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ERRATUM

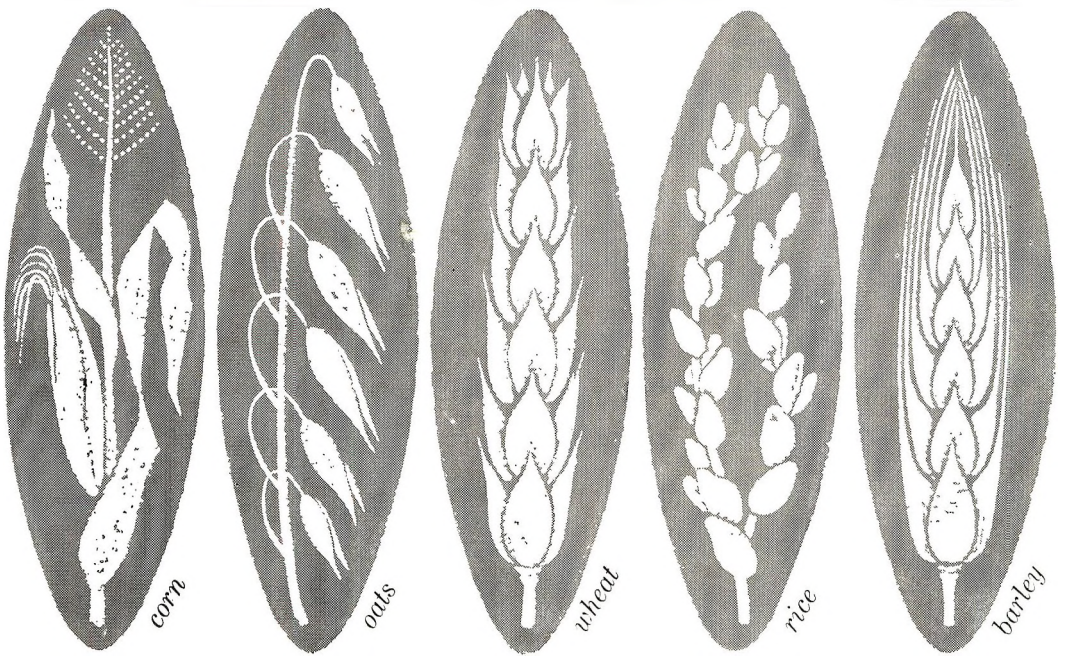
Journal of Nutrition, volume 66, number 3

Page 394, Third line from bottom is incorrect. The line should read —

gossypol solution was poured over 1600 gm of meal in the

For those who may wish to correct their copies, the last three lines of page 394 are reprinted below for convenience in pasting over top of these lines.

gossypol solution was poured over 1600 gm of meal in the mixing bowl of a mechanical mixer and mixed for 15 minutes at low speed at room temperature. A steam-heated water



a moderate reduction in fat indicates cereal and milk serving

Medical and nutrition authorities recommend moderation and reasonableness in food choice as a best guide to good health. When a moderate reduction of dietary fat is indicated, a good choice is the cereal and milk serving for breakfast and the late evening snack.

Both the breakfast cereal and the milk contribute well-balanced nourishment and provide quick and lasting energy. This serving, as shown below, is a good source of many nutrients and provides about 10 per cent of the daily recommendations of high quality protein, important B vitamins, and essential minerals. Served with nonfat milk, the fat content is very low.*

nutritive composition of average cereal serving

	Cereal, 1 oz. Whole Milk, 4 oz. Sugar, 1 teaspoon	Cereal** 1 oz.	Whole Milk 4 oz.	Sugar 1 teaspoon
CALORIES	203	104	83	16
PROTEIN	7.3 gm.	3.1 gm.	4.2 gm.	
FAT	5.3 gm.	0.6 gm.	4.7 gm.*	
CARBOHYDRATE	32.2 gm.	22 gm.	6.0 gm.	4.2 gm.
CALCIUM	0.169 gm.	0.025 gm.	0.144 gm.	
IRON	1.5 mg.	1.4 mg.	0.1 mg.	
VITAMIN A	195 I. U.	—	195 I. U.	
THIAMINE	0.16 mg.	0.12 mg.	0.04 mg.	
RIBOFLAVIN	0.25 mg.	0.04 mg.	0.21 mg.	
NIACIN	1.4 mg.	1.3 mg.	0.1 mg.	
ASCORBIC ACID	1.5 mg.	—	1.5 mg.	
CHOLESTEROL	16.4 mg.	0	16.4 mg.*	

*Nonfat (skim) milk, 4 oz., reduces the Fat value to 0.1 gm. and the Cholesterol value to 0.35 mg.
**Based on composite average of breakfast cereals on dry weight basis.

Bowes, A. deP., and Church, C.F.: Food Values of Portions Commonly Used. 8th ed. Philadelphia: A. deP. Bowes, 1956.
Cereal Institute, Inc.: The Nutritional Contribution of Breakfast Cereals. Chicago: Cereal Institute, Inc., 1956.
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TESTICULAR CHANGES IN PANTOTHENIC ACID-DEFICIENT RATS [1]

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(Received for publication June 23, 1958)

INTRODUCTION

Pantothenic acid deficiency in rats has been reported as leading to pathological changes in several organs. The most often reported damage concerns the hemorrhagic necrosis of adrenal glands (Daft and Sebrell, '39; Nelson, '39). Ashburn ('40) later described some changes in the testes of pantothenic acid-deficient rats, namely large multinuclear or else abnormal spermatids. In several experiments conducted in this laboratory, pantothenic acid-deficient animals were seen with damaged germinal epithelium and with impaired spermiogenesis (Barboriak et al., '57a, b).

The present investigation deals with the influence of pantothenic acid deficiency on the development of testicular pathology. As most of the "classical" signs of pantothenic acid deficiency, e.g., alopecia and hemorrhagic necrosis, were reported missing in animals fed bis-(N-pantoyl-β-aminoethyl)-disulfide (PAET), a pantothenic acid antagonist (Boxer et al., '50), a group of animals injected with this compound was included in this series of experiments to determine if, under the influence of this reagent, the testes would also be affected.

[1] This study was supported by a grant-in-aid from the National Vitamin Foundation, Inc.

METHODS

Sixty male albino weanling rats of the Sprague-Dawley strain, with an average initial weight of 35 gm, were divided into two groups: 40 animals were fed a pantothenic-acid deficient diet, whereas the remaining animals received a pantothenic acid-supplemented control diet. The deficient diet consisted, in percentage, of vitamin-free casein, 25;[2] salts IV,[3] 4; corn oil, 5; choline chloride, 0.2; cystine, 0.2 and sucrose, 65.6. Vitamins were supplied in milligrams per 100 gm of diet as follows: thiamine, 1.0; riboflavin, 1.0; pyridoxine, 1.0; nicotinic acid, 10.0; l-inositol, 20; p-aminobenzoic acid, 20.0; folic acid, 0.1; biotin, 0.1; menadione, 1.0; and α-tocopherol acetate, 10.0. Vitamins A and D were supplied in the form of halibut oil and a vitamin D concentrate.[4] The control diet was supplemented with 10.0 mg of calcium pantothenate per 100 gm of diet.

The animals were housed in individual screen-bottom cages, in an air-conditioned room at a constant temperature of 70°F. and constant relative humidity of 45%. Food and water were offered ad libitum. Body weights were determined once weekly.

The anti-metabolite, PAET, was administered subcutaneously once a day to one half of the animals in both the experimental and control groups; the dose was 10 mg per animal. At regular intervals — weekly in the deficient groups, biweekly in the control group — three to 4 animals in each group were sacrificed and their adrenals and testes preserved in 10% buffered formaldehyde for subsequent histological examination. The tissues were stained by the hematoxylin-eosin method.

RESULTS

The administration of the antimetabolite did not influence the weight gains of the control groups (fig. 1). The average

[2] Labco, Borden Company.
[3] Hegsted et al., '41.
[4] Drisdol, Winthrop-Stearns, Inc.

weight gain of the injected group was 146 gm in 28 days, as compared with 139 gm for the non-injected animals. In the deficient groups, however, the injected drug interfered markedly with growth. During the first week of experiment the injected group gained an average of 10 gm, the non-injected

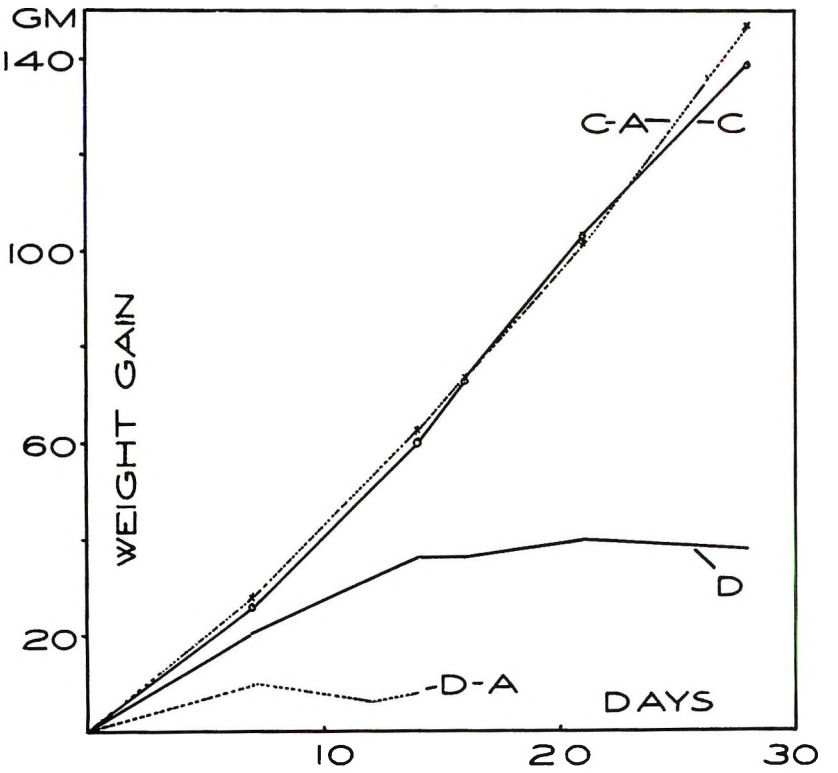

Fig. 1 Weight gains of young rats in pantothenic acid deficiency. C, controls; D, deficient groups; A, injected with bis-(N-pantoyl-β-aminoethyl) disulfide, a pantothenic acid antagonist.

group gained 20 gm. After two weeks some of the injected animals had already succumbed and none survived to the end of the third week. Histological examination of the adrenals from both control groups did not reveal any pathological changes. The relative thickness of the cortical zones was not affected and the zona fasciculata showed the usual lipid con-

tent. Few cases of light congestion were noted in the zona reticularis.

In the pantothenic acid-deficient groups, the adrenal glands of the non-injected animals showed alteration beginning the 4th week of deficiency: the lipid content of the zona fasciculata diminished markedly and in two animals the glands were necrotic. In the deficient group injected with the antimetabolite, the deficiency signs appeared during the second

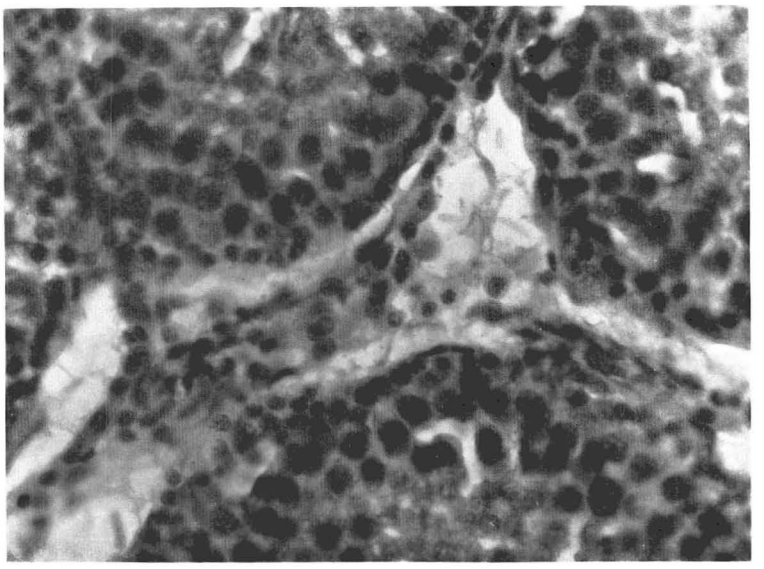

Fig. 2 Testicular tissue of control rat. × 400.

week of the deficiency. Again, the fat content of the zona fasciculata decreased and the congestion in the zona reticularis was quite marked. With one exception, none of the animals of this group showed signs of adrenal hemorrhagic necrosis.

Similar to the findings with the adrenals, the histological picture of testes in the control groups was not affected by the antimetabolite (fig. 2). In both the injected and non-injected animals, the spermiogenesis was quite active at the end of the second week, with an increasing production of sperm toward the end of the experiment. The layers of germinal cells did

not show pathological changes. In a few glands in both groups the intertubular tissue was rather underdeveloped or showed signs of slight degeneration.

In the deficient, antimetabolite-injected group, no spermiogenesis whatsoever was noted. At the end of the first week, the layers of germinal cells were so closely packed together that no lumen was discernible; they later broke up and giant

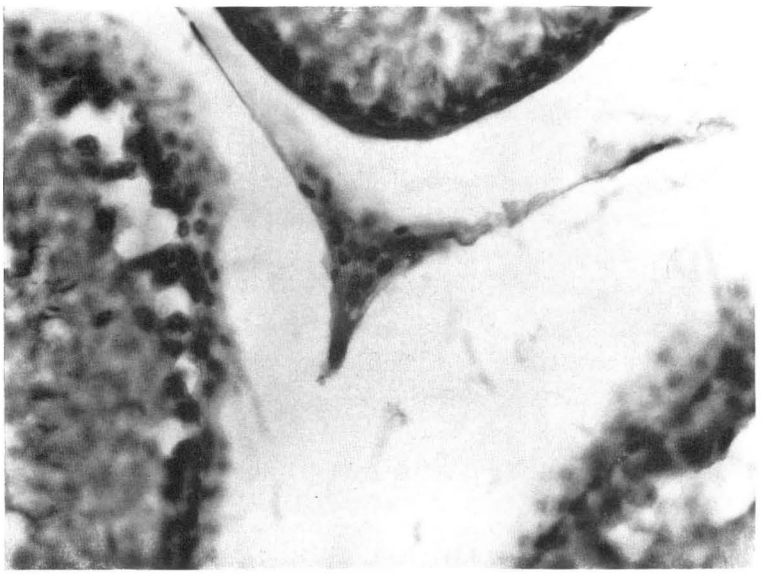

Fig. 3 Testicular tissue in pantothenic acid deficiency, showing breakdown of inter-tubular tissue. × 400.

cells appeared. The intertubular tissue began to degenerate during the second week. In the non-injected deficient group, spermiogenesis appeared during the third week; it was mostly slight with a moderate number of sperm. Germinal layers showed slight degeneration at the end of the second week. They later disintegrated in about one half of the tubules. The connective tissue was normal up to the third week. Slight to marked damage then appeared in all the animals examined.

The changes in the intertubular tissue developed gradually. In the first stage the tubules were pushed apart, but the net-

work of connective tissue and interstitial cells was left intact. Later the strands of connective tissue between the triangular masses were broken and degenerated; the interstitial cells decreased in number (fig. 3). In the final stage, the threads of connective tissue were entirely lost, leaving only a few cells surrounding or mixed with the shrunken interstitial cells.

DISCUSSION

Some of the pathological changes of testicular tissues observed in the present study may have been caused by the decreased food intake reported with pantothenic acid-deficient animals (Voris et al., '42). Closely packed layers of germinal cells and the presence of giant cells were also noted by Siperstein ('20) and Mason ('33) in their experiments with chronic and acute inanition. It would seem from this that the specific testicular lesion due to pantothenic acid deficiency is found in the degeneration or loss of intertubular tissue. Similar testicular damage has also been observed in previous experiments with pantothenic acid-deficient animals (Barboriak et al., '57a). Deficiencies of several other vitamins have been reported to lead to pathological changes in testes, among them vitamins A and E (Mason, '33), riboflavin(Shaw and Phillips, '41), and biotin (Katsh et al., '55). The loss of intertubular tissue in testes was not recorded in any of these reports.

The administration of the pantothenic acid antagonist, PAET, suppressed the growth of animals, accelerated the appearance of histological changes in adrenals and testes, and appreciably increased the mortality rate, but was not associated with any external signs of pantothenic acid deficiency.

SUMMARY

Severe pantothenic acid deficiency in the growing rat leads to histopathological changes in the testes. Especially characteristic of this condition seems to be degeneration of the glandular interstitial tissue. In pantothenic acid-deficient ani-

mals administered bis-(N-pantoyl-β-aminoethyl)-disulfide, an antagonist of pantothenic acid, the testicular damage appeared earlier and was more severe in nature.

ACKNOWLEDGMENTS

The authors wish to thank Dr. R. J. Floody, Hoffman-La Roche, Inc. for the generous supply of vitamins and Dr. N. S. Ritter, Merck Sharp and Dohme, for the antimetabolite used.

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ESSENTIAL FATTY ACID DEFICIENCY

III. EFFECTS OF CONJUGATED ISOMERS OF DIENOIC AND TRIENOIC FATTY ACIDS IN RATS [1]

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(Received for publication June 26, 1958)

INTRODUCTION

Miller and Burr ('37) fed tung oil to mature rats. They found that eleostearic acid is readily converted to dienoic acid in several organs, followed by a gradual decline in the amount of both acids. Reiser ('51) observed similar changes in lipides of eggs from hens fed conjugated trilinolein or tung oil. In experiments with essential fatty acid (EFA)-deficient rats, purified conjugated linoleic acid was not converted to polyunsaturated acids (Holman, '51). Furthermore, its administration caused decrease of weight and the early death of the rats. In later experiments, Holman and Greenberg ('54) found that conjugated linoleate and products of linoleate oxidation which contained conjugated double bonds had similar effects upon rats fed a fat-free diet. The supplements were unable to induce arachidonate synthesis; they made the dermal symptoms of deficiency more severe, and they decreased growth.

[1] Hormel Institute publication no. 174. Supported in part by grants from The Hormel Foundation, National Institutes of Health, Grant no. RG-4952, and the National Dairy Council.

[2] Fulbright Fellow, 1956–58. Permanent address: Department of Biochemistry and Nutrition, Polyteelmic Institute, Østervoldgade 10L[2], Copenhagen K, Denmark.

Hydrogenation of edible oils and fats can induce a variety of positional and geometrical isomers of the unsaturated fatty acids. The presence of such isomers has been suggested as a possible explanation for some of the "stressing" effect exerted by this type of fat upon the development of EFA deficiency (Aaes-Jørgensen, '54; Aaes-Jørgensen, Funch, Engel and Dam, '56; and Funch, Aaes-Jørgensen and Dam, '57). Although the content of conjugated fatty acids in hydrogenated fats was very small, (the order of 0.1% or less), an accumulation of conjugated dienoic acids was reported by Christensen, Dam and Engel ('57) in the adipose tissue of rats fed 28% of hydrogenated peanut oil as the sole dietary fat.

The present investigation was designed to study the effect of purified conjugated dienoic and trienoic fatty acid esters incorporated in an EFA-free, low-fat diet for weanling rats. Primary emphasis was given to their influence upon the polyunsaturated fatty acid pattern in the lipides of heart, testis, brain, and depot fat.

It should be pointed out that the present investigation concerned itself only with *conjugated cis* and *trans* isomers of unsaturated fatty acids. These isomers should not be confused with *trans* isomers of fatty acids in which the double bonds are not conjugated. The two groups of isomers are chemically and biologically dissimilar. The biological effects of a few of the non-conjugated *trans* isomers have been reported previously (Holman, '51; Holman and Aaes-Jørgensen, '56).

MATERIALS AND METHODS

Ethyl palmitate. Crude palmitic acid was crystallized twice from alcohol and the ethyl esters were prepared. Paper chromatographic examination showed the presence of a small amount of unsaturated acid. Therefore, the ester was brominated, fractionally distilled, and treated with Al_2O_3 and charcoal. The colorless ester showed a negative Beilstein test for bromine, and did not contain unsaturated components

detectable by alkaline isomerization or by paper chromatography.

Ethyl linoleate. This was prepared from the fatty acids of safflower oil by urea crystallization and low temperature crystallization, followed by esterification and fractional distillation. This preparation contained ethyl oleate (ca. 5%) as its major contaminant, as demonstrated by paper chromatography (Schlenk, Gellerman, Tillotson and Mangold, '57).

Conjugated cis, trans-*ethyl linoleate.* A concentrate of linoleic acid was obtained by urea crystallization of the fatty acids from safflower oil. This preparation (500 g) then was isomerized with ethylene glycol (2 l) and KOH (250 gm) by heating the mixture for 30 minutes at 180°. The fatty acids were extracted after acidification with HCl and subjected to low temperature crystallization for removal of linoleic acid. The ethyl esters were prepared for use in the feeding experiment. The esters had $\alpha_{233} = 98$; infrared analysis showed absorption at 10.18 μ and 10.54 μ and no absorption at 10.36 μ, indicating the absence of isolated *trans* double bonds.

Conjugated trans, trans-*linoleate.* To a sample of the above-described *cis, trans*-conjugated linoleic acid a few iodine crystals were added in petroleum ether solution under inert gas and the preparation was left exposed to sunshine for two days. It then was treated with charcoal, and the filtrate cooled to −18 to −20°. The crystals formed were filtered off on a chilled funnel (−20°), redissolved in 95% ethyl alcohol, cooled to 0°C, and the crystals were collected. This crystallization was repeated twice. The acid was converted to the ethyl ester, and the washed and dried ester was distilled after removal of the solvent. The ester had $\alpha_{233} = 100$ and $n^{24}{}_D = 1.4715$. Infrared analysis showed absorption at 10.14 μ, and a very faint indication of conjugated *cis, trans* absorption at 10.54 μ and 10.18 μ but no maximum at 10.33 μ, indicating that compounds with isolated *trans* double bonds were absent.

Ethyl α- and β-eleostearates. α-Eleostearic acid was prepared by cold saponification of tung oil, followed by separation of the acids by low temperature crystallization. After washing, the crystals were redissolved in ethanol and kept at −20°C for 24 hours, filtered, and washed. Then the crystals were redissolved in ethanol and kept at +5°C for 24 hours, and the precipitate was filtered and washed with cold ethanol. The α-eleostearic acid obtained melted at 48°C. The β-eleostearic acid was prepared from the α-acid by treatment with a catalytic amount of an alcoholic KI solution and exposed to diffused daylight for three days. The β-eleostearic acid was crystallized as described above until its melting point was at 71°C. Both acids were esterified with ethanol. The infrared spectrum of the ethyl α-eleostearic preparation showed two peaks, at 10.09 and 10.38 μ, identifying it as the α-isomer (*cis* 9, *trans* 11, *trans* 13-octadecatrienoic acid) (Paschke, Tolberg and Wheeler, '53). The β-eleostearic ester preparation had a strong maximum, 10.07 μ, indicating it to be, largely, the β-isomer. This preparation also had a small maximum at 10.38 μ, indicating a slight content of the α-isomer. The β-ethyl-eleostearate had $\alpha_{268} = 200$.

