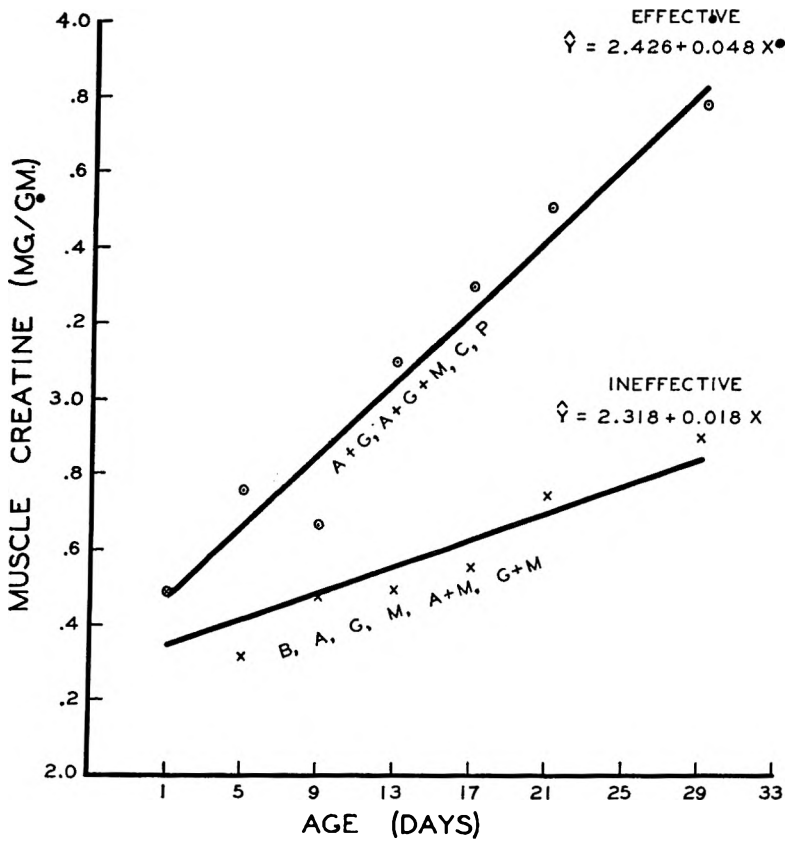

Fig. 3 Least squares regression lines for the muscle creatine-age relationship.

centage. Values farther apart than two standard deviations were considered significantly different.

Using this criterion of evaluation, the minor fluctuations along the creatine curves can be attributed to experimental error and individual variation. Compared to the basal, significantly higher muscle creatine levels at 4 weeks of age

were obtained by these effective supplements: arginine + glycine, arginine + glycine + methionine, creatine and the practical ration. The remainder of the supplements have been termed ineffective; these did not promote muscle creatine levels significantly higher than did the basal. The creatine curve for arginine supplementation (lot 2) approached significance, but in view of the curve for arginine + methionine (lot 6) arginine alone could not be considered an "effective" supplement.

The creatine values for the effective and ineffective dietary supplements have been separately pooled by periods. Figure 3 shows the curves that resulted from these pooled values fitted to straight lines by the method of least squares. In both cases the fit is an excellent one and the slopes are highly significant ($P < 0.01$) indicating an increase in muscle creatine with age. Optimum creatine synthesis was obtained only from combined arginine + glycine supplementation; however, all diets supported the significant increase in muscle creatine with age.

DISCUSSION

Although only a fraction of the glycine requirement was supplied in contrast to a considerable proportion of the arginine requirement, the response to arginine was greater. There is evidently some glycine biosynthesis by the chick, and, although glycine supplementation of the casein basal ration is necessary for optimum growth, arginine is by far the most limiting amino acid. This is in agreement with previous work. The lack of response to methionine is contrary to the findings of Briggs et al. ('42). Their positive response to methionine may be explained by their use of a lower level of casein (18%). The calculated methionine content of our basal was 0.69%. Glista et al. ('51) have reported the total sulfur amino acid requirement at 0.64%, which would explain the lack of response to methionine in these experiments.

Supplemental creatine did not improve growth on this 20% protein ration in contrast to the good growth response reported by Wietlake et al. ('54) using a 35% casein ration.

This may be explained on the basis that creatine will spare only that amount of arginine which normally is utilized for the biosynthesis of creatine. Thus the arginine available for tissue protein synthesis on the 20% casein diet still remains limiting, while considerably more was available in the higher casein diet of Wietlake.

The curves for creatine vs. age indicate an increase in muscle creatine which reaches a maximum beyond the 4-week experimental period, in contrast to the work of Mellanby ('08). This explains, in part, the higher arginine and glycine requirements of the chick during the first 4 weeks of life (Snyder, '54; Fisher et al., '55) in contrast to their requirement after 4 weeks of age (Almquist and Merritt, '50). Cohen et al. ('53) reported an increase in muscle creatine as the prematurity of infants decreased and as full term infants aged. The results of this chick experiment generally parallel these observations.

While there appears to be a correlation between growth rate and muscle creatine, this relationship is probably an artifact since the same amino acids required for optimal creatine formation are also required for tissue protein synthesis. The "normal" muscle-creatine levels of the creatine-fed groups were not concomitant with good growth and there appeared to be little or no arginine-sparing effect by dietary creatine under these experimental conditions. There was no correlation between body weight and creatine level in individual birds.

SUMMARY

A purified diet containing 25% casein employed in this study was supplemented with creatine, factorially with arginine, glycine and methionine, and was further compared to a practical starting ration. Two replicates, one of each sex, were used and weights and muscle creatine levels determined at 4-day intervals over a 4-week period. For growth, arginine was the most limiting amino acid. Glycine and methionine alone gave no response. Glycine in combination with arginine gave optimum growth equal to growth on the practical diet.

Methionine in double or tripl combination gave a slight but non-significant growth response. Creatine showed but a slight improvement in growth above the unsupplemented casein diet. For creatine formation, arginine plus glycine was required for optimum results. The muscle creatine level increased linearly with age over the 4-week period studied.

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PROTEIN INTAKE AND LIVER CHOLESTEROL: EFFECTS OF AGE AND GROWTH OF THE TEST ANIMAL[1]

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ONE FIGURE

Most of the studies of the effect of dietary protein on liver lipid retention deal primarily with glyceride fatty livers. Attention has been centered on the kind and amount of protein fed, rather than on the animal in which its effect has been measured. As a result, discrepancies in the findings, and especially in the cholesterol data, are difficult to evaluate.

Treadwell et al. ('44) recognized the importance of the test animal when they concluded that young animals used methionine preferentially to support growth and only secondarily to prevent fatty livers. While the present study was under way, Harper and his coworkers ('54) reported that liver fat in rats fed diets low in protein decreased with increased age.

The effect of dietary protein on liver lipid is ascribed largely to the labile methyl furnished by its methionine. Choline has been considered an equally good source of methyl and therefore equally effective as a lipotropic agent. Lucas and Ridout ('55) have recently pointed out, however, that protein not only may furnish lipotropic substances other than methionine, but that the tissue building function of protein may also be important in relation to its lipotropic activity.

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The use of protein for tissue building will vary with age and rate of growth. Singal's work (Singal et al., '53) suggests that protein or threonine may function in the synthesis of nucleic acids as well as of phospholipids.

Observations in our own laboratory had indicated that the effects of dietary protein and choline on liver cholesterol might not be entirely interchangeable. In growing rats, with a fully adequate protein intake, a moderate increase in dietary choline (0.5 to 2.5 mg/gm diet) resulted in increased liver cholesterol values (Okey and Turner, '51). Rats injected with choline methyl C^{14} excreted more $C^{14}O_2$ and had less urinary radioactivity when fed 30% of protein than when fed 15% of protein (Okey and Goossen, '53). However, very young, adolescent, and adult rats showed different degrees of response to protein intake in terms of effect on liver cholesterol storage.

While the present study was undertaken originally in order to determine the most desirable conditions for measurements of the effect of other dietary constituents on the use of food cholesterol, the findings also seem important because of the possible clinical implications. Obviously it is one thing if cholesterol deposition in the tissues of an older person can be altered by a few weeks on a diet high in protein, another if the alteration can be secured only by lifetime feeding of the diet, and still another if protein is lipotropic toward cholesterol only during the time that large amounts are being used for growth.

EXPERIMENTAL

General plan. The study of the effect of age and duration of feeding on lipotropic response to protein *per se* was planned as a basis for further work with methionine and choline. The ranges of protein concentration chosen were therefore not so extreme as to be inconsistent with good nutrition. Choline levels just high enough to prevent fatty livers and kidney injury in young rats fed the cholesterol-free control diets were considered desirable.

Three "interval" studies were carried out with matched groups of rats fed, from weaning, control and cholesterol-rich diets. The animals were sacrificed in subgroups after intervals varying from three to 12 weeks on diet. Approximately equal numbers of males and females were included in each subgroup. Diets for one typical series are given in table 1.

TABLE 1

Composition of diets

CONSTITUENT	10% Protein	15% Protein	30% Protein
	parts	*parts*	*parts*
Egg albumin	5.0	10.0	25.0
Casein (vitamin free)	5.0	5.0	5.0
Fat [1]	13.5	13.5	13.5
Sucrose	69.5	64.5	49.5
Salts [2]	4.0	4.0	4.0
Cholesterol [3]	(1)	(1)	(1)
"B" mix [4]	1.0	1.0	1.0
"A" mix [5]	1.0	1.0	1.0

[1] Primex.

[2] Hubbell, Mendel and Wakeman ('37).

[3] Controls received no cholesterol; experimental diets had 1% cholesterol substituted for 1% sucrose.

[4] "B" mix furnished in milligrams per kilogram: thiamine, 2.5; riboflavin, 4.0; folic acid, 2.0; pyridoxine, 2.0; niacin, 10; para-amino benzoic acid, 10.0; Ca pantothenate, 10; biotin, 1.0; inositol, 5; vitamin B_{12} triturate, 20; later series had ascorbic acid added to stabilize thiamine. Choline was ground with sucrose and added separately. It constituted 0.07% of the diet.

[5] "A" mix, per kilogram: "A", 15,000 I.U.; "D", 1,000 I.U.; mixed tocopherols, 0.3 gm; menadione, 0.05 gm.

Diets for the other two series differed in that they furnished 17 and 28% of protein with respectively 0.05 and 0.07% of choline. Data for the first of these series (fed 0.05% of choline) are therefore not strictly comparable to those reported.

Data for an additional "single-run" experiment with 120 adolescent rats fed the 10, 15, and 30% of protein diets (table 1) for three weeks are reported (table 2). Several studies with older animals fed similar diets have been evaluated for comparison.

Procedures for the care of animals, for autopsy, and for determination of tissue constituents have been described (Okey and Lyman, '54).

RESULTS AND DISCUSSION

Food intake and growth rate. Mean weight gains for the groups which constituted one "interval" series are shown in

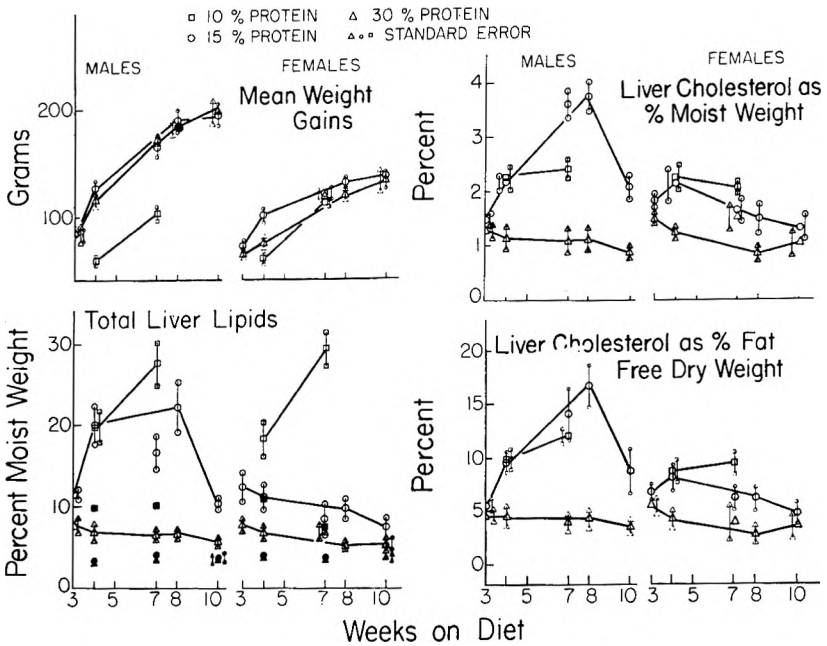

Fig. 1 Variations in liver lipids and cholesterol during growth. Open figures represent rats fed diets containing 1% cholesterol, solid figures, those fed cholesterol-free diets. Points represent means and standard errors for subgroups of 10 to 12 rats each.

the first part of figure 1. All diets were isocaloric, and individual growth curves showed that the relationships between food intakes and weight gains were quite consistent for given ages and diets. Both were very slightly lower for rats fed 30% of protein than for those fed 15%. Males fed 10% of protein ate less and grew less well than did those fed higher

protein levels. Sex differences in food intake and growth rate began to be evident at about the 4th week after weaning. Cholesterol-fed rats tended to eat slightly more than the controls of the same diet groups, especially in the period just after weaning. As a result of an effort to eliminate the effects of differences in food intake by pair-feeding, the food intakes of the 7-week subgroups were very slightly restricted.

Interval series – control rats

In addition to the control rats for which liver lipid data are charted in figure 1, controls on cholesterol-free diets were studied at intervals of 3, 4, 5, 7, 9, and 12 weeks after weaning as part of the two other "interval" studies. Liver lipid and cholesterol data were consistent, and varied within narrow ranges for any level of protein fed. For this reason, and because of limited caging facilities, only three control subgroups wore included in the series for which data are charted.

Total lipids ranged from 7.3 to 11.8% for the groups fed 10% of protein, from 3.7 to 4.1% for those fed 15% of protein, and from 3.3 to 4.0% for those fed 30% of protein. Rats fed 17 and 23% of protein showed intermediate and narrower ranges of variation. There was no consistent variation with age.

Total liver cholesterols were consistently high for the groups fed 10% of protein [0.38% (♂s) and 0.42% (♀s) at 4 weeks, and 0.39% (♂s) and 0.30% (♀s) at 7 weeks]. If the 7-week groups are excepted, means for the 15 and 17% protein groups ranged from 0.22 to 0.26%, and those for the 28 and 30% groups from 0.18 to 0.23%. The high means observed at the 7-week interval for the 15 and 17% protein groups (i.e., 0.37 and 0.33%) were first rated as of doubtful significance. However, almost identical figures have since been observed in 7-week controls fed 15% of protein ad libitum, and used for a later experiment.

Some year-old control rats on 12.5 to 17% protein diets have had liver cholesterols as high as 0.5%. However, older

control rats fed 20% or more protein usually had liver cholesterol values within the range shown by the 10-week groups charted. The control diets were almost entirely cholesterol free. Therefore, the liver cholesterol probably represents accumulation of synthesized cholesterol.

Cholesterol-fed rats

The liver lipid and cholesterol data presented in figure 1 represent means for sub groups of 10 males and 10 or 11 females sacrificed at each of the indicated intervals. Because of the different ages and sizes of the rats, the total lipid and cholesterol figures are charted as percentages rather than total amounts. In addition, they have been calculated to percentage of dry weight, milligrams per liver, and milligrams per 100 gm rat, and the cholesterol has been computed as percentage of fat-free dry weight, i.e., ratio of cholesterol to non-fat solids. The data show that variations in response to protein after different intervals on diet are of much the same relative magnitude, however expressed. It should be noted, however, that comparisons of medians and modes have frequently shown greater contrasts between the liver lipids of rats fed different levels of protein than have means and standard errors. This was because the larger variations from the median were often shown by only one or two rats per group. Variability within groups was undoubtedly increased by occasional short periods of hot weather and by the fact that only part of the rats could be caged at exactly the same time. The reported variations are typical of those from all the groups given 0.07% of choline.

Total lipids. As figure 1 shows, the livers of the cholesterol-fed rats given 30% of protein with cholesterol never became excessively fatty. Total lipids increased sharply between the third and 8th weeks after weaning in males fed 15% of protein. At least four-fifths of the lipid was evidently glyceride. A similar but less marked rise had been observed in rats fed 17% of protein. The fall in liver lipid be-

tween the 8th and 10th week began somewhat later than that observed by Harper's group in rats fed 9% of casein without cholesterol (Harper et al., '54). Cholesterol-fed females as well as males given 10% of protein had very large amounts of liver glyceride in spite of their relatively low food intakes.

Liver cholesterol during the first three weeks after weaning varied only slightly with the protein content of the diet. Indeed, in one series of cholesterol-fed rats, values at three weeks were a little higher in rats fed 28% of protein than in the corresponding groups fed 17%. This is a period of high choline and probably of high methionine requirement. At 4 to 5 weeks after weaning, the liver cholesterol fell gradually in the groups fed 28 and 30% of protein, and virtually leveled off at about 8 weeks. On the other hand, liver cholesterols in the rats fed 15% of protein increased rapidly up to the 8th week after weaning and then fell sharply. However, the level reached at 10 weeks by the males fed 15% of protein remained higher than that for males fed 30%. The lower food intakes and slower growth rates of the male rats fed only 10% of protein are reflected in liver cholesterols which were lower than for the rats fed 15% of protein.

Females, on the other hand, stored a little more liver cholesterol with 10% of protein than with 15%. This was accompanied by a much higher liver glyceride storage than was shown by the animals fed 15% of protein. Weight gains in females fed 10% of protein were not greatly retarded after the 4th week, but there was considerable evidence that extra fat storage was contributing to the weight gain.

Cholesterol-fed rats: "single-run" studies

Table 2 shows data for a three-week experiment with rats changed from stock to experimental diet when the females weighed 135 gm and the males 150 gm. Because experiments of this description have so frequently been used to measure lipotropic effect of diet, the extent to which the results show the same trends as the longer feeding experiments with wean-

lings assumes significance. The stock diet in this case contained 30% of mixed protein, including a considerable amount of dried whole milk, wheat germ and alfalfa leaf meal. Hence it is not surprising that the rats fed 10% of protein ate more and had slightly higher liver lipid and cholesterol values than did those fed 15%; and that liver lipid differences between the 15 and 30% groups were relatively smaller than those observed in rats fed the experimental diets from weaning. The

TABLE 2

Liver lipids

Three-week experiment with adolescent rats [1]
Diets with cholesterol

PROTEIN IN DIET	NO. AND SEX	WEIGHT GAIN		LIVER LIPID		TOTAL LIVER CHOLESTEROL			
		Mean	SE [2]	Mean	SE	Mean	SE	Mean	SE
		gm	*gm*	*% moist wt.*		*% moist wt.*		*% fat free dry wt.*	
10%	10 males	88	3.8	15.3	1.8	1.74	0.15	7.20	0.87
	10 females	44	3.8	4.9	0.1	0.72	0.05	2.66	0.25
15%	10 males	95	5.1	13.0	1.9	1.30	0.15	4.96	0.66
	11 females	46	4.2	5.9	0.5	0.96	0.13	3.29	0.48
30%	10 males	97	6.0	5.2	0.4	0.79	0.08	2.62	0.25
	11 females	40	3.0	4.4	0.2	0.87	0.10	3.01	0.37

[1] Rats transferred from stock to experimental diets when males weighed 150 gm and females 135 gm. Liver lipid and cholesterol values for stock rats 6 weeks old computed to percentage of moist weight were 4.1 (SE 0.1), and 0.25 (SE 0.01) respectively.

[2] $SE = \sqrt{\frac{\Sigma d^2}{\frac{n-1}{\sqrt{n}}}}$

failure of the females fed 10 and 15% of protein to accumulate excess liver fat might reflect a smaller need for protein than was the case with rats of the same age fed a low-protein diet from weaning. Certainly the food intakes and weight gains of the females were comparatively low. Females are obviously not adapted for short-time measurements of lipotropic response to protein or amino acid at this age.

Two similar "single-run" experiments, starting with rats 9 months to one year old and lasting 47 and 56 days, re-

spectively, were carried out, as well as a 9-month experiment in which weanling rats were fed control and cholesterol-rich diets furnishing, respectively, 12.5, 25, and 35% of casein from weaning. Some of the findings seem pertinent, although interpretation of the figures from these studies is difficult because of the frequency with which older rats developed a low-grade lung infection. Incidence and severity were highest in the groups fed least protein; and liver cholesterols were always lower in individual rats with grossly evident infection than in rats of the same group which did not show lung lesions. Surviving and apparently healthy males fed 12.5 to 15% of protein with 1% of cholesterol (for 6 to 8 weeks) had grossly fatty livers (with 15 to 30% of lipid), and cholesterol values which sometimes reached 4%. But liver cholesterol values were frequently lower in the apparently healthy, cholesterol-fed adults given 22 to 25% of protein (mean for group, 1.7%) than in those given 30 to 35% of protein (mean for corresponding group, 2.4%). Total liver lipids in males were, as a rule, less than 10% (moist weight) if the diets furnished more than 15% of protein. Values for females were usually lower. However, variation with changes in protein content of the diet was not entirely consistent. It was often difficult to estimate the extent of the lung pathology at autopsy, or to judge which animals could legitmately be considered comparable.