The composition of the diets used is shown in table 1. The basal diet, to which 5% sucrose or 5% of the various fat components was added, contained: vitamin test casein, 20%; sucrose, 65%; α-cellulose, 4%; vitamin mixture [3] in casein, 1%; Wesson salt mixture, 4%; and choline chloride [3] in casein, 1%.

Groups of 6 weanling male rats were given diet and water ad libitum for 17 weeks. The animals were weighed and inspected weekly. At the end of the experimental period, blood was drawn from the heart during ether anesthesia. The animals then were killed and an autopsy was performed. Testis, kidney, liver, aorta and heart samples were taken for histological examination; heart, testis, brain and abdominal depot fat samples were taken for analysis.

[3] Aaes-Jørgensen and Holman, ('58).

Polyunsaturated nonconjugated acids were measured in tissue lipides by the method of Holman and Hayes ('58). Conjugated acids were estimated by the ultraviolet absorption at 233 and 268 mμ in the non-isomerized lipide. The data are reported at relative densities (tables 3, 4, 5, 6), which are used as background absorption corrections in the isomerization procedure. Plasma cholesterol concentration was determined by the method of Abell, Levy, Brodie and Kendall ('52).

TABLE 1

Composition of diets [1]

GROUPS	70	71	72	73	74	75	76	77	78
Basal diet	95	95	95	95	95	95	95	95	95
Ethyl palmitate	5	4	4	4	4	4			
Ethyl linoleate		1							
Conjugated *cis, trans*-ethyl linoleate			1						
Conjugated *trans, trans*-ethyl linoleate				1					
α-Ethyl eleostearate					1				
β-Ethyl eleostearate						1			
Cottonseed oil							5		
Hydrogenated coconut oil [2]									5
Sucrose								5	

[1] All amounts given in percentage.

[2] Hydrol, Durkee's Famous Foods, Chicago, Illinois.

RESULTS

Growth. At the end of the experiment, significant differences were found in weight between the animals fed either cottonseed oil or ethyl linoleate (groups 76 and 71) and all the other groups (table 2). The poorest growth was obtained by feeding β-eleostearate plus ethyl palmitate (group 75). However, differences in growth rates between the groups fed no fat (group 77), ethyl palmitate (group 70), conjugated *cis, trans*-linoleate plus palmitate (group 72), conjugated *trans, trans*-linoleate plus palmitate (group 73), α- or β-eleostearate plus palmitate (groups 74 and 75), and hydrogenated coconut oil (group 78), were not significant. The growth of the latter 7 groups was very much the same throughout the experiment.

TABLE 2

Average growth, skin signs, yellow-brown pigment, and blood cholesterol of rats fed various fatty acid isomers

GROUP	DIET CHARACTERISTICS	AVERAGE INITIAL WEIGHT	FINAL WEIGHT (17 WKS.)	SKIN SIGNS [1] Weeks				YELLOW-BROWN PIGMENT Weeks				BLOOD CHOLESTEROL
				4	8	12	17	4	8	12	17	
		gm	*gm*									*mg/100 ml*
70	Palmitate	48.4	271 ± 21.2	1.0 [5]	1.4 [5]	1.4 [5]	1.7 [5]	0.1	0.3	0	0.1	71
71	Linoleate	48.2	357 ± 11.7	0.6 [6]	0.4 [6]	0.1 [6]	0. [6]	0.8	1.8	2.4	2.3	66
72	Conj. *cis, trans* linoleate	49.8	296 ± 14.4	0.5 [4]	1.5 [4]	1.7 [4]	1.6 [4]	0.9	0.3	0	0.1	66
73	Conj. *trans, trans* linoleate	48.8	277 ± 6.4	0.6 [5]	1.6 [5]	1.6 [5]	1.9 [5]	0.5	0	0.1	0	67
74	α-Eleostearate	48.2	283 ± 15.3	1.0 [6]	1.8 [6]	1.8 [6]	1.8 [6]	0.8	0	0.2	0.1	73
75	β-Eleostearato	48.8	265 ± 6.9	0.8 [5]	1.8 [5]	1.8 [5]	1.8 [5]	1.0	0	0	0	59
76	Cottonseed oil	49.0	367 ± 18.4	0. [4]	0. [4]	0. [4]	0. [4]	0.4	1.3	2.4	2.3	92
77	Fat-free	47.5	279 ± 10.1	0.5 [4]	1.3 [4]	2.1 [4]	2.3 [4]	0.6	0	0	0	51
78	Hydrogenated coconut oil	48.0	293 ± 14.5	0.1 [6]	0.9 [6]	1.9 [6]	2.0 [6]	0.9	0.3	0.1	0.2	63

[1] Many of the animals had slight scaliness of either tail or hindlegs, or both, at the beginning of the experiment. The average score was about 0.1/0.2. The superscript number after the score indicates the number of animals alive.

Dermal symptoms of EFA deficiency. Table 2 gives a summary of the gross symptoms observed throughout the 17 weeks of the experiment. At the beginning of the experiment, many of the animals showed a slight scaliness, either on the tip of the tail or on the feet. A steady increase in scaliness was seen in all groups except those fed ethyl linoleate or cottonseed oil. The symptoms became rather pronounced around the 7th and 8th weeks, in the rats supplemented with the isomers. However, there was almost no difference between the average skin scores of the rats in the several supplemented groups and the fat-free group, but there was a significant difference when compared with the groups fed EFA.

Yellow-brown pigmentation on the backs of the rats (Sinclair, '57; Aaes-Jørgensen, Funch and Dam, '57) developed only in those rats which were given ethyl linoleate or cottonseed oil in the diet (groups 71 and 76). Judging by the skin score, it is obvious that none of the isomers had any activity as essential fatty acids.

Tissue polyunsaturated fatty acids. Heart lipides were found to contain the most conjugated dienoic acids in those groups which were fed *cis, trans*-linoleate and α-eleostearate. The content of conjugated dienoic acids in the heart lipides was so variable that, aside from these two groups, little difference between groups could be discerned (table 3). Conjugated trienoic acids did not appear to be deposited in heart lipides to a significant degree.

Weanling rat hearts were found to contain more total nonconjugated polyunsaturated acids than any other group (table 3). This high amount of total acids is due primarily to high contents of dienoic and tetraenoic acids, which include the essential fatty acids, linoleic and arachidonic. However, the content of pentaenoic and hexaenoic acids was found to be as much as 10 times that found in adult rat heart tissue. In contrast to the high total polyunsaturated acid contents of hearts from weanling rats, that of the rats fed a fat-free diet for 17 weeks was only one-third as great, and that from all the groups receiving fat supplements ranged in between these amounts.

TABLE 3

Polyenoic acid pattern of heart tissue

GROUP	DIET CHARACTERISTICS	NO. OF ANIMALS ANALYZED	CONJUGATION		NON-CONJUGATED POLYENOIC ACIDS					
			k_b233 [1]	k_b268 [1]	Di.	Tri.	Tetr.	Pent.	Hex.	Total
70	Palmitate	5	46	29	6	486	91	13	18	614
71	Linoleate	6	70	57	259	2	173	56	14	504
72	Conjugated *cis*, *trans* linoleate	4	164	48	78	573	161	32	28	872
73	Conjugated *trans*, *trans* linoleate	3	88	52	23	559	107	15	23	727
74	α-Eleostearate	6	112	45	—	502	124	18	13	657
75	β-Eleostearate	5	78	37	24	519	96	17	19	675
76	Cottonseed oil	4	64	81	393	—	156	60	12	621
77	Fat-free	4	43	28	5	230	48	9	10	302
78	Hydrogenated coconut oil [2]	6	55	27	99	341	157	26	21	644
Initial control	Dam's milk	5	32	10	286	42	346	124	114	912

[1] k_b233 and k_b268 are the densities at 233 mμ and 268 mμ of 1 gm of tissue/1 ml of solvent before isomerization.

[2] Hydrol, Durkee's Famous Foods, Chicago, Illinois.

The non-conjugated dienoic acid content of heart lipides decreased drastically in all groups which did not receive EFA supplements. Those which did retained the contents of dienoic acids similar to that of weanling rats. The effects of diet upon non-conjugated trienoic acid contents were just the inverse of those upon the contents of dienoic acid; when linoleate was fed, trienoic acid content was low, and when it was not fed, the amount of trienoic acid in the heart rose significantly. These results are in agreement with previous results (Aaes-Jørgensen and Holman, '58, and Aaes-Jørgensen et al., '58).

The tetraenoic, pentaenoic, and hexaenoic acids found in the heart tissue of all groups were distinctly less than those found in the hearts of weanling rats. Dietary conjugated unsaturated acids were found to exert no specific effect upon these patterns of non-conjugated polyunsaturated acids of heart tissue; that is, no effects which 5% palmitate did not induce.

Testis lipides showed minor variations only in the content of conjugated dienoic and trienoic acids (table 4). No tendency toward accumulation of conjugated acids was indicated.

In weanling animals the dominant polyunsaturated acids in testis lipide are tetraenoic and pentaenoic acids. The total polyunsaturates of testis tissue are considerably less than those found in heart tissue. Dietary supplements of fatty substances did not markedly change the totals. Although not of the same magnitude, the changes in non-conjugated dienoic and trienoic acids followed the same pattern observed in heart tissue. The pentaenoic acid content of testis tissue remained as high as that in weanling rats, even when EFA was not given, but supplementation with linoleate doubled the amount of pentaenoic acid.

Brain lipides apparently do not accumulate dietary conjugated unsaturated fatty acids (table 5), because the conjugated dienoic and trienoic acid contents of brain lipides from all groups of rats were very similar.

TABLE 4

Polyenoic acid pattern of testis

GROUP	DIET CHARACTERISTICS	NO. OF ANIMALS ANALYZED	CONJUGATION		NON-CONJUGATED POLYENOIC ACIDS					
			k_b233 [1]	k_b268 [1]	Di.	Tri.	Tetr.	Pent.	Hex.	Total
					(*mg/100 gm tissue*)					
70	Palmitate	4	36	21	16	184	106	71	24	401
71	Linoleate	6	29	16	69	45	202	233	5	554
72	Conjugated *cis*, *trans* linoleate	4	38	14	35	149	138	108	10	440
73	Conjugated *trans*, *trans* linoleate	4	45	17	23	177	138	82	10	430
74	α-Eleostearate	4	37	17	29	153	130	78	12	402
75	β-Eleostearate	4	49	19	23	219	149	103	12	506
76	Cottonseed oil	3	22	13	105	34	236	283	9	667
77	Fat-free	3	46	27	26	204	173	112	18	533
78	Hydrogenated coconut oil [2]	5	48	29	55	143	148	164	13	523
Initial control	Dam's milk	5	14	3	60	31	200	150	28	469

[1] k_b233 and k_b268 are the densities at 233 mμ and 268 mμ of 1 gm of tissue/1 ml of solvent before isomerization.

[2] Hydrol, Durkee's Famous Foods, Chicago, Illinois.

TABLE 5

Polyenoic acid pattern of brain

GROUP	DIET CHARACTERISTICS	NO. OF ANIMALS ANALYZED	CONJUGATION		NON-CONJUGATED POLYENOIC ACIDS					
			k_b233 [1]	k_b268 [1]	Di.	Tri.	Tetr.	Pent.	Hex.	Total
					(*mg/100 gm tissue*)					
70	Palmitate	3	54	38	42	194	183	89	181	689
71	Linoleate	5	45	22	17	83	436	194	253	983
72	Conjugated *cis*, *trans* linoleate	3	45	16	23	111	188	91	169	582
73	Conjugated *trans*, *trans* linoleate	3	67	79	33	190	223	112	220	778
74	α-Eleostearate	4	54	31	—	304	249	118	266	937
75	β-Eleostearate	4	47	21	—	245	237	120	260	862
76	Cottonseed oil	4	47	25	61	67	461	220	247	1056
77	Fat-free	3	49	28	15	250	208	118	219	810
78	Hydrogenated coconut oil [2]	5	37	19	10	186	224	128	250	798
Initial control	Dam's milk	5	15	4	35	41	401	88	354	919

[1] k_b233 and k_b268 are the densities at 233 mμ and 268 mμ of 1 gm of tissue/1 ml of solvent before isomerization.

[2] Hydrol, Durkee's Famous Foods, Chicago, Illinois.

The dominant non-conjugated polyunsaturated acids of brain lipides from weanling rats are tetraenoic and hexaenoic; the contents of dienoic and pentaenoic are relatively small. Total polyunsaturated acids were not greatly affected by the dietary supplements. EFA deficiency did not appear to affect the non-conjugated dienoic acid content of brain very much, whereas a definite increase in non-conjugated trienoic acid was found in rats which were fed no linoleate. The high tetraenoic acid content of the weanlings' brains remained in those groups which were fed linoleate, but decreased in all other groups. The pentaenoic acid content was increased only upon linoleate supplementation. The high hexaenoic acid content in weanlings decreased somewhat in all groups.

Adipose tissue (lumbar) from rats fed supplements of conjugated dienoic and trienoic acids contained large amounts of conjugated dienoic acid (table 6). These findings agree with those of Christensen et al. ('57), who found an accumulation of conjugated dienoic acid in the depot fat of rats fed a diet containing hydrogenated peanut oil having less than 0.1% conjugated dienoic acid. Conjugated dienoic acid in adipose tissue from rats fed linoleate was somewhat higher than that of rats fed a fat-free diet or hydrogenated coconut oil, but was much less than that found in tissue from rats fed conjugated isomers.

Supplementation of the animals with linoleate caused deposition of very large amounts of non-conjugated dienoic acids in the lumbar fat depot (table 6). Compared with the composition of other tissues analyzed, the presense of relatively large amounts of non-conjugated dienoic acids in the fat depots of the animals given either palmitate, hydrogenated coconut oil, or no fat in the diet was rather surprising. However, it should be pointed out that the fat content of adipose tissue is higher than that of other tissues. Also, Dam and Engel ('58) found a 1% content of dienoic acid in the subcutaneous fat from rats reared on a fat-free diet. The amounts of non-conjugated trienoic acids in the several

TABLE 6

Polyenoic acid pattern of depot fat

GROUP	DIET CHARACTERISTICS	NO. OF ANIMALS ANALYZED	CONJUGATION		NON-CONJUGATED POLYENOIC ACIDS					
			k_b233 [1]	k_b268 [1]	Di.	Tri.	Tetr.	Pent.	Hex.	Total
					(*mg/100 gm tissue*)					
70	Palmitate	4	207	26	216	103	14	4	27	364
71	Linoleate	5	311	78	3808	76	49	13	29	3975
72	Conjugated *cis*, *trans* linoleate	4	2011	35	—	316	20	4	14	354
73	Conjugated *trans*, *trans* linoleate	3	2421	53	91	95	14	4	17	221
74	α-Eleostearate	4	2023	74	—	525	12	3	3	543
75	β-Eleostearate	3	2363	52	6	165	13	3	3	190
76	Cottonseed oil	4	529	178	7397	157	140	34	57	7785
77	Fat-free	2	158	19	159	129	20	6	18	332
78	Hydrogenated coconut oil [2]	4	254	17	229	111	18	5	6	369

[1] k_b233 and k_b268 are the densities at 233 mμ and 268 mμ of 1 gm of tissue/1 ml of solvent before isomerization.

[2] Hydrol, Durkee's Famous Foods, Chicago, Illinois.

groups were not strikingly different, as was the case in other tissues examined. The highest content of trienoic acids was found in the rats supplemented with conjugated *cis, trans*-ethyl linoleate or α-ethyl eleostearate. Different from all the other tissues analyzed, the depot fat was found to contain only small amounts of tetraenoic acids. The largest amounts of penta- and hexaenoic acids occurred in the rats supplemented with linoleate.

Inclusion of 5% ethyl palmitate in the fat-free diet had no effect upon growth but tempered the dermal symptoms somewhat (groups 70 and 77). The polyenoic acid pattern of the tissues was changed only slightly by the palmitate. However, in the heart, trienoic and tetraenoic acids were doubled by feeding palmitate.

Plasma cholesterol varies slightly from group to group, indicating no specific relation to the variations in the dietary fat (table 2).

Histological examinations of the testis and epididymis revealed random and very slight degenerations of the spermatogenic epithelium, characteristic of essential fatty acid deficiency. In the epididymes of the rats supplemented with α- or β-eleostearate, a small number of multinuclear cells was observed, and the amount of spermatozoa appeared to be somewhat reduced. Aside from a few calculi at the corticomedullary border, occurring at random in all the experimental groups, no indication was seen of impairment of the kidneys related to the dietary fat supplements. Extensive histological studies of heart and aorta tissues revealed no abnormalities.[4]

DISCUSSION

Agreement seems to exist concerning the lack of EFA activity of geometric and positional isomers of the dienoic and trienoic fatty acids. Some question exists regarding a possible toxic or "stressing" effect of these compounds upon the EFA deficiency syndrome. Holman ('51) found that conjugated

[4] These were done by Dr. S. Hartroft of Washington University, St. Louis, Missouri.

linoleic acid caused a decrease in weight and the early death of two rats which had been kept on an EFA-free diet for 10 to 12 months previous to the oral supplementation of the purified substance. The early death may have been related to the severe stress of prolonged EFA deficiency. Holman and Greenberg ('54) found conjugated linoleate to have an adverse effect upon EFA-deficient young rats when the supplement (40 to 50 mg) was given orally in 75 to 100 mg of fat. In the present investigation, the conjugated substances were fed as 1% of the diet with 4% ethyl palmitate. This method of supplementation may have caused less local irritant effect than was the case in the experiments quoted above.

Although, in the present experiments, no significant adverse effect was observed on growth rate or on the development of the dermal symptoms, examination of the polyenoic acid pattern in various tissues revealed interesting changes induced by the individual conjugated isomers. In general, only the content of conjugated dienoic acids in the tissues was affected markedly by the dietary supplementation of the conjugated compounds. The content of conjugated trienoic acids appeared to be almost unaffected by these supplements. The effect of supplementation was found to vary widely, depending upon the tissue studied. As a rule, dietary conjugated dienoic and trienoic esters caused a marked increase in the amount of conjugated dienoic acids in the depot fats. In the lipides of hearts, conjugation of dienoic acid was increased by dietary *cis, trans*-conjugated dienoic acid, and, to a lesser extent, by α-eleostearic acid. In the lipides of testis or of brain, no increase in conjugation of dienoic or trienoic acid was found as the result of feeding any of the supplements. It should be pointed out that the data for conjugated dienoic and trienoic acids, expressed as k_b, include other substances which absorb at 233 or 268 mμ. Therefore, small differences between groups or tissues may not be due to differences in conjugation of dienoic and trienoic acids alone. However, the changes measured in preformed conjugation of adipose tissues, are so pronounced that there seems to be no doubt that they are due to the conjugated dietary supplements.

The pattern of non-conjugated polyunsaturated fatty acids in heart, testis, and brain indicated that EFA deficiency was induced in all groups except those fed linoleate (Aaes-Jørgensen and Holman, '58; Aaes-Jørgensen et al., '58). The effect on the contents of non-conjugated acids of the dietary supplementation with the conjugated compounds was similar to that exerted by ethyl palmitate alone.

In the depot fat it was surprising to find relatively high amounts of non-conjugated dienoic acids in the animals fed ethyl palmitate, hydrogenated coconut oil, or no fat in the diet. However, it is not known whether the dienoic acid measured is actually linoleic acid. The negative or small values for non-conjugated dienoic acids observed in the depot fat of animals supplemented with conjugated material may not be valid, because of the presence of large amounts of conjugated dienoic acids which may interfere with the measurement of non-conjugated dienoic acids.

The contrast between adipose tissue and the other tissues examined in their responses to EFA deficiency is striking. The polyunsaturated acid composition of the adipose tissue from EFA-deficient rats did not reveal the marked increase in content of trienoic acids which has been found characteristic of the lipides of other organs. Thus, adipose tissue apparently cannot be used for chemical evaluation of EFA deficiency in the same manner as can lipides from heart or testis.

Isomers of the polyenoic acids have been suggested as a partial cause of the adverse effect of hydrogenated oils as a sole source of dietary fats (Aaes-Jørgensen, '54; Aaes-Jørgensen et al., '56; Funch et al., '57; Christensen et al., '57). Hydrogenated fats are known to contain only a small amount of conjugated isomers of linoleic and linolenic acids, but appreciable and variable amounts of isolated *trans* unsaturation. In the present experiment, the conjugated fatty acid esters were fed as 1% of the diet. This is many times the amount obtained by rats fed on diets containing hydrogenated fat. Thus, the adverse effect observed in experiments with hydrogenated fats apparently cannot be explained by their content of conjugated unsaturated fatty acids.

SUMMARY

Weanling male rats were fed a semi-synthetic, fat-free diet with or without supplements. The supplements, replacing 5% of sucrose, were: (a) 1% of conjugated *cis, trans*-ethyl linoleate plus 4% of ethyl palmitate; (b) 1% of conjugated *trans, trans*-ethyl linoleate plus 4% of ethyl palmitate; (c) 1% of ethyl α-eleostearate plus 4% of ethyl palmitate; (d) 1% of ethyl β-eleostearate plus 4% of ethyl palmitate; (e) 5% of ethyl palmitate; (f) 5% of cottonseed oil; and (g) 5% of hydrogenated coconut oil; (h) 1% of ethyl linoleate plus 4% of ethyl palmitate.

The experiments showed that the conjugated di-and trienoic fatty acid ester supplements did not significantly intensify the retardation of growth or the development of dermal symptoms of EFA deficiency. On the other hand, the results agreed with previous reports that these conjugated acids do not have EFA activity.

Conjugated *cis, trans*-ethyl linoleate, and, to a lesser extent, ethyl α-eleostearate, increased the amount of conjugated dienoic acids in the lipides of hearts. Apparently none of the conjugated compounds increased the content of conjugated dienoic and trienoic acids in the lipides of testis or brain, whereas all of these compounds markedly increased the amount of conjugated dienoic acid in adipose tissue.

The pattern of non-conjugated polyenoic acids of the lipides from heart, testis, brain, and adipose tissue from EFA-deficient animals was not markedly changed by dietary supplementation of the conjugated unsaturated fatty esters. It is concluded that the presence of small amounts of conjugated polyenoic acid does not explain the dietary effects of hydrogenated fat upon rats. The polyenoic acid pattern of adipose tissue differed markedly from that of other tissues examined.

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SUPPLEMENTATION OF CEREAL PROTEINS WITH AMINO ACIDS

I. EFFECT OF AMINO ACID SUPPLEMENTATION OF CORN-MASA AT HIGH LEVELS OF PROTEIN INTAKE ON THE NITROGEN RETENTION OF YOUNG CHILDREN [1]

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The recent report of the F.A.O. Committee on protein requirements reviews the need for taking into consideration the amino acid content of individual proteins and of diets in estimating human protein requirements (F.A.O., '57). It is now well recognized that the relative lack of one or more of the essential amino acids reduces the biological value of food protein (Flodin, '57). Similarly, the correction of this deficiency by a combination of foods whose amino acid content is adequate or by the addition of the missing amino acids in synthetic form has been found in countless animal experiments to result in improved biological value (Flodin, '57). It is also known from studies in experimental animals that the addition of too much of certain of the synthetic amino acids can result in an imbalance which depresses the nutritive value of the protein of the food or diet when fed at relatively low protein levels (Elvehjem, '56; Elvehjem and Harper, '55).

The application of this knowledge to the formulation or supplementation of human diets is greatly hindered by the lack of certainty as to the optimum pattern of amino acids for

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human nutrition. One approach has been to use the amino acid proportions found in those proteins known to be of high biological value, notably egg and milk protein. The results of studies of amino acid requirements in children (Holt and Snyderman, '56; Albanese, '50) and in adults, reviewed by Rose ('57) and Scrimshaw et al. ('58), have also been used but entail considerable uncertainty. The F.A.O. Committee tried to solve this problem by using all available data in the establishment of a theoretical "reference protein" whose amino acid content would serve as a "reference pattern" (F.A.O., '57).

If the proportions of the individual essential amino acids in this reference protein were really optimal for human growth and development, human diets could theoretically be devised and supplemented by making their amino acid pattern coincide with that of the reference pattern. Such a simple approach would not be entirely satisfactory, however, because the availability of the amino acids in a diet does not necessarily coincide with its amino acid content. Furthermore, the optimum pattern may vary with the physiological state of the individual. Finally, there is no assurance that the pattern developed by the F.A.O. Committee is really biologically superior to the patterns found in good natural protein such as egg and milk. Nevertheless, the development of a reference pattern through the use of amino acids in the diet is a valuable experimental approach to the determination of optimal amino acid relationships for man.

The present paper reports the first in a series of studies designed to evaluate the F.A.O. "reference pattern" by using it as a base for the supplementation of food proteins fed to children recovering from severe protein malnutrition. The effect of progressively supplementing corn protein with those amino acids indicated to be limiting by comparison with the F.A.O. "reference pattern" has been measured by studying the subsequent nitrogen retention in children fed approximately 3.0 gm of protein and 100 Cal. per kilogram of body weight per day. The technique provides direct information

as to the effects of amino acid imbalances as well as simple amino acid additions on nitrogen retention in these children.

MATERIALS AND METHODS

In this series of studies, hospitalized children who have recently recovered from kwashiorkor have been kept in metabolic beds which permit the quantitative collection of urine and feces. All food consumed was weighed or measured and aliquots of the complete diet were analyzed as well as the pooled urine and feces from each experimental period. Carmine was fed at the start and at the end of each period to guide the collection of the feces. The Kjeldahl method was used for the nitrogen determinations (A.O.A.C., '50).

The basal diet contained in grams per 100 gm: corn-masa, 85; corn gluten, 5; glutamic acid, 2; glycine, 3; and cornstarch, 5. The preparation of corn-masa has been previously described as consisting of the treatment of whole corn with a heated lime solution before grinding (Bressani and Scrimshaw, '58). Corn gluten and glutamic acid were used to raise the total nitrogen to a level of 2.3% so that sufficient protein could be fed in a total bulk of diet which was well tolerated by the child. The amino acids were substituted for the glycine so that the diets remained isonitrogenous. A multiple vitamin and mineral capsule[2] was given daily to each child.

The essential amino acid content of the corn-masa, corn gluten, and the basal diet as well as that of the F.A.O. "reference protein," as determined in this laboratory, is given in table 1. According to this pattern, the order of deficiency in the basal diet from greatest to least was as follows: tryptophan, lysine, methionine, valine, isoleucine and threonine. These were added step-wise to the basal diets in amounts shown in table 1, which were calculated to make up the difference between the amino acid content of the basal diet and that of the FAO "reference pattern." Because initial calculations involving the amino acid content of corn-masa were based on

[2] Gevral, donated by Lederle Laboratories, American Cyanamid Company.

literature values which subsequently proved too low, the quantities added were actually slightly in excess of the amounts required by the pattern. Only for methionine, however, was this excess observed to have possible significance.

TABLE 1

Amino acid content of corn-masa, corn gluten and the basal diet used in determining the effect of amino acid supplementation on the retention of nitrogen in young children

AMINO ACID	CORN-MASA	CORN GLUTEN	BASAL DIET	FAO "REFERENCE PROTEIN" PATTERN	ADEQUACY OF BASAL DIET	AMOUNT ADDED/GM NITROGEN [1]
	mg amino acid/gm nitrogen				%	*mg*
Arginine	198	184	130	—	—	—
Histidine	177	99	104	—	—	—
Isoleucine	292	245	187	270	69	196
Leucine	607	1152	511	306	—	—
Lysine	200	115	118	270	44	243
Methionine	150	123				
Cystine	83	68	149	270	55	148
Phenylalanine	271	458				
Tyrosine	195	344	376	180	—	—
Threonine	253	232	165	180	91	96
Tryptophan	16	24	12	90	13	148
Valine	282	285	190	360	53	391

[1] Lysine was added in the L-form; all other amino acids in the DL-form. D-isomers were assumed not to be utilized except in the case of methionine.

Corrections were made for the D-form of the amino acids employed by doubling the amount added, except for DL-methionine, which was assumed to be fully utilized and lysine, which was added in the natural form. In these initial studies each experimental combination was fed for a two-day adaptation period followed by a three-day period during which the exact nitrogen intake and output were determined.

In this first study, the relatively high protein intake of 3.0 gm and 100 Cal. per kilogram of body weight was fed throughout. Four balance periods were carried out with a boy, PC-56, 3 years and 7 months old weighing 9.89 kg with a height of 82 cm at the beginning of the trials, and 18 balance periods were completed with PC-57, a boy 4 years and 4 months old weighing 12.0 kg with a height of 86 cm at this time.

RESULTS

Case PC–56. The changes in nitrogen absorption, nitrogen retention and in body weight in case PC-56 are shown in figure 1. Supplementary data are contained in table 2. This child was studied through 4 consecutive nitrogen balance periods. After being in nitrogen equilibrium on the basal diet, the child's nitrogen balance became positive upon the addition to the

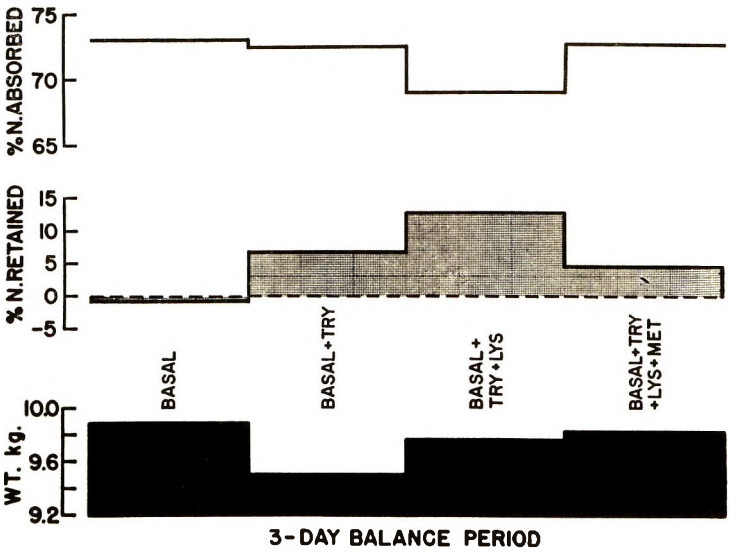

Fig. 1 Effect of amino acid supplementation of a corn-masa basal diet on the per cent nitrogen absorbed and retained by child PC-56 fed approximately 3 gm of protein per kilogram of body weight.

basal diet of 148 mg of DL-tryptophan per gram of nitrogen. The nitrogen balance became more strongly positive when the basal + tryptophan diet was supplemented with 243 mg of L-lysine per gram of nitrogen. The last diet tested, basal + tryptophan + lysine + 148 mg of DL-methionine per gram of nitrogen, did not improve the retention; instead, the methionine supplementation had a negative effect on nitrogen retention. The nitrogen absorption stayed fairly constant throughout the 4 periods. At this point the balance studies had to be discontinued because the child developed chicken pox. During

TABLE 2

Effect of amino acid supplementation of a corn-masa basal diet on the daily intake, excretion and retention of nitrogen by child PC-56

PERIOD	DIET	NITROGEN INTAKE	NITROGEN IN FECES	NITROGEN ABSORBED	NITROGEN ABSORBED	NITROGEN IN URINE	NITROGEN RETAINED	NITROGEN RETAINED	WEIGHT
		mg/kg/day			*% intake*	*mg/kg/day*		*% intake*	*kg*
1	Basal	449	122	327	73.0	330	— 3	— 0.67	9.89
2	Basal + tryptophan	450	124	326	72.5	296	+ 30	+ 6.67	9.52
3	Basal + tryptophan + lysine	460	143	317	69.0	248	+ 69	+ 12.82	9.77
4	Basal + tryptophan + lysine + methionine	456	124	332	72.8	311	+ 21	+ 4.62	9.83

the basal period the child dropped from 9.89 kg to 9.52 kg. This weight then increased to 9.77 kg during the third period and was 9.83 kg at the end of the experiment.

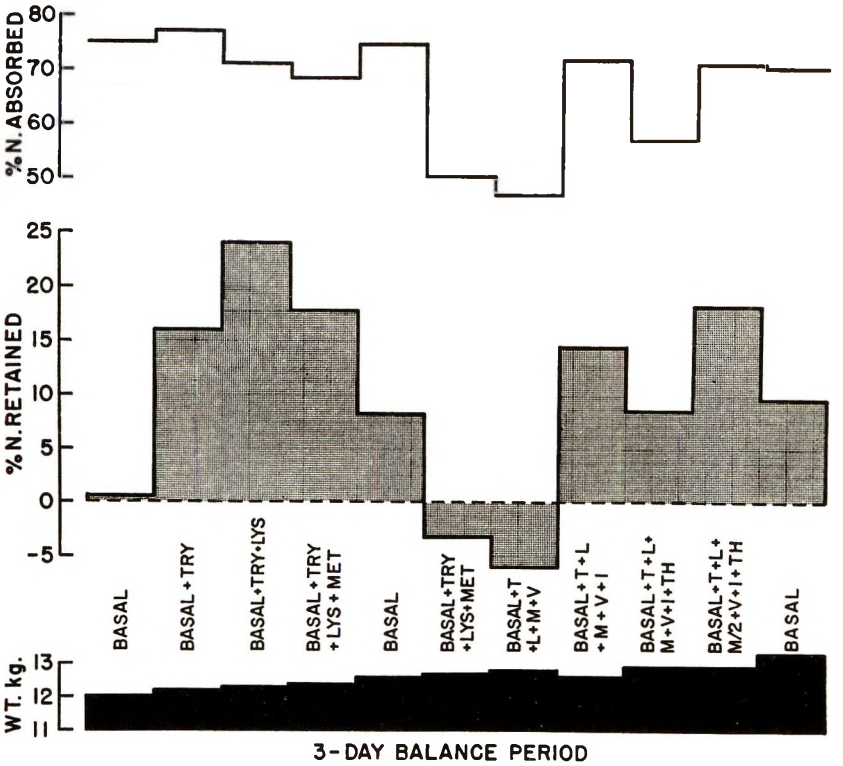

Fig. 2 Effect of amino acid supplementation of a corn-masa basal diet on the per cent nitrogen absorbed and retained by child PC-57 fed approximately 3 gm of protein per kilogram of body weight, Series "A."

Case PC-57. Series A. The same sequence of amino acid additions was tested simultaneously on case PC-57. The results are shown in figure 2. The responses prior to supplementation with methione were qualitatively identical with those of case PC-56, although the responses are quantitatively greater, especially with tryptophan supplementation. The depressing effect of methionine was also apparent in this patient, but to a lesser extent. The child was then returned to the

basal diet, and a marked reduction in nitrogen retention occurred. The child was again placed on the basal + tryptophan + lysine diet with 148 mg of DL-methionine per gram of nitrogen added. In this period the nitrogen balance shifted to negative. Furthermore, the nitrogen absorbed, which had been relatively constant up to this point, also decreased sharply.

The addition to the previous diet (basal + tryptophan + lysine + methionine) of DL-valine, the next limiting amino acid, at the rate of 391 mg per gram of nitrogen, made the nitrogen retention even more negative, and the absorption of nitrogen remained poor. On the other hand, the addition of 196 mg of DL-isoleucine per gram of nitrogen to the previous diet (basal + tryptophan + lysine + valine) had a remarkable effect in increasing both nitrogen absorption and retention. The addition of DL-threonine, the least limiting amino acid, at the rate of 96 mg per gram of nitrogen, did not improve nitrogen retention. On the contrary, both absorption and retention decreased in comparison with the previous period.

To investigate the possibility that the level of methionine was too high, a diet with slightly less than half as much added DL-methionine (61 mg per gram of nitrogen) plus the same amount of the other previously tested amino acids was fed. This reduction in the amount of methionine improved nitrogen retention, but not to the level obtained with basal + tryptophan + lysine alone. Feeding only the basal diet again resulted in a decrease in nitrogen retention. Except for the period following the second basal trial, the child continued to gain weight throughout this series of experiments.

Supporting data are given in table 3. In this series of studies with PC-57, the maximum retention of nitrogen, 112 mg per kilogram per day, was obtained on the basal + tryptophan + lysine diet. It was reduced to minus 16 mg per kilogram per day when the same diet + methionine was fed following a basal period and was reduced to its lowest, minus 29 mg per kilogram per day when valine as well as methionine was added to the basal + tryptophan + lysine diet.

TABLE 3

Effect of amino acid supplementation of a corn-masa basal diet on the daily intake, excretion and retention of nitrogen by child PC-57 (Series A)

PERIOD	DIET	NITROGEN INTAKE	NITROGEN IN FECES	NITROGEN ABSORBED	NITROGEN ABSORBED	NITROGEN IN URINE	NITROGEN RETAINED	NITROGEN RETAINED	WEIGHT
		mg/kg/day			*% intake*	*mg/kg/day*		*% intake*	*kg*
A-1	Basal	478	119	359	75.1	356	+ 3	+ 0.6	12.0
2	Basal + tryptophan	464	105	359	77.4	283	+ 76	+ 16.2	12.2
3	Basal + tryptophan + lysine	470	134	336	71.5	224	+ 112	+ 24.0	12.3
4	Basal + tryptophan + lysine + methionine [1]	462	146	316	68.4	233	+ 83	+ 18.1	12.4
5	Basal	471	117	354	75.0	315	+ 39	+ 8.3	12.6
6	Basal + tryptophan + lysine + methionine [1]	451	223	228	50.5	244	— 16	— 3.5	12.7
7	Basal + tryptophan + lysine + methionine [1] + valine	454	241	213	47.0	242	— 29	— 6.2	12.8
8	Basal + tryptophan + lysine + methionine [1] + valine + isoleucine	460	128	332	72.2	265	+ 67	+ 14.6	12.6
9	Basal + tryptophan + lysine + methionine [1] + valine + isoleucine + threonine	447	190	257	57.4	218	+ 39	+ 8.6	12.9
10	Basal + tryptophan + lysine + methionine [2] + valine + isoleucine + threonine	450	129	321	71.5	238	+ 83	+ 18.4	12.9
11	Basal	478	138	340	71.3	294	+ 46	+ 9.7	13.3

[1] DL-Methionine, 0.34%.

[2] DL-Methionine, 0.14%.

Case PC-57. Series B. In order to test the effect of the same amino acids, added in a different sequence, a second series of trials was conducted with PC-57 after keeping him on a milk diet for 12 days. The results are shown in figure 3. Nitrogen retention was measured during a 5-day balance period on milk, followed by two three-day balance periods

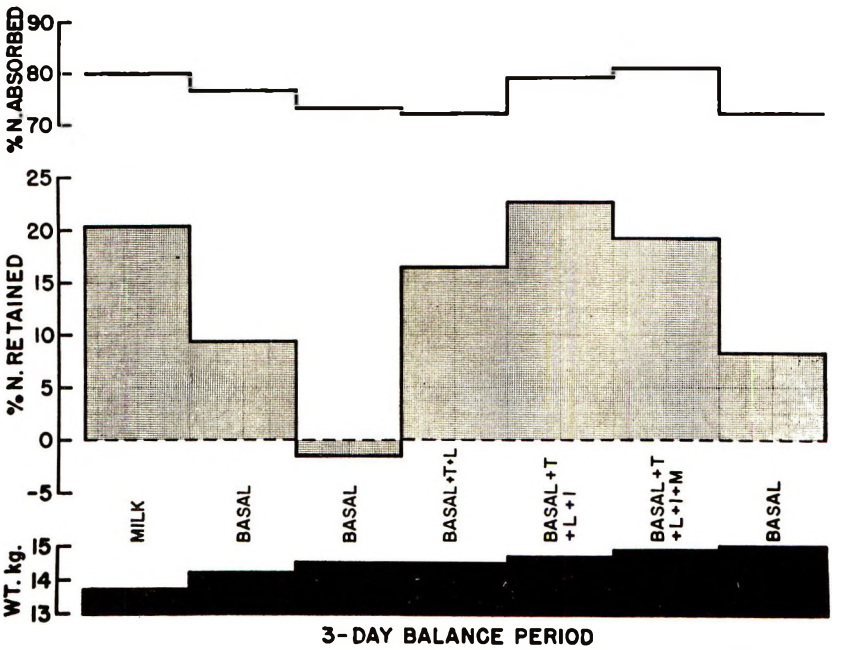

Fig. 3 Effect of amino acid supplementation of a corn-masa basal diet on the per cent nitrogen absorbed and retained by child PC-57 fed approximately 3 gm of protein per kilogram of body weight, Series "B."

during which the basal diet was fed. It can be seen that in the first three day period during which the basal diet was administered, nitrogen retention decreased sharply although the child remained in positive balance. However, during the second three-day period on this same diet, the balance became negative. Since supplementation with tryptophan alone had given positive results each time at this level of protein intake, the basal + tryptophan combination was omitted and the basal +

tryptophan + lysine diet was fed. The balance immediately became strongly positive. The addition of isoleucine improved the retention still further to a level exceeding that of the initial period with milk. The 148 mg of DL-methionine per gram of nitrogen subsequently added to the latter diet had a depressing effect although it was relatively slight. When the child was then returned to the basal diet, nitrogen retention again decreased markedly. Nitrogen absorption remained relatively constant throughout the experiment, and the child continued to gain weight.

From the supporting data given in table 3, it is apparent that the milk diet resulted in a retention of 97 mg per kilogram per day, and a nitrogen absorption of 79.8%. However, a similar absorption of 79.2% on the basal + tryptophan + lysine diet induced a maximum retention of 108 mg of nitrogen per kilogram per day. Upon methionine supplementation, this figure was reduced to 90 mg with no change in nitrogen absorption. On feeding the last basal diet shown in this sequence, the retention decreased to 39 mg, a figure similar to that for the nitrogen retained when the basal diet was fed after the milk period.

DISCUSSION

Although the number of trials is limited and the periods short, the results are remarkably consistent and clear in their implications. Under the experimental conditions, children are sensitive to small changes in the amino acid content of their diets and reflect them in the nitrogen retention and some times in the nitrogen absorption on an isonitrogenous diet. When the nitrogen balance is positive, gain in weight usually occurs. When the first two of the amino acids predicted to be deficient by comparison with the F.A.O. "reference pattern" were added in proportions to approximate the latter, nitrogen retention was markedly improved in each of the three trials. It is noteworthy that the addition of these two amino acids, tryptophan and lysine, in amounts above those calculated to be necessary to reach the level of the next limiting amino acid, produced no detectable imbalance but rather increased nitrogen retention.

TABLE 4

Effect of amino acid supplementation of a corn-masa basal diet on the daily intake, excretion and retention of nitrogen by child PC-57 (Series B)

PERIOD	DIET	NITROGEN INTAKE	NITROGEN IN FECES	NITROGEN ABSORBED	NITROGEN ABSORBED	NITROGEN IN URINE	NITROGEN RETAINED	NITROGEN RETAINED	WEIGHT
		mg/kg/day			*% intake*	*mg/kg/day*		*% intake*	*kg*
B-1	Milk [1]	476	96	380	79.8	283	+ 97	+ 20.4	13.7
2	Basal	463	106	357	77.2	314	+ 43	+ 9.4	14.2
3	Basal	453	123	330	73.0	338	— 8	— 1.8	14.5
4	Basal + typtophan + lysine	463	129	334	72.4	258	+ 76	+ 16.5	14.5
5	Basal + tryptophan + lysine + isoleucine	475	99	376	79.2	268	+ 108	+ 22.7	14.7
6	Basal + tryptophan + lysine + isoleucine + methionine	469	90	379	80.8	289	+ 90	+ 19.2	14.9
7	Basal	474	132	342	72.2	303	+ 39	+ 8.2	15.0

[1] Five-day balance period.

However, when methionine, the third limiting amino acid by this criterion, was added, it had a consistently adverse effect on retention. The most obvious explanation is that the "reference pattern" is in error by calling for too much methionine in proportion to the other essential amino acids. Using literature data for estimating the methionine content of the basal diet, the total sulphur-containing amino acids, including the 0.34% methionine supplement, was calculated to be the 270 mg/gm of nitrogen called for by the "reference pattern." Actual analysis subsequently gave a value of 297 mg of methionine + cystine per gram of nitrogen. When the amount of the methionine supplement was reduced to 61 mg per gram of nitrogen, giving a total content of 210 mg of sulphur amino acids per gram of nitrogen, a significant increase in nitrogen retention was observed in comparison with the previous period in which the diet contained 297 mg of these amino acids per gram of nitrogen. The proportion of sulphur amino acids to tryptophan was 3.3 to 1 at the higher level of methionine addition and 2.3 to 1 at the lower, a point of interest since the F.A.O. pattern uses tryptophan, the most deficient of the amino acids in corn, as a reference base. It is also significant that both valine and threonine addition appeared to decrease nitrogen retention, although it should be noted that the levels of these amino acids in the "reference pattern" were very slightly exceeded.

It is a point of major interest and importance that children are immediately sensitive to such small changes in amino acid proportions that they are readily detectable within a single three-day period in nitrogen balance experiments of this type. When the effect of the change on nitrogen retention is large, however, the full effect may not be stabilized in so short a time and hence may influence the magnitude of the response in the succeeding period. This is apparent in the balance studies with PC-57. In series A the combined supplementation with tryptophan, lysine and methionine results in a much lower retention immediately after the second basal period than just before it. In series B retention was much lower in the second

of the two consecutive basal periods following the initial trial with milk. In neither series was any infection, gastro-intestinal upset or other possible contributory cause noted. In order to obtain more quantitative estimates of the amounts of amino acid supplements, our further studies employ two consecutive three-day balance periods for each combination tested. That this does not alter the qualitative findings will be demonstrated in subsequent papers in this series.