Evaluation of the data from all the groups of rats studied indicates that correlation between food intake and liver cholesterol values was by no means clean cut. Individual rats of any group, when arranged in quartiles in the order of food intakes, were likely to be found in the same quartiles when arranged in the order of percentages of liver cholesterol. But neither scatter diagrams nor correlation coefficients indicated significant interdependence of liver cholesterol and food intake in rats of the same age, sex and diet groups. This is the more noteworthy because food intake was synonymous with fat and cholesterol intake.

Computation of grams gained per gram of food eaten showed the highest mean values for the rats fed 15 and 17% of protein. For the 15% group values for the third week after weaning were 0.44 (♂s) and 0.37 (♀s), decreasi g to 0.14 (♂s) and 0.06 (♀s) for the 10th week. Corresponding figures for the 30% protein groups were 0.37 (♂s) and 0.32 (♀s) for the third week and 0.10 (♂s) and 0.05 (♀s) at the 10th week. For males fed the cholesterol-rich diets ad libitum, values at 7 weeks were 0.14 for the 10% of protein diet, 0.21 for the 15% diet and 0.12 for the 30% diet. There seemed therefore to be some evidence that the rats with the highest liver cholesterols were also those which were making the most efficient use of their food for growth.

Age differences in the lipotropic effect of food protein might be expected to result from variation in the proportion of that protein used for growth. If only the protein ingested in excess of need has a lipotropic effect, there ought to be some inverse relationship between this excess and the amount of liver fat and cholesterol stored at any given age.

Computation of the approximate amount of this protein "excess" from food intake and growth data gave overall figures which increased with the amount of protein in the diet and with the age of the test animal. However, when the figures for protein "excess" were divided by the body weights of the animals, variations were insufficient to account for the age differences in liver cholesterols noted.

Sex differences in liver cholesterol in response to protein intake at the 15% level were consistent and large, especially in rats just attaining sexual maturity. Only when the protein intake was as low as 10% and the liver glycerides proportionately high did liver cholesterols reach really high levels in females. Possibly the female needs a lower percentage of protein than does the male in order to tolerate a diet high in cholesterol. Possibly she uses dietary cholesterol at a more rapid rate and therefore has a faster liver cholesterol turnover.

The data as a whole suggest that protein is most actively lipotropic toward cholesterol when an animal is growing rapidly. Also, an actively growing animal given a diet which requires a rather large caloric intake in order to secure enough protein to support growth is likely to have a higher rate of liver cholesterol storage than is the animal fed a diet which furnishes enough protein to supply the need for growing tissue without stimulating an excessive caloric intake. A cholesterol-rich diet too low in protein to support optimal growth may be conducive to rapid development of glyceride fatty livers, but result in lower liver cholesterols than o e furnishing more adequate protein.

SUMMARY

Rats were fed, from weaning, isocaloric control and cholesterol-rich diets containing, respectively, percentages of protein (casein and egg albumin) varying from 10 to 30. They were sacrificed after intervals varying between three and 12 weeks on diet. Several "single-run" experiments with older animals are summarized for comparison. Relationships between growth, food intake and liver lipid storage are discussed.

Control rats on cholesterol-free diets showed some decrease in liver cholesterol with increased percentages of dietary protein. Mean values varied from about 0.4% for those fed 10% of protein tc 0.21 for the 30%-protein rats. With the exception of high values noted for rats fed 15 and 17% of protein at 7 weeks, changes with age were minor.

Cholesterol-fed rats placed on a 15%-protein diet at weaning had the highest observed concentrations of liver cholesterol at about 7 to 8 weeks thereafter. At 10 weeks, liver cholesterols were much lowered. With 30% of protein, at three weeks after weaning, values were almost the same as for 15%. However, with the higher protein intake, liver cholesterol values fell rather than rose during the period of rapid preadolescent growth. With only 10% of protein, food intakes andrg owth rates were lowered. Liver glycerides were much

higher than with 15% of protein, but liver cholesterol did not accumulate proportionately.

Males fed 15% of protein with 0.07% of choline stored more liver cholesterol than females. With 10% of protein, sex differences were less marked. Protein was a less effective lipotropic agent in adults than in rapidly growing adolescents.

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CAROTENE UTILIZATION AS INFLUENCED BY THE ADDITION OF VITAMIN B_{12} TO DIETS CONTAINING YEAST OR A SYNTHETIC VITAMIN MIXTURE [1]

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In an experiment previously reported, rats fed a diet containing a mixture of synthetic vitamins utilized carotene more efficiently than did those fed a diet with yeast as the source of the B vitamins (Mayfield and Roehm, '56). A comparison of the two diets, together with the work of High and Wilson ('53), suggested that the vitamin B_{12} content of the synthetic vitamin mixture might be the factor responsible for this greater utilization of carotene. Data were also presented in this earlier report suggesting that yeasts from different sources might vary in their influence on the utilization of carotene. The purpose of this investigation was to study carotene utilization as affected by (1) various yeasts, with and without vitamin B_{12}, and (2) a synthetic vitamin mixture, with and without vitamin B_{12} supplementation.

EXPERIMENTAL PROCEDURE

Young rats [2] were assigned to the experimental groups, depleted of vitamin A and fed one of the test rations ad libitum.

[1] This study was a part of the Western Regional Cooperative Project W-4, Nutritional Status and Dietary Needs of Population Groups in Selected Areas of the West, Subproject 2, Biological Availability and Interrelationships of Nutrients in Foods. It was financed in part from funds appropriated under the Research and Marketing Act of 1946. Contribution from Montana State College, Agric ltural Experiment Station. Paper no. 366 Journal Series.

[2] Obt ined from Holtzman Rat Company, Madison, Wisconsin.

Each group, consisting of 4 females and 4 males, received the same basic test diet during the depletion period as in the 14-day test period. During the test period each rat was fed a daily supplement of 60 μg of carotene [3] dissolved in 0.2 ml of oil containing 0.50 mg of alpha-tocopherol. These feedings were pipetted directly into the mouths of the rats. Food consumption was recorded. The rats were killed 24 hours after the last supplement was fed and kidneys and livers were frozen and held at −10°F. until analyzed for vitamin A. The method used for the vitamin A analyses was essentially that used by Vavich and Kemmerer ('50). Techniques of feeding and other procedures have been previously described (Mayfield and Roehm, '56). The data were treated statistically according to the analysis of variance (Ostle, '54).

When the yeast was the source of the B vitamins, the test diet consisted of vitamin A-free casein 18% [4]; salt mixture 4% [5]; brewers' yeast (a, b, c or d) 8% [6]; cornstarch 65% and cottonseed oil 5%.[7] Vitamin D [8] was added to the oil to make 5 U. S. P. units of vitamin D per gram of diet. Four of the diets containing yeast-a, -b, -c or -d were fed without the addition of vitamin B_{12} to groups 1, 2, 3 and 4, respectively. Using the vitamin B_{12} potency given by the various yeast producers, vitamin B_{12} [9] was added to the diets containing yeast-a, -b or -c so that the resulting values were calculated to be 3.0 μg per 100 gm of diet. These diets were fed to groups 7, 8 and 9,

[3] Carotene, 90% beta and 10% alpha, obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.

[4] "Vitamin-Free" test casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

[5] Salt Mixture, U. S. P. XIV, Nutritional Biochemicals Corporation, Cleveland, Ohio.

[6] Yeast-a, Brewers' Yeast U. S. P., Nutritional Biochemicals Corporation, Cleveland, Ohio; yeast-b, Brewers' Yeast Powder, General Biochemicals, Inc., Chagrin Falls, Ohio; yeast-c, Fleischman's Pure Primary Grown Dry Yeast, Type 90-B, Standard Brands, Inc., New York, N. Y.; yeast-d, Torula Food Yeast, Type B, Lake States Yeast Corporation, Rhinelander, Wisconsin.

[7] Wesson Oil.

[8] Viosterol, Irradiated Ergosterol, 400,000 U. S. P. units of vitamin D per gram. Nutritional Biochemicals Corporation, Cleveland, Ohio.

[9] Vitamin B_{12}, 0.1% Trituration (with mannitol), Nutritional Biochemicals Corporation, Cleveland, Ohio.

respectively. This amount of vitamin B_{12} was that used in the synthetic vitamin-mixture diets previously reported (Mayfield and Roehm, '56) and was based on the report by Betheil and Lardy ('49) The vitamin B_{12} analyses of these yeasts made at a later date [10] were not identical to those supplied by the producer; hence, the resulting diets containing yeast, with and without vitamin B_{12} supplementation, varied as follows in their vitamin B_{12} content: yeast-a diets 4 mμg (no vitamin B_{12} added), 2644 mμg (vitamin B_{12} added); yeast-b diets 6 mμg and 3006 mμg; yeast-c diets 20 mμg and 3020 mμg; and yeast-d diet 6 mμg (no vitamin B_{12} added) per 100 gm diet. These values are based on the figures obtained by assay with *Lactobacillus leichmannii.*

Rats in groups 5, 6, 10 and 11 received diets similar to the yeast diets with the following exceptions: 5% of one of the synthetic vitamin mixtures was substituted for the 8% of brewers' yeast and the cornstarch was increased from 65 to 68%. Group 5 received the following vitamin mixture with no vitamin B_{12} added: pyridoxine hydrochloride 0.63 mg, calcium pantothenate 5.0 mg, choline chloride 250.0 mg, niacin 0.63 mg, *p*-aminobenzoic acid 30.0 mg, inositol 100 mg, vitamin K 0.21 mg, biotin (free acid) 10.0 μg, folic acid 0.20 mg, riboflavin 0.40 mg, thiamine hydrochloride 0.50 mg and cornstarch to make 5 gm. This mixture contained the vitamins, other than vitamin B_{12} in amounts believed to be necessary and adequate for the normal nutrition of the rat.

A comparison of the vitamin content of the yeast and synthetic vitamin-mixture diets indicated that the niacin content of the yeast diet was approximately 4.6 times greater than that of the synthetic vitamin-mixture diet. Since there was a possibility that this larger amount of niacin might retard the conversion of carotene, rats in group 6 received a vitamin mixture similar to that used for group 5 but with 4.0 mg of

[10] Vitamin B_{12} analyses of yeasts made by the Wisconsin Alumni Research Foundation. Assay values by the *Lactobacillus leichmannii* and *Ochromonas malhamensis* method respectively were: yeast-a, 0.50 and < 1 mμg; yeast-b, 0.80 and ca. 1 mμg; yeast-c, 2.43 and 4 mμg, and yeast-d, 0.78 and ca. 1 mμg per gram of yeast

niacin per 5 gm of mix. The resulting diet approximated the yeast diet in niacin content.

Rats in group 10 were fed a diet similar to that given group 5 but with 3.0 μg of vitamin B_{12} added to 5 gm of vitamin mixture or to 100 gm of diet. This was approximately the same amount of vitamin B_{12} as in the yeast diets supplemented with this vitamin, namely 2.644, 3.006 and 3.020 μg per 100 gm of diet.

A vitamin mixture containing vitamin B_{12} and a low amount of thiamine was used in the study which prompted the present investigation. Rats receiving the diets with this vitamin mixture utilized carotene more effectively than did those receiving the yeast diets. Though there was no indication that this low level of thiamine might be the influencing factor in the greater utilization of carotene, it seemed advisable to repeat this group. Hence, rats in group 11 were fed a diet containing a vitamin mixture similar to that used for group 10 containing vitamin B_{12} but with only 0.025 mg of thiamine hydrochloride per 5 gm of vitamin mixture or 100 gm of diet. This amount approximates that used in the previous work in which each rat was fed daily supplements of 2.5 μg of thiamine.

RESULTS AND DISCUSSION

Data showing the influence on carotene utilization of adding vitamin B_{12} to diets containing yeasts or synthetic vitamin mixtures are shown in table 1. The B_{12} content of the diets, calculated from the *leichmannii* assay figures, are given together with the average food consumption of each group for the 14-day test period.

When the average amounts of vitamin A in the livers plus kidneys of the various groups were treated according to the analysis of variance [11] the influence of the various factors studied could be evaluated. Since previous work (Mayfield and Roehm, '56) indicated an inverse response by males and females to the influence of ascorbic acid on the utilization of

[11] Calculations were performed by the Statistical Laboratory, Montana State College.

TABLE 1

Influence of adding vitamin B_{12} to diets containing yeast or a synthetic-vitamin mixture on vitamin A storage in livers and kidneys of rats fed 60 μg of carotene daily

GROUP NO.[1]	SOURCE OF B VITAMINS [2]	VITAMIN B_{12} CONTENT OF DIET	GAIN IN BODY WT.	FOOD EATEN	WEIGHT OF LIVER	VITAMIN A IN	
						Liver	Liver plus kidneys [3]
		mμg/100 gm	*gm*	*gm*	*gm*	*μg*	*μg*
				Diets without added vitamin B_{12}			
1	Yeast-a	4	40	119	6.2	60.0	70.9 ± 7.2 [4]
2	Yeast-b	6	57	146	8.0	82.5	93.7 ± 9.3
3	Yeast-c	20	50	133	6.1	85.9	94.6 ± 8.2
4	Yeast-d	6	54	148	7.1	81.0	91.9 ± 9.4
5	Synthetic-vitamin mix High thiamine	0	54	151	7.2	90.8	99.0 ± 12.4
6	Synthetic-vitamin mix High thiamine Niacin to equal yeast diets	0	55	154	6.6	96.7	104.1 ± 6.7
				Diets with added vitamin B_{12}			
7	Yeast-a	2644	48	149	6.7	115.1	121.2 ± 11.9
8	Yeast-b	3006	59	174	7.1	116.8	125.2 ± 15.2
9	Yeast-c	3020	57	161	7.0	94.8	106.0 ± 7.5
10	Synthetic-vitamin mix High thiamine	3000	53	158	6.4	102.9	109.4 ± 5.0
11	Synthetic vitamin mix Low thiamine	3000	24	110	4.8	96.2	100.2 ± 11.9

[1] Eight rats per group, 4 females and 4 males.

[2] Yeasts and synthetic vitamin mixtures described in experimental procedure.

[3] Statistical evaluation of data on total vitamin A in livers plus kidneys:

(a) No significant difference among groups 2, 3, 4, 5 and 6.

(b) Rats in groups 2, 3 and 4 stored significantly more vitamin A than those in group 1 ($P < 0.01$).

(c) Rats in groups 7 and 8 (with added vitamin B_{12}) stored significantly more vitamin A than did those in corresponding groups 1 and 2 (without added B_{12}) ($P < 0.01$).

(d) Rats in groups 7 and 8 (yeast with added vitamin B_{12}) stored significantly more vitamin A than did those in groups 10 and 12 (synthetic-vitamin mix with B_{12}) ($P < 0.05$).

( ) No significant difference between groups 3 and 9, or among groups 5, 6, 10 and 11.

[4] Standard error of the mean.

carotene, the present data were also analyzed to investigate the response by sex to the various factors. In most cases, males and females responded to the variations in the diets in a similar manner. However, analysis of the data suggested the need for further study of the response by sex to the addition of vitamin B_{12} and niacin to the diets containing the synthetic vitamin mixture. All groups were balanced for sex and only the mean responses per group are shown in table 1. The following evaluations are based on the mean amount of vitamin A in the livers plus kidneys of the various groups.

Different yeasts and carotene utilization. Rats receiving diets containing yeasts-b, -c or -d, without additional vitamin B_{12}, utilized carotene in a similar manner, having from 91.9 to 94.6 μg of vitamin A in liver plus kidneys. Those fed yeast-a had significantly less vitamin A storage, a mean of 70.9 μg, than did those receiving the other yeasts ($P < 0.01$). This finding supports the indication from previous work (Mayfield and Roehm, '56) that yeasts-a and -b had different effects on the utilization of carotene.

Niacin and carotene utilization. The larger amount of niacin (4.0 mg/100 gm diet) in the synthetic vitamin mix used in group 6 as compared to that used in group 5 (0.63 mg/100 gm diet) did not affect the utilization of carotene. This larger amount approximated that in the yeast diets and was fed to investigate the possibility that, at this level, niacin might retard the conversion of carotene and hence be responsible for the difference in carotene utilization of rats receiving the B vitamins from yeast or a synthetic vitamin mixture.

Yeast versus a mixture of synthetic vitamins. Rats in groups 5 and 6, receiving the B vitamins from a synthetic vitamin mixture without added vitamin B_{12} and with either the low or high level of niacin, stored significantly more vitamin A than did those on the yeast-a diet, group 1. The study from this laboratory previously referred to showed a highly significant difference between a yeast diet and a synthetic vitamin-mixture diet containing vitamin B_{12}. The fact that this difference is significant even when vitamin B_{12} was not included

in the synthetic mix indicates that vitamin B_{12} was not the influencing factor in the case of the yeast-a diet. However, when yeasts-b, -c or -d were fed there was no difference in the vitamin A storage by these rats, groups 2, 3 and 4, and those receiving the synthetic vitamin mixture, groups 5 and 6.

Yeast diets with added vitamin B_{12}. The utilization of carotene was significantly increased when vitamin B_{12} (approximately 3.0 μg/100 gm of diet) was added to the yeast-a or -b diets ($P < 0.01$). There was no significant increase when this vitamin was added to the yeast-c diet. The vitamin B_{12} content of the yeast-c diet without added B_{12} is 20 mμg per 100 gm of diet or from three to 5 times more than the amount present in the yeast-a or yeast-b diets (table 1). The amount of vitamin B_{12} included in the synthetic vitamin mix and added to the yeast diet was that used by Betheil and Lardy ('49). Assuming a daily diet consumption of about 10 gm per rat, the rats in the present study received approximately 0.30 μg of vitamin B_{12} per day. High and Wilson ('53) injected intramuscularly saline solutions containing vitamin B_{12} in an amount which equaled 0.14 μg per day and found a significant increase in carotene utilization.

Vavich, Stull, Raica and Kemmerer ('55), studying the effect of nonfat milk on the utilization of carotene, fed daily supplements of 0.05 and 0.60 μg of vitamin B_{12} in water to determine if the B_{12} content of the milk was the influencing factor in the greater utilization of carotene. They found no significant responses to these supplements of vitamin B_{12}. Rats in the present study given the yeast-c diet with 20 mμg of vitamin B_{12} /100 gm of diet received about 0.002 μg per day. The report of Vavich et al. ('55) indicates that it is improbable that this small amount of B_{12} (0.002 μg/day), even though three to 5 times greater than that in the other yeast diets, supplied the physiological needs of the rats for this factor in such a way that they did not respond to the vitamin B_{12} supplements. The data presented offer no explanation for the

lack of response to the vitamin B_{12} supplementation when rats were fed yeast-c.

Synthetic vitamin-mix diets with added vitamin B_{12}. The addition of vitamin B_{12} (3.0 μg/100 gm diet) to the synthetic vitamin mixture diets did not increase carotene utilization Rats receiving these diets with added vitamin B_{12} stored significantly less vitamin A than did the rats fed the yeast-a or yeast-b diets with added B_{12}. These findings suggest that some factor or factors in addition to vitamin B_{12} may be involved in obtaining the results discussed in this paper.

TABLE 2

B vitamins present in (1) the synthetic vitamin-mixture diet fed to group 5 and (2) the yeast diets fed to groups 1–4 (based on approximate vitamin analysis of yeast).

B VITAMINS	SYNTHETIC VITAMIN-MIXTURE DIET	YEAST DIETS
	per 100 gm	*per 100 gm*
Choline chloride	250.0 mg	28.5 mg
Niacin	0.63 mg	2.9 mg
Folic acid	200.0 μg	400.0 μg
Riboflavin	0.40 mg	0.44 mg
Vitamin B_{12}	0.00	4 to 20 mμg
Thiamine	0.50 mg	0.78 mg

Comparison of diets used. The following comparison of known differences among the diets used and the possibility of their influence on carotene utilization are presented in order to review factors, other than vitamin B_{12}, that may have influenced this study. Table 2 shows the B vitamin content of the synthetic vitamin-mixture diet fed to group 5 and the approximate amount of the same vitamins in the yeast diets fed to groups 1 to 4. The latter is based on an approximate vitamin analysis of yeast. A comparison of the two diets shows the following general relationships: (1) niacin 4.6 times greater in yeast diet, (2) folic acid two times greater in yeast diet, (3) riboflavin about the same in both diets and (4) choline 8.9 times greater in the synthetic vitamin-mixture diet. These diets with or without additional vitamin B_{12} were

similar in this factor. Feeding the larger amount of niacin to rats in group 6 has shown that this amount of niacin did not influence the utilization of carotene.

Popper and Chinn ('42) found that choline deficiency impairs the utilization of both carotene and vitamin A for hepatic deposition. There is also evidence that vitamin B_{12} and folic acid may be involved in the metabolism and synthesis of choline and may serve as a sparer of choline (Schaefer, Salmon and Strength, '49; Schaefer, Salmon, Strength and Copeland, '50; Bennett, Joralemon and Halpern, '51 and Gillis and Norris, '51). The possible interrelationships of these factors have been discussed by High and Wilson ('53).

Both the yeast and the synthetic vitamin-mix diets contained 18% of casein. The yeast diets contained approximately 4% of additional yeast protein which under certain circumstances may have influenced the carotene utilization. James and El Gindi ('53) found that rats fed diets containing lactalbumin or gluten excreted more carotene in the feces than did those which received diets containing casein or zein. However, they did not feed lactalbumin or gluten in combination with casein so it is not known if the addition of these proteins to the regular level of casein would have influenced carotene utilization.