The data thus far presented emphasize the importance of establishing a proper balance between the essential amino acids if a maximum retention of nitrogen is to be obtained. They also indicate that although the F.A.O. "reference pattern" will not prove ideal for all experimental conditions or physiologic states, this attempt to formulate a provisional pattern is a valuable experimental approach to the problems of amino acid supplementation.

SUMMARY

A simplified basal diet in which corn is the only source of protein was fed to two boys recently recovered from severe protein malnutrition (kwashiorkor) at a level to provide 3 gm of protein and 100 Cal. per kilogram of body weight. Nitrogen balance was determined in a total of 22 three day periods. The children, aged three years and 7 months, and 4 years and 4 months, weighed 9.9 and 12.0 kg and measured 82 and 86 cm, respectively. They continued to gain weight during the progressive supplementation of the basal diet with the amino acids indicated to be deficient by comparison with the amino acid pattern of the F.A.O. "reference protein." The amounts of tryptophan and lysine required to bring the amino acid intake to the reference level resulted in marked improvement in nitrogen retention. This amount of methionine brought about a decrease which could be reversed so that retention became strongly positive by supplementation with isoleucine. Valine and threonine also appeared to have a negative effect on retention at least until after isoleucine had been given. The results show that by the supplementation of a vegetable protein with

the appropriate essential amino acids, good nitrogen retention and satisfactory gain in weight can be obtained, at least with an intake of 3.0 gm of protein and 100 Cal. per kilogram per day in young children.

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SUPPLEMENTATION OF CEREAL PROTEINS WITH AMINO ACIDS

II. EFFECT OF AMINO ACID SUPPLEMENTATION OF CORN-MASA AT INTERMEDIATE LEVELS OF PROTEIN INTAKE ON THE NITROGEN RETENTION OF YOUNG CHILDREN [1]

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The first paper in this series (Scrimshaw et al., '58) presented the results of supplementing corn protein fed to young children at a level of 3.0 gm per kilogram of body weight with synthetic essential amino acids to match the amino acid pattern of the F.A.O. "reference protein" ('57). Although nitrogen retention was poor on the basal diet alone, it was progressively improved by the step-wise addition of tryptophan, lysine, and isoleucine. On one case the basal diet supplemented with tryptophan, lysine and isoleucine was compared with one in which the same amount of protein was furnished by milk; greater nitrogen retention was found with the former than with the milk-based diet. In general, the children tended to gain weight on the corn-masa diet when the amino acid retention became or remained positive as the result of the amino acid supplementation.

Recent reports suggest that to some extent, merely increasing the intake of a poor quality protein can compensate for part of its qualitative deficiency and result in greater absolute

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nitrogen retention (Allison and Anderson, '45; De Maeyer and Vanderborght, '57; Sure, '57). At the same time, with high levels of intake, the relative percentage of retention was improved and the distinction between the nitrogen retention of proteins of widely differing biological value was shown to be less marked. In other words, when fed at high intake levels, differences in the amino acid composition of the diet appear to become less critical and less easy to determine.

This second paper explores the effect of amino acid supplementation at levels of 2.0 and 1.5 gm of protein per kilogram which are relatively low for children of this age and physiological state (Béhar, Viteri and Scrimshaw, '57). In general the results confirm those obtained previously at the level of 3.0 gm of protein per kilogram of body weight, but the responses to the amino acid additions are of smaller magnitude.

MATERIAL AND METHODS

The techniques and diet employed were identical with those described previously (Scrimshaw et al. '58) except that less protein was supplied, and two successive three-day collection periods were employed with each combination tested. Two comparison periods in which the only protein in the diet came from milk were available as part of other experimental work. In these two cases 5-day balance periods were begun at the end of 7 days on the milk diet so that the results should represent stabilized values. Case PC-66, a boy one year and 10 months old weighing 8.9 kg and measuring 71.5 cm at the beginning of the trials and child PC-67, one year and 6 months of age weighing 9.2 kg and measuring 72.0 cm were both fed 2.0 gm of protein and 90 Cal. per kilogram per day. PC-57, a 4-year and 9-months old boy weighing 15.6 kg with a height of 89.5 cm, when the series of trials was begun, was fed a diet providing 1.5 gm of protein and 80 Cal. per kilogram of body weight per day.

RESULTS

Case PC-67. The responses of child PC-67 are shown in figure 1 in which the data for each pair of three-day periods are averaged. The initial milk balance based on a 5-day period showed a nitrogen retention of 36 mg per kilogram per day or 8.2% of the amount consumed, compared with —15 mg per kilogram per day and —5.7% during the 6 days on the

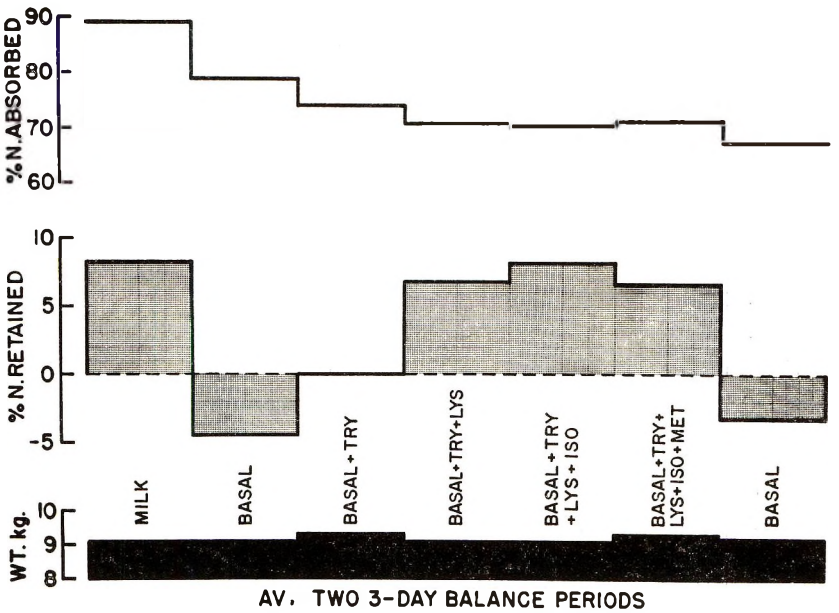

Fig. 1 Nitrogen absorption and retention and weight changes in child PC-67 recently recovered from kwashiorkor and fed milk or a corn-masa basal diet progressively supplemented with amino acids.

basal diet. When tryptophan was added in an amount designed to bring the total of this amino acid in the diet to that of the F.A.O. "reference protein" ('57), nitrogen retention improved to the point where the child was in nitrogen balance. The addition of 0.56% of lysine to the tryptophan supplement resulted in a marked further improvement in nitrogen retention; adding isoleucine brought about a slight additional increase to a level of retention nearly comparable to that found with milk protein at the beginning of the trials. Adding

TABLE 1

Daily intake, excretion and retention of nitrogen by PC-67 with three-day periods of supplementation

THREE-DAY PERIOD	DIET	NITROGEN INTAKE	NITROGEN IN FECES	NITROGEN ABSORBED	NITROGEN ABSORBED	NITROGEN IN URINE	NITROGEN RETAINED	NITROGEN RETAINED	WEIGHT
		mg/kg/day			*% intake*	*mg/kg/day*		*% intake*	*kg*
	Milk [1]	438	47	391	89.3	355	+ 36	+ 8.2	9.10
1	Basal	319	61	258	80.9	260	— 2	— 0.6	9.10
2	Basal	319	70	249	78.2	277	— 28	— 8.6	9.10
3	Basal + tryptophan	319	73	246	77.1	235	+ 11	+ 3.4	9.29
4	Basal + tryptophan	319	93	226	70.8	237	— 11	— 3.4	9.29
5	Basal + tryptophan + lysine	322	86	236	73.3	196	+ 40	+ 12.4	9.12
6	Basal + tryptophan + lysine	322	102	220	68.3	215	+ 5	+ 1.6	9.12
7	Basal + tryptophan + lysine + isoleucine	322	99	223	69.2	199	+ 24	+ 7.4	9.12
8	Basal + tryptophan + lysine + isoleucine	322	91	231	71.7	204	+ 27	+ 8.4	9.12
9	Basal + tryptophan + lysine + isoleucine + methionine	317	94	223	70.4	197	+ 26	+ 8.2	9.26
10	Basal + tryptophan + lysine + isoleucine + methionine	317	87	230	72.6	212	+ 18	+ 5.7	9.26
11	Basal	319	98	221	69.3	220	+ 1	+ 0.3	9.20
12	Basal	319	112	207	64.9	228	— 21	— 6.6	9.20

[1] Five-day balance period.

methionine to the mixture of supplementary amino acids brought about a small drop which is apparent in figure 1, and returning the child to the basal diet resulted once again in negative nitrogen balance. The results are more variable when the data are examined for the two separate three-day periods averaged for figure 1. The data for each three-day

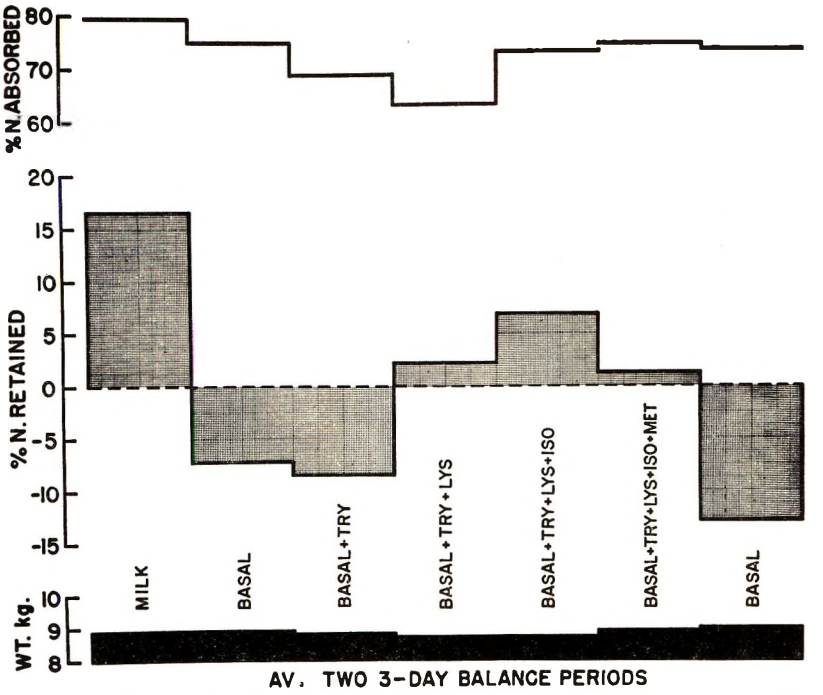

Fig. 2 Nitrogen absorption and retention and weight change in child PC-66 recently recovered from kwashiorkor and fed milk or a corn-masa basal diet progressively supplemented with amino acids.

period as tabulated in table 1 suggest a tendency for the responses to be greater in the first three-day period on an experimental treatment than during the second, although the results are not entirely consistent. Absorption of nitrogen varied from 65 to 81% without a clear pattern emerging.

Case PC-66. The response of PC-66 to progressive supplementation of corn-massa protein with synthetic amino acids is shown in figure 2. The changes proved similar to

TABLE 2

Daily intake, excretion and retention of nitrogen by PC-66 with three-day periods of supplementation

THREE-DAY PERIOD	DIET	NITROGEN INTAKE	NITROGEN IN FECES	NITROGEN ABSORBED	NITROGEN ABSORBED	NITROGEN IN URINE	NITROGEN RETAINED	NITROGEN RETAINED	WEIGHT
		mg/kg/day			*% intake*	*mg/kg/day*		*% intake*	*kg*
	Milk [1]	432	90	342	79.2	270	+ 72	+ 16.7	8.90
1	Basal	321	82	239	74.3	265	— 26	— 8.3	8.77
2	Basal	321	61	260	81.0	278	— 18	— 5.6	8.77
3	Basal + tryptophan	321	101	220	68.5	241	— 21	— 6.5	8.75
4	Basal + tryptophan	321	97	224	69.7	253	— 29	— 9.0	8.75
5	Basal + tryptophan + lysine	320	118	202	63.2	199	+ 3	+ 0.9	8.69
6	Basal + tryptophan + lysine	320	114	206	64.5	194	+ 12	+ 3.8	8.69
7	Basal + tryptophan + lysine + isoleucine	320	89	231	72.3	209	+ 22	+ 6.8	8.69
8	Basal + tryptophan + lysine + isoleucine	320	81	239	74.6	216	+ 23	+ 7.2	8.69
9	Basal + tryptophan + lysine + isoleucine + methionine	312	87	225	72.1	235	— 10	— 3.2	8.92
10	Basal + tryptophan + lysine + isoleucine + methionine	312	70	242	77.5	223	+ 19	+ 6.1	8.92
11	Basal	320	83	237	74.1	278	— 41	— 12.9	9.00

[1] Five-day balance period.

those in figure 1, except that there was no significant response to the initial addition of tryptophan. The supporting data tabulated by three-day periods are shown in table 2. There was the same tendency as in the preceding series for the first three-day period of each pair to show a more marked response to the changes in the diet than the second. With the exception

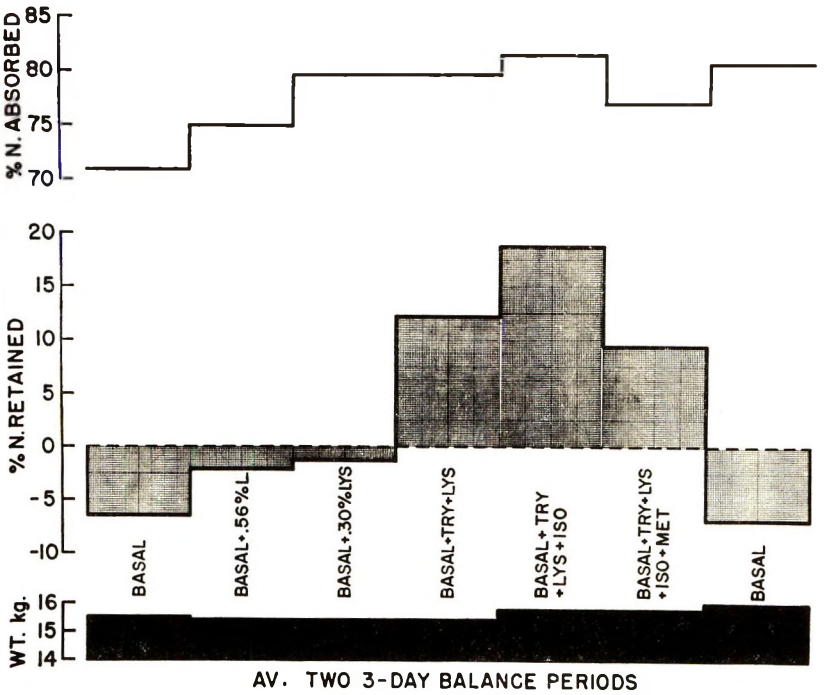

Fig. 3 Nitrogen absorption and retention and weight changes in child PC-57 recently recovered from kwashiorkor and fed a corn-masa basal diet progressively supplemented with amino acids.

of the initial supplementation with tryptophan, essentially no difference in nitrogen retention appeared between each of the two three-day periods when using combinations of tryptophan, lysine and isoleucine. The amount and percentage of nitrogen retained was greater with milk than with any pair of experimental periods involving the basal diet in this series of trials. Nitrogen absorption was 79% during the initial milk period and ranged from 63 to 81% thereafter.

TABLE 3

Daily intake, excretion and retention of nitrogen by PC-57 with three-day periods of supplementation

THREE-DAY PERIOD	DIET	NITROGEN INTAKE	NITROGEN IN FECES	NITROGEN ABSORBED	NITROGEN ABSORBED	NITROGEN IN URINE	NITROGEN RETAINED	NITROGEN RETAINED	WEIGHT
		mg/kg/day			*% intake*	*mg/kg/day*		*% intake*	*kg*
1	Basal	241	73	168	69.9	190	— 22	— 8.8	15.6
2	Basal	241	69	172	71.6	185	— 13	— 5.2	15.6
3	Basal + 0.56% lysine	239	68	171	71.5	183	— 12	— 5.0	15.5
4	Basal + 0.56% lysine	239	51	188	78.5	185	+ 3	+ 1.3	15.5
5	Basal + 0.30% lysine	239	52	187	78.4	196	— 9	— 3.5	15.5
6	Basal + 0.30% lysine	239	45	194	81.0	191	+ 3	+ 1.2	15.5
7	Basal + tryptophan + lysine	239	49	190	79.5	151	+ 39	+ 16.3	15.5
8	Basal + tryptophan + lysine	239	46	193	80.8	173	+ 20	+ 8.4	15.5
9	Basal + tryptophan + lysine + isoleucine	240	45	195	81.2	141	+ 54	+ 22.5	15.8
10	Basal + tryptophan + lysine + isoleucine	240	43	197	82.2	160	+ 37	+ 15.3	15.8
11	Basal + tryptophan + lysine + isoleucine + methionine	240	55	185	76.9	152	+ 33	+ 13.6	15.8
12	Basal + tryptophan + lysine + isoleucine + methionine	240	55	185	76.9	172	+ 13	+ 5.2	15.8
13	Basal	235	49	186	79.1	188	— 2	— 0.9	16.1
14	Basal	235	42	193	82.1	199	— 6	— 2.6	16.1

Case PC-57. This child differed from the others by being older and heavier. Thus the child's protein intake level of 1.5 gm per kilogram probably has a similar relation to requirement as did the 2.0 gm per kilogram for the two younger children. The results as shown in figure 3 are similar in general findings to those found in the previous trials. In this case, however, the diet was supplemented first with lysine instead of tryptophan. Adding sufficient lysine to bring the total of this amino acid up to the amount present in the "reference protein" pattern had a small positive effect on nitrogen retention, and reducing the lysine supplementation by roughly half did not result in any significant change. The addition of tryptophan to the basal diet plus lysine, however, produced a much greater response and the addition of isoleucine increased the net nitrogen retention to a level comparable to that which would have been expected with milk.

The addition of methionine again produced a distinct drop in nitrogen retention and retention became strongly negative when the child was returned to the basal diet. The data for individual three-day periods shown in table 3 reveal some variation between pairs but not enough to alter the conclusions drawn from the average of both. Nitrogen absorption varied from 70 to 82% in this series of trials.

DISCUSSION

The nitrogen retention data presented show that the responses of children fed corn-masa protein supplemented with various synthetic amino acid combinations are qualitatively the same at intake levels of 2.0 and 1.5 gm as those previously reported (Scrimshaw et al., '58) for experiments at 3.0 gm of protein per kilogram per day. However, at the intermediate levels of protein intake employed in the present study, the basal diet resulted in strongly negative balances in each case. As reported previously for higher protein intakes step-wise supplementation of the basal diet with tryptohan, lysine and isoleucine to match the amino acid pattern of the F.A.O.

"reference protein" ('57) resulted in step-wise increases in the average nitrogen retention for two three-day periods. In general, the second three-day period gave a higher retention than the first when the change resulted in a net improvement. Conversely, the second three-day period gave a lower retention when the change resulted in a net decrease as with the addition of methionine or the return to the basal diet alone.

It is clear from the data that single three-day periods are sufficient to indicate the direction of the response. However, these tendencies are emphasized by the use of two three-day periods. For most purposes it would be sufficient to determine nitrogen balance for three days after a 5-day adjustment period; the results of amino acid supplementation would be qualitatively the same as with the use of a two-day adjustment period but would present even greater quantitative contrasts. Therefore, the effects of amino acid supplementation on nitrogen retention are more consistent when the results of the second three-day balance period are examined. This observation was also noted by Barness et al. ('57) who state that less variable retentions are obtained in the second three-day interval of their two three-day period studies with children. From a physiological point of view, a better picture of the mechanism of protein absorption and utilization is obtained when information from the two periods is used separately.

Although the weight gains were not as consistent as previously reported from the higher level of protein intake, it is important to note that these children, consuming corn-masa protein as a sole protein source at a rate of 1.5 to 2.0 gm of protein per kilogram, showed no significant loss of weight. It is of considerable interest that the children lost more nitrogen on the basal diet at the levels used in this study than when 3.0 gm per kilogram per day were employed as in the first paper in this series. Similarly the addition of the amino acids tryptophan or lysine or both, did not have as great an effect on the actual amount of nitrogen retained as that reported previously for the higher levels of protein intake. It

is possible that adding a single amino acid to the level of the F.A.O. pattern ('57), without at the same time correcting deficiencies of other amino acids, could create a new imbalance; under such circumstance nitrogen retention could be stationary or even decreased. An imbalance associated with the addition of a single amino acid would be most apparent in diets low in nitrogen (Harper, '58). This may be an explanation for the poorer response to supplementation of the basal diet with tryptophan alone at the lower nitrogen intake of the present study.

The combination of tryptophan, lysine and isoleucine produced excellent results in two of the cases, PC-57 and PC-67. In the trials with PC-66, however, unlike the other experiments to date, this combination of amino acids did not bring the nitrogen retention near the range previously obtained with milk. We cannot at present offer any explanation for the consistently lower retention figures of this child with the basal diet and its supplemented combinations unless it reflects genetic variation, incomplete recovery, or some lingering damage from the severe disease which he has experienced. It may also be significant that the protein intake of this child was believed to be lower in proportion to his body needs than in any of the other trials.

In interpreting any of the results using children of varying ages recovering from malnutrition, it is important to note that a given level of protein intake may be high for one child, intermediate for another and low for a third depending on weight and state of nitrogen stores. It must also be noted that when these children begin the study they may still be relatively depleted in protein so that the percentage of nitrogen retained on a given intake slowly drops as nitrogen stores are replenished. This had been taken into account in both the experimental design and the interpretation of the data. The rate of this change varies greatly from child to child and disappears after prolonged treatment. As experience accumulates, it will perhaps be possible to analyze and generalize more on the quantitative significance of these changes result-

ing from amino acid supplementation. From a qualitative stand point, meanwhile, the results show that the supplementation of a cereal protein of relatively poor biological value such as corn with appropriate amino acids results in a consistent improvement in the nitrogen retention of young children fed the intermediate levels for their age and physiological state of 1.5 to 2.0 gm of protein, and 80 to 90 Cal. respectively per kilogram of body weight.

SUMMARY

Two boys aged 22 and 18 months, weighing 8.9 and 9.2 kg, respectively and recently recovered from severe protein malnutrition (kwashiorkor), were fed a simplified basal diet providing 2.0 gm of protein per kilogram in which corn-masa was the only protein source. A third post-kwashiorkor boy aged 4 years and 9 months and weighing 15.6 kg was given the basal diet at a level of 1.5 gm per kilogram per day. Nitrogen absorption and retention were measured with each combination for two successive three-day periods as this diet was supplemented step-wise with essential amino acids to match the amino acid pattern of the F.A.O. "reference protein." Initial iso-caloric, iso-proteic milk protein comparison periods were included for the two younger children.

At the levels of protein intake used, the single addition of either tryptophan or lysine did relatively little to restore the negative nitrogen balance occurring with the basal diet, but giving tryptophan and lysine together resulted in markedly increased nitrogen retention in each case. Nitrogen retention was further improved by isoleucine addition but decreased by methionine added at a level intended to match that of the "reference protein." Nitrogen absorption varied from 63 to 81%. The children tended to maintain or gain weight when nitrogen balance was positive and to lose weight slightly during or immediately following periods of negative balance.

The results show that even at an intermediate level of protein intake, the supplementation of vegetable protein with the

appropriate essential amino acids can result in good nitrogen retention by young children.

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THE EFFECT OF DIETARY PROTEINS ON THE LEVEL OF PLASMA CHOLINESTERASE OF RATS [1]

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INTRODUCTION

It is generally believed that the nutritional adequacy of a dietary protein is dependent upon the presence of sufficient amounts of the so-called essential amino acids. However, the studies of Woolley ('46) and Womach and Rose ('46) suggest that, in addition to the essential amino acids, some unknown nutritional factors may be needed for optimal growth. In another series of experiments, Chow ('50) found that the feeding of casein hydrolysate to dogs, depleted by prolonged protein-free feeding, stimulated the production of both plasma albumin and globulins, whereas the feeding of lactalbumin hydrolysate resulted in the regeneration of only plasma albumin. Fractionation of the casein hydrolysate yielded a crude fraction, which, when added in minute quantities to the lactalbumin hydrolysate, stimulated the production of both plasma albumin and globulins. These data indicate that, in addition to adequate amounts of the essential amino acids, some as yet unidentified factors present in dietary protein preparations are needed for optimal protein nutrition.

[1] The data of this paper are taken from a dissertation submitted by the author in partial fulfillment of the requirements for the degree of Doctor of Science in the Department of Biochemistry in the School of Hygiene and Public Health of Johns Hopkins University.

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Since all enzymes thus far isolated are proteins, it seems reasonable to expect that the synthesis of enzymes, such as plasma cholinesterase, could likewise be affected by the ingestion of dietary proteins. To test this hypothesis, weanling rats were fed diets containing various protein preparations for several months and the plasma cholinesterase activities of these groups of animals were determined. The results are presented in this communication.

EXPERIMENTAL

Animals. All rats were born and maintained in our animal quarters. Unless otherwise stated, 23-day-old rats with an average weight of 46 gm (range 35 to 65) for males and 42 gm (range 32 to 56) for females were employed. The animals were offered the various test diets (see below) and water ad libitum. They were housed in individual cages with large wire screens. All animals were weighed weekly for the first two months of the experimental period and bimonthly thereafter.

Following the feeding period all animals were bled by cardiac puncture under light ether anesthesia. The blood specimens were drawn into a heparinized syringe and immediately centrifuged. Plasma was quickly frozen and stored in a freezer at −25°F. until analyzed.

Composition of test diets. The diets contained 20% protein (Kjedahl nitrogen × 6.25), 12% corn oil,[3] 4% salts IV (Hegsted et al., '41), sucrose to make up 100% and adequate amounts of the known vitamins (Barrows et al., '52). In experiments in which the protein contents of the diets were increased, sucrose was withdrawn in amounts equal to the weight of the added protein preparation.

Determination of plasma cholinesterase. The cholinesterase activity in 0.3 ml of plasma was determined by the method of Ammon ('30) as modified by Mazur and Bodansky ('46). Duplicate determinations of cholinesterase activity were carried out on all experimental samples. In order to make the

[3] Mazola.

cholinesterase activity of samples determined on different days more comparable, all determinations were referred to a standard solution of a normal horse serum. The cholinesterase activity in 0.3 ml of this solution (mean = 316 mm^3 CO_2/hr., $\sigma = 12.8$, N(days) = 20) was assumed to be 1.0 rat plasma (RP) unit.

RESULTS

Reliability of cholinesterase determination. Duplicate determinations of cholinesterase activity were carried out on 53 different plasma samples. The enzymic activities of these samples ranged from 0.42 RP units/0.3 ml to 2.00 RP units/0.3 ml. In 45 of the 53 samples (approximately 90%) the duplicates did not differ from the mean value by more than $\pm 5\%$.

Stability of cholinesterase at −25°F. In order to determine the stability of cholinesterase during storage at −25°F., the enzymic activities of plasma samples obtained from 10 rats were determined immediately and after one month of storage. The cholinesterase activities of 9 of the 10 frozen samples did not vary by more than $\pm 5\%$ of the enzymic activities of the fresh samples. Since there was an equal distribution of negative and positive deviations, there is no evidence for a systematic change in the cholinesterase activity of plasma during storage under these conditions.

Individual variability. Five young adult female rats were bled on 5 consecutive days and the plasma cholinesterase activities of these samples were determined. The variation of the enzymic activities of samples obtained from individual rats was not greater than that of the method of analysis (table 1). Therefore this enzyme is a stable characteristic of individual adult female rats at least over the period tested.

Effect of various dietary proteins on plasma cholinesterase. An initial experiment indicated that feeding certain proteins results in significant differences in the plasma cholinesterase activities of rats after three months whereas the effect of the feeding of other proteins was demonstrable only after 5

months. Therefore, in a subsequent experiment all animals (90 males and 90 females) were fed the test diets for 5 months. Animals of each sex were divided into 9 groups of 10 animals each and fed one of the test diets. All diets contained 20% protein (Kjeldahl nitrogen $\times$ 6.25). The following 8 protein preparations were used: wheat gluten, wheat gluten supplemented with lysine and methionine,[4] soybean meal, casein, egg albumin, lactalbumin, whole desiccated liver, and benzol-extracted beef muscle. In addition, the stock ration of our laboratory (Barrows et al., '52) was fed to the 9th group of animals. The animals were bled on the 150th day of feeding. The growth rates of the female and male rats are shown in figures 1 and 2

TABLE 1

Daily variations in the plasma cholinesterase [1] of adult female rats

RAT	MEAN	OBSERVED RANGE [2]	CALCULATED RANGE [3]
1	1.36	1.53 — 1.24	1.43 — 1.29
2	1.06	1.17 — 0.96	1.11 — 1.01
3	1.01	1.14 — 0.86	1.06 — 0.97
4	1.88	2.05 — 1.82	2.07 — 1.79
5	2.09	2.15 — 2.04	2.21 — 1.98

[1] Cholinesterase in RP units/0.3 ml of plasma.
[2] Enzymic activity determined on 5 consecutive days.
[3] Based on $\pm$ 5% of mean value.

respectively. The growth of the animals varied according to the nutritive quality of dietary protein preparation offered. For example, both male and female rats fed the diets containing wheat gluten or whole desiccated liver grew poorly in comparison with animals fed diets containing any of the other proteins of higher biologic value. The growth responses to the latter proteins were indistinguishable in the female but not in the male rats. For example, male animals fed the stock diet or the diets containing egg albumin or soybean did not

[4] Supplemented in amounts to equal the lysine and methionine contents of the casein diet.

grow as well as the rats fed the 4 remaining diets. These results may be explained in part on the basis of an inadequate supplementation of biotin to the egg albumin diet and insufficient amounts of vitamin B_{12} present in the soybean diet.

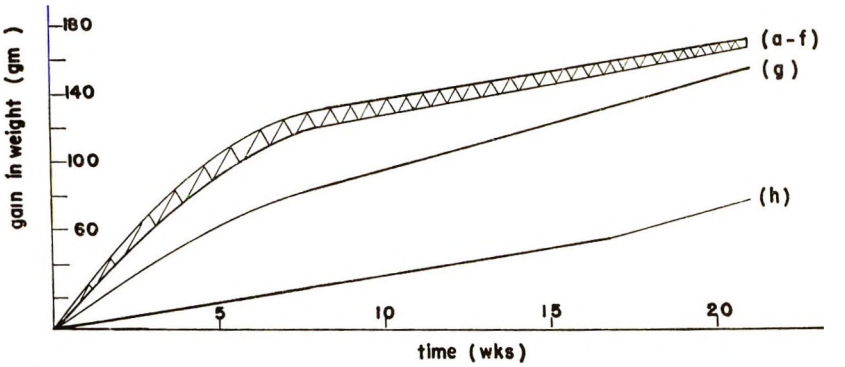

Fig. 1 Growth of female rats fed various proteins. Diets: a = beef muscle; b = casein; c = wheat gluten supplemented; d = lactalbumin; e = stock; e' = soybean; f = egg albumin; g = whole desiccated liver; h = wheat gluten.

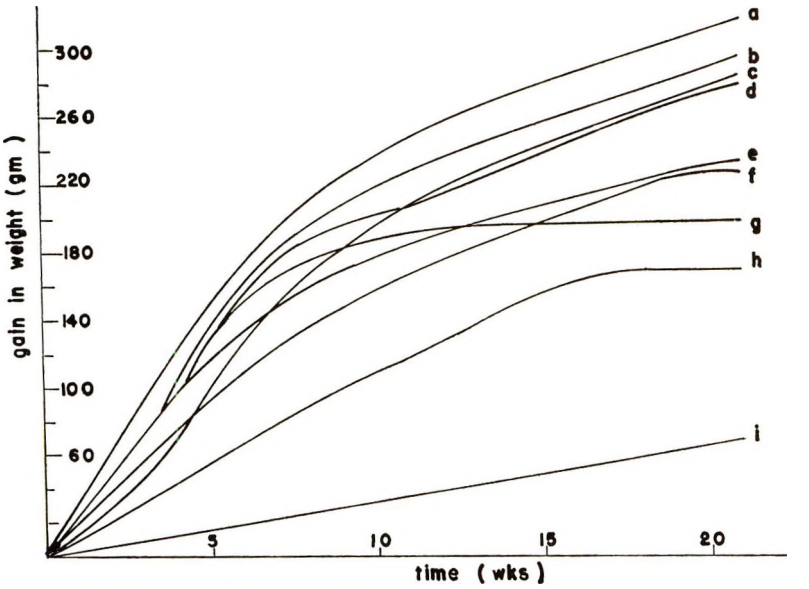

Fig. 2 Growth of male rats fed various proteins. Diets: a = beef muscle; b = casein; c = wheat gluten supplemented; d = lactalbumin; e = stock; f = soybean; g = egg albumin; h = whole desiccated liver; i = wheat gluten.

The ingestion of various dietary proteins results in marked differences in the cholinesterase levels in the plasma of female rats (table 2). For example, weanling female rats fed a poor protein, wheat gluten, had both inferior growth rates and low plasma cholinesterase activities. The addition of lysine and methionine to the diet improved the growth rates and at the same time elevated the levels of the enzyme. Furthermore, the plasma cholinesterase activities of female rats fed diets containing proteins which support adequate growth do

TABLE 2

The effect of dietary proteins on the plasma cholinesterase of rats

DIETARY PROTEIN PREPARATIONS [1]	PLASMA CHOLINESTERASE (RP UNITS/0.3 ML)	
	Females	Males
Wheat gluten	0.60 ± 0.04 [2]	0.56 ± 0.04
Wheat gluten plus supplement [3]	1.30 ± 0.10	
Soybean meal	1.18 ± 0.11	
Casein	1.31 ± 0.07	0.70 ± 0.04
Stock diet	1.36 ± 0.13	
Egg albumin [4]	1.40 ± 0.10	
Whole desiccated liver	1.42 ± 0.15	
Lactalbumin	1.59 ± 0.11	0.67 ± 0.05
Beef muscle	1.88 ± 0.07	0.62 ± 0.03

[1] All diets contained 20% protein (Kjeldahl N × 6.25).

[2] Mean and $S.E._M$.

[3] Supplement contained 17.6 gm L-lysine·HCl·H_2O and 3.5 gm L-methionine per kilogram of diet.

[4] Supplemented with 1 mg biotin.

not differ significantly from that of the animals fed the diet containing casein. The outstanding exception to this is the high level of the cholinesterase activity in the plasma of female rats fed the beef muscle diet. It is difficult to ascertain from the growth curves in figure 1 whether there are differences in growth of the female rats fed diets containing proteins of high nutritive value. Therefore, in order that this increment may not be attributed to differences in the protein quality between beef muscle and proteins such as casein and egg albumin, a comparison of the gain in body weight of

female rats fed diets containing these proteins for three, 5 and 20 weeks is shown in table 3. No significant differences in the growth of these animals were observed. Thus, even though the ages, body weights and growth rates were indistinguishable, the plasma cholinesterase activity was higher in the females fed the beef muscle diet. It is interesting to note that in spite of the impairment of growth the enzymic activity of the female rats fed the whole desiccated liver diet was essentially equal to that of animals fed the casein diet.

No marked differences in the plasma cholinesterase activities of male rats were observed (table 2), although the growth

TABLE 3

The effect of feeding protein preparations of high nutritive value on the growth of female rats

DIETS	GAIN IN BODY WEIGHT		
	3 wks.	5 wks.	20 wks.
	gm	*gm*	*gm*
Casein	64.2 ± 2.9 [1]	100.7 ± 2.3	165.0 ± 6.2
Egg albumin	69.0 ± 2.8	100.0 ± 2.2	167.0 ± 3.8
Beef muscle	69.0 ± 3.6	105.4 ± 4.8	170.0 ± 4.2

[1] Mean and $S.E._M$.

rates of the animals differed strikingly. In general, the plasma cholinesterase activity of male rats is markedly lower than that of the females fed the same diet. The exception to this is the enzymic activity of the animals fed the wheat gluten diet. This lack of sex difference may be attributed to the low level of plasma cholinesterase of the female rats.

Effect of the supplementation of a casein diet with various protein preparations on the plasma cholinesterase levels of female rats. In the following experiment the 20% casein diet used previously served as the basal diet to which beef muscle, whole desiccated liver, egg albumin or additional casein was added. The results of this experiment (table 4) demonstrate that the gain in body weight of the animals fed the various diets for 150 days was essentially the same regardless of the

protein supplement or the percentage of protein in the diet. However, marked differences in the plasma cholinesterase activity of the various groups were observed. For example, supplementation of the basal casein diet with beef muscle or whole desiccated liver elevated the level of the enzymic activity. Although feeding a diet supplemented with 4% of whole desiccated liver resulted in a significantly increased plasma cholinesterase activity, 13% of beef muscle was needed to bring about an equivalent increment. Therefore, whole

TABLE 4

The effect of supplementing a casein diet with dietary proteins on the plasma cholinesterase of female rats

DIETS	PROTEIN [1]	GAIN IN BODY WEIGHT [2]	PLASMA CHOLIN-ESTERASE
	%	*gm*	*RP units/ 0.3 ml*
24% Casein	20	189 ± 8.6 [3]	1.32 ± 0.10
38% Casein	32	181 ± 6.1	1.17 ± 0.09
24% Casein + 15% egg albumin [4]	32	186 ± 6.2	1.27 ± 0.05
24% Casein + 3% beef muscle	23	180 ± 6.0	1.31 ± 0.09
24% Casein + 13% beef muscle	32	188 ± 7.0	1.58 ± 0.07
24% Casein + 4% whole desiccated liver	23	194 ± 6.9	1.60 ± 0.09
24% Casein + 17% whole desiccated liver	32	208 ± 4.0	1.95 ± 0.14
21% Beef muscle	20	174 ± 6.3	1.74 ± 0.09

[1] Protein content based on Kjeldahl N × 6.25.
[2] After 150 days of feeding.
[3] Mean and $S.E._M$.
[4] Supplemented with 1 mg of biotin.

desiccated liver is believed to be richer than beef muscle in the nutrients responsible for this effect. The increase in the enzymic activity is not due to the protein content of the diet since increasing the casein to 38% did not elevate the plasma cholinesterase level. Furthermore, it seems unlikely that this increment in enzyme level results from changing the pattern of amino acids in the diet by the supplementation of another protein since the addition of egg albumin to the basal casein diet was ineffective. Increasing the whole desiccated liver supplement approximately fourfold (from 4% to 17%) resulted

in only slightly more than a twofold additional increase in the enzymic activity over that of the animals fed the basal diet (0.28 RP units/0.3 ml to 0.65 RP units/0.3 ml). These results suggest that a concentration of enzymic activity is approached which can no longer be effectively increased by dietary means. Furthermore, again the differences in enzymic activities cannot be attributed to the quality of the protein ingested as indicated by the increment in weight. The gain in body weight of the animals fed the diet containing 24% of casein was not significantly different from that of those offered the diet supplemented with beef muscle or whole desiccated liver, although marked differences in the plasma cholinesterase activities existed.

Other testing procedures. Testing the activity of various liver fractions in an attempt to isolate the active ingredient was impractical by the procedure thus far described due to the long feeding period and the large amount of material needed. Two approaches to this problem were investigated. The first was based on the observation that the level of plasma cholinesterase of female rats appears to be related to the estrogen level (Sawyer and Everett, '46; Everett and Sawyer, '46) and, therefore, animals approaching sexual maturity may be more responsive to the test diets than weanling rats. Thus, this method (method A) employs 45- to 65-day-old female rats. Prolonged protein-free feeding is known to deplete animals of various enzymes (Millman, '51; Wainio et al., '53) and the repletion of these tissue components can be brought about by feeding adequate diets (Miller, '50; Litwak et al., '50). Therefore, the second method (method B) measures the effect of the test diets on the rate of return of cholinesterase activity in the plasma of mature female rats following a period of protein depletion.

Method A. In the first experiment 24 45-day-old female rats were divided equally into two groups and fed either the 24% casein or 21% beef muscle diet. After 30 days of feeding the plasma cholinesterase activities of these two groups were

found to be 0.71 ± 0.04 RP units/0.3 ml and 0.92 ± 0.07 RP units/0.3 ml respectively. On the basis of the difference in enzyme levels, an additional experiment was carried out in which 65-day-old female rats were fed the test diets for 60

TABLE 5

The effect of the ingestion of casein or beef muscle on the plasma cholinesterase of 65-day-old female rats

DIET [1]	PROTEIN [2]	30 DAYS OF FEEDING		60 DAYS OF FEEDING	
		GAIN IN BODY [3] WT.	PLASMA CHOLIN-ESTERASE	GAIN IN BODY WT.	PLASMA CHOLIN-ESTERASE
	%	*gm*	*RP units/ 0.3 ml*	*gm*	*RP units/ 0.3 ml*
24% Casein	20	54 ± 7.1 [4]	1.22 ± 0.10	69 ± 7.0	1.29 ± 0.10
38% Casein	32	45 ± 2.5	1.01 ± 0.10	63 ± 3.8	1.32 ± 0.13
21% Beef muscle	20	60 ± 6.7	1.49 ± 0.08	77 ± 7.9	1.70 ± 0.14
34% Beef muscle	32	51 ± 6.2	1.52 ± 0.10	76 ± 4.2	1.72 ± 0.05

[1] All diets contained 300 μg vitamin B_{12} per kilogram.
[2] Protein content based on Kjeldahl N × 6.25.
[3] Six animals per group with initial body wt. of 149 ± 3.1 gm.
[4] Mean and $S.E._M$.

TABLE 6

The effect of dietary proteins on the level of plasma cholinesterase of rats depleted in proteins

DIET	PROTEIN [2]	PLASMA CHOLINESTERASE [1]		
		Depleted	14th day of repletion	28th day of repletion
	%			
24% Casein (1.72 ± 0.13) [3]	20	32 ± 6 [4]	51 ± 1	64 ± 6
24% Casein + vitamin B_{12} [5] (1.68 ± 0.18)	20	32 ± 4	51 ± 3	65 ± 7
21% Beef muscle (1.77 ± 0.12)	20	32 ± 5	62 ± 4	76 ± 5

[1] Based on initial level taken as 100%.
[2] Protein content based on Kjeldahl N × 6.25.
[3] Initial levels of cholinesterase.
[4] Mean and $S.E._M$.
[5] Vitamin B_{12}, 300 μg per kilogram of diet.

days. Vitamin B_{12} was added to all diets. The animals were bled on the 30th and 60th days of feeding and the concentration of plasma cholinesterase was determined. These data (table 5) demonstrate that feeding a 21% beef muscle diet

to 65-day-old female rats for 30 or 60 days results in significantly higher plasma levels of the enzyme. Increasing the percentage of beef muscle in the diet to the 34% level did not further elevate the level of plasma cholinesterase.

Method B. In this experiment the animals were bled initially and following the period of protein-free feeding (45 days). On the 14th anl 28th day of feeding the test diets the rats were again bled. Feeding a protein-free diet resulted in a marked reduction in the plasma cholinesterase activity (table 6). Furthermore, the repletion of the enzymic activity in the plasma of animals fed the beef muscle diet was more rapid than in the animals fed the casein diet regardless of the addition of vitamin B_{12}.

All these procedures reduce the time of feeding necessary to demonstrate differences in the plasma cholinesterase levels of rats fed various protein preparations. However, the method in which 65-day-old rats are fed the test diets for 30 or 60 days is the one of choice, since this procedure yielded greater differences in enzymic activities. The disadvantages of the depletion-repletion method are: (1) the additional bleedings and enzyme determinations and (2) the minimum feeding period of 75 days. Two additional points of interest are found in these data. This phenomenon can be demonstrated under another experimental condition, namely, repletion of the enzymic activity in mature protein-depleted female rats. In addition, these data offer no evidence that the nutrients which stimulate the elevation of the plasma cholinesterase level are related to vitamin B_{12}.

Assay for active fraction in whole desiccated liver. In these experiments 65-day-old female rats were fed the basal casein diet (24%) to which various liver fractions were added. After 60 days of feeding the plasma cholinesterase activities were determined. In order that observed differences in feeding the various liver fractions might not be attributable to differences in the protein or vitamin contents of the diets, an experiment was carried out in which one group of animals was fed a diet of 24% casein plus 17% whole desiccated liver and the other

group fed a 38% casein diet containing an amount of the water-soluble vitamins equal to that of the former diet. The level of plasma cholinesterase of female rats fed the casein diet supplemented with additional vitamins failed to equal

TABLE 7

The effect of the ingestion of whole desiccated liver on the plasma cholinesterase of female rats

DIETS	PROTEIN [1]	INITIAL BODY [2] WT.	GAIN IN BODY WT.	PLASMA CHOLIN-ESTERASE
	%	*gm*	*gm*	*RP units/ 0.3 ml*
38% Casein [3]	32	188 ± 4.5 [4]	59 ± 2.0	1.22 ± 0.06
24% Casein + 17% whole desiccated liver	32	186 ± 8.8	59 ± 2.0	1.83 ± 0.11

[1] Protein content based on Kjeldahl N × 6.25.

[2] Six animals per group.