Further work is needed to determine what factor (or factors) other than vitamin B_{12} may have influenced the carotene utilization of the groups of rats used in this study.

SUMMARY

Rats fed 60 μg of carotene daily together with a diet containing yeast-b, -c or -d, without additional vitamin B_{12}, stored significantly more vitamin A than did those on a diet containing yeast-a.

The addition to the synthetic vitamin-mixture diet of an amount of niacin approximating that in the yeast diet (4.0 mg/100 gm of diet) did not influence carotene utilization.

Rats receiving the B vitamins from the synthetic vitamin mixture without added vitamin B_{12} stored significantly more

vitamin A than did those on the yeast-a diet. However, their storage was similar to that of the rats receiving the yeast-b, -c or -d diets.

The utilization of carotene was significantly increased when vitamin B_{12} was added to the yeast-a or -b diets. There was no significant increase when this vitamin was added to the yeast-c diet.

The addition of vitamin B_{12} (3.0 μg/100 gm diet) to the diet containing a mixture of synthetic vitamins did not increase carotene utilization. Rats receiving this diet stored significantly less vitamin A than did those fed the yeast-a or -b diet with added vitamin B_{12}.

The results of this investigation indicate that some factor (or factors) in addition to vitamin B_{12} influenced the utilization of carotene.

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AMINO ACID REQUIREMENTS OF YOUNG WOMEN BASED ON NITROGEN BALANCE DATA

I. THE SULFUR-CONTAINING AMINO ACIDS [1]

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The pioneer investigations of Holt ('41) and of Rose ('42) and their collaborators on the amino acid requirements of man represent a rational approach to problems of protein nutrition. Among the amino acids they have designated as being indispensable dietary components for adult man is the sulfur-containing amino acid, methionine. The present studies were undertaken to provide more information as to the amounts of sulfur-containing amino acids necessary in human nutrition.

Reports from Rose's laboratory ('49, '50) set a figure of 1.10 gm as the minimum daily intake of methionine for normal man. It is indicated that this value was obtained by nitrogen balance studies on young men fed a diet where amino acid mixtures furnished 6.7 to 10 gm of nitrogen per day and represents, in the case of variations in individual requirements, the highest level of the amino acid necessary to establish nitrogen equilibrium.

Harte and Travers ('47), using three different proteins, calculated from the amount of protein required for nitrogen balance and its amino acid composition that the human requirement was 0.5 gm methionine and 0.2 gm cystine per day.

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Jones et al. ('55) reported the methionine requirement to be 150 to 230 mg per day when the diet contained 500 mg of cystine. These figures were obtained in a nitrogen balance study of women maintained on a diet of amino acid mixtures supplemented with some natural foods.

In the study to be reported here, on 8 healthy young women fed a diet containing nitrogen as a mixture of L-amino acids, glycine, and peanut protein, the amounts of cystine plus methionine necessary to achieve nitrogen equilibrium were 350 mg or less in 5 out of 8 subjects with the highest requirement being 550 mg.

EXPERIMENTAL

The experimental procedures of Rose ('49) and Leverton et al. ('52, '53) were followed but these authors' diets were modified to contain peanut protein and, contrasted to other reported studies, amino acids exclusively as the L-isomers.

The subjects were placed on a natural diet until they were in nitrogen balance and then were fed the experimental diet containing an equivalent amount of nitrogen as peanut protein, L-amino acids and glycine. After 7 days on the experimental diet the latter was modified by removing methionine from the amino acid supplement. After 7 days, supplements of methionine in 100 mg increments were added to the methionine-low diet of subjects in negative nitrogen balance.

Subjects. The subjects were 8 healthy young college women. During the study they maintained their normal schedule, but were requested to engage in no unusual physical exertion. All except one individual maintained constant weights during the experimental period. Their caloric and nitrogen needs were determined on a pre-treatment diet of natural foods. It was found that one individual, subject 4 needed an adjustment in calories from 2360 to 2600 when placed on the experimental diet. The total nitrogen of the diet varied somewhat with the individual and ranged from 7.3 to 8.4 gm per day. The age and weight of each subject at

the beginning and end of the experiment, and the caloric and nitrogen content of the diets are recorded in table 1.

Preparation and purification of L-*amino acids.* Glycine, L-cystine, L-histidine, L-leucine, L-lysine, and L-tryptophan were commercial products. L-methionine, L-phenylalanine, L-threonine and L-valine were prepared by selective hydrolysis with acylase of the acetyl derivatives of the commercial DL-forms using the procedures of Birnbaum and associates ('52). L-Isoleucine was prepared from the epimeric mixture of L-

TABLE 1

The age and weight of each of 8 women subjects at the beginning and the end of the experiments together with the caloric and nitrogen content of the diets

SUBJECT	AGE	WEIGHT BEGINNING EXPERIMENT	WEIGHT END EXPERIMENT	ENERGY VALUE OF DIET	NITROGEN IN DIET
		kg	*kg*	*Cal.*	*gm*
1	22	73.5	70.4	2080	7.35
2	24	63.2	63.7	2400	7.84
3	25	49.7	49.6	1760	7.36
4	30	71.8	71.5	2360 2600	8.38
5	21	49.4	48.8	1730	7.30
6	21	54.1	54.4	2400	8.32
7	23	51.3	52.0	1750	7.32
8	21	57.5	56.9	1750	7.32

isoleucine and D-alloisoleucine [2] by the acylase method of Greenstein et al. ('53). All of the amino acids were purified and analyzed by the procedures described by Dunn and Rockland ('47). The purified products were shown to contain negligible quantities of heavy metals and other inorganic impurities and to be of high purity as determined by specific rotation and semi-micro Kjeldahl determination of nitrogen.

Dietary regime. The diet was patterned after that of Leverton et al.[3] and contained some natural foods (orange juice,

[2] Dow Chemical Company product.

[3] Unpublished experiments.

TABLE 2

Daily menu plan for complete basal diet with variable calorie and nitrogen content

BREAKFAST	LUNCH	DINNER
100 gm Orange juice	100 gm Canned peaches	100 gm Canned pears
Muffin [1] (½ recipe)	Muffin [1] (½ recipe)	Muffin [1] (⅓ recipe)
10 gm Butter [2] (centrifuged)	10 gm Butter [2] (centrifuged)	5 gm Butter [2] (centrifuged)
10 gm Lemon juice (filtered)	10 gm Lemon juice (filtered)	10 gm Lemon juice (filtered)
10 gm Sucrose [2]	10 gm Sucrose [2]	10 gm Sucrose [2]
		Pudding [3] (double recipe)
3.44 gm Peanut protein	3.44 gm Peanut protein	5.16 gm Peanut protein
30 gm Jelly [2]	20 gm Butterscotch [2]	20 gm Butterscotch [2]
Vitamin capsule (1)	Mineral mix capsule (2)	Carbonated beverage [4] (7 oz. bottle)
Mineral mix capsule (1)		Gum drops [2]
		Mineral mix capsule (1)
1.56 gm Amino acids	1.80 gm Amino acids	1.80 gm Amino acids
Glycine [5]	Glycine [6]	Glycine [5]

[1] Recipe for one muffin: Cornstarch, 50 gm; sugar, 20 gm; salt, 2 gm; agar, 3 gm; baking powder, 5.6 gm; water, 60 ml; corn oil, 7 gm; butter fat, 10 gm.

[2] Amount varied somewhat depending on caloric requirements of individual.

[3] Recipe for one double pudding: Cornstarch, 16 gm; sugar, 60 gm; salt, 2 gm; water, 180 ml; butter fat, 20 gm; flavoring as desired.

[4] Squirt and Seven-up were used.

[5] Amount varied to give ¼ of quantity required per day to equal nitrogen content of pre-treatment diet.

[6] Amount varied to give ⅜ of quantity required per day to equal nitrogen content of pre-treatment diet.

canned peaches, and canned pears, all low in nitrogen content) to increase palatability. It also included amino acids, peanut protein, filtered lemon juice, centrifuged butter, cornstarch and agar or mucilose flakes. Some of these constituents were fed in muffins and puddings. Additional sources of calories were sugar, carbonated beverages, gum drops and butterscotch topping. Vitamins [4] were given daily and were supplemented with Vitamin E once a week. The minerals, prepared according to a formula of Rose et al. ('50), were also given in capsule form three times daily except for sodium chloride and baking powder which were incorporated in the muffins. A typical daily menu is shown in table 2.

Except for the 0.3 to 0.4 gm present in natural foods, the protein nitrogen of the diet was supplied by peanut protein, glycine and L-amino acids in amounts required to simulate the essential amino acid composition of whole egg protein. The content of peanut protein and egg protein with respect to the essential amino acids plus cystine, histidine, and tyrosine was determined by microbiological assay procedures (Dunn et al., '49; Murphy and Dunn, '50). Two grams of nitrogen as peanut protein were incorporated in the diet and the amino acid supplement was added in amounts necessary to achieve the amino acid composition of egg protein (with the exception that all of the sulfur-containing amino acids were added as methionine) and bring the nitrogen level to 3 gm. This portion of nitrogen in the diet was unvarying for all subjects. Values obtained for the amino acid assays of whole egg protein and peanut protein and the resultant calculated composition of the amino acid supplement are shown in table 3. Sufficient glycine, 7 to 8 gm, was added to the diet of each individual in order to bring the total to the levels in the pre-treatment period, when natural foods were supplied.

The diet with the complete amino acid supplement is referred to as the complete basal diet and is calculated to contain, as determined by microbiological assays, 1000 mg of sulfur-containing amino acids, 200 mg as cystine and 800 mg as

[4] Abbott Vitacaps.

methionine. The term "basal diet without added methionine" signifies a diet containing no methionine except the 150 mg derived from the peanut protein and the 14 mg from the natural food protein. The latter value is relatively small and is disregarded hereafter.

Preparation of samples and methods of assay. Nitrogen analyses on food, urine and feces were made by the macro-Kjeldahl method. Aliquots of the daily diet were homogenized in the Waring blendor and were taken for analysis whenever

TABLE 3

Values for "essential" amino acids in peanut and whole egg proteins and amino acids supplements

AMINO ACID	WHOLE EGG PROTEIN 3 GM N	PEANUT PROTEIN 2 GM N	SUPPLEMENT OF AMINO ACIDS (CALCULATED)
	gm	*gm*	*gm*
L-Cystine	0.45	0.20	...
L-Histidine [1]	0.43	0.25	0.18
L-Isoleucine	1.28	0.50	0.78
L-Leucine	1.72	0.84	0.88
L-Lysine [2]	1.39	0.39	1.00
L-Methionine	0.56	0.14	0.67
L-Phenylalanine	0.96	0.68	0.28
L-Threonine	0.77	0.42	0.35
L-Tryptophan	0.19	0.12	0.07
L-Tyrosine	0.60	0.55	0.05
L-Valine	1.29	0.59	0.70

[1] Fed as the equivalent quantity of L-histidine monohydrochloride monohydrate.
[2] Fed as the equivalent quantity of L-lysine monohydrochloride.

the diet composition was changed. Twenty-four-hour urine collections and fecal collections, the latter marked for 5 day periods with carmine, were made throughout the dietary study. Preparation and assay of the food materials for amino acid content were carried out according to procedures developed by Dunn and co-workers ('49).

RESULTS

The results of the study are shown in table 4 in terms of nitrogen balance. The figures, obtained by subtracting the

Nitrogen balance values obtained in 8 healthy women on a diet containing varying amounts of methionine

DIET	DAY	NITROGEN BALANCE IN GRAMS PER DAY							
		Subjects							
		1	2	3	4	5	6	7	8
	1	+ 0.03	— 0.90	— 0.05	+ 0.47	+ 0.43	— 1.15	— 0.27	— 0.44
	2	+ 0.26	— 0.12		+ 0.09	+ 0.43	— 0.16	— 0.27	+ 0.24
Period I	3	— 0.26	+ 1.10	— 0.05	— 0.62	+ 0.96	— 0.86	— 0.30	+ 0.77
	4	— 0.62	— 0.28	+ 0.17	+ 0.70	+ 0.43	+ 1.50	+ 0.90	— 1.04
Complete basal	5	+ 0.44	...	+ 0.25	— 0.58	+ 0.46	+ 1.06	— 1.43	— 0.17
800 mg methionine	6	— 0.75	— 0.68	— 0.47	— 0.37	— 0.72	+ 2.22	+ 0.08	+ 0.13
200 mg cystine	7	+ 0.52	— 0.13	— 0.31	— 0.06	+ 0.18	0.10	+ 0.34	+ 1.03
	8	+ 0.29	+ 0.05	— 0.16	— 0.13	+ 0.87	+ 1.24	— 0.49	— 0.85
	9	+ 0.69	— 0.06						
	10		— 0.17						
Average		+ 0.07	— 0.13	— 0.09	— 0.06	+ 0.38	+ 0.43	— 0.18	— 0.04
	1	— 1.50	— 0.76	— 0.50	— 0.62	+ 1.24	— 0.18	+ 0.28	— 0.58
Period II	2	— 0.19	— 0.61	+ 0.11	+ 0.27	— 0.28	...	...	+ 1.12
	3	— 0.46	— 0.85	+ 0.31	— 0.20	+ 0.70	+ 0.66	+ 0.56	+ 0.78
Basal without added	4	+ 0.01	+ 0.70	+ 0.01	— 0.60	+ 0.20	— 0.89	— 0.61	— 0.09
methionine	5	— 1.10	+ 0.00	— 0.92	— 0.10	+ 0.09	— 0.18	+ 0.07	+ 0.58
150 gm methionine	6	— 1.05	+ 0.50	— 1.06	— 0.75	+ 0.18	+ 0.87	— 0.52	+ 0.37
200 gm cystine	7	— 0.78	+ 0.49	— 0.82	— 1.40	+ 0.35	— 0.29	— 0.32	— 0.40
	8	— 0.48	+ 0.41	— 0.27	— 0.76	+ 0.02	+ 0.26		
	9	— 0.68	+ 0.54	— 0.40	— 1.41				
Average		— 0.70	+ 0.05	— 0.40	— 0.62	+ 0.31	+ 0.04	— 0.05	+ 0.25
	1	+ 0.13		+ 0.32	— 0.80				
	2	+ 0.36		+ 0.25	— 0.98				
Period III	3	— 0.37		— 0.60	— 0.45				
	4	+ 0.55		+ 0.58	— 0.70				
100 mg methionine added	5	+ 2.39		+ 1.58	+ 0.10				
to period II diet	6			+ 0.36	— 0.59				
	7			+ 0.60					
	8			— 0.08					
Average		+ 0.61		+ 0.38	— 0.57				
	1				— 0.11				
Period IV	2				— 0.08				
	3				+ 0.11				
200 mg methionine added	4				— 0.14				
to period II diet	5				+ 0.31				
	6				+ 0.12				
	7				+ 0.42				
Average					+ 0.09				

values for urinary and fecal nitrogen from those for food nitrogen, show considerable day-to-day variability. Similar fluctuations have been found by other workers (Rose, '49) For this reason nitrogen balance has been measured for each level of methionine over a period of at least 7 days; the fluct uations then average out. In the table, the days are numbered from the beginning of each period but they represent consecutive experimental days. An average value for nitrogen balance is considered to represent equilibrium unless it is negative by an amount greater than 5% of the total nitrogen in the diet.

In period I, during which the complete basal diet with 1000 mg of sulfur-containing amino acids was fed, the mean nitrogen balance value for the 8 to 10 experimental days was well within 5% of the total nitrogen of the diet for every subject and they were therefore all in nitrogen equilibrium.

In period II, the methionine was removed from the amino acid supplement, glycine being added in an amount necessary to keep the nitrogen level constant. This left in the diet only the 350 mg of the sulfur-containing amino acids present in the peanut protein and this was sufficient to maintain 5 of the 8 subjects, 2, 5, 6, 7 and 8, in nitrogen equilibrium. Three subjects, 1, 3 and 4, went into negative nitrogen balance by an average daily value that was greater than 5% of the total nitrogen intake. For these individuals, 350 mg of sulfur-containing amino acids did not suffice to maintain nitrogen equilibrium. When 100 mg of methionine was added to the diet (period III), and the glycine reduced by an appropriate amount to equalize the nitrogen content, subjects 1 and 3 promptly went into nitrogen balance. Thus, 450 mg of sulfur-containing amino acids were required for these two individuals. Subject 4 (period III) needed an additional 100 mg of methionine making a total of 550 mg of sulfur-containing amino acids required to maintain nitrogen balance.

For 3 of the 5 subjects who remained in nitrogen equilibrium on the 350 mg level, the amount of peanut protein was reduced to 1 gm and additional amino acids were supplied

to maintain the pattern and the amounts found in 3 gm of whole egg protein nitrogen (table 3). The total nitrogen of the diet was adjusted with glycine. Under these conditions where 175 mg of sulfur-containing amino acids were present in the diet, the average daily nitrogen balance values for periods of 5 days were: subject 6, + 0.48; subject 7, — 0.14; subject 8, — 0.24. In these three individuals, 175 mg of sulfur-containing amino acids (75 mg present as methionine) were apparently sufficient to maintain nitrogen equilibrium.

DISCUSSION

The problem of establishing the nutritional requirements for the sulfur-containing amino acids is of considerable practical importance. Many of the plant proteins such as those of peanut and soybean, widely used as nutritional sources of nitrogen, are relatively low in cystine and methionine. The results of the present study, insofar as nitrogen balance studies for limited periods can be translated into nutritional requirements, indicate that the needs of the healthy adult for cystine-methionine may actually be quite modest. They help to explain the results of Cox et al. ('47) who were unable to achieve any beneficial effect from methionine supplements in nitrogen balance studies of casein in humans in contrast to various experimental animals.

Even under rigid dietary control, the authors' subjects varied considerably in the amounts of cystine-methionine required to maintain nitrogen balance. The amounts cannot be correlated with weight since, although two of the three subjects requiring more than 350 mg had the largest weights of the group, the third subject weighed the least.

The highest requirement value obtained in this study is considerably below the figure of 1.1 gm set by Rose ('49) as the minimum optimum requirement of methionine and is more in line with the results of the Wisconsin investigators (Jones, Baumann and Reynolds, '55).

SUMMARY

The amounts of the sulfur-containing amino acids required to maintain nitrogen equilibrium in 8 healthy young women have been determined. When these subjects were placed on a diet containing 7 to 8 gm of nitrogen in the form of peanut protein, the essential L-amino acids, and glycine, it was found that the cystine-methionine requirement was 350 mg or less for 5 subjects, 450 mg for two subjects and 550 mg for one subject.

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AMINO ACID REQUIREMENTS OF YOUNG WOMEN BASED ON NITROGEN BALANCE DATA

II. STUDIES ON ISOLEUCINE AND ON MINIMUM AMOUNTS OF THE EIGHT ESSENTIAL AMINO ACIDS FED SIMULTANEOUSLY [1]

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Rose ('49) has shown that a dietary source of isoleucine is necessary to maintain a state of nitrogen equilibrium in adult man. He further indicates that by his criteria a value of 700 mg for this amino acid is the minimum daily requirement.

In the study to be reported here, data will be given on the amount of isoleucine required by young women who were fed a diet where the essential L-amino acids and glycine served as the chief nitrogen source. It was found that under the conditions of the experiment, for 7 subjects investigated, the amount of isoleucine required to maintain nitrogen equilibrium varied from 250 to 450 mg per day.

This study was undertaken as part of a cooperative project designed to investigate the amino acid requirements of young women and at the conclusion of the study on isoleucine, data were available on the minimum required amounts of all the essential amino acids. Taking advantage of this opportunity, minimum amounts of the 8 amino acids were fed simultaneously to the same subjects who participated in the isoleucine study. The data obtained are included in this report.

[1] This project was supported in part by a U. S. Dept. of Agriculture contract sponsored by the Human Nutrition Research Branch, Agricultural Research Service. It was supported in part by grants to one of us (M.S.D.) from Swift and Company, the U. S. Public Health Service and the University of California.

EXPERIMENTAL

The preparation and purification of the L-amino acids, preparation of samples and methods of assay as well as the general plan of the experiment have been discussed in a previous paper (Swendseid, Williams and Dunn, '56).

Subjects. The subjects were 7 healthy college women who maintained their usual schedule during the course of the experiment. Their caloric and nitrogen needs were determined on a pretreatment diet of natural food. It was found that an upward adjustment in calories was necessary to maintain nitrogen equilibrium when the subjects were changed from

TABLE 1

The caloric range, the nitrogen content of the diet and the weight and age of each of 7 women subjects

SUBJECT	AGE	WT. BEGINNING EXPERIMENT	WT. END EXPERIMENT	CALORIC RANGE IN DIET	NITROGEN IN DIET
		kg	*kg*	*Cal.*	*gm*
1	25	48.6	48.6	2150–2500	5.97
2	20	60.0	59.1	2200–2500	5.97
3	24	60.1	59.5	2200–2500	5.97
4	36	56.8	56.3	2200–2550	7.08
5	22	50.5	50.4	2450–2700	7.08
6	24	75.0	76.3	2150–2550	7.08
7	27	50.9	50.9	2200–2350	7.08

natural food to the amino acid-containing diet of isonitrogen content. This phenomenon of an increased caloric requirement when nitrogen is fed in the form of amino acids was also observed by Rose ('49) and has been discussed in a recent paper (Rose et al., '54). The caloric range, together with the nitrogen content of the diet, the weight and age for each subject are shown in table 1.

The subjects all experienced extreme discomfort when isoleucine was removed from the amino acid supplement of the diet. Their symptoms included: loss of appetite, a feeling of nausea, dryness of skin and mucous membranes, easy fatigue, and headaches. The addition of isoleucine restored their sense of well-being.

Dietary regime. The 7 subjects were placed first on a diet of natural food. Calories and nitrogen content were adjusted over a 5 to 10 day period until nitrogen equilibrium was established. The nitrogen content of the diet was set at either 6 or 7 gm per day. After a two-day transition period where one or two experimental meals were given, the subjects were fed entirely on the experimental diet of the same nitrogen content as the preceding natural food diet. This so-called basal synthetic diet was patterned after the diets used by Leverton et al.[2] and was similar to that described in a previous report (Swendseid, Williams and Dunn, '56) except that no peanut protein was used. In the daily menu plan of the preceding study (Swendseid, Williams and Dunn, '56) the following substitutions were made for peanut protein and amino acid supplement: breakfast, 2.44 gm of amino acid mixture (table 2); lunch, 3.66 gm of amino acid mixture and dinner, 3.66 gm of amino acid mixture. The dietary nitrogen was present chiefly as the essential L-amino acids and glycine with approximately 0.4 gm of nitrogen in the natural food portion. The essential L-amino acids plus histidine were supplied in a mixture in amounts simulating those contained in 18.75 gm of whole egg protein (3 gm of nitrogen) as determined by microbiological assay (Dunn and co-workers, '49). This portion of nitrogen in the diet was unvarying for all subjects and the composition of the amino acid mixture in amounts per day as it was present in the complete basal synthetic diet is given in table 2. Glycine was added to this mixture in a quantity sufficient to bring the total nitrogen content of the diet to the level of that in the natural foods, a value of approximately 6 or 7 gm. Calories were adjusted for each subject by varying the amount of pure carbohydrate food until the weight was stabilized and the appetite satisfied.

After a period of at least 7 days on the complete amino acid mixture the isoleucine level was either reduced to 200 mg (two subjects) or removed completely from the amino acid mixture (5 subjects). The isoleucine requirement of each sub-

[2] Unpublished experiments.

ject was determined within 100 mg by either increasing or decreasing the level for 5-day periods until nitrogen equilibrium was established. In the data to be reported, the basal

TABLE 2

Composition of amino acid mixture for complete basal diet

AMINO ACID	GM PER DAY
L-Cystine	0.45
L-Histidine [1]	0.43
L-Isoleucine	1.28
L-Leucine	1.72
L-Lysine [2]	1.39
L-Methionine	0.56
L-Phenylalanine	0.96
L-Threonine	0.77
L-Tryptophan	0.19
L-Tyrosine	0.60
L-Valine	1.29
Total	9.64

[1] Fed as the equivalent quantity of L-histidine monohydrochloride monohydrate.
[2] Fed as the equivalent quantity of L-lysine monohydrochloride.

TABLE 3

Amino acid composition of minimum-level mixture and of the natural-food portion of the diet

AMINO ACID	AMINO ACID MIXTURE	NATURAL FOOD
	mg per day	*mg per day*
L-Isoleucine	200 or 300	44
L-Cystine	450	
L-Leucine	600 [1]	67
L-Lysine	800 [2]	94
L-Methionine	200	15
L-Phenylalanine	200 [1]	45
L-Threonine	275 [1]	56
L-Tryptophan	150 [1]	
L-Tyrosine	900 [1]	
L-Valine	550 [1]	60
L-Histidine		20
L-Arginine		140
Total	4,325	541

[1] From data obtained by Dr. Ruth Leverton, University of Nebraska.
[2] From data obtained by Dr. May Reynolds, University of Wisconsin.

diet with the complete amino acid supplement will be referred to as the complete basal diet. It contained a total of 1330 mg of isoleucine, 1280 mg being present as synthetic L-isoleucine and 50 mg (determined by microbiological assay) occurring in the natural food.

When the isoleucine level was established and the subjects were in nitrogen equilibrium, the amino acid mixture equivalent to the essential amino acids in 18.75 gm of whole egg protein was removed from the diet and a mixture of the essential amino acids at their predetermined minimum levels was substituted. The composition of this minimum-level mixture is given in table 3. Since the natural food portion of the diet contained significant amounts of some of these amino acids as assayed by microbiological methods, these amounts in milligrams per day are also recorded in table 3. Glycine was added to keep the nitrogen level constant with that in the preceding experimental periods. Six subjects were kept on this minimum-level mixture for a period of 5 days.

RESULTS

The results of the experiment on isoleucine requirements are shown in table 4 in terms of nitrogen balance, the figures being obtained by subtracting the values for urinary and fecal nitrogen from those for food nitrogen. As in the previous paper (Swendseid, Williams and Dunn, '56) an average value for nitrogen balance was considered to represent equilibrium unless it was negative by an amount greater than 5% of the total nitrogen in the diet. The various periods shown in the table each represent a specific level of isoleucine. They were not run consecutively in some subjects due to certain exigencies in the experimental procedure.

In period I, where the complete basal diet containing 1280 mg of isoleucine was fed, the mean nitrogen balance value for the 6 to 12 experimental days was within 5% of the total nitrogen of the diet for all 7 subjects and they were therefore all considered to be in nitrogen equilibrium.

TABLE 4

Nitrogen balance values obtained in seven healthy women on a semi-synthetic diet containing varying amounts of isoleucine

DIET	DAY	NITROGEN BALANCE IN GRAMS PER DAY						
		Subjects						
		1	2	3	4	5	6	7
Period I	1	— 0.60	— 0.62	+ 0.27	— 0.01	— 0.16	+ 0.40	+ 0.03
Complete basal with 1280	2	+ 0.46	— 0.25	+ 0.24	+ 0.31	— 1.25	— 0.21	+ 0.48
mg of isoleucine +	3	+ 0.39	— 0.71	— 0.07	+ 0.20	+ 0.40	+ 0.49	+ 1.19
50 mg isoleucine in	4	+ 0.37	+ 0.92	+ 0.32	— 0.18	— 0.39	+ 0.53	+ 0.95
natural food (1330 mg	5	— 0.27	— 0.45	+ 0.24	— 0.37	— 0.23	— 0.66	— 0.14
isoleucine)	6	— 1.06	— 0.64	+ 0.34	+ 0.34	+ 0.02	+ 0.44	+ 1.31
	7	+ 0.54	— 0.20	— 0.24		— 0.15	+ 0.86	+ 0.56
	8	+ 0.03	— 0.62			+ 0.34	— 0.34	
	9	— 0.58	— 1.04					
	10		+ 0.14					
	11		+ 1.30					
	12		+ 0.21					
Average		— 0.08	— 0.16	+ 0.16	— 0.09	— 0.18	+ 0.19	+ 0.63
Period II	1	— 0.31	— 0.27	— 0.82	— 0.71	— 0.51	— 0.91	— 0.36
Basal without added	2	— 1.33	— 0.95	— 1.58	+ 0.22	— 0.66	— 0.56	— 0.73
isoleucine (50 mg iso-	3	— 2.06	— 0.80	— 1.12	— 1.46	— 1.86	— 2.27	— 1.16
leucine in natural	4	— 2.28	— 1.64	— 1.46	— 1.30			— 1.08
food)								
Average		— 1.49	— 0.91	— 1.25	— 0.81	— 1.01	— 1.25	— 0.83
Period III	1	+ 0.33	— 0.28	+ 0.12				
100 mg of isoleucine	2	— 0.18	— 0.99	+ 0.19				
added to diet of	3	— 0.25	— 0.83	— 0.54				
period II	4	— 0.51	+ 0.24	— 0.71				
	5		— 1.44	— 0.32				
Average		— 0.06	— 0.66	— 0.25				
Period IV	1	+ 0.12	+ 0.13	+ 0.48	+ 0.06	+ 0.16	— 1.36	— 0.80
200 mg of isoleucine	2	— 1.38	+ 0.07	— 0.08	— 0.92	— 1.40	— 0.20	— 0.98
added to diet of	3	+ 1.07	— 0.02	— 0.06	— 1.18	— 0.36	— 1.49	— 1.05
period II	4	— 0.70	— 0.11	+ 0.20	— 0.76	— 0.72	— 1.68	— 0.51
	5	— 0.31	— 0.56	— 0.23	— 0.07	— 0.60		
	6	— 0.05						
	7	— 0.26						
Average		— 0.23	— 0.10	+ 0.06	— 0.57	— 0.58	— 1.18	— 0.83
Period V	1				— 0.28	— 0.27	+ 0.08	+ 0.87
300 mg of isoleucine	2				+ 0.26	+ 0.22	— 0.85	+ 0.28
added to diet of	3				— 0.83	— 0.96	— 0.87	+ 0.26
period II	4				— 0.22	— 1.30	— 0.31	+ 0.24
	5				+ 0.81	— 0.67	+ 0.24	+ 0.15
	6					— 0.72	+ 0.82	
	7					— 0.75	+ 0.08	
	8						+ 0.86	
Average					— 0.05	— 0.66	+ 0.08	+ 0.36
Period VI	1					+ 0.00		
400 mg of isoleucine	2					— 0.19		
added to diet of	3					— 0.10		
period II	4					— 0.45		
Average						— 0.18		

In period II, isoleucine was removed from the amino acid mixture, glycine being added to keep the nitrogen level of the diet constant. This left 50 mg of isoleucine that was present in natural food. All of the subjects immediately went into strong negative balance and experienced such discomfort that they could not be maintained on this diet for longer than three to 4 days. They were losing nitrogen at the rate of approximately 1 gm per day.

TABLE 5

Nitrogen balance values obtained in six healthy women fed a semi-synthetic diet containing minimum amounts of all eight essential amino acids

DIET	DAY	NITROGEN BALANCE IN GRAMS PER DAY					
		Subjects					
		1	2	3	4	6	7
Basal without added isoleucine supplemented with enough isoleucine to maintain nitrogen equilibrium							
Average		— 0.23	— 0.10	+ 0.06	— 0.05	+ 0.08	+ 0.36
Minimum level of all essential amino acids	1	+ 0.06	— 0.02	— 0.66	— 0.28	+ 0.79	+ 0.01
	2	— 0.49	— 0.87	— 0.50	— 0.54	— 0.38	+ 0.61
	3	+ 0.23	— 0.09	+ 0.07	+ 0.40	— 0.83	+ 0.79
	4	— 0.04	+ 0.21	— 0.38	— 1.11	— 0.02	— 0.22
	5	+ 0.03	— 0.11	— 0.29	+ 0.29	— 0.30	— 0.68
	6		— 0.34				
Average		— 0.04	— 0.20	— 0.35	— 0.25	— 0.15	+ 0.10

In period III, during which 150 mg of isoleucine were fed to three subjects, there was a definite negative balance in one individual and equivocal results in the other two. One individual could be maintained on this level for only 4 days and the other showed a trend toward a negative balance during the last days of the period.

Three subjects, 1, 2, and 3, were definitely in nitrogen equilibrium in period IV, the 250 mg level; but 4 subjects, 4, 5, 6, and I, were in negative balance. For these 4 subjects, another

100 mg of isoleucine was added (period V). In this period, subjects 4, 6, and 7 went into nitrogen equilibrium. These three individuals had an isoleucine requirement of 350 mg. Subject 5 required an additional 100 mg (period VI) making a total of 450 mg isoleucine for nitrogen balance maintenance

When the subjects went into nitrogen balance, 6 of them (subject 5 was not included) were placed on a iso-nitrogen diet where all 8 of the essential amino acids were fed simultaneously at their predetermined minimum level. Table 5 records the data obtained. For purposes of comparison the average nitrogen balance of the preceding period is included in the table. For a 5-day period on this diet, all the subjects remained in nitrogen balance with the possible exception of subject 3 who may have been in slight negative balance.

DISCUSSION

The experiments with isoleucine demonstrate very clearly that restriction of this amino acid not only results in a negative nitrogen balance but also profoundly affects the health of the individual. The severity of symptoms indicates that deprivation of isoleucine must involve definite metabolic changes the nature of which at the present time are entirely unknown. Investigation of these metabolic changes may provide information as to some special role of isoleucine in tissue metabolism.

For the subjects studied, the amounts of isoleucine required to maintain nitrogen balance varied from 250 to 450 mg. These values are significantly lower than Rose's ('49) figure of 700 mg as the minimum requirement, although a final evaluation cannot be made until his complete data are published. A lower level for methionine requirement has also been found in a study run under comparable conditions (Swendseid, Williams and Dunn, '56). These findings suggest that some differences in the two experimental studies may be influencing the amino acid requirement. Possible factors include sex of the subjects, the use of D-amino acids in Rose's experiment, the amino acid pattern and the level of nitrogen in the diet.

The level of nitrogen in Rose's experiments was 10 gm instead of 6 to 7 gm in the present experiments. In this regard, studies with rats (Sauberlich and Salmon, '55) have shown that the tryptophan requirement of this animal is influenced by the type and level of protein fed. It may be suggestive that in the isoleucine experiment the three subjects who were in nitrogen balance at the lowest level of isoleucine, 250 mg, were also fed the diet containing the least amount of nitrogen.

The experiments showing maintenance of nitrogen balance during simultaneous administration of the 8 essential amino acids or their counterparts supplemented with glycine as the nitrogen source reaffirm the conclusions of Rose ('49) as to the number and kinds of amino acids required by the adult for nitrogen equilibrium. Arginine and histidine, essential for the growing rat, are apparently not necessary for the human adult, at least under these experimental conditions. The study also furnishes additional proof that the minimum levels of these amino acids for young women as established in individually reported experiments (Leverton et al., '52, '53, and '55; Jones, Baumann and Reynolds, '55; Swendseid, Williams and Dunn, '56) are essentially correct. The total amount of the essential amino acids representing approximately 0.6 gm of nitrogen demonstrates the remarkable ability of the body tissues in synthesizing the other amino acids required for protein formation.

In the opinion of the present investigators, these studies on amino acid requirements under strictly prescribed conditions have their greatest value in providing a starting point for a systematic study of factors that may cause variations in the needs of the body for the essential amino acids. The factors to be studied should include age, sex, state of health, level of dietary nitrogen, and amino acid pattern. Until these results are available the translation of requirements of amino acids to maintain nitrogen balance under certain specified conditions into the concept of nutritional requirements will be subject to modification.

SUMMARY

The amount of isoleucine required to maintain nitrogen equilibrium in 7 healthy young women has been determined. When these subjects were placed on a diet containing 6 to 7 gm of nitrogen in the form of the essential L-amino acids and glycine, it was found that the isoleucine requirement varied from 250 to 450 mg per day. Three of the subjects required 250 mg, three 350 mg, and one 450 mg.

In the second part of the experiment, minimum required amounts of all 8 essential amino acids were fed simultaneously to 6 subjects. The remainder of the nitrogen content of the diet was chiefly glycine. It was found that nitrogen balance was maintained in these subjects for the experimental period of 5 days with the exception that equivocal results were obtained in one individual.

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MATERNAL DIET AND RESISTANCE TO DENTAL CARIES IN THE COTTON RAT [1]

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Much evidence (Shaw, '55) has accumulated to show that diet affects the development of dental caries in experimental animals. Such evidence has been obtained chiefly with weanling young. Little information is available on the effects of maternal diet or stage of development (age) upon susceptibility to experimentally induced dental caries. In rodents it has been shown that the older the tooth the less susceptible it is to dental caries (Hodge, '43; Braunschneider et al., '48; and Constant et al., '54). These phases of the problem have been studied in relation to calcium and protein levels in the diet, and to the age of young cotton rats fed cariogenic diets post-weaning.

EXPERIMENTAL

Ten breeding females paired with a male were alloted each to three experimental groups, table 1, during three experimental periods of dietary change. Each female underwent reproduction to yield at least two litters per experimental period. The offspring, weaned at 17 days of age, were then used to ascertain the influence of the maternal diet upon dental caries development. Litters containing too few young or those born immediately after transfer between periods were discarded.

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The basal diet (30% protein and 0.6% Ca) used for the breeding females as the maternal diet is presented in table 2 with the modifications for periods B and C shown in table 1. Caries experience was determined in the young rat by the

TABLE 1

Experimental design

EXPERIMENTAL PERIOD	GROUP		
	1	2	3
A	30% protein 0.6% calcium	30% protein 0.6% calcium	30% protein 0.6% calcium
B	30% protein 0.2% calcium	15% protein 0.6% calcium	15% protein 0.2% calcium
C	30% protein 0.1% calcium	15% protein 0.1% calcium	15% protein 0.1% calcium

TABLE 2

Basal maternal diet

(30% protein : 0.6% calcium)

CONSTITUENT	AMOUNT	DIGESTIBLE PROTEIN	CALCIUM	PHOSPHORUS
	%	%	%	%
Corn meal	25.1	1.90	0.005	0.070
Linseed meal	10.0	3.08	0.039	0.087
Soybean meal [1]	10.0	3.37	0.025	0.059
Wheat germ meal	8.0	2.19	0.006	0.080
Brewers' yeast	3.0	1.27	0.004	0.047
Iodized salt	1.0			
Liver powder [2]	1.0			
Irradiated yeast [3]	0.01			
Casein (crude)	18.2	18.2		
Calcium carbonate	0.33		0.132	
Calcium phosphate (dibasic)	1.36		0.401	0.310
Dextrin	18.0			
Butter	4.0			
Stabilized vitamin A [4]	40 I.U./100 gm			

[1] Expeller process.

[2] Wilson's Liver Powder N. F.

[3] Fleischmann's Irradiated Dry Yeast. Standard Brands Inc., New York, N. Y.

[4] Nopecay 10, Type IV. National Oil Products Co., Harrison, N. J.

feeding of diet 302 (Shaw et al., '44) and 802 modified to reduce the calcium from 0.56 to 0.20% (802A) for 14 weeks. In order to test the age factor in caries resistance, young rats in a third group were maintained upon their mothers' respective diets until they were 6 weeks of age and were then transferred to diet 802A for 14 weeks. All diets were fed ad libitum and city water (1.2 ppm of fluorine) was available at all times. The experimental animals were weighed weekly.

At the close of each experiment, dental caries scores were obtained by established procedures (Shaw et al., '44). Post-mortem observations were made upon each animal. Extra weanling rats were used for analyses of molar teeth and femurs.

RESULTS

Growth data for the offspring showed that there was a remarkable similarity in the gains per week for 6 and 14 weeks respectively for both males and females in all groups. The former reached an average weight of about 150 gm at 17 weeks of age while females averaged 130 gm at the same age. A perceptible tendency toward a reduced growth rate was observed in the low-calcium-15% protein group but it was without significance.

In the breeding females it was evident that coincident with advance in age a reduced dietary calcium seriously curtailed reproduction beyond that of controls with adequate calcium. Fifteen per cent of protein with low-calcium diets further depressed reproduction.

A stepwise decrease in femur ash followed the reduction in dietary calcium in all cases. The ash content of femurs of young rats was 56.7 $\pm$ 3.2%, 53.9 $\pm$ 3.3%, 48.9 $\pm$ 0.2%,respectively, for maternal dietary levels of 0.6, 0.2 and 0.1% of calcium. No differences were obtained in the ash of molar teeth as the result of the dietary regimen. Increased frequency of developmental defects was observed in the offspring as the maternal diets were lowered in calcium. Such defects as

uneven occlusal surfaces, "tilted molars," and retarded tooth development were observed.

The data in tables 3, 4, and 5 show consistent trends in dental caries incidence and in lesion size in all groups of young rats fed diets 802 or 802A (cariogenic) and placed on experiment at 17 days of age. A reduction of the dietary calcium of the weanling rat from 0.56 to 0.2% (period A, a and b, period

TABLE 3

The effect of the level of calcium in the maternal diet upon dental caries in the offspring (group 1)

MATERNAL DIET EXPERIMENTAL PERIOD	WEANLING DIET	NO. OF ANIMALS	AVERAGE NO. LESIONS [1]	AVERAGE EXTENT [2]
1-A – 30% protein (0.6% Ca)	a – 802 (0.56% Ca)	17	26 ± 1.3 [3]	53 ± 4.8 [3]
	b – 802A (0.2% Ca)	17	32 ± 1.0	75 ± 4.8
	c – 802A (0.2% Ca) [4]	19	15 ± 1.7	27 ± 3.8
1-B – 30% protein (0.2% Ca)	a – 802 (0.56% Ca)	25	24 ± 1.2	43 ± 3.7
	b – 802A (0.2% Ca)	26	30 ± 1.0	63 ± 4 6
	c – 802A (0.2% Ca) [4]	21	10 ± 1.1	14 ± 1.8
1-C – 30% protein (0.1% Ca)	a – 802 (0.56% Ca)	9	22 ± 1.1	40 ± 3.5
	b – 802A (0.2% Ca)	9	29 ± 2.1	66 ± 9.6
	c – 802A (0.2% Ca) [4]	8	10 ± 1.5	15 ± 3.4

[1] Lesions represent average number of carious fissures per capita.

[2] Extent is an expression of the average total caries involvement per capita. Individual lesions are evaluated 1 + to 5 + with increasing severity of lesions. See Constant et al. ('54a).

[3] Standard error of the means. The "t" test was used in testing the significance of the mean. Differences due to maternal dietary treatments 1-A, 1-B, and 1-C, are not significant (99% probability). Average lesions and extent are significantly different for post-weaning diets a, b, and c, in all experimental periods.