[3] Additional vitamins added per kilogram diet: 17.0 mg riboflavin, 68.0 mg niacin, 2.6 mg pyridoxine HCl, 34.0 mg calcium pantothenate, 2560 mg choline chloride, 3.4 mg folic acid, 60 mg inositol, 2.3 mg thiamine, 120 μg vitamin B_{12}.

[4] Mean and $S.E._M$.

TABLE 8

The effect of the ingestion of liver fractions on the plasma cholinesterase of female rats

DIETS	NO. OF RATS	INITIAL BODY WT.	GAIN IN BODY WT.	CHOLIN-ESTERASE
		gm	*gm*	*RP units/ 0.3 ml*
24% Casein	6	174 ± 5.9 [1]	54 ± 4.7	1.38 ± 0.12
24% Casein + 17% WDL [2]	6	181 ± 5.4	49 ± 4.7	1.77 ± 0.08
24% Casein + 3% LC [3]	7	175 ± 7.3	47 ± 7.1	1.77 ± 0.10
24% Casein + 14% LR [4]	7	176 ± 4.7	56 ± 4.8	1.50 ± 0.06

[1] Mean and $S.E._M$.

[2] WDL = Whole desiccated liver.

[3] LC = Liver concentrate.

[4] LR = Liver residue.

that of animals fed a diet containing whole desiccated liver (table 7). Therefore, it seems unlikely that the nutrients present in whole desiccated liver responsible for this phenomenon are identical to any of the known water-soluble vitamins. In the second experiment two fractions obtained from whole

liver, namely, concentrate (LC) and liver residue (LR), were assayed for their ability to increase the level of the enzyme. Liver residue (LR) is the hot-water-insoluble fraction which remains after extraction to remove liver concentrate (LC). Each 17 gm of whole desiccated liver yielded 14 gm of liver residue (LR) and 3 gm of liver concentrate (LC). Feeding a 24% casein diet supplemented with 3% of liver concentrate (LC) but not 14% liver residue (LR) increased the level of plasma cholinesterase to that of animals fed the diet supplemented with 17% of whole desiccated liver (WDL) (table 8).

DISCUSSION

The plasma cholinesterase level is a stable characteristic of young adult female rats over a short period of time. However, the concentration of the enzymic activity increases during growth. For example, in these various experiments the mean cholinesterase activity of the plasma of 75-, 90- and 120-day-old female rats fed a 20% casein diet was 0.71 ± 0.04, 1.22 ± 0.10 and 1.29 ± 0.10 RP units/0.3 ml respectively. It may be observed that the increases in the enzymic activity diminished as the growth rate was retarded. These results are in agreement with the findings of Sawyer and Everett ('46) and Mundell ('44), which demonstrate a higher cholinesterase activity in the plasma of mature as compared to immature female rats. Moreover, the plasma cholinesterase of female rats is markedly affected by the dietary protein preparation ingested.

The higher plasma cholinesterase activity of the animals fed beef muscle in comparison with those fed casein cannot be explained on the basis of hemoconcentration, since the concentrations of plasma proteins of the two groups in the first experiment were 6.05 ± 0.16 gm/100 ml and 5.80 ± 0.20 gm/100 ml, respectively. In addition, the experimentally determined cholinesterase activities of mixtures of plasma of rats fed the casein and beef muscle diets were equal within experimental error to the sum of the activities of each plasma

sample. Thus, no evidence is available to indicate that the observed differences in the plasma cholinesterase activities may be attributed to differences in the concentration of activators or inhibitors. Since the differences attributed to diet were equally as great when the enzymic activity was determined by a colorimetric (de la Heurga et al., '52) rather than by the manometric method, these results are not merely artifacts of the method of analysis. Therefore, it seems likely that the increased plasma cholinesterase activity represents an increased amount of the enzyme *per se*.

However, two forms of cholinesterase, namely, true and pseudocholinesterase exist in the plasma of rats (Everett and Sawyer, '47). The relative amounts of these enzymes have been found to be dependent upon sex (Everett and Sawyer, '47) and the greater amount of plasma cholinesterase activity in mature females in comparison with mature males or immature females has been attributed to a higher level of pseudocholinesterase. The method used in the present studies determines primarily pseudocholinesterase, although true cholinesterase is not completely inhibited. Therefore, the lack of a sex difference in the concentration of plasma cholinesterase of animals fed wheat gluten for 5 months indicates an unusually low level of pseudocholinesterase in these female rats. Furthermore, the effect of the ingestion of various dietary protein preparations on plasma cholinesterase activities was not evident in male animals. Taken as a whole these results suggest that the amount of plasma pseudocholinesterase rather than true cholinesterase is increased by the ingestion of certain dietary protein preparations. Finally, it should be pointed out that the effect of certain dietary proteins on the plasma cholinesterase activity of female rats is not restricted to the young growing animals since similar effects have been demonstrated in protein depleted adult female rats.

The feeding of an incomplete dietary protein, wheat gluten, resulted in low enzymic activities as well as poor growth rates. Supplementation of this diet with the essential amino acids,

lysine and methionine, to equal the levels found in casein, or feeding a complete dietary protein such as casein or egg albumin improved the growth rates and increased the levels of plasma cholinesterase. Therefore, adequate amounts of the essential amino acids are a prerequisite for the production of plasma cholinesterase. However, differences attributable to the ingestion of certain dietary protein preparations could not be explained on the basis of differences in the total amount or pattern of the essential amino acids. For example, supplementation of a 24% casein diet with 4% of whole desiccated liver resulted in a significantly increased level of plasma cholinesterase whereas supplements of 14% of casein or 15% of egg albumin were ineffective.

The nature of the nutrients responsible for this phenomenon is unknown. However, since the beef muscle was treated by extraction with benzol and contained little fat by analysis, the active material is probably not a fat-soluble component of the diet. In addition, there is no evidence that any of the known water-soluble vitamins are responsible for this phenomenon. Although amounts of the inorganic salts known to be required for adequate nutrition were included in all diets, experiments were not carried out to test other ions such as molybdenum which has been demonstrated to be essential for the synthesis of xanthine oxidase (DeRenzo, '54; Westerfeld and Richert, '54). Thus, these data indicate that the ingestion of some, as yet unknown, factors found in beef muscle and whole desiccated liver results in an increased plasma cholinesterase activity of female rats. Efforts to fractionate the active substance from whole liver showed that it was in the hot-water-soluble fraction.

CONCLUSIONS

The concentration of plasma cholinesterase of female but not of male animals was found to differ according to the protein preparation offered in the diet. Animals fed wheat gluten had low plasma cholinesterase levels. The addition

of lysine and methionine to this diet elevated the level of the enzymic activity. Although the growth rates of the female rats fed protein preparations of high nutritive quality were indistinguishable, the plasma cholinesterase activities of the animals fed diets containing beef muscle or whole desiccated liver were markedly higher than those of animals fed other protein preparations. This increased concentration of plasma cholinesterase activity is believed to be due to the presence of some factors present in beef muscle and whole desiccated liver rather than to differences in the amino acid contents of the dietary proteins. Fractionation of whole liver yielded a fraction which when added to a 24% casein diet at a level of only 4% resulted in a definite elevation of plasma cholinesterase activity.

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STUDIES OF AMINO ACID SUPPLEMENTATION AND AMINO ACID AVAILABILITY WITH OATS [1]

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INTRODUCTION

Mitchell and Smuts ('32) demonstrated that the primary amino acid deficiency of oat protein was lysine. However, to assess the nutritive value of oat protein when fed with other protein sources or with amino acid supplements it will be necessary to know what other essential amino acids are limiting.

The studies of Pecora and Hundley ('51) with rice and those of Sauberlich, Chang and Salmon ('53) with corn show that the amino acid deficiencies of a grain can not be predicted from the amino acid levels found in the protein. It is necessary that the grain be fed to experimental animals with amino acid supplements to determine which amino acids will improve the utilization of the protein.

Tormo ('57) demonstrated that a supplement of lysine, methionine and threonine to an oat diet caused more efficient protein utilization than a supplement of lysine alone. The present study was designed to demonstrate the relationships among these three amino acids and to determine if other amino acid supplements would improve the nutritive value of oat protein. The biological availability of lysine, methionine and threonine was also studied to determine if poor availability might be the cause of the apparent amino acid deficiencies.

[1] Published with the approval of the Director of the Oklahoma Agricultural Experiment Station.

EXPERIMENTAL

The oats used in these studies were supplied by the Agronomy Department of Oklahoma State University. The oats used in the experiment presented in tables 1 and 2 were mixtures of winter oat varieties which were known to contain protein of high nutritive value (Weber, Thomas, Reder, Schlehuber and Benton, '57). The oat sample used in the experiments presented in tables 3 and 5, which is designated as "oat mixture," was a mixture of equal quantities of Cimarron, Forkedeer and Tennex varieties.

The whole oats were ground in a Wiley Mill before being incorporated into the diets. Each diet contained, in grams per 100 gm of diet: salts IV (Hegsted, Mills, Elvehjem and Hart, '41), 4; corn oil, 5; and choline chloride, 0.15. Vitamins were added to provide, in milligrams per 100 gm of diet: thiamine, 0.4; riboflavin, 0.6; pyridoxine hydrochloride, 0.3; nicotinic acid, 2.0; calcium pantothenate, 2.0; inositol, 2.0; folic acid, 0.05; vitamin B_{12}, 0.002; biotin, 0.01 and 2-methyl, 1-4 naphthoquinone, 0.05. Each rat received orally two drops weekly of a supplement which contained 15,000 U.S.P. units of vitamin A, 1700 U.S.P. units of vitamin D and 20 mg of α-tocopherol per gram. The remainder of each diet was made up of the protein source and sucrose. When supplements were added to the diets they replaced equal amounts of sucrose.

Weanling male rats of the Sprague-Dawley strain weighing from 40 to 50 gm were used in the growth studies. The rats were divided into groups of 6 animals each and were housed in individual cages with raised screen bottoms. Diets were supplied to the rats ad libitum. The experimental period in experiment 1 (table 1) was three weeks while in all other experiments it was 4 weeks.

To measure the availability of certain amino acids from oat protein, the method of Carrol, Hensley and Graham ('52) was used. Male white rats of the Sprague-Dawley strain weighing from 200 to 300 gm were used in these studies. The diets contained the same levels of salts and vitamins as those used in

the growth studies. The samples of DeSoto oats and the oat mixture were the same as used in the experiment presented in table 3. They were incorporated as 94% of the diet, omitting the corn oil and sucrose used in the growth studies. Chromic oxide, as 2% of the diet, was added to serve as an index of digestibility. The animals were fed this diet for three or 4 days and were then killed with ether and the contents of the last two inches of the small intestine was collected. The intestinal contents from three rats fed the same diet were pooled and immediately frozen. This material was dried in the frozen state under vacuum. Three pooled samples were prepared from animals receiving the same diet and the results from these samples are referred to in table 5 as experiments 1, 2 and 3.

The endogenous amino acids in the intestinal contents were estimated by feeding a low-nitrogen diet (9 mg N/100 gm) in which cornstarch replaced the oats. The samples were collected in the same manner as above but it was necessary to use the intestinal contents from 6 rats instead of three to obtain a satisfactory amount for analysis.

Each of the three diets and each of the samples of intestinal contents were analyzed for chromic oxide by the method of Bolin, King and Klosterman ('52). Also, samples were hydrolyzed with 3 N HCl (approximately 0.04 ml of acid to 1 mg of protein) by autoclaving at 120°C for 16 hours. Nitrogen was determined on the hydrolyzates by the micro Kjeldahl method of Ma and Zuazaga ('42). The hydrolyzates were analyzed for lysine, methionine and threonine by a modification of the method of Henderson and Snell ('48) in which the sodium salts of the medium were replaced by equivalent amounts of potassium salts.[2] *Leuconostoc mesenteroides* was used for the determination of lysine and methionine while *Streptococcus faecalis* was used for the determination of threonine.

The coefficients of "true digestibility" were calculated by the formula, $(A - B + C)/A \times 100 =$ coefficient of true di-

[2] R. J. Sirny, unpublished data.

gestibility, where A is the ratio of nutrient to chromic oxide for the oat diet, B is the ratio for the intestinal contents of the rats fed the oat diet and C is the ratio for the intestinal contents of the rats fed the low-protein diet.

The individual growth studies were analyzed by the multiple range test of Duncan ('55). The coefficients of digestibility were analyzed by an analysis of variance according to the method of Snedecor ('46).

RESULTS

The weight gains of rats fed oat diets supplemented with lysine, methionine and threonine are summarized in table 1. In both experiments significant growth responses ($p < 0.01$) were obtained when lysine was added to the oat diet. A supplement of methionine to the oat diet caused a statistically significant ($p < 0.01$) depression in experiment 1. In experiment 2 the weight gain of the group fed methionine was the same at the end of 4 weeks as that of the group fed the unsupplemented oat diet although at two weeks a growth depression

TABLE 1

Effect of amino acid supplements on the weight gains of rats fed oat diets

GROUP NO.	PROTEIN SOURCE	L-LYSINE [1]	DL-METHIONINE	DL-THREONINE	WEIGHT GAIN [2]	
					Experiment 1	Experiment 2
		%	%	%	*gm/wk*	*gm/wk*
1	85% oats [3]	—	—	—	19.3 ± 0.7	25.5 ± 1.7
2	85% oats	0.60	—	—	23.4 ± 1.3	33.5 ± 1.1
3	85% oats	—	0.50	—	14.1 ± 0.8	25.8 ± 1.6
4	85% oats	—	—	0.75	19.7 ± 0.8	29.5 ± 0.9
5	85% oats	0.60	0.50	—	24.7 ± 1.4	33.8 ± 1.2
6	85% oats	0.60	—	0.75	22.3 ± 1.2	
7	85% oats	—	0.50	0.75	16.0 ± 0.3	
8	85% oats	0.60	0.50	0.75	26.4 ± 0.6	38.4 ± 1.5

[1] Fed as L-lysine·monohydrochloride.

[2] The mean ± the standard error of the mean of groups of 6 rats for an experimental period of three weeks in experiment 1 and 4 weeks in experiment 2.

[3] The oats (85%) supplied 2.08% nitrogen in experiment 1 and 1.99% nitrogen in experiment 2.

was apparent. A reduction in the weight gain was also obtained when methionine and threonine were added to the diet in experiment 1. This was probably caused by the methionine supplement, for a threonine supplement alone did not effect the growth rate of the rats. When lysine, methionine and threonine were added to the basal oat diet the weight gain was greater than that of the other groups which received the oat diets. In both experiments statistically significant ($p < 0.05$) differences were obtained between the growth rates of the group fed lysine, methionine and threonine and that of the

TABLE 2

Weight gains of rats fed various levels of oats supplemented with amino acids

GROUP NO.	PROTEIN SOURCE	L-LYSINE [1]	DL-METHIONINE	DL-THREONINE	WEIGHT GAIN [2]
		%	%	%	*gm/wk*
1	55% oats [3]	0.40	0.32	0.49	26.8 ± 1.2
2	70% oats	0.50	0.41	0.62	38.2 ± 0.9
3	85% oats	0.60	0.50	0.75	37.0 ± 0.5
4	Diet fed group 1 + 0.13% DL-tryptophan and 0.24% L-histidine·HCl				33.4 ± 1.1
5	20% casein	—	—	—	37.8 ± 2.1

[1] Fed as L-lysine·monohydrochloride.

[2] The mean ± the standard error of the mean of groups of 6 rats for an experimental period of 4 weeks.

[3] The oats used in this experiment contained 2.34% nitrogen.

group which received lysine alone. It was apparent from these results that the oat diet supplemented with lysine, threonine and methionine was a protein source which would support rapid growth in the rat.

To test further the adequacy of this protein source the amount of the protein in the diet was reduced and comparison was made with rats fed a 20% casein diet. The results of this experiment are presented in table 2. The levels of the amino acid supplements were reduced in proportion to the reductions in the level of oats to maintain the same balance of amino acids. The group fed 70% of oats with the amino acid supplements gained weight at the same rate as the groups fed 85%

of oats with similar supplements. The 70% oat diet contained 1.84% of nitrogen. The weight gains of these two groups were the same as that of the group which received the diet containing 20% of casein. Reducing the level of oats to 55% and the level of nitrogen to 1.45% caused a significant depression in the weight gain ($p < 0.01$). The addition of tryptophan and histidine to this diet caused a marked growth response ($p < 0.01$). In an experiment not presented in the tables no growth response was obtained by adding tryptophan and histidine to the 85% oats diet with added lysine, methionine and threonine.

TABLE 3

Growth responses of rats to amino acid supplements when fed oats of different nutritive values

GROUP NO.	PROTEIN SOURCE	L-LYSINE [1]	DL-METHIONINE	DL-THREONINE	WEIGHT GAIN [2]
		%	%	%	*gm/wk*
1	85% DeSoto oats [3]	—	—	—	19.2 ± 1.2
2	85% DeSoto oats	0.60	—	—	23.0 ± 1.1
3	85% DeSoto oats	0.60	0.50	0.75	34.7 ± 0.6
4	78.8% oat mixture [3]	—	—	—	22.1 ± 0.5
5	78.8% oat mixture	0.60	—	—	28.5 ± 0.7
6	78.8% oat mixture	0.60	0.50	0.75	37.6 ± 0.7
7	20% casein	—	—	—	37.3 ± 1.1

[1] Fed as L-lysine monohydrochloride.

[2] The mean ± the standard error of the mean of groups of 6 rats for an experimental period of 4 weeks.

[3] The oats in diets 1 to 6 supplied 1.86% of nitrogen.

The growth responses to amino acid supplements were studied with two oat samples of different nutritive values in hopes that the cause of the difference might be learned. The weight gains are presented in table 3 and the analyses of the oat samples for nitrogen, lysine, threonine and methionine are presented in table 4. The DeSoto oat variety was previously shown to contain protein of lower nutritive value than other oat varieties (Weber et al., '57). The levels of the two oat samples were adjusted to give the same level of nitrogen supplied by oats in each diet.

The group fed the oat mixture showed a greater weight gain than the group fed the DeSoto oats when no amino acid supplements were added to the diets ($p < 0.01$). The lysine supplement caused marked growth responses with both oat samples ($p < 0.01$). Both groups which received lysine, methionine and threonine gained more weight ($p < 0.01$) than the comparable group which received lysine alone. In both cases the group fed the oat mixture gained more weight ($p < 0.05$) than that fed the DeSoto oats and the same amino acid supplement. From these results it would appear that each of the growth-limiting amino acids is more deficient in the DeSoto oat diet. However, the amino acid analyses (table 4) show that the oats in the diets would supply the same levels of each of these amino acids.

TABLE 4

Nitrogen and amino acid levels in oats

	PROTEIN	LYSINE	METHIONINE	THREONINE
	$\frac{mg\ N \times 6.25}{100\ mg}$	*gm/16 gm N*	*gm/16 gm N*	*gm/16 gm N*
DeSoto Oats [1]	13.1	4.6	1.7	2.6
Oat mixture [1]	13.5	4.4	1.7	2.8

[1] These are the oat samples used in the experiment presented in table 3.

The availabilities of the lysine, methionine and threonine from oats were investigated to determine if availability was a factor in the amino acid deficiencies of oats or in the differences in nutritive value. The digestibility coefficients were determined by analysis of contents of the small intestine to avoid the bacterial destruction or synthesis of amino acids which might occur in the large intestine. The results are presented in table 5.

It is apparent from the analysis of variance presented with table 5 that there were no statistically significant differences in the coefficients of digestibility among the three experiments or between the oat mixture and the DeSoto oats. It is of interest that the mean coefficients of digestibilities were higher

in each case for the DeSoto oats although it was of lower nutritive value.

Since no significant differences were found between diets in the coefficients of digestibility for amino acids or nitrogen it appears valid to consider as representative of oats the coefficients of digestibility obtained from the two diets. The value in the analysis of variance under treatments indicates

TABLE 5

Coefficients of digestibility of amino acids of oats

OAT SAMPLE	EXPERIMENT [1]	COEFFICIENT OF DIGESTIBILITY			
		Nitrogen	Lysine	Threonine	Methionine
Winter mixture	1	83	85	74	92
	2	87	78	60	82
	3	88	79	75	78
DeSoto	1	88	87	77	91
	2	90	86	80	93
	3	82	91	65	72
Mean winter mixture		86	81	70	84
Mean DeSoto		87	88	74	85
Mean all values		86	84	72	85

Analysis of variance

SOURCE	DEGREES OF FREEDOM	MEAN SQUARE	F
Between experiments	2	69	1.6
Between diets	1	70	1.6
Between treatments	3	269	6.4 [2]
Diets × treatments	3	14	.3
Error	14	42	

[1] Each experiment is the result of analysis of the pooled sample from three rats.
[2] Significant at the 1% level.

that a highly significant difference ($p < 0.01$) exists between the mean coefficients of digestibility of nitrogen, lysine, threonine and methionine. It is apparent from consideration of these values that this difference is due to the low mean coefficient of digestibility for threonine. This suggests that the threonine in oats is less available than nitrogen, lysine or methionine.

DISCUSSION

In these studies lysine was found to be the amino acid most limiting for the growth of young rats fed oats as the sole source of protein. This confirms the work of Mitchell and Smuts ('32). Methionine and threonine were also required to obtain a rapid rate of growth. However, when these amino acids were fed individually with an oat diet containing added lysine they did not cause growth responses. Apparently methionine and threonine are equally limiting for growth under these conditions.

A diet containing 85% of oats and supplements of lysine, methionine and threonine supported as rapid a rate of growth as a diet containing 20% of casein as the protein source. Since 20% of casein is considered a good protein source for the growing rat under normal conditions, the oats must supply nearly adequate levels of the other essential amino acids. Observing that reducing the level of oats from 85 to 70% of the diet supplemented with lysine, methionine and threonine did not reduce the growth rate of the rats is further evidence for the adequacy of this diet to support growth in the young rat.

The difference in nutritive value between the DeSoto oat variety and other winter varieties (Weber et al., '57) was due neither to differences in the levels of the three growth-limiting amino acids nor to differences in the availability of these three amino acids. This makes it necessary to postulate that the difference either is the result of an unknown amino acid inter-relationship or that some material in the diet other than protein is causing the effect. Weber et al. ('57) eliminated palatability and fiber content as the causes since they used a restricted intake feeding procedure and adjusted the crude fiber contents of the diets to similar levels.

If the balance of the essential amino acids is compared to the rat's requirements, as Flodin ('53) has done with a number of grains, it is apparent that lysine should be the most limiting amino acid for the growth of the white rat. Methionine and cystine, threonine, tryptophan and histidine should

each be equally limiting. The growth studies confirm that lysine is the most limiting in oat protein. However, the sulfur-containing amino acids and threonine were found to be more limiting than tryptophan and histidine. This may be the result of poor availability of the threonine and either methionine or cystine.

These results are similar to those obtained by Pecora and Hundley ('51) with rice. They found that supplements of both lysine and threonine were required to improve the growth of rats fed rice as the sole source of protein. Flodin ('53) calculated that about 40% of the threonine of rice protein must be unavailable to the rat if these amino acids are equally limiting for growth.