[4] Maternal diet until 59 days old, then cariogenic diet 802A for 14 weeks.

B, a and b, in groups 1, 2, and 3, and in period C in group 1) caused a significant increase in caries scores. A similar trend was observed in period C, groups 2 and 3, but because of the limited number of animals and high variability it was found to be not significant statistically. Delayed feeding of diet 802A until the young were 59 days of age sharply reduced both the incidence and extent of caries in all cases (groups 1, 2, and 3, and periods A, B, and C). These results reaffirm previous

work reported from this laboratory (Constant et al., '54a, '54b). It is equally apparent that the maternal diet used in these experiments had much less effect upon experimentally induced caries than the post-weaning diets. A shift of the calcium downward from 0.6 to 0.2 and to 0.1% in the maternal diet had no detrimental effect upon the teeth of offspring subsequently fed diets containing 0.56 or 0.2% of calcium as far

TABLE 4

The effect of concentration of protein in the maternal diet upon caries susceptibility of the offspring (group 2)

MATERNAL DIET EXPERIMENTAL PERIOD	WEANLING DIET	NO. OF ANIMALS	AVERAGE NO. LESIONS [1]	AVERAGE EXTENT [2]
2-A – 30% protein (0.6% Ca)	a – 802	18	26 ± 1.0 [3]	53 ± 4.6 [3]
	b – 802A	19	32 ± 1.2	79 ± 5.4
	c – 802A [4]	17	14 ± 1.8	26 ± 3.8
2-B – 15% protein (0.6% Ca)	a – 802	18	26 ± 1.5	54 ± 3.9
	b – 802A	19	31 ± 0.9	75 ± 4.7
	c – 802A [4]	13	18 ± 2.3	28 ± 4.1
2-C – 15% protein (0.1% Ca)	a – 802	10	28 ± 1.3	63 ± 4.3
	b – 802A	10	29 ± 1.3	65 ± 6.3
	c – 802A [4]	10	19 ± 1.7	30 ± 4.3

[1] Lesions represent average number of carious fissures per capita.

[2] Extent is an expression of the average total caries involvement per capita. Individual lesions are evaluated 1 + to 5 + with increasing severity of lesions. See Constant et al. ('54a).

[3] Standard error of the means. Differences due to maternal dietary treatments 2-A, 2-B, and 2-C, are not statistically significant (99% probability). Differences between post-weaning diets a, b, and c, are significant except between 2 Ca and 2 Cb. The data in this case are consistent with the general trend.

[4] Maternal diet until 59 days old, then cariogenic diet 802A for 14 weeks.

as increasing susceptibility to caries was concerned. Though there is a consistent trend toward a slightly reduced susceptibility as the maternal diet was lowered in calcium, the results were not statistically significant. Thus the data here lead to the conclusion that the maternal diet is less important than that of the weanling cotton rat in its influence upon caries development.

There was some evidence that the 15% of protein fed with 0.1% of calcium in the maternal diet (2-C, a and b) checked the caries increase observed as the result of the lowered dietary calcium mentioned above. The protein content of the maternal diet otherwise (30 to 15%) had no apparent effect upon the caries score of the offspring. This fact emphasized

TABLE 5

The effect of simultaneous variations in calcium and protein contents of the maternal diet upon the caries susceptibility of the offspring (group 3)

MATERNAL DIET EXPERIMENTAL PERIOD	WEANLING DIET	NO. OF ANIMALS	AVERAGE NO. LESIONS [1]	AVERAGE EXTENT [2]
3-A – 30% protein (0.6% Ca)	a – 802	20	26 ± 0.9 [3]	49 ± 3.5 [3]
	b – 802A	22	30 ± 0.9	70 ± 3.5
	c – 802A [4]	21	10 ± 1.3	15 ± 2.5
3-B – 15% protein (0.2% Ca)	a – 802	21	18 ± 1.3	33 ± 5.2
	b – 802A	19	23 ± 1.4	44 ± 4.0
	c – 802A [4]	17	10 ± 1.8	15 ± 2.0
3-C – 15% protein (0.1% Ca)	a – 802	4	22 ± 4.1	46 ± 1.3
	b – 802A	3	27 ± 3.6	58 ± 1.4
	c – 802A [4]	2	7 ± 1.0	7 ± 2.0

[1] Lesions represent average number of carious fissures per capita.

[2] Extent is an expression of the average total caries involvement per capita. Individual lesions are evaluated 1 + to 5 + with increasing severity of lesions. See Constant et al. ('54a).

[3] Standard error of the means. The difference in caries susceptibility of the young on diets a and b was highly significant when the maternal diet was 3-A or 3-B. The caries susceptibility of rats weaned from period C was not significantly different from those in period A.

Animals placed on the various post-weaning diets showed consistent and significant differences in caries susceptibility. The means of group 3 Ca and 3 Cb were not statistically significant although the trend appears to be consistent.

[4] Maternal diet until 59 days old, then cariogenic diet 802A for 14 weeks.

again that manipulation of the diet of the dam had less effect upon the dental scores in the young cotton rat than did the dietary regimen during the post-weaning period. A simultaneous lowering of the protein to 15% and calcium to 0.2% resulted in significant reductions in the caries in the offspring receiving post-weaning semi-synthetic diets 802 and 802A. This

may have been caused by a better nutritive balance between protein and calcium insofar as caries susceptibility was concerned. Constant et al. ('54) reported that raising the protein from 24 to 34% in a low-mineral-low-calcium diet increased dental caries. These dietary effects on teeth seem to be associated with growth rate, tooth development and maturation. The maternal diets used contained calcium and phosphorus in ratios ranging from 1: 1.1 to 1: 3.4 respectively. It is possible that the lower Ca : P ratio improved the nutritive mineral balance sufficiently to reduce dental caries susceptibility. Sobel et al. ('48, '53) reported that the mineral content of the diet altered the composition of the teeth and that resistance to caries was associated with the lowering of the calcium to phosphorus ratio. Our data would tend to support in part the observations and report of Sobel and his co-workers. In these studies it appears that the absolute calcium level in the maternal diet was not the critical factor *per se*.

Young rats fed diet 802A at weaning occasionally had convulsive seizures and exhibited dark tar-like feces. The incidence of convulsive seizures was increased in the young from mothers fed the low-calcium diets. Young which exhibited these symptoms also showed less nerve sensitivity, paralysis of the hind quarters and internal hemorrhage. Animals which survived the period of rapid growth ceased to have the convulsive seizures.

Gross post-mortem changes were observed in some kidneys of the animals fed diet 802A. Animals fed diet 802 exhibited calcinosis of the heart as described by Constant et al. ('52), and frequently calcinosis of the liver and kidney. No calcinosis was found in the rats fed diet 802A.

It is recognized that the use of the same females to produce successive litters for periods A, B, and C probably furnished a less variable heredity in test offspring but that it introduced another variable, increasing age, which became evident in period C. Furthermore, in period B the ration was less adequate nutritionally than the one used in period A. Hence, breeding females started period C with certain degrees of

residual nutritional and reproductive stresses. Therefore, one would expect less satisfactory results during this period. While the results of groups 1, 2, and 3 in period C are comparable, they cannot be compared directly with those obtained during period B, groups 1, 2, and 3. However, the general pattern of results both with respect to the maternal diet and the dental caries assay follows that obtained in the two previous periods. In this report, the interpretation of the results has been made with these facts in mind.

SUMMARY AND CONCLUSIONS

A study has been made of the effect of maternal diets upon dental caries resistance of young cotton rats. The variables studied were the intake of calcium and protein and the age of exposure to cariogenic diets. Symptoms of convulsion and the passage of black tarry feces indicated that the animals in question had calcium intakes too low to permit good growth. The results may be summarized as follows:

1. Reproduction was disturbed and less adequate when the calcium content of the diet was reduced to 0.2 or 0.1%. A reduction of the protein of the diet to 15% caused a further decline in reproductive performance.

2. Growth rates were similar in all cases even though the ash content of the femur was reduced in the groups with lower calcium intakes.

3. Reduction of dietary calcium of the weanling rat from 0.56 to 0.2% resulted in a consistent increase in dental caries score. Caries was greatly reduced if the weanlings were fed their mothers diet for 6 weeks prior to a transfer to diet 802A (cariogenic). The influence of the diet of the young cotton rat was much greater in relation to experimentally induced dental caries than the maternal diet. The simultaneous lowering of protein to 15% and calcium to 0.2% in the maternal diet slightly decreased dental caries incidence and extent. It is suggested that nutritive balance is essential to maximum dental caries resistance in this species and that the

calcium to phosphorus and calcium to protein ratios seem to be important.

4. Cariogenie diet 802 caused calcinosis of the heart muscle and to a lesser degree in the kidney and liver. This diet with the calcium reduced (802A) to 0.2% eliminated the calcinosis but it appeared to induce kidney pathology. These pathologic conditions also seem to be dependent upon mineral balance.

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EFFECT OF ADDING CARBOHYDRATE TO MILK DIETS

I. GROWTH AND BODY COMPOSITION

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Cow's milk need be supplemented only with trace minerals in order to promote good growth when fed as the sole diet of weanling rats (Kemmerer et al., '32). Using a mineralized milk diet, these authors have shown that weanling rats reach 200 gm in weight in about 36 days, gaining an average of 3.9 gm per day. Good growth of rats has also been obtained by Scott and Norris ('49) using dried cow's milk supplemented with vitamins and minerals. A recent report by Bixby et al. ('54) confirms the above findings for both fluid and powdered milk.

The successful use of modified cow's milk formulas in infant feeding has suggested that a study be made in growing animals, comparing the nutritive properties of milk with those of milk to which various carbohydrates had been added.

The present report deals with the weight gain, food and water consumption, organ weights, and body composition of rats grown on powdered whole milk diets, with and without added carbohydrates, supplemented with vitamins and trace minerals.

EXPERIMENTAL

In each experiment 6 groups of 10 male weanling rats each (McCollum-Wisconsin strain) were selected on the basis of litter origin and weight and assigned to the 6 experimental

diets. The composition of the diets, the trace mineral supplement, and the vitamin supplement are shown in table 1. Diet A consisted of powdered whole milk with vitamin and trace mineral supplements, and in diets B to F one-third of

TABLE 1

Composition of powdered milk diets

	DIET A	DIETS B TO F
	%	%
Powdered whole milk [1]	99.5	66.2
Added carbohydrates [2]	...	33.3
Minerals [3] and vitamins [4]	0.5	0.5

Caloric distribution

	DIET A			DIETS B TO F		
	gm	Calories	% Total Calories	gm	Calories	% Total Calories
Protein (N × 6.25)	24.4	97.6	19.3	16.2	64.8	13.8
Fat	27.5	247.5	48.9	18.3	164.7	35.1
Carbohydrate	40.2	160.8	31.8	60.0	240.0	51.1
Ash	6.0	....	...	4.1	....	...
Vitamins	0.4	....	...	0.4	....	...
Moisture	1.5	....	...	1.0	....	...
	100.0	505.9	100.0	100.0	469.5	100.0

[1] Powdered whole milk was obtained from Golden State Creamery.

[2] Diet B, glucose; diet C, lactose; diet D, sucrose; diet E, dextrin (Amidex, medium grade, Corn Products Refining Company); and diet F, starch (Clinton Brand cornstarch).

[3] Mineral mixture based on Kemmerer et al. ('32); iron pyrophosphate, 50.8 mg, copper sulfate, 4.9 mg, manganese sulfate, 6.3 mg per 100 gm of diet.

[4] Vitamins — Thiamine hydrochloride 0.4 mg, riboflavin 0.5 mg, niacinamide 5.0 mg, pyridoxine hydrochloride 0.25 mg, calcium pantothenate 2.0 mg, choline bitartrate 200.0 mg, inositol 100.0 mg, p-aminobenzoic acid 10.0 mg, folic acid 0.20 mg, biotin 0.02 mg, vitamin B_{12} 0.01 mg, menadione 0.2 mg, ascorbic acid 20.0 mg, α-tocopherol 5.0 mg, Oleum Percomorphum 0.015 ml per 100 gm of diet.

the powdered milk was replaced by carbohydrate. The carbohydrates used in the present experiments included a monosaccharide, glucose, two disaccharides, lactose and sucrose and two complex carbohydrates, dextrin and starch.

The animals were housed in individual screen-bottom cages in an air-conditioned room maintained at 74 to 76°F. They

were allowed food and water ad libitum, and records were kept of food and water intake and of weight gain during the 6-week growth period. The experiment was repeated 4 times so that carcass and other analyses could be made at the end of some of the experiments.

At the termination of experiment 104 the animals were fasted for 24 hours and then sacrificed.[1] The liver and kidney weights of each animal were recorded and the livers taken for analysis of lipid, nitrogen and dry matter. The dry weight and lipid were determined by the method of Sarett and Jandorf ('47), and the nitrogen of the fat-free dried liver by the Kjeldahl method. Nitrogen values were multiplied by 6.25 for calculation of protein content and converted to a fresh liver weight basis. The carcass of each animal (minus liver and intestinal tract) was weighed in a tared pint Mason jar, autoclaved at 15 lbs. pressure for 4 hours, and saved for analysis of dry matter, lipid, nitrogen and ash. Each carcass was homogenized in a Waring blendor and samples taken for the determination of nitrogen by the Kjeldahl method, and of dry weight and total lipid (ether-soluble material) by methods previously described (Sarett and Jandorf, '47).

In order to calculate the lipid, protein and ash laid down on each diet during the experiment, 12 20-day-old weanling rats (average weight 48.9 gm) were similarly sacrificed following a 4-hour fast, the intestinal tract removed, and the remainder analyzed for total lipid, protein, ash and dry matter.

At the conclusion of experiment 106 the animals were fasted and sacrificed and each adrenal gland was rapidly excised and weighed. The adrenal gland from the left side of each animal was homogenized in a McShan-Erway homogenizer with trichloracetic acid solution and the extract analyzed for total ascorbic acid by the method of Lowry, Lopez and Bessey ('45). Cholesterol determinations on the other adrenal glands were carried out by the method of Sperry and Webb ('50).

[1] By intraperitoneal injection of Nembutal (Abbott) solution.

RESULTS

The weight gains of the rats on the powdered milk diets with and without added carbohydrates are summarized in table 2. The findings in eaoh of the 4 experiments are shown, and the average values for all the experiments are given with the standard deviations. The significance of the differences in

TABLE 2

Average weight gains of male weanling rats[1] *in 6 weeks on powdered milk diets*

DIET	ADDED CARBOHYDRATE	EXP. 101	EXP. 103	EXP. 104	EXP. 106	ALL EXP. ± S.D.
		gm	*gm*	*gm*	*gm*	*gm*
A	None	184.5	186.0	185.1 (9)	208.6	191.2 ± 26.2 (39)
B	Glucose	178.6	175.4	175.6	191.5	180.3 ± 22.1
C	Lactose	150.1 (9)	142.7 (9)	138.1 (8)	158.8	148.0 ± 24.7 (36)
D	Sucrose	187.8 (9)	199.0	181.6	207.5	194.1 ± 20.2 (39)
E	Dextrin	182.0	173.4	173.3	186.8	178.9 ± 22.0
F	Starch	182.1	193.1	174.5	195.1	186.2 ± 20.6
		p values from "t" test (Snedecor, '46)				
						ALL EXPS.
Diets A vs E		....	< 0.3	< 0.2	< 0.1	< 0.05
Diets B vs D		< 0.4	< 0.05	< 0.5	< 0.1	< 0.01
Diets C vs E		< 0.02	< 0.02	< 0.01	< 0.02	< 0.001
Diets D vs E		....	< 0.05	< 0.3	< 0.05	< 0.01

[1] Average starting weights of groups in experiment 101 were 48 to 51 gm, experiment 103, 51 to 53 gm, experiment 104, 55 to 56 gm, and experiment 106, 48 to 49 gm. Each experiment was started with 10 rats per group. Where mortality occurred, the figure in parenthesis shows the number of surviving rats.

weight gain between groups on different diets is shown as the p value for some of the individual experiments and for the average of the 4 experiments. The data show that the gain in weight on diet A (powdered milk) is about the same as that obtained with diet D (sucrose). These values are slightly greater than those obtained with diets B, E and F (glucose, dextrin and starch). In the individual experiments the differences are not significant but when all the experiments are taken together the probability that the difference in weight gain on diets A and E is due to chance is less than

0.05. The weight gain with sucrose (diet D) is significantly greater than with glucose or dextrin when all 4 experiments are considered. There is no significant difference between the growth obtained on the diets containing glucose, dextrin and starch, even when all 4 experiments are taken together.

With lactose as the added carbohydrate (diet C), growth was significantly less than that obtained on any of the other diets (table 2). Comparison with the dextrin diet (E) shows a p value of < 0.001. Diarrhea persisted for a few weeks in most of the rats on the high lactose diet. The poor growth on this diet confirms previous findings on the toxicity of high lactose diets for the growing rat (Day, '36; Handler, '47; Mitchell and Dodge, '35). The level of lactose present in milk does not appear to be toxic for the rat (Kemmerer et al., '32; Boutwell et al., '45; Bixby et al., '54).

The average caloric intake of the rats (fed ad libitum) and gain in weight per 100 calories are shown in table 3. The food intake is given in terms of calories rather than grams since the caloric value of diet A differed from that of the other diets. There was no significant difference between the caloric intakes on any of the diets. The average caloric intakes for the 6-week period ranged from 2,225 Cal. on the added lactose diet (diet C) to 2,420 on the diet with added sucrose (diet D). The greater caloric intake on the sucrose diet accounts in part for the greater weight gain on this diet. The gain in weight per 100 calories was slightly greater on the powdered milk diet than on the added carbohydrate diets, presumably due to the higher protein and mineral content of the powdered milk diet. The efficiency of food utilization on the lactose diet was significantly lower than that found on any of the other diets. There were no significant differences between the other added carbohydrate diets in weight gain per 100 Cal. With powdered whole milk, diet A, 2.35 gm of milk solids were required per gram of weight gain during the 6-week period. This is similar to the figure of 2.25 gm of milk solids per gram gain reported by Kemmerer et al. ('32) for a

TABLE 3

Calories, protein and water intake of male weanling rats in 6 weeks on powdered milk diets [1]

(From experiments 101, 103, 104 and 106)

DIETS	ADDED CARBOHYDRATE	NO. OF RATS	CALORIE INTAKE	GM GAINED/ 100 CAL.	PROTEIN INTAKE	GM GAINED/ GM PROTEIN	WATER INTAKE	ML WATER/ 100 CAL.	ML WATER/ GM PROTEIN
			cal.		*gm*		*ml*		
A	None	39	2290 ± 295	8.4 ± 0.9	110.3 ± 14.2	1.74 ± 0.20	946 ± 139	41.5 ± 5.1	8.6 ± 1.1
B	Glucose	40	2240 ± 251	8.1 ± 0.7	77.4 ± 8.7	2.33 ± 0.20	736 ± 117	32.8 ± 3.6	9.5 ± 1.1
C	Lactose	36	2225 ± 304	6.7 ± 0.9	76.9 ± 10.5	1.93 ± 0.26	1307 ± 416	59.0 ± 17.5	17.1 ± 5.1
D	Sucrose	39	2420 ± 260	8.0 ± 0.5	83.6 ± 9.0	2.33 ± 0.15	769 ± 85	32.1 ± 3.0	9.3 ± 0.8
E	Dextrin	40	2350 ± 248	7.6 ± 0.5	81.1 ± 8.6	2.21 ± 0.14	795 ± 147	33.6 ± 4.5	9.8 ± 1.3
F	Starch	40	2290 ± 259	8.1 ± 0.7	79.2 ± 9.0	2.36 ± 0.21	735 ± 100	32.1 ± 4.0	9.3 ± 1.1

p values from "t" test (Snedecor, '46)

	Water Intake, ml
Diets A vs C	< 0.001
Diets A vs E	< 0.001

[1] Values shown with standard deviations.

TABLE 4

Lipid, protein, ash and solids in carcasses and livers of male rats grown for 6 weeks on powdered milk diets [1]

(From experiment 104; diet A, 9 rats, diet C, 8 rats, other diets, 10 rats)

DIET	ADDED CARBOHYDRATE	CARCASS				LIVER			
		Lipid	Protein	Ash	Dry weight	Percentage of body weight	Lipid	Protein	Dry weight
		%	%	%	%		%	%	%
A	None	9.4 ± 1.9	21.0 ± 0.4	4.2 ± 0.3	33.8 ± 1.4	3.95 ± 0.57	5.3 ± 1.7	20.7 ± 0.6	27.5 ± 2.0
B	Glucose	11.1 ± 2.4	21.1 ± 0.7	3.9 ± 0.4	35.1 ± 2.0	4.30 ± 0.38	4.1 ± 0.7	20.4 ± 0.2	26.0 ± 0.5
C	Lactose	7.0 ± 1.1	21.0 ± 0.4	4.3 ± 0.4	31.2 ± 1.2	4.65 ± 0.30	3.9 ± 0.5	20.6 ± 0.7	26.0 ± 0.9
D	Sucrose	11.5 ± 2.2	21.1 ± 0.7	3.8 ± 0.3	35.1 ± 1.7	4.25 ± 0.60	4.0 ± 0.8	20.9 ± 0.8	26.4 ± 1.4
E	Dextrin	10.8 ± 2.0	21.1 ± 0.7	4.1 ± 0.3	35.0 ± 1.6	4.88 ± 0.62	5.3 ± 1.8	20.6 ± 0.3	27.5 ± 1.8
F	Starch	11.4 ± 2.3	21.6 ± 0.7	3.9 ± 0.4	35.4 ± 1.8	4.19 ± 0.55	4.1 ± 0.8	20.7 ± 0.6	26.3 ± 1.1

[1] Values shown with standard deviations.

5-week period, using fluid milk, and to 2.32 gm reported by Scott and Norris ('49) using dried milk.

The average protein intake and the gain in weight per gram of protein consumed are also shown in table 3. On diet A, the protein intake was about 40% greater than that found on the added carbohydrate diets, and consequently, the weight gain per gram of protein was much lower on diet A than on the diets with added carbohydrate. The protein intakes did not vary widely on the added carbohydrate diets, and the utilization of protein for growth was uniformly good on these diets except for that with added lactose.

The water intake of the animals on diet A was significantly higher than that of the animals on the diets B, D, E or F ($p < 0.01$, table 3). The increased water requirement on diet A is related to the high protein and mineral content of the diet and is also evident when the data are calculated as milliliters of water per 100 Cal. of food (table 3). The addition of carbohydrates other than lactose has a water-sparing effect. On the added lactose diet (C), diarrhea and other upsets of metabolism result in a water intake almost twice that found with the other added carbohydrate diets and about 50% greater than that observed on the powdered milk diet.

Data on liver and carcass analyses of the animals in experiment 104 are shown in table 4. The carcasses of the animals on the diets containing added carbohydrate, other than lactose, contained higher levels of lipid, 10.8 to 11.5%, than those of the animals on diet A, 9.4%. The differences between this value on diet A and those of diets B, D, E or F were not statistically significant, but have been observed several times in similar experiments in this laboratory. The total amount of lipid laid down in the tissues during growth was higher on diets B, D, E or F than on diet A (table 5). With diet E, added lactose, 7.0% lipid was found in the carcass and only 7.1 gm of lipid was retained during growth. These values were significantly lower than the corresponding figures for the other groups.

The level of protein in the carcasses of the animals on all of the diets was quite similar, ranging from 21.0 to 21.6% with a small standard deviation in each group (table 4). The animals on diet A retained 38.4 gm of protein during the 6 weeks in which they consumed 107.5 gm of protein, while the animals on diets B, D, E and F retained 36.7 to 38.2 gm of the 77.3 to 80.1 gm consumed (table 5). (The above figures for protein intake are taken from experiment 104 and differ slightly from the average figures given in table 2.) Protein retention was lowest in the animals on diet C.

TABLE 5

Increase in lipid, protein and ash content of male rats grown for 6 weeks on powdered milk diets [1]

(From experiment 104)

DIET	ADDED CARBOHYDRATE	LIPID INCREASE	PROTEIN INCREASE	ASH INCREASE
		gm	*gm*	*gm*
A	None	15.6	38.4	7.6
B	Glucose	18.1	36.7	6.5
C	Lactose	7.1	28.9	5.6
D	Sucrose	19.9	38.2	6.7
E	Dextrin	17.8	37.1	6.9
F	Starch	18.9	38.1	6.5

[1] The increases in lipid, protein and ash were calculated by subtracting average amounts found in weanling rats from those found in the carcasses and livers of the experimental animals. The weanling rats (minus intestinal tract) were found to contain (with standard deviation) $10.5 \pm 1.7\%$ lipid, $17.4 \pm 0.7\%$ protein, $3.06 \pm 0.1\%$ ash, and $30.2 \pm 1.3\%$ dry weight.

The percentage of ash and the grams of ash laid down in the animals on diet A were slightly higher than those found on diets B, D, E and F. The animals on diet A retained 7.6 gm of the 26.5 gm of minerals consumed and those on diets B, D, E and F, retained 6.5 to 6.9 gm of the 19.5 to 20.3 gm consumed.

The percentage of dry weight of the carcass was highest in those groups with the higher percentage of lipids (table 4). The average concentration of water in the carcasses (100 minus % dry weight) was 68.8% on the lactose diet, 66.2%

on the powdered milk diet and 64.6 to 65.0% on the diets with the other added carbohydrates.

The livers of the animals on the added dextrin diet were much larger than in any of the other groups (table 4). The livers of the animals on the diets with added glucose, sucrose and starch comprised a slightly greater percentage of body weight, than did those of rats on the powdered milk diet. On the diet with added lactose the livers of the animals were large in proportion to the weight of the rats. The composition of the livers of the animals on all the above diets was approximately the same in level of protein, lipid and percentage of dry weight. The percentage of lipib in the liver is similar to that reported by Bixby et al. ('54) in rats grown on mineralized powdered whole milk.

The average kidney weights of the animals sacrificed in experiment 104 are shown in table 6 and are also calculated as percentage of body weight. The relative weight of the kidneys of the animals on the added lactose diet was much greater than that found in the animals on each of the other diets. Little effect of diet on relative kidney size is seen in the other groups.

The weights of the adrenal glands and their ascorbic acid and cholesterol content as determined in experiment 106 are also shown in table 6. With the added lactose diet, the relative size of the adrenal glands was greatest. In the animals on diet A, the adrenal weights were slightly but not significantly greater than those found in the rats on diets B, D, E and F. The level of ascorbic acid was lowest in the adrenals of the animals on the lactose diet, 4.24 μg per milligram, and varied only from 4.62 to 4.78 μg per milligram in the other 5 groups. The concentration of cholesterol in the adrenal glands varied widely among the animals in each group and the average level was highest in the animals receiving added lactose (table 6). The levels of cholesterol in the adrenal glands of all of the rats in this experiment and of stock ani-

TABLE 6

Kidney weights and adrenal gland analyses in male rats grown for 6 weeks on powdered milk diets [1]

DIET	ADDED CARBOHYDRATE	KIDNEYS [2]		ADRENAL GLANDS [3]			
		Weight	% of body wt.	Weight	mg/100 gm body wt.	Ascorbic acid	Cholesterol
		gm		*mg*		*μg/mg*	*μg/mg*
A	None	1.76 ± 0.12	0.80 ± 0.06	34.2 ± 5.0	14.6 ± 2.4	4.62 ± 0.44	19.9
B	Glucose	1.61 ± 0.21	0.76 ± 0.05	29.3 ± 5.3	13.1 ± 1.3	4.74 ± 0.61	18.2
C	Lactose	1.51 ± 0.15	0.89 ± 0.10	31.0 ± 3.9	16.7 ± 2.6	4.24 ± 0.69	23.7
D	Sucrose	1.77 ± 0.22	0.81 ± 0.09	31.5 ± 4.3	13.1 ± 1.6	4.78 ± 0.53	16.8
E	Dextrin	1.68 ± 0.11	0.79 ± 0.03	30.9 ± 5.0	14.2 ± 2.3	4.71 ± 0.63	18.5
F	Starch	1.69 ± 0.16	0.80 ± 0.05	30.5 ± 2.4	13.5 ± 1.3	4.74 ± 0.58	20.4

[1] Values shown with standard deviations.

[2] Data of experiment 104.

[3] Data of experiment 106.

mals from the same colony are much lower than values reported from other laboratories.[2]

DISCUSSION

Powdered whole milk, when supplemented with the trace minerals, iron, copper and manganese, and a mixture of known vitamins, supports good growth of rats. Growth and food utilization of the same order of magnitude as shown in the present report were previously found by Kemmerer et al. ('32) using fluid milk and trace minerals, by Scott and Norris ('49) using powdered milk with vitamins and minerals, and by Bixby et al. ('54) using both fluid and powdered milk with trace minerals.

When carbohydrate replaces one-third of the milk solids, the carbohydrate content of the diet is 60%, of which 27% comes from the lactose in the milk. When lactose is the carbohydrate added, the level of lactose in the diet is too high for the rat and leads to diarrhea, high water requirement, and poor growth. Other deleterious effects of high lactose diets for the rat have been described previously (Day, '36; Handler, '47; Mitchell and Dodge, '35; Boutwell et al., '45).

Good growth, approximately equal to that obtained with powdered whole milk, is obtained when carbohydrates such as glucose, sucrose, dextrin or starch replace one-third of the milk powder. This addition of carbohydrate to milk reduces the percentage of protein in the diet from 24.4 to 16.2 and the percentage of calories supplied by protein from 19.3 to 13.8. The higher level of protein in the powdered milk diet

[2] The level of cholesterol in the adrenal glands of the experimental and stock rats from this colony (McCollum-Wisconsin strain) is low as compared with values reported from other laboratories. This is probably due to a difference in the strain of rats. The animals used in this laboratory are obtained from the colony which was started here over thirty years ago with rats obtained from Dr. E. V. McCollum. Dr. F. J. Agate, Jr., of Columbia University has analyzed the adrenal glands of some of the animals in this colony and has found values similar to those reported here. We have analyzed the adrenal glands from some of Dr. Agate's animals (Long-Evans strain) and have found levels of cholesterol similar to those which he finds in his rats, namely 40 to 60 μg per mg.

results in only slightly better food efficiency than is found with the added carbohydrate diets. The retention of protein in the carcass is about the same on both types of diet. The gain in weight per gram of protein consumed is lower on the powdered milk diet than on the added carbohydrate diets.

The high protein and mineral content of the powdered milk diet increases the excretory load and consequently the water requirement of the animals. A detailed study of this aspect has been made (Sarett and Snipper, '56). The increased weight of the adrenal glands of the rats on diet A as compared with those on the added carbohydrate diets may also be related to the higher protein and mineral content of diet A.

Analysis of the carcasses of the experimental animals shows that the protein content of the tissues was about the same regardless of whether or not carbohydrate was added to the diet. The level of fat in the carcass was 10.8 to 11.5% on the added carbohydrate diets as compared with 9.4% in animals on the powdered milk diet. Other investigators have found 11 to 15% of fat in carcasses of animals on stock or control diets (Beare et al., '53; Pickens et al., '40; Light et al., '34).

It appears from the present data that a nutritionally sound animal can be grown with a diet of powdered milk alone or of two-thirds powdered milk and one-third glucose, sucrose, dextrin or starch. The lowering of the level of protein and minerals in the diet by the addition of carbohydrate may be beneficial in that the water requirement and the load on the kidneys and adrenal glands are diminished. Further experiments along these lines are in progress.

SUMMARY

1. Data are presented indicating that good growth and nutritionally sound rats are obtained with a diet of powdered milk or of two-thirds powdered milk and one-third added carbohydrate (glucose, sucrose, dextrin or starch) when each of the diets is supplemented with vitamins and trace minerals.

2. In 6-week studies animals on the powdered milk diet (24.4% protein) gained approximately the same weight as those on the added carbohydrate diets (16.2% protein). The caloric intakes were not significantly different on the various diets.

3. The water intakes on the diets with added carbohydrate were considerably less than that on the powdered milk diet.

4. The concentration of lipid in the carcasses of the animals on diets with added carbohydrate was higher than that found in the animals on the powdered milk diet.

5. The concentration of protein in the carcasses of the animals was about the same on all of the diets studied. The animals on the powdered milk diet laid down only slightly more protein during growth than those on the added carbohydrate diets.

6. A diet of one-third lactose and two-thirds powdered milk supports poor growth of rats due to its high lactose content.

7. The effects of the above diets on growth, food and water consumption, body composition, and organ weights are discussed.

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EFFECT OF ADDING CARBOHYDRATE TO MILK DIETS

II. WATER RESTRICTION [1]

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ONE FIGURE

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In previous experiments it was shown that the weight gain of male weanling rats on a diet of powdered whole milk plus vitamins and trace minerals was about the same as that found when one-third of the powdered milk in the diet was replaced by carbohydrates such as glucose, sucrose, dextrin or starch (Sarett and Snipper, '56). It was also observed that the animals on the powdered milk diet drank 25 to 30% more water than those on the diets with added carbohydrate.

The present experiment was designed to show the effects of restricting the water intake of rats on both types of diet. The weight gain, food and water consumption, organ weights and body composition of rats grown on the powdered milk diet and on the diet with added starch were determined, with the water allowance limited to (1) the full amount of water which the animals usually consumed with each diet, (2) 80% or (3) 60% of this amount of water. The data show that the animals on the added starch diet were less adversely affected by water deprivation than those on the powdered milk diet.

[1] Presented before the American Institute of Nutrition at San Francisco, California, April 10–16, 1955 (Fed. Proc., *14:* 448).

EXPERIMENTAL

Two groups of 30 male weanling rats each (average weight, 50 gm) were selected on the basis of litter origin and weight, and placed in individual screen-bottom cages in an air-conditioned room maintained at 74 to 76°F. One group was given the powdered milk diet (diet A) and the other the powdered milk diet with added starch (diet F). The composition of these diets, including the added vitamin and trace mineral mixtures, has been described (Sarett and Snipper, '56). For two weeks the animals were allowed food and water ad libitum and records were kept of food and water intake and of weight gain.

For the final 4 weeks the animals on each diet were separated into three groups of 10 each. The water allowance for the animals in each group was restricted so that with each diet there were groups receiving 100, 80 and 60% of the average ad libitum water intake on that diet. The animals were weighed twice a week, and the daily water allowance for each animal was readjusted on the basis of its new weight. Any water remaining the following morning was removed and measured.

The water allowance was calculated from previous records of food and water consumption of animals on these diets maintained in the same room at the same temperature (Sarett and Snipper, '56). From the average weekly weights and average food and water consumption for each weekly interval, a curve relating water intake to body weight was constructed for each diet. From this curve a table was drawn up showing the milliliters of water per day for animals weighing from 90 to 250 gm. These figures were taken as 100% of water allowance and were multiplied by 0.8 and 0.6 respectively, to determine the water allowances for the other groups. The water allowances for the three groups on diet A and for those on diet F are shown in table 1.

Records were kept of food intake, water intake, and weekly weight gain for the 4-week period. The animals were then fasted for 24 hours, during which time they received their

usual water allotment, and sacrificed [2]. The livers, kidneys, and adrenal glands were weighed. The carcasses (including adrenals and kidneys) were analyzed for dry matter, lipid, total nitrogen and ash and the livers were analyzed separately for dry weight, lipid, and total nitrogen by methods previously described (Sarett and Jandorf, '47; Sarett and Snipper, '54).

TABLE 1

Daily water cllowances [1]

WEIGHT OF RAT	DIET A			DIET F		
	Group 1 (100%)	Group 2 (80%)	Group 3 (60%)	Group 4 (100%)	Group 5 (80%)	Group 6 (60%)
gm	*milliliters of water per day*					
90	21.0	17.0	12.5	16.5	13.0	10.0
100	21.5	17.5	13.0	16.5	13.5	10.0
110	22.0	17.5	13.0	17.0	14.0	10.5
120	22.5	18.0	13.5	17.5	14.0	10.5
130	23.5	19.0	14.0	18.0	14.5	11.0
140	24.5	19.5	14.5	18.5	15.0	11.5
150	25.0	20.0	15.0	19.0	15.5	11.5
160	25.5	20.5	15.5	19.5	16.0	12.0
170	26.0	21.0	15.5	20.0	16.0	12.0
180	26.5	21.5	16.0	20.5	16.5	12.5
190	27.5	22.0	16.5	21.0	17.0	13.0
200	28.0	22.5	16.5	21.5	17.5	13.0
210	28.5	23.0	17.0	22.0	17.5	13.5
220	29.0	23.5	17.5	22.5	18.0	13.5
230	29.5	23.5	18.0	23.0	18.5	14.0
240	30.0	24.0	18.0	23.0	18.5	14.0
250	30.5	24.5	18.5	23.5	19.0	14.5

[1] Each animal in groups 1 and 4 received the amount of water usually consumed when food and water were allowed ad libitum with diets A and F, respectively. Groups 2 and 3 were given 80 and 60%, respectively, of that allowed for group 1, and groups 5 and 6 were given 80 and 60%, respectively of that allowed for group 4.

RESULTS

The weight gains and food and water consumption of the animals during the 4-week experimental period are summarized in table 2. On diet A the average water intake of group 1 was 700 ml, the animals of group 2, restricted to 80%

[2] By intraperitoneal injection of Nembutal (Abbott) solution.

TABLE 2

Weight gain, water and food intake of growing rats during 4 weeks on restricted water intake [1]

FACTOR OF INTEREST	DIET A			DIET F		
	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
WATER ALLOWANCE, %	100	80	60	100	80	60
Number of rats	10	10	10	9	10	10
Body weight:						
Av., 2 wks., gm [2]	114	113	113	119	117	117
Av., 6 wks., gm	256	238	211	263	244	222
Av. gain, gm	142 ± 13	125 ± 12	98 ± 31	144 ± 17	127 ± 8	105 ± 12
Water:						
Intake, ml	700 ± 56	567 ± 14	410 ± 19	539 ± 14	443 ± 14	324 ± 11
Intake, %	100	81.5	58.6	100	80.4	60.2
Food:						
Intake, cal.	2110 ± 140	1800 ± 200	1640 ± 310	1930 ± 180	1810 ± 110	1740 ± 150
Intake, %	100	85	78	100	94	91
Water:						
Ml/100 cal.	34.2 ± 5.7	31.9 ± 3.4	25.8 ± 4.8	28.1 ± 2.5	24.6 ± 1.4	18.7 ± 1.1
Ml/gm protein	7.1	6.6	5.4	8.2	7.1	5.4
Ml/gm gain	4.9	4.5	4.2	3.7	3.5	3.1
Body weight gain:						
Gm/100 cal.	7.0 ± 1.1	7.0 ± 0.5	5.9 ± 1.6	7.5 ± 0.7	7.0 ± 0.4	6.0 ± 0.4
Gm/100 ml water	20.4 ± 1.4	22.1 ± 1.9	23.8 ± 7.2	26.7 ± 2.7	28.6 ± 1.8	32.4 ± 3.2
Gm/gm protein	1.44	1.45	1.22	2.17	2.03	1.75

[1] Including standard deviations.

[2] Start of water restriction. Animals were on same diets with water ad libitum for two weeks after weaning.

of full allowance, consumed 567 ml (81.5%) and those of group 3, restricted to 60%, consumed 410 ml (58.6%). On diet F the average intake at full allowance was 539 ml (group 4), and groups 5 and 6 consumed 443 ml or 80.4% and 324 ml or 60.2%, respectively. On both diets, weight gain was markedly reduced by water restriction. The average weight gains

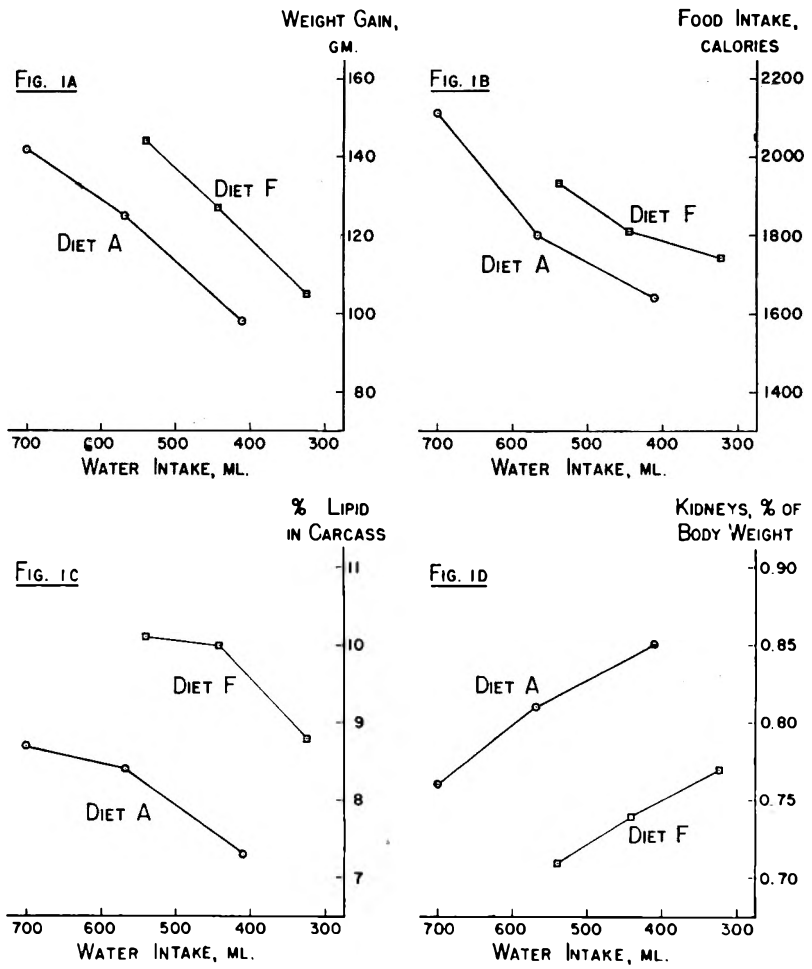

Fig. 1 Relationship of weight gain, food intake, relative kidney weight and percentage of carcass lipids to water intake in animals on diets A and F for 4 weeks. Diet A, powdered milk; diet F, powdered milk with added starch.

at each relative level of water allowance were about the same on both diets (fig. 1 a). However, the absolute water intakes of the animals on the powdered milk diet were much higher than those of the rats receiving the corresponding percentages of water allowance on the added-starch diet. With diet A, an intake of 567 ml of water (group 2) permitted a weight gain of 125 gm; whereas with diet F, an intake of 539 ml of water (group 4) led to a gain of 144 gm.