The hypothesis that the threonine of oat protein is poorly available to the rat is supported by the observation that it is not removed from the intestine as rapidly as other amino acids. Although differences in availability were not large they are probably great enough to explain the requirement for threonine with the oat diet.

SUMMARY

In growth studies on rats fed oat diets supplemented with amino acids, lysine was found to be the primary amino acid deficiency in oat protein. Supplements of lysine, methionine and threonine were required to obtain a rapid rate of growth. Rats fed the oat diet supplemented with lysine, methionine and threonine grew as well as rats fed a diet containing 20% of casein. Reducing the level of oats in the diet from 85 to 70% did not affect the growth rate when the three amino acids were added to the diet. At 55% of oats in the diet the growth rate was reduced and either histidine or tryptophan became limiting.

Studies of the availability of the three limiting amino acids of oat protein showed that the threonine was not as available to the rat as the nitrogen of the diet. Lysine and methionine were found to be approximately as available as nitrogen.

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EVALUATION OF LYSINE SUPPLEMENTATION OF INFANT FORMULAS FED TO RATS

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In a review of the effects of heat on milk, Heineman ('53) pointed out that the data on the loss of lysine during preparation of sterilized and dried milks are inconclusive. Controlled animal feeding trials by Hodson ('52) showed that there is no loss of amino acids during normal heat processing of milk sufficient to diminish the nutritive value of the protein. Schroeder, Iacobellis and Smith ('53), using dogs as test animals, found that autoclaving whole milk at 10 or 15 pounds pressure for 15 or 30 minutes had no detrimental effect on the digestibility, biological value or nutritive index of the protein. Whitnah ('43) and more recently Bixby et al. ('54) reported that rats grow adequately on both evaporated and spray-dried milk supplemented with minerals, indicating little or no amino acid inactivation during processing.

In contrast, Mauron and co-workers ('55) showed that 3.6% of the lysine in spray-dried milk and 8.4% of that in evaporated milk is lost during processing. Application of a digestibility test to the latter product *in vitro* indicated that perhaps twice that amount of lysine is inactivated. Cook et al. ('51) concluded that the nutritive value of evaporated milk for rats is inferior to that of fresh milk. Differences in loss of lysine reported to be due to heat processing of milk have been attributed to differences in processing techniques (Cook et al., '51; Mauron et al., '55). It has been suggested by Albanese ('56) that the destruction of lysine during processing of infant foods based on cow's milk may be great enough to impair their protein value.

To gain more precise knowledge on the possible loss of amino acids during processing of milk based foods, this investigation was undertaken. The purpose was to examine, through animal growth studies, possible changes brought about by effects of heat processing on lysine, as well as possible nutritional differences when lysine is added to infant feeding formulas.

MATERIALS AND METHOD

The test animals were 21-day-old male Sprague-Dawley strain rats. Average weight was 47.4 $\pm$ 4.3 gm. They were randomized by weight into groups of 10 and housed individually in screen-bottom cages in an air-conditioned room. Records of food and water intake (supplied ad libitum) and weight gain were made at weekly intervals. Since rats receiving diets patterned after human milk have a high incidence of diarrhea, presumably due to a high lactose content, (Fischer and Sutton, '49; De Groot and Engel, '57), observations for diarrhea were made at weekly intervals. Diarrhea was considered to be present when the catch pans showed watery, unformed stools.

The groups of rats were given different feedings under varying circumstances. Feeding trials were carried on for 6 weeks, after which the animals were sacrificed by decapitation, allowed to bleed completely, and the livers and kidneys removed and weighed.

Experiment I

In phase A of experiment I, the basic diet consisted of powdered infant food A.[1]

In phase B, the diet consisted of concentrated liquid infant food A, virtually identical to the powder in proportions of solids; the total solids were at 24.1%.[2]

[1] Similac Powder: Cow's milk protein 13.75%, lactose 53.4%, fat 26.85% (mixture of corn, coconut, olive, milk fat and cocoa butter), minerals 4.00% including milk ash plus added Na, K, and Ca salts, fish liver oil concentrate, ascorbic acid, niacin and thiamine.

[2] Similac Liquid: Cow's milk protein 3.45%, lactose 13.1%, fat 6.8% (mixture of corn, coconut, olive, milk fat and cocoa butter), minerals 0.75% including milk ash plus added potassium citrate, fish liver oil conc., ascorbic acid, niacin, thiamine, and pyridoxine.

Both products contained 1.3% of L-lysine on a dry, solids basis.[3] To these foods was added L-lysine in the form of the hydrochloride,[4] in amounts sufficient to increase the level by as much as 200%, as shown in table 1. All diets were supplemented with 1.0% of a copper, iron and manganese salt mixture.[5] The powdered diets were fed dry; the liquids as the concentrate. Dry diets were prepared at frequent intervals and stored in the refrigerator. Only the approximate amounts consumed in 24 hours were placed in the feeding cups. All soiled food was weighed and replaced daily. The liquid diets were prepared fresh daily, and all unconsumed food was measured and discarded at the end of 24 hours.

Experiment II

In the second experiment all rats were fed a lysine-deficient diet, described in table 2, for 12 days. After depletion, the average weight of the rats was 42.8 $\pm$ 3.7 gm. Twelve different groups were fed the diets shown in table 3, for 4 weeks. Infant food A, powder and liquid, was the same as used in experiment I; infant food B was similar in composition.[6] The value for lysine in each diet is shown in table 3, on a dry basis. A lysine supplement was calculated to increase the original level in the diets by approximately 50.0%. Since infant food B is regularly enriched with methionine, bringing its sulfur-bearing amino acid content to approximately 0.7%, sufficient DL-methionine was added to powdered food A and liquid food A to raise their content of this acid to the same level.

Commercial evaporated milk was diluted with water and homogenized with extra fat (corn, coconut and olive oil mixture) and lactose to approximate the gross composition of

[3] As determined by microbiological assay.

[4] du Pont's Darvyl.

[5] Percentage composition of salt mixture: $FeSO_4 \cdot 7H_2O$, 12.0; $CuSO_4 \cdot 5H_2O$, 0.8; $MnSO_4 \cdot 4H_2O$, 14.4; Cornstarch, 72.8.

[6] Bremil Powder: Cow's milk protein 11.7%, lactose 54.6%, fat 27.5% (mixture of palm, coconut and peanut oils), minerals 3.9% including milk ash plus added Na, K, Ca, and Fe salts, methionine, vitamin A palmitate, crystalline vitamin D_3, ascorbic acid, niacin and riboflavin.

TABLE 1

Diets, weight gains, food and nitrogen efficiency of rats on experiment I

DIET NO.	BASIC FOOD	LYSINE CONTENT	WEIGHT GAIN	FOOD EFFICIENCY [1]	NITROGEN EFFICIENCY [2]
		%	gm		
Phase A					
1	Powdered infant food A [3]	1.3	105(1)[4]	25.6	11.8(5)
2	Powdered infant food A + lysine	1.5	120(2)	25.7	11.8
3	Powdered infant food A + lysine	1.9	109	23.4	10.4
4	Powdered infant food A + lysine	2.6	111	26.5	11.1
5	Powdered infant food A + lysine	3.9	114	25.3	9.5(6)
Phase B					
6	Liquid infant food A [5]	1.3	99(3)	28.6	11.9(7)
7	Liquid infant food A + lysine	1.5	116(4)	29.2	11.9
8	Liquid infant food A + lysine	1.9	111	27.3	10.7(8)
9	Liquid infant food A + lysine	2.6	106	28.1	10.5(8)
10	Liquid infant food A + lysine	3.9	102	25.1	9.6(8)

[1] Grams gain/100 gm food intake.

[2] Grams gain/1.0 gm nitrogen intake.

[3] Similac Powder. See footnote 1, page 546 for composition.

[4] (1)–(8) Differences between 1 and 2, 3 and 4, 5 and 6, and 7 and 8 are statistically significant ($P < 0.05$).

[5] Similac Liquid. See footnote 2, page 546 for composition.

liquid food A. The evaporated milk was also enriched with sufficient vitamin A and D concentrate, niacin, vitamin B_6 and ascorbic acid to bring the levels of these nutrients to those of liquid food A. It was fed in this form and with the addition of lysine.

TABLE 2

Composition of depletion diet, experiment II

INGREDIENT	AMOUNT
	%
Cornstarch	50.8
Non-nutritive fibre [1]	10.9
Corn oil	8.0
Salt mix, U.S.P. XIV [1]	2.9
Zein [1]	24.2
DL-Tryptophan [1]	1.1
B complex dry mix [1]	2.1
A and D concentrate [2]	2.0 gm/100 lbs.

[1] Purchased from General Biochemicals, Inc.

[2] 20,000 U.S.P. units vitamin D and 140,000 U.S.P. units vitamin A/gm.

A mineral mixture [5] was added to all diets. Diets 11 and 12 were fed dry, 13 to 17 were diluted with water to 25% solids, and 18 to 22 were fed in the original concentration. The test diets were prepared fresh daily, and all food not consumed by the rats in 24 hours was measured and discarded.

Significance of differences was determined by application of Student's "t" test.

RESULTS

All rats except one [7] survived and grew on all diets except the depletion diet. At necropsy, the occurrence of a large, gas-filled cecum was an almost constant finding. On gross examination, fatty livers were present in approximately 10% of the rats, but there was no significant correlation of this condition with a particular dietary regimen.

[5] Same as footnote 5, see p. 547.

[7] One animal on diet 20 was accidentally killed at the end of the third week.

TABLE 3

Diets, weight gains, food and nitrogen efficiency of rats on experiment II

DIET NO.	DIET	LYSINE CONTENT	WEIGHT GAIN	FOOD EFFICIENCY [1]	NITROGEN EFFICIENCY [2]
		%	*gm*		
11	Depletion	Negligible	— 2		
12	Laboratory chow [3]	—	191		
13	Powdered infant food A [4]	1.30	85	32.2	14.9(7)
14	Powdered infant food A + lysine	1.95	81	31.6	13.9(7)
15	Powdered infant food A + methionine	1.30	101	34.4	15.7(7)
16	Powdered infant food B [5]	0.91 [6]	86	33.6	18.4(6)
17	Powdered infant food B + lysine	1.56 [6]	80	32.5	16.6(7)
18	Evaporated milk base	1.14 [6]	71	33.5	15.1(7)
19	Evaporated milk base + lysine	1.79 [6]	66	30.0	13.8(7)
20	Liquid infant food A [7]	1.30	73(2) [8]	31.9(4)	13.9(7,8)
21	Liquid infant food A + lysine	1.95	80	32.4	13.5(7)
22	Liquid infant food A + methionine	1.30	89(3)	39.0(5)	16.7(9)

[1] Grams gain/100 gm food intake.

[2] Grams gain/1.0 gm nitrogen intake.

[3] Purina.

[4] Similac Powder. See footnote 1, page 546 for composition.

[5] Bremil Powder. See footnote 6, page 547 for composition.

[6] Calculated from protein content of food.

[7] Similac Liquid. See footnote 2, page 546 for composition.

[8] (2)–(9) Differences between 2 and 3, 4 and 5, 6 and 7, and 8 and 9 are statistically significant ($P < 0.05$).

Diarrhea was a complicating factor in these experiments. After the first week of feeding, all animals, with the exception of those on diets 11 and 12, had severe diarrhea. After three weeks, the rats on diet 6 adapted completely and had no more diarrhea. In all other groups, from 10 to 100% of the rats still had diarrhea at time of sacrifice.

Experiment I

The data on weight gain and food and protein efficiency in experiment I are summarized in table 1. In phase A, the only significant differences in growth occurred between the animals on diets 1 and 2, and in phase B, between those on diets 6 and 7. The extra growth was apparently due to increase in appetite stimulated by small amounts of lysine, since there was no significant improvement in effective utilization of food or nitrogen between these respective groups of rats. The addition of lysine in larger amounts, however, impaired nitrogen efficiency. Rats on diet 5 utilized protein less effectively than those on diet 1; those on diets 8, 9 and 10 less effectively than those on diet 6.

There were no significant differences between kidney or liver weights of the control animals and of those on diets with supplementary lysine, in relation to body weight.

Experiment II

In table 3 are shown the findings of experiment II. The 10 rats remaining on the depletion diet for the 4-week test feeding period survived, but lost an average of 0.5 gm per week. The animals on the stock chow made an average gain of 48 gm per week. Following depletion, these rats realized normal growth potential when fed a complete diet that did not stress the animals with persistent diarrhea.

The added lysine was in no instance associated with a significant increase or decrease in weight. The addition of methionine to infant food A, powder and liquid, tended to increase weight, but only in the case of the liquid was the difference

significant ($P < 0.05$). This weight increase was not unexpected, since methionine is known to be one of the limiting amino acids for rats fed milk protein. The only significant increase in food efficiency occurred with liquid infant food A plus methionine.

In nitrogen efficiency, infant food B showed a significant increase over all feedings, except liquid food A plus methionine. There are two probable reasons for this: the protein content of this feeding was even further below optimum for the rat than that of the other infant formulas; and added methionine improves the amino acid balance of milk protein for the rat. Addition of methionine to liquid food A increased nitrogen efficiency significantly.

Amino acid supplementation was without significant effect on kidney or liver size.

DISCUSSION

Several investigators have pointed out the shortcomings of the rat as a test animal for foods patterned after human milk (Cox et al., '55; Scott and Norris, '49). Handler ('47) suggested that failure of rats to grow on high lactose-containing diets is due to the inability of the animal to metabolize the carbohydrate. When the dietary level is between 60 and 70%, the blood galactose reaches a toxic level and the animals die. Fischer and Sutton ('49) furthermore point out that lactose is irritating to the gastrointestinal tract of the rat, leading to increased motility with diarrhea. De Groot and Engel ('57) ascribe the diarrhea to the presence of undigested lactose in the caudal section of the intestinal tract, leading to water retention and gas formation. De Groot and Hoogendoorn ('57) point out that intestinal lactase activity is only 10% of initial value at time of weaning, and that no increase of lactase activity results from continued overfeeding of lactose. It is not clear whether the stress of persistent diarrhea or lactose intolerance was the more dominant factor affecting the growth performance of our rats.

The differences in loss of lysine reported for various types of milk processing were not evident in this study. Rats grew equally well whether fed evaporated milk, dry infant formula or liquid infant formula. Because the rats were stressed by suboptimal protein intake, persistent diarrhea, and, for some, lysine depletion, differences in nutritional value among these feedings would be more readily manifest than they would under more nearly normal conditions. Even so, rats fed diets patterned after human milk are under dietary stress, and thus do not represent the most appropriate medium for testing foods of this type.

Lysine addition to cow's milk protein furnishes no significant improvement in nitrogen efficiency. Similar results with rats were reported by Sarett ('56), who added lysine to a milk and cereal mixture, by Block et al. ('56), who supplemented a soybean infant food product with lysine, and by Thiessen and Reussner ('58), who added lysine to a cereal and milk diet.

With higher levels of lysine supplementation, nitrogen efficiency was significantly reduced. A lowered growth response in rats to high supplementary levels of lysine has been reported by Russell, Taylor and Hogan ('52) and in dogs by Gessert and Phillips ('56).

CONCLUSIONS

1. Weanling rats live on infant formulas patterned after human milk, but growth is considerably below optimum levels. Furthermore, the composition of these feedings stresses the rats with diarrhea, and possibly with lactose intolerance.

2. Small supplements of lysine to the diets of the rats so stressed appear to stimulate appetite, and, secondarily, growth, but do not improve food or nitrogen efficiency. Larger supplements do depress food and nitrogen efficiency.

3. Growth of animals depleted of lysine was essentially similar on several infant formulas. Supplementation of these diets with lysine was without effect on growth.

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ESSENTIAL FATTY ACIDS IN INFANT NUTRITION

II. EFFECT OF LINOLEIC ACID ON CALORIC INTAKE [1]

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Although a relationship between dietary unsaturated fatty acids and utilization of Calories has been recognized in young rats (Wesson and Burr, '31; Burr and Beber, '37), scant attention has been given to the metabolic role of the essential fatty acids in infant nutrition. In a study which was designed to relate the intake of linoleic acid to the concentration of unsaturated fatty acids in the blood serum, it was observed that Caloric consumption varied with the amount of linoleic acid in the diet. It is the purpose of this report to present data concerning the influence of dietary fat on Caloric intake in healthy infants.

MATERIAL AND METHODS

The 18 infants under study were among those for whom blood serum fatty acids have been reported. Data regarding these infants and their diets have been summarized in the previous paper (Wiese and coworkers, '58). Sixteen subjects, two weeks to 4 months of age, were given various cow's milk mixtures which included skim, half-skim, evaporated, a special

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compounded mixture [3] and a proprietary milk preparation.[4] Two infants were fed directly from the breast. In addition, the skim and half-skim milk mixtures were supplemented with linoleic acid given as the methyl or ethyl ester or triglyceride. The linoleic acid intake in the entire group varied from <0.1 to 8% of the Calories (<0.01 to 0.56 gm/100 ml). Records of the daily food intake as well as daily weights were kept. To smooth the curves for Caloric intake and weight, the results were plotted to fit a straight line by the method of least squares using the formula $A = y + bx$, where A is the intercept, b is the slope of the line and x is the age in days. Although calculations based on body surface also were made, it was found more convenient to express Caloric intake as Calories per kilogram per day.

RESULTS

Caloric consumption in relation to dietary linoleic acid

A statistical comparison regarding the Calories consumed per unit body weight was made between the infants who were fed on the skim and evaporated milk mixtures. At the end of an average of 3.5 weeks the 12 infants who received skim milk were consuming food at the rate of 155 Cal./kg/day, whereas the 6 infants who received evaporated milk were consuming food at the rate of 106 Cal./kg/day. The difference between the two groups was significant ($P < 0.001$).[5]

The number of individual subjects receiving each of the other 10 milks was too few for statistical analysis, hence the data concerning Caloric intakes were considered in relation to stepwise increases in the linoleic acid content of the diet.

[3] Skim and evaporated milk, lard and Dextri-Maltose.

[4] Varamel, a proprietary milk preparation in which vegetable fats are substituted for butter fat and to which it is necessary to add carbohydrate to make the Caloric value equal to that of whole cow's milk. Baker Laboratories, Inc., Cleveland, Ohio.

[5] "P" was derived from calculated "t" values (when $N < 30$) using a two tail test of the one tail probability table in Dixon and Massey ('51).

As noted in table 1 the general trend was for decreasing Caloric intakes with increasing amounts of linoleic acid in the diet. For example, one infant who was maintained on a half-skim milk mixture which provided only 0.5% of the Caloric intake as linoleic acid, at the end of 5 weeks, was consuming food at the rate of 110 Cal./kg/day. This was

TABLE 1

Summary of Caloric intake in relation to linoleic acid in diet

DIET	LINOLEIC	NUMBER OF INFANTS	AV. DURATION	CAL./KG AT END OF DURATION
	% of Cal.		*wks.*	
Skim milk	< 0.1	12	3.5	155
Half-skim milk	0.5	1	5.0	110
Evaporated milk	0.9	6	3.5	106
Skim + 1% linoleic acid	1.0	3	3.0	109
Half-skim + 1% linoleic acid	1.5	1	5.0	105
Special compound [1]	1.7	1	7.0	92
Skim + 3% linoleic acid	3.0	2	3.0	122
A [2]	3.0	1	4.0	95
Breast milk	4.0	2	9.0	85
Special compound + B [3]	4.5	1	2.0	72
Skim + 5% linoleic acid	5.0	1	3.0	105
Half-skim + 7.5% linoleic acid	8.0	1	2.0	85

[1] Skim and evaporated milk, lard and dextri-maltose.

[2] Varamel ®, a form of evaporated milk in which vegetable fats are substituted for butter fat and which requires the addition of carbohydrate for the usual 20 Cal./oz. formula. The Baker Laboratories, Inc., Cleveland, Ohio.

[3] Lipomul ®, a 40% emulsion of vegetable oils. Upjohn Co., Kalamazoo, Michigan.

considerably less than the mean intake for the infants who received skim milk for 3.5 weeks. When skim milk was supplemented with 1% of the Calories as linoleic acid, the Caloric consumption decreased greatly compared with the rate when on skim milk alone. On the other hand, two infants who received skim milk with a supplement of 3% of the total Calories as linoleic acid were consuming an average of 122 Cal./kg/day. Nevertheless, the decrease in intake for each infant was marked compared to their intakes when on the low-fat mixture

alone: case 7, from 168 to 114 Cal./kg/day; case 19, from 174 to 130 Cal./kg/day. The lowest Caloric consumption rates were obtained with Varamel, breast milk, the special compounded milk, and half-skim milk which was supplemented so that 8% of the Calories was derived from linoleic acid. It is apparent that with linoleic acid intakes of <0.1% of the Calories, the daily food consumption is greater than when linoleic acid in the milk mixtures constitutes 0.5% or more of the Calories. This phenomenon was consistent in 87% of the cases studied and was independent of the total amount of fat in the milk mixtures.

Caloric consumption and weight gain for individual infants

Typical examples of the progress of infants fed varying levels of linoleic acid are presented in figures 1 through 4. Depicted are the type of milk with the percentage of Calories provided by linoleic acid and fat, weight and Caloric intake at the beginning and at the end of each diet. Also included are the levels of dienoic acid in the serum.

The effect of a low-fat diet may be noted in figure 1. After 13 weeks the Caloric consumption was at the rate of 153 Cal./kg/day. Weight gain was satisfactory. When the diet provided 1.7% of the Calories as linoleic acid from a special compounded milk mixture and cereals, the Caloric consumption decreased to 92 Cal./kg/day. The addition of a commercial fat emulsion to the milk mixture which increased the intake of linoleic acid to 4.6% of the Calories resulted in a further drop to 72 Cal./kg/day. Again the weight gain was steady. Dienoic acid in the serum remained at a low level of 4 to 5% of the total fatty acids when on the low fat diet. It rose sharply to a level of 17% of the total fatty acids in one week when the diet supplied 1.7% of the Calories as linoleic acid.