With full allowance of water, the animals on diet A consumed 2110 Cal.; whereas those on diet F consumed 1930 Cal. On diet A the restriction of water to 80 and 60% decreased the food intake to 85 and 78% respectively, and on diet F to 94 and 91% respectively. With the added-carbohydrate diet, restriction of water did not curtail the food intake as much as that found with the powdered milk diet (fig. 1 b). The animals on diet F required only 19 to 28 ml of water per 100 Cal. at the three levels of water allowance, as compared with 26 to 34 ml per 100 Cal. for those on diet A.

On both diets the weight gain per 100 Cal. was either unaffected or only slightly decreased by restriction of water to 80%, but was markedly diminished with 60% water intake.

The weight gains calculated as grams gained per gram of protein were much higher for the animals on diet F (even with the lowest water intake) than were found with all three levels of water allowance on diet A. A similar relationship is found when the weight gain is calculated per 100 ml of water consumed.

The organ weights and the composition of the livers and carcasses of the animals following a 24-hour fast at the end of the experiment are summarized in table 3. Group 1 lost a larger percentage of its body weight during the fast than did the comparable group (4) on diet F. This probably reflects the lower fat level in the carcasses of the animals fed the powdered milk diet. In groups restricted to 80 and 60% of the water intake the percentage of weight loss was the same regardless of diet and of lipid content of the carcass. It should be noted that during the fast, the groups on diet

TABLE 3

Carcass analyses and organ weights of growing rats on restricted water intake [1]

FACTOR OF INTEREST	DIET A			DIET		
	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
WATER ALLOWANCE, %	100	80	60	100	80	60
Number of rats	10	10	10	9	10	10
Body weight:						
Av., 6 wks., gm	256	238	211	263	244	222
Av., after fast, gm	233	224	201	244	229	212
Fasting loss, %/24 hrs.	9.1 + 1.2	6.2 + 1.2	4.8 + 1.3	7.4 + 1.2	6.3 + 1.4	4.8 + 0.5
Carcass:						
Dry weight, %	33.3	32.9	32.2	34.4	34.1	32.9
Protein, %	21.3 ± 0.4	21.2 ± 0.8	21.4 ± 0.5	21.5 ± 0.5	21.2 ± 0.8	20.9 ± 0.9
Lipid, %	8.7 ± 1.5	8.4 ± 1.9	7.3 ± 2.0	10.1 ± 1.7	10.0 ± 1.4	8.8 ± 2.4
Ash, %	3.9	4.0	4.3	3.9	4.0	4.1
Liver:						
Weight, gm	7.7	7.1	6.0	7.5	7.0	6.1
Per cent of body wt.	3.3 ± 0.2	3.2 ± 0.2	3.0 ± 0.2	3.1 ± 0.2	3.1 ± 0.2	2.9 ± 0.1
Dry weight, %	28.7	28.6	28.5	28.7	28.9	28.9
Protein, %	21.7 ± 0.5	21.4 ± 0.9	21.9 ± 0.7	21.3 ± 1.2	22.0 ± 0.4	22.1 ± 0.5
Lipid, %	4.1 ± 1.1	4.4 ± 1.2	4.1 ± 0.7	4.9 ± 0.8	4.6 ± 0.9	4.9 ± 0.6
Kidney:						
Weight, gm	1.78	1.79	1.68	1.74	1.70	1.63
Per cent of body wt.	0.76 ± 0.06	0.80 ± 0.07	0.85 ± 0.10	0.71 ± 0.04	0.74 ± 0.08	0.77 ± 0.06
Adrenals:						
Weight, mg	46.8	45.6	41.1	43.2	46	41.5
Mg/100 gm body wt.	20.2 ± 2.5	20.4 ± 2.0	20.7 ± 2.6	17.7 ± 2.8	20.2 ± 1.5	19.7 ± 2.5

[1] Including standard deviations.

A were allowed more water than the comparable groups on diet F.

In the carcasses the percentage of protein was approximately the same in all 6 groups of animals. The percentage of lipid was higher in all the groups receiving diet F than in those receiving diet A. This was found previously with the same diets using ad libitum water intake (Sarett and Snipper, '56). The animals in group 6, restricted to 60% of the water intake of those on diet F, had as great a percentage of lipid in the carcass as did the animals in group 1 with full allowance of water on diet A (fig. 1 c). The differences in carcass lipid account for the small differences in percentage of dry weight in the carcasses of the various groups. The relatively high level of ash in the carcasses of animals on restricted water intake on both diets is due to the fact that the animals grew less but that skeletal development was not hampered as much as other tissue development by water restriction.

The relative weights of the livers of the animals on diet A were slightly higher than those of the animals on the same percentage of water intake on diet F. The level of protein in the livers was approximately the same in all 6 groups, and the percentage of liver lipid was slightly higher in the animals fed the added carbohydrate diet.

The kidney weights of the animals in group 1 averaged 0.76% of the body weight as compared with 0.71% for the animals in group 4. As water was restricted with each diet, the relative kidney weights increased to 0.80 and 0.85% on diet A and to 0.74 and 0.77% on diet F (fig. 1 d). The relative weights of the kidneys of the rats on diet F with 60% water restriction (324 ml) was approximately the same as was found in animals on diet A with full allowance of water (700 ml). In groups receiving approximately the same amount of water (groups 2 and 4), the relative kidney weights were significantly lower on diet F than on diet A ($p < 0.01$).

The weight of the adrenal glands in terms of milligrams per 100 gm of body weight was higher in the animals on the

powdered milk diet than on the added-starch diet with full allowance of water. When water was restricted there was a slight increase in relative adrenal weights on both diets.

DISCUSSION

The increased water requirement of high protein and mineral-containing diets and the water sparing effect of carbohydrate have long been known (Adolph, '33; Gamble et al., '29). In the present experiment the effects of limiting the water intake of growing rats on a powdered milk diet which is high in protein and mineral content, and on the same diet with the protein and minerals reduced one-third by adding starch were studied. When water intake is unrestricted on these diets growth is good and is usually about the same on both diets. Under these conditions the animals consume approximately one-third more water with the powdered milk diet (700 ml) than with the added-starch diet (539 ml). When water was restricted on both diets to 80 and 60% of the usual water intake for that diet, many adverse effects were noted. In some respects the effects were similar in both diets, i.e., the weight gains were curtailed to about the same extent; there was a diminution in body lipid, and an increase in relative size of the adrenals as well as a decrease in relative size of the liver. However, the curtailment in food intake as a result of water restriction was much greater on the powdered milk diet than on the added-starch diet. On the powdered milk diet restriction of water to 80 and 60% resulted in a curtailment of food intake to 85 and 78%, respectively, of that found with normal water allowance. However, on the added-starch diet similar water restriction curtailed food intake to only 94 and 91%, respectively. Crampton and Lloyd ('54) have reported that restriction of water intake by 50% in growing rats on a diet containing 25% protein and 8% minerals, resulted in a curtailment of food intake to 73%. The ability of the rats on the added-starch diet to eat a larger percentage of their normal food intake when water was re-

stricted emphasizes the water sparing effect of this diet as compared with the powdered milk diet.

It is interesting to compare the findings in group 2 (80% water allowance on diet A) with those in group 4 (full water allowance on diet F) since both groups took in about the same amount of water during the study, 567 and 539 ml, respectively. The animals in group 4 gained more weight than those in group 2, used the food more efficiently, had a higher level of carcass lipids, and had much smaller kidneys and adrenal glands than did the rats of group 2. These comparisons are evident in figure 1 and clearly show the difference in water requirement on the two diets.

The kidneys of the animals on the powdered milk diet were larger than those of the animals on the added-starch diet with full allowance of water on each diet. Restriction of water intake led to an increase in relative kidney weight on both diets. With only 60% of water allowance (324 ml) permitted the animals receiving diet F the kidney weights were 0.77% of the body weight, which is similar to the 0.76% found for the kidneys of animals which received full water allowance (700 ml) on diet A.

These findings in the growing rat are similar to those in the infant in which comparisons have been made of unmodified milk diets and of milk with added carbohydrate (Pratt and Snyderman, '53; Calcagno and Rubin, '54). Pratt and Snyderman ('53) showed that the renal water requirement of rapidly growing infants was approximately 85% greater on a milk diet than on a diet of milk with added carbohydrate. These authors have pointed out that added carbohydrate provides a safety factor which would be available when water intake was decreased or when extra renal losses from the gastrointestinal tract or from the lungs and skin in hot weather were increased. This safety factor is seen in the rat which is able to consume more food and gain more weight on low water intakes with the added-starch diet as compared with the powdered milk diet. Calcagno and Rubin ('54) have made similar comparisons in infants and have shown a lower blood

urea nitrogen and a decrease in renal osmolar load when carbohydrate was added to milk diets. In diarrhea, water loss through the intestine decreases the amount of water left for the kidneys (Darrow et al., '49). Cooke, Pratt and Darrow ('50) found that in heat stress infants lose much more of their water through the lungs and skin leaving less for excretion of metabolic end products in the urine. Darrow et al. ('54) showed that dilution of cow's milk with water and addition of carbohydrate are necessary to provide a low osmolar load which provides a margin of safety if water intake is low, if extrarenal expenditure of water is high, or if renal ability to concentrate the urine is limited.

SUMMARY

The effects of water restriction in growing rats were studied using diets of powdered milk and of two-thirds powdered milk and one-third starch, supplemented with vitamins and minerals. Groups of rats were placed on these diets and restricted each day to (1) the full allowance of water usually consumed with each diet (2) 80% or (3) 60% of this amount of water. The following observations were made:

1. When water was unrestricted (or 100% allowance), growing rats drank approximately 30% more water on the powdered milk diet (A) than on the same diet with added starch (F). The gain in weight was approximately the same on both diets.

2. Water restriction led to a greater decrease in food consumption in the groups receiving diet A than in those receiving diet F.

3. The weight gains were reduced to about the same extent on each diet when water was restricted to the same relative level of water allowance for each diet.

4. A comparison of animals receiving approximately the same amount of water with each diet showed a greater weight gain, superior food efficiency, more carcass lipid and less kidney hypertrophy in those on the diet with added starch.

5. At all levels of water intake, the weight gain per gram of protein consumed was much higher on the diets with added starch than was found on any of the levels of water intake with the powdered milk diet.

6. The composition of the livers and carcasses was not markedly affected by water restriction. The carcasses of the animals on the powdered milk diet contained less lipid than did those of animals on the added-starch diet.

7. The kidneys and adrenal glands of the animals on the powdered whole milk diet were relatively heavier than those in animals fed the added-starch diet. Water restriction increased kidney size on both diets. The relative kidney size of animals allowed 324 ml of water on diet F was about the same as that found in animals consuming 700 ml of water on diet A.

8. The implications of these findings in terms of water requirements for growth are discussed.

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THE BIOLOGICAL UTILIZATION OF VARIOUS FAT-SOLUBLE ESTERS OF PYRIDOXINE AND 4-DESOXYPYRIDOXINE BY RATS[1,2]

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INTRODUCTION

The long-chain fatty acid derivatives of vitamin B_6 (Sakuragi and Kummerow, '56b) have been shown to possess a high solubility in fats and a high stability toward heat (Sakuragi and Kummerow, '55). It has also been shown that the absorption, excretion and utilization of the long-chain fatty acid esters of pyridoxine differ from the water soluble forms (Sakuragi and Kummerow, '56a). It is conceivable that the interrelationship of the biological antagonism between pyridoxine and 4-desoxypyridoxine may further be clarified by a study of the fat-soluble derivatives of these substances. In the present study, therefore, feeding experiments were carried out with rats, and the activity of pyridoxine tripalmitate, trilinoleate, hydrochloride and triacetate compared with the 4-desoxypyridoxine derivatives. Previous studies on the biological preparations of water-soluble pyridoxine have shown that the antiacrodynic potency of pyridoxine was enhanced when an animal was also supplemented with highly un-

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[2] Portion of a thesis presented by T. Sakuragi as partial fulfilment of the requirment for the degree of Doctor of Philosophy in Food Technology.

saturated long-chain fatty acids (Salmon, '40; Quackenbush et al., '42; Witten and Holman, '52; Sinclair, '52). It, therefore, appeared possible that the linoleic acid ester of pyridoxine might possess enhanced biological activity.

EXPERIMENTAL

Feeding experiments with rats on diets supplemented with optimal amounts of various derivatives of pyridoxine

Four 21-day-old male and female weanling rats in each group were kept on the various derivatives of pyridoxine with or without 10% of corn oil in the basal ration, and the change in body weight was recorded weekly. The basal ration, the composition of which is shown in table 1-I, was supplemented with pyridoxine hydrochloride, pyridoxine triacetate and pyridoxine tripalmitate at levels that supplied 1.5 μg of the vitamin calculated as pyridoxine hydrochloride per gram of the ration. To prevent the possible oxidation of pyridoxine trilinoleate, this compound was dissolved in a highly hydrogenated coconut oil and administered weekly with a calibrated medicine dropper in amounts equivalent to 70 μg as pyridoxine hydrochloride.

Feeding experiments with rats on diets supplemented with suboptimal amounts of pyridoxine hydrochloride and of pyridoxine tripalmitate

Male 21-day-old weanling rats were first kept on the vitamin B_6- free basal ration (table 1 diet II) for two weeks. The composition of the diet was essentially the same as that proposed by Sarma et al. ('46) for the vitamin B_6 assay. The rats were then divided into groups of 4 to 5 rats each, and transferred to the same ration supplemented with pyridoxine hydrochloride or pyridoxine tripalmitate with or without 1% of sulfaguanidine [3] in the diet. The vitamin B_6 levels used for

[3] Kindly supplied by the American Cyanamid Company, Fine Chemical Division.

the assay were 30 µg and 60µg as pyridoxine hydrochloride per 100 gm of the diet, which was fed to the rats ad libitum. The test was conducted over a period of three weeks.

Growth response to a single dose of pyridoxine preparations

The male rats which were used in this phase of work had been kept, for 6 weeks, on a pyridoxine-deficient diet (table 1 diet I) with or without 10% of corn oil. At the end of this conditioning period, the rats on the fat-free ration started to show acrodynic symptoms, but the animals on the 10% corn oil diet were still increasing in body weight. Four to 5 rats in each group were then supplemented with a single dose of 70 µg of pyridoxine hydrochloride, and an equivalent

TABLE 1

Composition of the basal ration per 100 gm of ration

CONSTITUENT	DIET I	DIET II
Glucose (cerelose)	68 gm (78 gm for fat-free diet)	75 gm
Corn oil	10 gm	3 gm
Vitamin-free casein	18 gm	18 gm
Wesson salts	4 gm	4 gm
Inositol	100 mg	10 mg
Choline chloride	200 mg	100 mg
Niacin	10 mg	2.5 mg
Thiamine hydrochloride	0.45 mg	0.2 mg
Riboflavin	0.9 mg	0.3 mg
p-Aminobenzoic acid	0.3 mg	..
Folic acid	0.08 mg	..
Calcium pantothenate	1.0 mg	2.0 mg
Menadione	..	1.0 mg
Biotin	0.4 µg	10.0 µg
Vitamin A	160 I.U./week	
Vitamin D	1.6 µg/week	
Vitamin E	295 µg/week	

Fat soluble vitamins were dissolved in a highly hydrogenated coconut oil and administered by a medicine dropper weekly.

amount of pyridoxine tripalmitate or pyridoxine trilinoleate dissolved in a highly hydrogenated coconut oil. Pyridoxine hydrochloride was supplied as an aqueous solution with a calibrated medicine dropper The rats in a control group were fed one drop of plain hydrogenated coconut oil. The body weight change was recorded at proper time intervals.

Growth response to a single dose of various mixtures of the pyridoxine preparations and the 4-desoxypyridoxine preparations

Male 21-day-old weanling rats were kept on the vitamin B_6-free basal ration without corn oil (table 1 diet I) for 7 weeks. At the end of this conditioning period, the animals showed rather severe acrodynic symptoms with an average dermal index of 3.5 (Quackenbush et al., '39). The rats were then evenly divided, with respect to body weight and the severity of dermal lesions, into 5 groups of 6 rats each, and supplemented with various combinations of pyridoxine and desoxypyridoxine derivatives. Pyridoxine hydrochloride and 4-desoxypyridoxine hydrochloride were fed as aqueous solutions and the esters were administered as solutions in ethyl laurate with calibrated medicine droppers. In all the groups, the desoxypyridoxine level administered was 5 mg as desoxypyridoxine hydrochloride and the pyridoxine level was 70 μg as pyridoxine hydrochloride per rat. The changes in the body weight and in the dermal index were recorded over a period of 7 days.

RESULTS AND DISCUSSION

The growth rates of the experimental groups for 7 weeks indicated that pyridoxine triacetate, pyridoxine tripalmitate and pyridoxine trilinoleate were utilized by rats as a source of vitamin B_6; no statistically significant difference in body weight gain was observed when an optimal level of the preparation was supplied (table 2). Pyridoxine triacetate has been reported to have an activity equal to that of pyridoxine hydrochloride for rats (Harris, '40; Unna, '40). During the

TABLE 2

Body weight gain of rats fed various pyridoxine derivatives

DIET	SUPPLEMENTS	FEEDING PERIOD	NO. AND SEX	SULFA-GUANIDINE	AVERAGE GAIN ± STANDARD ERROR OF MEAN
		wks		%	*gm*
I [1]	No fat, no vitamin B_6	7	4 M	..	16.5 ± 4.5
		7	4 F	..	13.3 ± 2.2
I	No fat, with pyridoxine trilineleate	7	4 M	..	144.5 ± 10.2
		7	4 F	..	107.5 ± 3.8
I	No fat, with pyridoxine hydrochloride	7	4 M	..	137.0 ± 5.9
		7	4 F	..	101.3 ± 7.7
I	10% Corn oil, no vitamin B_6	7	4 M	..	24.0 ± 6.6
		7	4 F	..	25.3 ± 3.7
I	10% Corn oil, with pyridoxine triacetate	7	4 M	..	160.8 ± 10.0
		7	4 F	..	107.8 ± 8.8
I	10% Corn oil, with pyridoxine tripalmitate	7	4 M	..	164.3 ± 4.5
		7	4 F	..	107.5 ± 10.1
I	10% Corn oil, with pyridoxine trilinoleate	7	4 M	..	163.3 ± 4.3
		7	4 F	..	124.0 ± 8.1
II	Pyridoxine hydrochloride, 30 μg [2]	3	4 M	..	25.0 ± 2.1
II	Pyridoxine tripalmitate, 30 μg	3	4 M	..	36.0 ± 2.2
II	Pyridoxine hydrochloride, 60 μg	3	4 M	..	52.8 ± 2.0
II	Pyridoxine tripalmitate, 60 μg	3	5 M	..	56.0 ± 2.0
II	Pyridoxine hydrochloride, 60 μg	3	4 M	1	41.5 ± 1.2
II	Pyridoxine tripalmitate, 60 μg	3	5 M	1	35.8 ± 1.4

[1] For diet I, pyridoxine hydrochloride, pyridoxine triacetate and pyridoxine tripalmitate were mixed in the diet at a level of 1.5 μg as pyridoxine hydrochloride per gram of ration. Pyridoxine trilinoleate was administered weekly to supply 70 μg as pyridoxine hydrochloride every week.

[2] The vitamin B_6 level is indicated as pyridoxine hydrochloride per 100 gm of ration.

assay period, the average daily intake of the food, fed ad libitum, ranged between 8 and 12 gm. Therefore, 12 to 18 μg. of pyridoxine hydrochloride, as such, or as the palmitoyl or acetyl derivatives, were consumed every day, whereas the intake of pyridoxine trilinoleate was 10 μg calculated as pyridoxine hydrochloride.

It has long been known that pyridoxine deficiency produces symptoms in rats superficially similar to those due to a lack of the essential fatty acids (Birch, '38; Quackenbush et al., '42; Sinclair, '52). Supplementation with either linoleate or vitamin B_6 cures the deficiency symptoms (Salmon, '40; Quackenbush et al., '42; Schneider et al., '40; Medes and Keller, '47), although the skin lesions in the two deficiencies are histologically quite distinct (Sinclair, '52). Furthermore, the deficiency symptoms disappeared more rapidly with linoleate plus pyridoxine (Quackenbush et al., '42; Witten and Holman, '52), and also the maximum growth of the rats was maintained in the presence of both vitamin B_6 and linoleic acid in the diet (Salmon, '40; Sinclair, '52).

The administration of pyridoxine trilinoleate simultaneously introduced linoleic acid. The body weight gain in the rats which had received pyridoxine trilinoleate, therefore, would be expected to be higher than that of the rats in the group in which pyridoxine hydrochloride was the sole source of vitamin B_6. The results, however, indicated that the linoleic acid moiety had no effect on growth. This is probably due to the fact that the requirement of linoleic acid is 50 to 100 mg for a male rat per day, and 10 to 20 mg for a female rat per day (Sebrell and Harris, '54; Holman et al., '54), whereas an amount of pyridoxine trilinoleate equivalent to 10 μg of pyridoxine hydrochloride per day per rat provided only 40.8 μg of linoleic acid.

The results on the assay with a suboptimal amount of vitamin B_6 are also shown in table 2. In the group supplemented with 60 μg of vitamin B_6 per 100 gm of ration, no significant difference in growth was observed between pyridoxine tripalmitate and pyridoxine hydrochloride. When supple-

mented with 30 μg of vitamin B_6, however, pyridoxine tripalmitate gave a higher body weight gain in three weeks than free pyridoxine. This difference was statistically significant.[4] In other groups, the diets were also supplemented with 60 μg of vitamin B_6 preparations per 100 gm of ration along with 1% of sulfaguanidine. The sulfa drug was incorporated into the ration at the expense of the glucose. The results indicated that when the sulfa drug was present in the diet, better growth was noted with free vitamin B_6 than with the tripalmitate. The difference was statistically significant.[4] This would suggest that the sulfa drug prevented the loss of pyridoxine hydrochloride presumably due to the intestinal flora (Sarma et al., '46; Carpenter et al., '48). Under these conditions, free pyridoxine seemed to be more readily available than the ester and the palmitate less susceptible to destruction by the intestinal organisms.

In all groups, the rats which received a single dose of various pyridoxine derivatives showed marked gain in body weight within 12 hours after supplementation (table 3). When the pyridoxine ester of long-chain fatty acids were fed, the growth response was as great as when an equivalent amount of pyridoxine hydrochloride was administered. Thus the pyridoxine moiety in the esters became available to the rats without delay.

In the rats supplemented with free 4-desoxypyridoxine and free pyridoxine, and those supplemented with 4-desoxypyridoxine dipalmitate and pyridoxine tripalmitate, no effect of supplemented vitamin B_6 was noted. This indicated the competitive activity of the desoxypyridoxine administered (table 4). In the rats supplemented with 4-desoxypyridoxine dipalmitate and free pyridoxine, the time lag in the availability

[4] The standard error of the mean was calculated by the formula,

$$\sqrt{\frac{\Sigma d^2}{n(n-1)}}$$

The ratio of mean difference (experimental group-control group) and $\sqrt{(S\,E\,M)^2_c + (S\,E\,M)^2_e}$ in excess of 3.0 was considered to be significant.

TABLE 3

Average growth response of rats to a single dose of pyridoxine hydrochloride, pyridoxine tripalmitate and pyridoxine trilinoleate

SUPPLEMENTS [1]	AFTER SUPPLEMENTATION					
	Fat-free diet			10% Corn oil diet		
	12 Hrs.	2 Days	10 Days	12 Hrs.	2 Days	10 Days
	gm	*gm*	*gm*	*gm*	*gm*	*gm*
None	— 0.8	+ 2.3	— 3.2	+ 3.8	+ 5.0	+ 8.4
Pyridoxine hydrochloride	+ 7.8	+ 13.3	+ 19.8 ± 2.9 [2] (4) [3]	+ 9.4	+ 14.6	+ 29.0 ± 3.0 (5)
Pyridoxine tripalmitate	+ 8.8	+ 14.0	+ 21.3 ± 1.8 (4)	+ 7.6	+ 14.6	+ 30.0 ± 2.3 (5)
Pyridoxine trilinoleate	+ 9.4	+ 17.2	+ 23.3 ± 2.8 (4)	+ 8.4	+ 17.6	+ 30.3 ± 1.1 (4)

[1] Seventy micrograms of pyridoxine hydrochloride and an equivalent amount of the ester were administered per rat.
[2] Standard error of mean.
[3] Number in parenthesis indicates the number of rats in each group.

TABLE 4

Average growth response of rats to a single dose of various mixtures of pyridoxine and 4-desoxypyridoxine preparations and the change in dermal lesions

SUPPLEMENTS [1,2]	AFTER SUPPLEMENTATION					AV. DERMAL INDICES	
	1 Day	2 Days	3 Days	4 Days	7 Days	0 Day	7th Day
	gm	*gm*	*gm*	*gm*	*gm*		
None	+ 0.4	— 0.4	— 1.4	— 0.4	— 5.2	3.6	4.8
4-Desoxypyridoxine · HCl + pyridoxine · HCl	+ 0.8	+ 0.3	— 5.3	— 5.7	— 7.0	3.5	6.2
Desoxypyridoxine dipalmitate + pyridoxine · HCl	+ 11.5	+ 11.8	+ 6.0	— 1.2	+ 2.7	3.5	5.2
4 Desoxypyridoxine · HCl + pyridoxine tripalmitate	— 1.8	+ 5.0	+ 1.4	+ 3.8	+ 3.0	3.3	4.0
Desoxypyridoxine dipalmitate + pyridoxine tripalmitate	— 3.8	+ 0.8	— 3.3	— 7.5	— 10.3	3.3	7.7

[1] Desoxypyridoxine preparations were supplemented at a level of 5 mg as 4-desoxypyridoxine hydrochloride per rat. Pyridoxine preparations were supplemented at a level of 70 μg as pyridoxine hydrochloride per rat.
[2] Six male rats were used in each group.

of the two active principles induced a quick and large growth response in the first part of the experimental period, and a loss in body weight in the latter part. The body weight gain on the first day was as high as when only 70 μg of pyridoxine preparations were administered (table 3) and the gain in body weight would have continued at least up to the 7th day if no desoxypyridoxine preparation had been included in the supplement. Reversed results were observed in the group fed free desoxypyridoxine and pyridoxine tripalmitate. In this group, a growth response was not in evidence during the first day. On the second day, however, substantial response in growth was noted indicating the slower effect of pyridoxine tripalmitate, or a quick disappearance of desoxypyridoxine which was fed in the free form. In the latter two groups, the activity of the desoxypyridoxine or the pyridoxine which were fed in the form of its ester appeared on the second or third day (table 4). This fact indicated that the supplements were stored in the body for a certain period of time, at least until all the water-soluble supplements were excreted from the body.

Prior to the identification and the synthesis of the antiacrodynic factor, Birch et al. ('36) treated a vitamin B_6 fraction with benzoyl chloride and reported that this treatment inactivated the vitamin. Thus their statement led to an impression that pyridoxine benzoate was biologically inactive (Rosenberg, '45). In the present study, the activity of pyridoxine tribenzoate (Ichiba and Michi, '38) as a supplement for vitamin B_6 was qualitatively tested by a single dose assay with acrodynic rats (Unna, '40). The results indicated that the tribenzoate still retained activity. Structurally similar compounds, the p-nitrobenzoate [5] and the m-nitrobenzoate [5]

[5] Pyridoxine tri-p-nitrobenzoate and pyridoxine tri-m-nitrobenzoate were prepared from pyridoxine hydrochloride, which was treated with the respective nitrobenzoic acid chloride in pyridine at room temperature. The trinitrobenzoates were recrystallized from pyridine-methanol. The tri-p-nitrobenzoate (Calculated for $C_{29}H_{20}O_{12}N_4$: N, 9.09%. Determined: N, 9.09%) melted at 199.5–200.0° (decomposition). Pyridoxine tri-m-nitrobenzoate (Calculated for $C_{29}H_{20}O_{12}N_4$: N, 9.09%. Determined: N, 9.06%) melted at 174.5°.

of pyridoxine were also tested for their activity. Alleviation of the acrodynic lesions indicated the availability of the trinitrobenzoates. The activity, however, appeared to be extremely low, since no appreciable response in body weight gain to the trinitrobenzoate was noted, whereas supplementation with pyridoxine tribenzoate induced a quick and marked gain in body weight. The extremely high melting points of the trinitrobenzoates would seem to explain their low physiological availability.

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SUMMARY

The biological activity of various esters of pyridoxine for the growth of rats has been tested. Pyridoxine triacetate, tripalmitate and trilinoleate were biologically utilized by rats as a source of vitamin B_6, and the activity was equal to that of pyridoxine hydrochloride. The results of the feeding experiments on diets supplemented with a suboptimal level of pyridoxine tripalmitate or pyridoxine hydrochloride appeared to indicate that the ester was less susceptible to destruction by the intestinal flora. The biological utilization of these supplements could not be differentiated by a single-dose assay. However, the growth-response tests to a single dose of a mixture of various preparations of pyridoxine and 4-desoxypyridoxine showed that, although the free forms of these active principles were more readily available than the esterified compounds, the latter were retained in the body for a longer period of time. The data appeared to indicate that at least a part of the long-chain fatty acid ester of vitamin B_6 could be stored in the body without complete hydrolysis. It was also shown that pyridoxine tribenzoate, pyridoxine tri-p-nitrobenzoate and pyridoxine tri-m-nitrobenzoate were biologically active as a source of vitamin B_6, though the activity of the latter two appeared to be extremely low.

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EFFECT OF CHOLINE, METHIONINE AND ETHIONINE ON FAT ABSORPTION [1]

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The exact role of the phospholipids in the absorption of fat is uncertain at this time. A number of explanations have been offered for the increased rate of fat absorption (Adlersberg and Sobotka, '43; Tidwell, '50) when lecithin or choline is ingested along with the lipide: (1) lecithin may increase the degree of emulsification which could aid either fat hydrolysis or particulate absorption (Augur et al., '47); (2) choline, when given as such or freed from lecithin may increase the speed of fat absorption solely as the result of an acetylcholine-like action, causing a stimulation of the rhythmic movements and an increased tonus of the intestine,[2] (3) fat absorption may be promoted if lecithin influences the passage of fat through the intestinal mucosa (Frazer, '46) or has any part in the resynthesis of triglycerides within the intestinal mucosa (Sinclair, '29; Williams, '49; Borgström, '51). In the latter case, choline would necessarily be involved if it is a limiting factor in the formation of lecithin (Artom and Cornatzer, '46), or affects phospholipid turnover (Artom and Cornatzer, '46; Patterson et al., '44; Friedlander et al., '45). Methionine or ethionine might influence choline formation.

Irwin et al. ('36) found that the addition of small amounts of various hydrotropic substances had little or no effect on the rate of fat absorption and that large amounts invariably

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[2] Best, C. H., Personal communication.

decreased the rate. More recently Shoshkes et al. ('50b) have reported the absorption by the rat of orally fed fat to be unaffected by the presence of soybean phosphatide. In intestinal instillation experiments, the absorptive process was found to be unaltered by the presence of large amounts of the phosphatide or choline. Also, the studies of Zilversmit et al. ('48) have cast some doubt on the correctness of the assumption that fat can pass through the intestinal wall only as a phospholipid intermediate.

Best [3] suggested that evidence might be obtained to clarify the role of choline in fat absorption by an investigation of the effect upon fat absorption of methionine and some substances which might have a physiological effect similar to that of choline. Methionine might aid indirectly by promoting choline synthesis at a more physiological rate, avoiding a possible cholinergic effect of an excess of choline and the emulsifying action of the preformed lecithin. The present study was designed to investigate further the findings regarding the effect of choline upon the rate of fat absorption. Additional evidence was obtained from the use of methionine, prostigmine, mecholyl and ethionine to aid in determining whether the effect was more likely the result of an acetylcholine-like action or of aiding in phospholipid formation.

EXPERIMENTAL

In the first part of this study (table 1), the blood levels of particulate fat were followed after fat ingestion and the injection of the test supplements. Adult male albino rats were fasted 24 hours and blood samples collected. After receiving by tube 0.3 ml of olive oil per square decimeter of body surface (Rubner, '02) the rats were injected intraperitoneally with equal volumes of solutions of either DL-methionine, choline chloride, prostigmine or mecholyl, neutralized and then made isotonic with saline, or with saline alone. Additional blood samples were drawn hourly for three hours after feeding the

[3] See footnote 2, page 569.

fat, and chylomicron counts were made as previously described (Burr et al., '54). The counts reported are the average numbers in 10 squares of the micrometer disc. It has been shown that the chylomicrograph and the area beneath the curve can

TABLE 1

Effect of methionine, choline, prostigmine and mecholyl on blood fat levels

NO. RATS	FAT GIVEN WITH		CHYLOMICRON COUNT Hours				AREA UNDER CURVE [1] (1–3 HRS.)	"t" VALUE [2]
			Fasting	1	2	3		
		mg						
15	Saline		9	45	61	48	105 ± 9	
10	Methionine	30	9	51	58	52	110 ± 7	0.95
8	Choline	15	6	52	82	58	137 ± 6	4.12
12	Prostigmine	0.0003	14	66	71	32	120 ± 6	1.81
15	Mecholyl	0.001	11	60	72	40	122 ± 8	2.18

[1] Including the standard error of the mean.

[2] All "t" values above 3 are statistically significant (Fisher, '38).

TABLE 2

Rate of fat absorption as affected by varying amounts of injected methionine as measured by the recovery method

NO. RATS	FAT WITH INJECTION OF	AMOUNT OF FAT ABSORBED			"t" VALUE [1]
		mg	%	*mg/dm²/hr.*[2]	
21	Saline		39.9	36.5 ± 1.4	
10	Methionine	60	29.1	26.6 ± 3.7	1.26
11	Methionine	25	44.7	40.8 ± 2.8	1.38
11	Methionine	10	45.9	42.0 ± 2.5	2.22

[1] All "t" values above 3 are statistically significant (Fisher, '38).

[2] Including the standard error of the mean.

be used as a relative measure of the absorbed fat in the blood and of the rate of the various processes involved during the absorptive period (Burr et al., '54).

In order to test the effect of decreasing amounts of methionine, the Cori technique as modified by Deuel et al. ('40) was used (table 2) as another method for measuring the rate of fat absorption. The rats were fasted 48 hours and fed by tube a similar amount of fat and then the supplement was

injected. They were sacrificed after a three-hour absorptive period and the unabsorbed fat was recovered and weighed.

Similarly, the rates of absorption were followed by two methods on much larger groups of animals. Both determinations were made after a single fat feeding and injection of small amounts of methionine, prostigmine and choline (table

TABLE 3

Fat absorption rate as measured by two methods

NO. RATS	FAT SUPPLEMENT		RATE OF FAT ABSORPTION AS MEASURED BY: Chylomicron counts Area under curve [1] (1–4 hrs.)	RATE OF FAT ABSORPTION AS MEASURED BY: Recovery of unabsorbed fat from tract [1]	"t" VALUE [2]
		mg		*mg/dm²/hr.*	
25	Saline		125 ± 14	24.4 ± 2.4	
25	Methionine	15	131 ± 8	26.4 ± 1.7	
25	Prostigmine	0.0003	128 ± 10	23.5 ± 2.0	
8	Choline	15		45.7 ± 3.7	5.7

[1] Including the standard error of the mean.

[2] All "t" values above 3 are statistically significant (Fisher, '38).

TABLE 4

Blood fat levels after repeated injections of supplements of methionine, choline and ethionine given with and without fat

GROUP NO.	NO. RATS	FAT SUPPLEMENT (TWICE DAILY) [1]		RATE OF ABSORPTION AS MEASURED BY CHYLOMICRON COUNTS: Hours 0	1	2	3	AREA UNDER CURVE [2]	"t" VALUE [3]
			mg						
1	11	Saline		10	21	20	20	41 ± 3	
	12	Choline	10	10	25	23	22	46 ± 3	
	5	Ethionine	20	8	16	21	18	38 ± 3	
2	10	Saline		8	26	44	59	87 ± 5	
	16	Choline	10	10	42	56	65	110 ± 4	5.10
	17	Ethionine	20	12	61	98	114	185 ± 9	7.98
3	13	Saline		13	25	48	52	86 ± 2	
	12	Methionine	5	12	29	50	56	92 ± 4	1.52
	10	Choline	10	13	35	52	59	99 ± 2	5.03
	10	Ethionine	20	11	33	71	106	141 ± 6	9.53

[1] Supplements given in milligrams per 100 gm of body weight. Only groups 2 and 3 received fat.

[2] Including the standard error of the mean.

[3] All "t" values above 3 are statistically significant.

3) The procedure differed only in that 50% glucose [4] was available during the preperiod, and the absorptive period was extended to 4 hours.

Finally, the chylomicron levels were followed on three groups of animals after a similar preperiod with the exception that the supplements or equal volumes of physiological saline were injected twice daily (table 4). In order to test the effect of the supplements alone on the particulate fat of the blood, the first group received no fat. The third group was run to confirm the results of the second one and to test a methionine supplement after its repeated injection.

RESULTS AND DISCUSSION

A major difficulty encountered in these experiments was the determination of a suitable level of the test supplements. Larger amounts of methionine, prostigmine and mecholyl than those employed in table 1 were found in preliminary experiments (Tidwell and Nagler, '51, '52) to depress the rate of fat absorption. It was to be expected that large amounts of all of these substances would inhibit the absorption of fat since Irwin et al. ('36) have shown that excesses of a wide variety of substances have this effect. The smaller quantities selected were those which in the earlier tests seemed most promising for increasing the rate of fat absorption. Repeated tests failed to confirm the results reported in the above mentioned preliminary experiments, that prostigmine and mecholyl might favorably influence the rate of fat absorption. An improvement in the technique of the method used might account for the contradictory results of these later studies.

However, these studies did confirm our earlier finding that choline increases the rate of the absorption of fat as shown in tables 1 and 4. Choline has been reported to be a limiting factor in the formation of lecithin (Artom and Cornatzer, '46) and free choline shown to be rapidly incorporated into

[4] The Cerelose was generously supplied by the Corn Products Refining Company, New York.

this phospholipid (Kennedy, '53). As given, it could not increase emulsification of the fat in the intestine, hence the role of choline in accelerating fat absorption may be that of promoting phospholipid formation. Our lack of agreement with Shoshkes et al ('50a) might be explained by the larger amount of choline they employed as well as the quite different experimental conditions.

Methionine is generally believed to play an important part in the formation of choline. If the rate of formation of choline could be sufficiently increased by having an excess of methionin present, the rate of fat absorption might also be accelerated. Then too, methionine is not believed to have the acetylcholine-like property of choline so its use would avoid having an unphysiological amount of choline present at any time. Methionine appeared to increase only slightly the rate of appearance of particulate fat in the blood in these experiments (tables 1 and 2). Although there was not a highly significant increase in the rate (P about 0.05) when the amount given was decreased to 10 mg, the results do suggest the possibility of a more favorable effect if the most effective amount were given under the right experimental conditions. The slight, but apparently consistent increase in rate with small amounts of methionine, may be the result of a synthetic process too slow to produce sufficient choline to be more effective.

Prostigmine and mecholyl were employed to obtain additional evidence regarding the promotion of fat absorption by choline as a result of its possible acetylcholine-like action. Prostigmine inhibits the action of cholinesterase while mecholyl has an acetylcholine-like effect and is not readily destroyed by this enzyme. Hence, both would tend to cause an excitation of the musculature of the digestive tract that might influence fat absorption. In repeated studies and unlike earlier preliminary results, even if these amounts did produce an excitation of the musculature of the digestive tract, there was no indication that such promoted fat absorption under the conditions of this study (tables 1 and 3). Hence the accelerat-

ing action of choline cannot be ascribed to a cholinergic effect on the basis of these findings.

In only the last two experiments (tables 3 and 4) was glucose available during the preperiod. In the first, the rats received single and, in the latter, multiple injections of the test supplements. The glucose was made available because it has been reported (Barrenscheen and Papadopoulou, '49) that the glycogen content of the liver has an important influence on the ability of the animal to synthesize choline from the necessary ingredients. Again under these conditions, all conclusions to be drawn from the data obtained are the same as those in the previous experiments without glucose and the multiple injections.

The observation that choline chloride produced a transient lipemia in rabbits (Schneider and Stutinsky, '49) suggested a test of the effect of choline without ingested fat. It is evident in group 1 of table 4 that there was not a significant increase in chylomicrons when choline or ethionine was given without fat.

The results with methionine as a fat supplement prompted the investigation of ethionine, a specific antagonist of methionine, which has been reported to affect a prompt reduction in level of serum lipids (Feinberg et al., '54). Surprisingly, the rate of fat absorption after fat feeding was markedly accelerated as indicated by the increased chylomicronemia following the injection of ethionine. This result as well as those on methionine and choline was confirmed on the third group of animals (table 4).

The report of an excess of free amino acids including high concentrations of free methionine, after the injection of ethionine (Wu and Bollman, '54; Levy et al., '55), might give some clue to the effect of a methionine supplement if it had been continuously supplied during fat absorption for an increased choline and phospholipid formation. Any effect upon the chylomicronemia from a decreased pancreatic secretion, resulting from the degenerative changes in the acinar tissue

after ethionine injection (Goldberg et al., '50), would appear to be ruled out by (1) the unchanged fat levels when ethionine was given without fat and (2) the small amounts of ethionine given over only a 48-hour period. However, there is also the po sibility that fat absorption occurred at a normal rate, but its remeval from the blood was slower as a result of the antilipotropic effect of ethionine. The latter view is not in agreement with the reported low fasting blood lipide level when ethionine was given (Feinberg et al., '54).

SUMMARY

In agreement with an earlier report, the rate of fat absorption was found by two methods to be accelerated when fat was supplemented with choline. The inability of the injected choline to aid emulsification as well as the failure of various hydrotropic substances to affect the rate of fat absorption suggests that lecithin as well as choline are otherwise involved in the absorptive process.

The supplements of prostigmine and mecholyl used failed to affect the rate of fat absorption. This supplied evidence to indicate that the effect of choline on the absorptive process cannot be that of a cholinergic one, but may be solely the result of an increased production of lecithin. The slight effect of a methionine supplement on the promotion of fat absorption might be the result of a failure to supply more physiological amounts continuously or of a synthetic process too slow to produce sufficient choline to be more effective.

The significantly increased blood fat levels after the use of ethionine as a fat supplement might be associated with its reported liberation of a considerable amount of free methionine. This might well account for the accelerated rate of fat absorption, if the latter were continuously available in suitable quantities for additional choline and phospholipid formation which may in some way aid the passage of the fat into the intestinal mucosa.

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