The infant whose findings are shown in figure 2 was observed first while receiving evaporated milk. At the end of

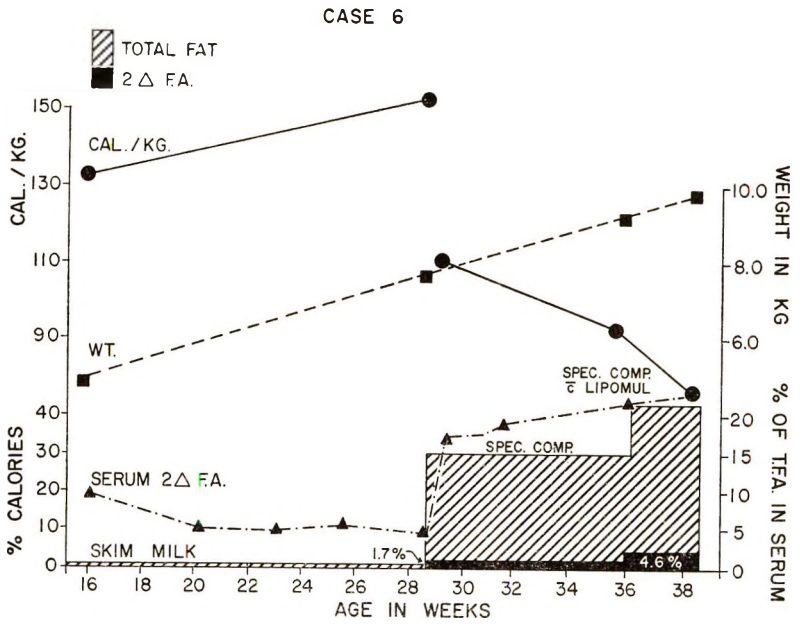

Fig. 1 Growth curve and Caloric intake for infant receiving skim milk and a specially prepared milk mixture containing fat with liberal amounts of linoleic acid.

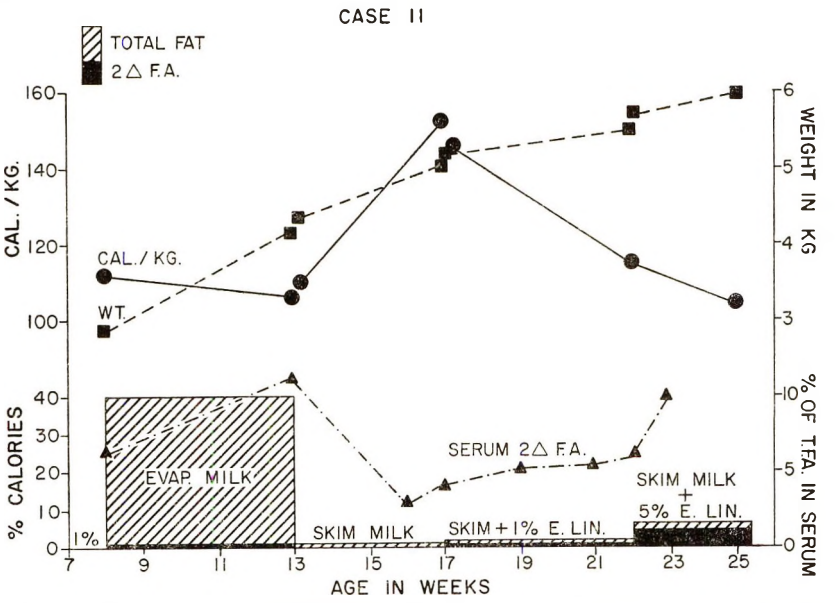

Fig. 2 Growth curve and Caloric intake for infant receiving evaporated milk followed by skim milk and subsequently supplemented with ethyl linoleate.

5 weeks, the Caloric consumption was at the rate of 106 Cal./kg/day. When he was changed to skim milk, the consumption at the end of 4 weeks had increased to 152 Cal./kg/day. The addition of 1% of the Calories as linoleic acid (ethyl linoleate) decreased the daily Caloric intake after 5 weeks to 112 Cal./kg/day. A further decrease to 105 Cal./kg/day was found at the end of three weeks of supplementation of skim

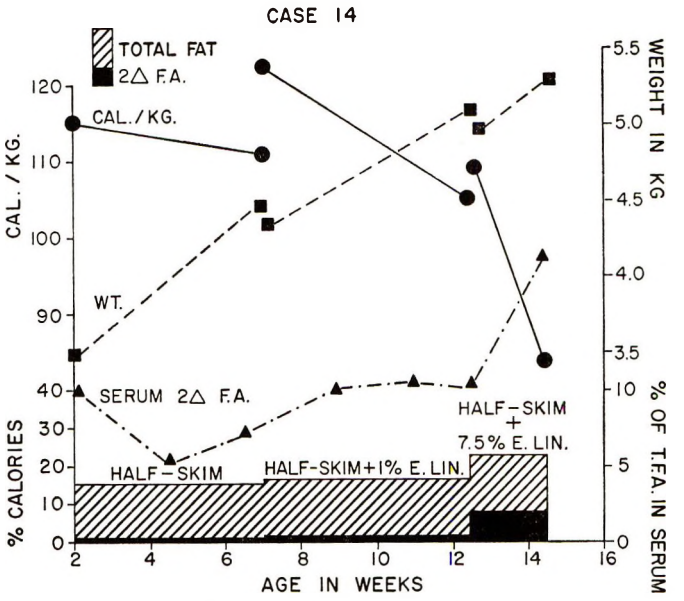

Fig. 3 Growth curve and Caloric intake for infant receiving half-skim milk and subsequently supplemented with ethyl linoleate.

milk with 5% of the Calories as linoleic acid provided in the form of ethyl linoleate. The weight gain throughout was steady and satisfactory. The dienoic acid levels of the serum were typical of those for evaporated and skim milk mixtures. With the addition of the ethyl ester of linoleic acid at a 1% Caloric level of intake the rise was not as great as with natural fat (evaporated milk mixture).

The findings in reference to the use of half-skim are shown in figure 3. During a 5-week interval weight gain was ade-

quate at an intake of about 110 Cal./kg/day. There was only a slight decrease in Caloric consumption with the addition of 1% linoleic acid; however, when the supplement of linoleic acid was increased to 7.5% of the Calories, there was a marked drop in food consumption within two weeks to 85 Cal./kg/day. The weight curve showed no essential change. Again, the serum levels for dienoic acid followed closely the progressive increase in linoleic acid intake.

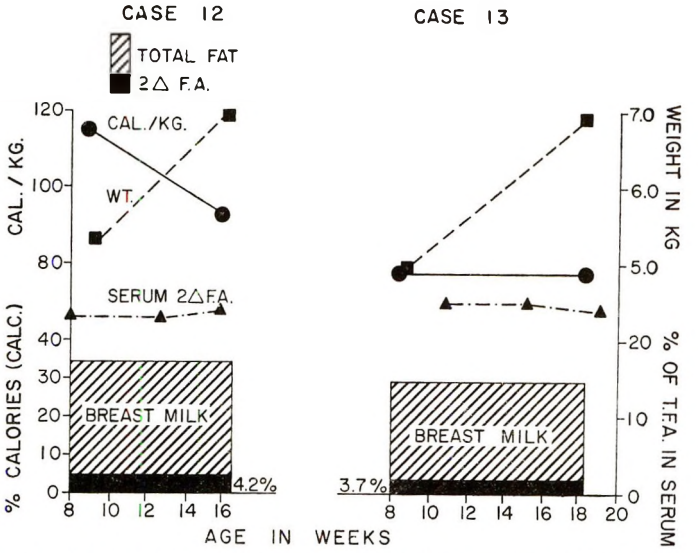

Fig. 4 Growth curve and Caloric intake for two breast fed infants.

Data are presented in figure 4 for two breast-fed infants. Both infants gained weight steadily during the periods of observation. One infant was consuming 93 Cal./kg/day at the end of 8 weeks and the other infant was satisfied with 78 Cal./kg/day. In addition to breast milk, the infants received cereals and fruits, however, the linoleic acid content of their diets was essentially the same at about 4% of the total Calories. The high serum dienoic acid levels also were comparable.

DISCUSSION

From the data presented concerning Caloric intake it is apparent that linoleic acid plays a definite metabolic role in infants. The addition of linoleic acid to the diet of infants given a low-fat milk mixture in most instances rapidly decreased Caloric intake and conversely, removal of linoleic acid from the diet resulted in increased Caloric consumption. The mechanisms involved whether enzymatic or otherwise must await elucidation.

In the consideration of Caloric expenditure, one must include evaluation of growth, physical activity, food absorption, specific dynamic action and basal metabolism. It has been shown that in feeding diets with and without fat, plateauing of the weight curve is a characteristic feature of essential fatty acid deficiency in rats (Burr and Burr, '29). Also, young dogs reared on diets low in fat develop marked emaciation in spite of Caloric intakes identical to those of littermate control animals with fat in the diet (Hansen and Wiese, '51). It is possible that if infants were maintained for long periods (years) on low-fat diets, effects on growth might be discernible. With regard to physical activity studies with rats by Burr and Beber ('37) disclosed no difference in activity between fat-deficient and control rats. In our infants physical activity was not influenced by diet as long as they were fed to their full satisfaction. Abnormal Caloric losses in the stool have not been reported in animals. It seems unlikely that the small differences in specific dynamic action of the various milk mixtures would account for large differences in Caloric consumption. On the other hand, increased metabolism has been demonstrated in young rats when the diet is lacking in fat (Wesson and Burr, '31; Burr and Beber, '37; Panos and coworkers, '56). Basal metabolism data were not obtained on our infants but early studies on an adult human subject on a low-fat diet (Brown and coworkers, '38) showed that the basal metabolic rate as well as the respiratory quotient reacted in the same manner as for rats on diets low in fat.

The daily Caloric requirement for young infants usually is given as 100 to 110 Cal./kg. Caloric consumption in this same range (106 to 109 Cal./kg/day) was found for 9 infants when the dietary intake of linoleic acid constituted 1.0% of the total Calories. If one metabolic function of linoleic acid is related to the utilization of Calories, it would appear from the data presented that a dietary level of 1.0% of the total Calories is sufficient to meet the so-called normal Caloric requirement of infants. In contrast to the findings for the di-, tri- and tetraenoic acids in the serum, (Wiese and coworkers, '58) no demonstrable differences in Caloric consumption were noted between the administration of the ester and glyceride forms of linoleic acid. Although a dietary level of 1.0% of the Calories as linoleic acid appears to be adequate for healthy infants under one year of age, it does not necessarily constitute the minimum or the ideal level. The lowest Caloric intakes were attained with diets which provided about 4% of the Calories as linoleic acid. This finding substantiates the conclusion from blood serum studies that an intake in the neighborhood of 4% of the Calories as linoleic acid is more nearly optimum than 1% for healthy young infants.

SUMMARY AND CONCLUSIONS

The Caloric intake was measured on 18 healthy infants under 4 months of age who, for one to 28 weeks, were fed on one or several of 12 different types of milk. The linoleic acid intake ranged from $<0.1\%$ to 8.0% of the total Calories. The diets very low in linoleic acid were given on 13 different occasions to 12 of the infants and in every instance the intake was above 125 Cal./kg/day. When linoleic acid as the ester, triglyceride or in a natural fat was present in the diet, on 31 occasions in 18 different infants in all but 6 instances the intake was less than 125 Cal./kg/day. In spite of differences of 20 to 40% in Caloric intake the slope of the weight curve did not change in the majority of the infants studied. On the basis of the data presented it appears that in young infants

optimum Caloric efficiency is attained when linoleic acid comprises about 4% of the Caloric intake.

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ESSENTIAL FATTY ACIDS IN INFANT NUTRITION

III. CLINICAL MANIFESTATIONS OF LINOLEIC ACID DEFICIENCY [1]

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An extensive clinical study is being made to evaluate the role of the essential fatty acids in infant feeding. After careful explanation of the nature of the study, the mothers of several hundred infants have chosen to participate. The chief criteria of selection are that the parents seem anxious to cooperate and that the infants are normal neonates. The individual subjects include children of physicians, medical students as well as those seen in the Well Baby Clinics. As an integral part of the study the healthy young infants are given one of 5 different milk mixtures which vary in their content of fat and linoleic acid. One group received a milk mixture practically devoid of fat and because of the unsatisfactory progress of a number of the infants, this phase of the study has been terminated. The findings seem worthy of presentation at this time.

[1] Supported in part by a grant from The Baker Laboratories, Inc., Cleveland, Ohio, who also prepared the special formulas used in the study.

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MATERIAL AND METHODS

Twenty-seven infants received a skim milk mixture which supplied 1.4% of the Calories as fat (0.1 gm%) with <0.1% of the Calories as linoleic acid (<0.04 gm%). Protein constituted 15% of the Calories with carbohydrate supplying the remainder of the Caloric intake. The milk contained added vitamins and iron and was prepared so that when diluted with equal parts of water there were 63 Cal./100 ml (19 Cal./oz.). Solid foods were added to the diet, beginning with cereals at three months of age, and at monthly intervals thereafter, fruits, vegetables and meats were given. The infants were maintained on the low-fat regimen for periods varying from two weeks to 12 months. Linoleic acid as trilinolein was added to the diet of three infants and as natural fat by changing to one of the other milk mixtures in 9 instances. Ethyl arachidonate was used in the diet of one infant. Tripalmitin was added to the diet of another. Three infants were shifted to a milk mixture containing only saturated fat. The subjects were examined regularly by the same pediatricians, particular attention being given to growth, development, illnesses, the number and character of the stools, and to the condition of the skin. Periodic determinations of the blood serum unsaturated fatty acids were made by the method of alkaline isomerization (Wiese and Hansen, '53).

RESULTS

Clinical features of the group as a whole

The infants readily accepted the milk low in fat content. Vomiting and regurgitation did not occur and weight gain was adequate in most instances. In general, progress was satisfactory except for the following 4 outstanding features:

Character of stools. The occurrence of frequent, large, dark-brown, sirupy bowel movements was a distinguishing symptom in 25 of the 27 infants. In three instances it was necessary to change the type of feeding because of loose stools.

Perianal irritation. Raw red exuding areas often developed in the diaper region. The usual therapeutic measures had no effect on the severe lesions in 14 of the infants.

Abnormalities of the skin. Of the 24 infants who were fed on the milk preparation low in fat for at least one month, changes in the skin were observed in 15. Characteristically, there developed dryness and leathery thickening of the skin which could be detected readily by inspection and palpation. Soon desquamation was noted. This was particularly apparent in the colored infants where the fine, flaky, white scales stood in contrast to the dark background (fig. 1). Annoying exudation often occurred in the body folds and the raw exuding surface in the intertriginous areas became a disconcerting feature to both the mothers and the attending physicians.

Serum lipid findings. At three months of age the serum lipids were determined in 16 infants receiving the low-fat diet. The levels of dienoic and tetraenoic fatty acids were uniformly low and those for the trienoic acid relatively high compared with three-month-old infants who received fat and linoleic acid in the diet. The mean values for the di-, tri- and tetraenoic acids were 3.3, 4.9 and 2.8% of the total fatty acids, respectively.

When symptoms of fat deficiency were marked, it was at the discretion of the pediatrician-in-charge to add specific fatty acids to the diet, or to change the milk preparation to one containing fat. In each instance wherein the milk mixture was changed to one containing linoleic acid or the low-fat diet was supplemented with linoleic acid, the diarrhea stopped, the rash in the diaper region disappeared, the raw exuding areas in the intertriginous folds cleared and the skin gradually returned to a normal soft velvety texture. The serum lipids reflected the dietary change. By the diligent use of local therapy and the addition of solid foods to the diet, 4 of the infants were maintained satisfactorily on the low-fat milk for 6 months and three for 12 months. After the addition of solid foods, the serum di- and tetraenoic acids were found to increase gradually and the trienoic acid to decrease.

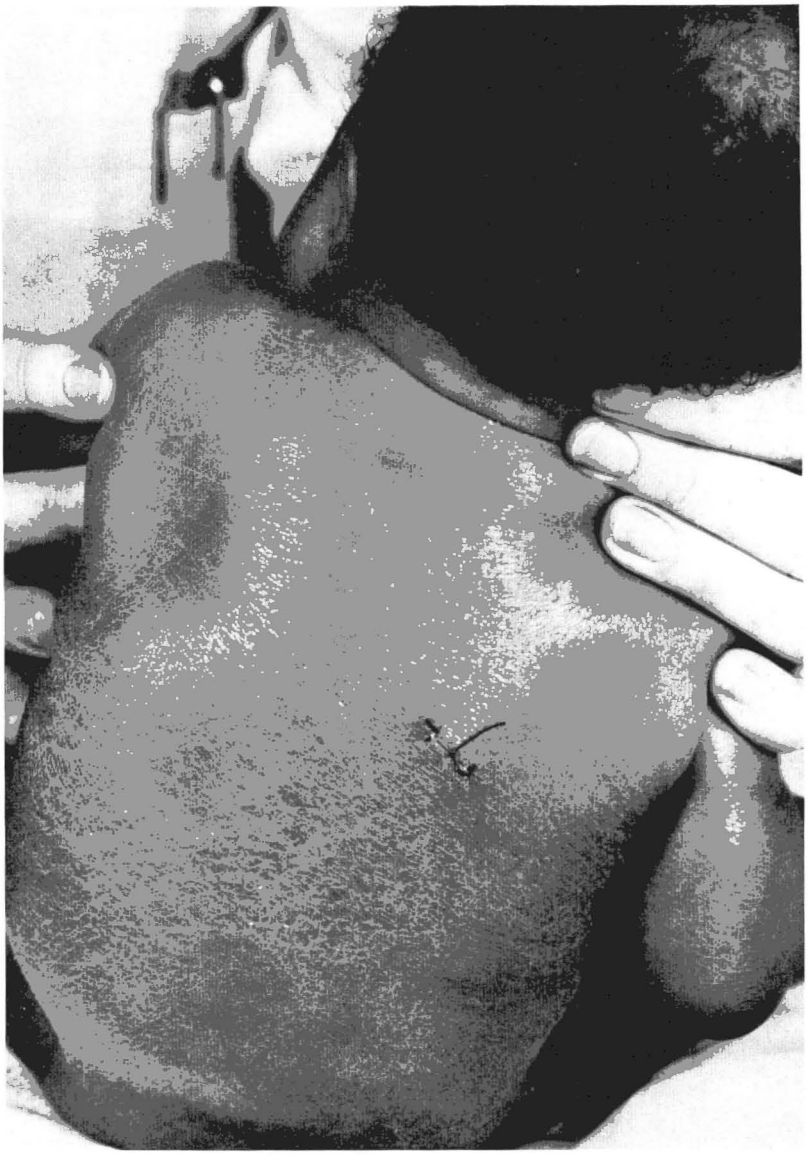

Fig. 1 Typical appearance of skin of 10-week-old infant given a skim milk mixture lacking linoleic acid.

Observations concerning individual infants

The relationship which exists between dietary linoleic acid and the condition of the skin in young infants, as well as the correlation between these two features and the blood serum levels of unsaturated fatty acids are illustrated by reference to the case histories of several infants.

Case 19: A female negro infant with a birth weight of 2850 gm was fed the milk mixture low in fat beginning on the third day of life. Her stools were semi-formed and numbered 5 to 6 daily. By 10 weeks of age her entire skin became dry and scaly as illustrated in figure 1. Later there developed a parchment-like texture with fissuring in the post-auricular areas. At three months cereal was added to the diet without any demonstrable change in the condition of the skin. Six weeks later linoleic acid, as trilinolein, was given by dropper directly into the mouth before each feeding in an amount to equal 2% of the daily Caloric intake. After one week of supplementation, the skin became soft and smooth except for some excoriation in the diaper area. After three weeks the linoleic acid was discontinued, and 10 days later pureed fruits and vegetables were added to the diet. The skin remained of normal texture although the low-fat milk was continued until one year of age. While fed the low-fat milk mixture alone the blood serum levels for the 2- and 4-double-bond fatty acids were characteristically low with a high level for the 3-double-bond fatty acid. With the addition of linoleic acid to the diet the dienoic acid rose as indicated in table 1.

Case 54: This female negro infant weighed 1680 gm at birth and was placed on the low-fat milk mixture on the third day of life. At one week of age (during an epidemic of staphylococcus aureus, phage type 81) she developed a breast abscess which responded to incision, drainage and antimicrobial therapy. After receiving the low-fat milk for 7 days, she began having large, loose brown stools (8 to 10 per day). Within 4 weeks the skin was noted to be taut, shiny, and marked excoriation was present in the diaper area. The skin

TABLE 1

Blood serum levels of di- tri- and tetraenoic acids for individual infants in relation to intake of linoleic acid

CASE NO.	DIET	DURATION	DIENOIC ACID [1]	TRIENOIC ACID [1]	TETRAENOIC ACID [1]
19	Skim milk	3 months	2.9	6.9	4.0
	Skim milk + 2% Cal. as linoleic acid (trilinolein)	2 weeks	10.1	4.0	2.8
	Skim milk + cereals, fruit, vegetables and meats	9 months	15.8	2.8	8.6
54	Skim milk	3 months	2.2	5.5	1.5
	Skim milk + 2% Cal. as palmitic acid (tripalmitin)	6 weeks	3.1	5.8	0.3
	Milk containing 7.3% Cal. as linoleic acid	6 weeks	37.9	2.8	10.2
39	Skim milk	6 weeks	1.1	5.7	1.2
	Skim milk + 2% Cal. as arachidonic acid (ethyl arachidonate)	1 month	1.4	6.3	3.4
22	Skim milk	3 months	2.8	7.1	2.0
	Skim milk, topical olive oil	3 weeks	3.0	6.3	1.9
	Milk containing 1.3% Cal. as linoleic acid	2 months	16.7	2.5	7.1

[1]As percentage of total fatty acids in serum.

changes became more severe with flaking and denudation of the intertriginous folds. At two months of age the infant was given a fat supplement of tripalmitin to equal 2% of the daily Caloric intake. Although supplementation was continued for 6 weeks, no improvement was noted nor did any significant change occur in the serum levels of unsaturated fatty acids. When 4 months of age, the infant was admitted to the hospital because of staphylococcal pyopneumothorax which gradually responded to appropriate therapy. After the first two weeks of hospitilization the low-fat milk mixture was changed to one containing 42% of the Calories as saturated fat. Again no improvement of the skin was noted. Next the diet was changed to a milk mixture containing liberal amounts of linoleic acid and within two weeks, the skin had become soft and moist with no desquamation. There was also a prompt increase in the di- and tetraenoic acid levels of the serum with a decrease in trienoic acid. The values for the serum fatty acids are shown in table 1.

Case 39: A male negro infant, one of twins, weighing 1550 gm at birth was fed the low-fat milk mixture beginning on the second day of life. After one week he developed voluminous stools (3 to 8 per 24 hours) and by 4 weeks of age, the diaper region was severely excoriated despite local therapeutic measures. At 6 weeks of age definite generalized skin changes were noted which consisted of desquamation, denudation and palpable thickening. Due to intermittent edema the other twin (case 40) was given a low-salt milk which contained liberal quantities of linoleic acid. No abnormalities of the skin occurred in this twin (fig. 2). Ethyl arachidonate [3] in an amount equal to 2% of the daily Calories was fed by dropper before each feeding to the affected infant with slow improvement over the next 5 weeks; however, the changes were by no means as striking as those seen in the infants who were given trilinolein. The relatively poor response to arachidonic acid may have been due to impurity of the product as

[3] Kindly supplied by Dr. Ralph T. Holman, Hormel Institute.

indicated in a personal communication from Dr. Ralph Holman. Serum levels for the unsaturated fatty acids initially reflected the low intake of linoleic acid, but there was only a slight rise in tetraenoic acid and no change in di- and trienoic acid levels during supplementation as indicated in table 1.

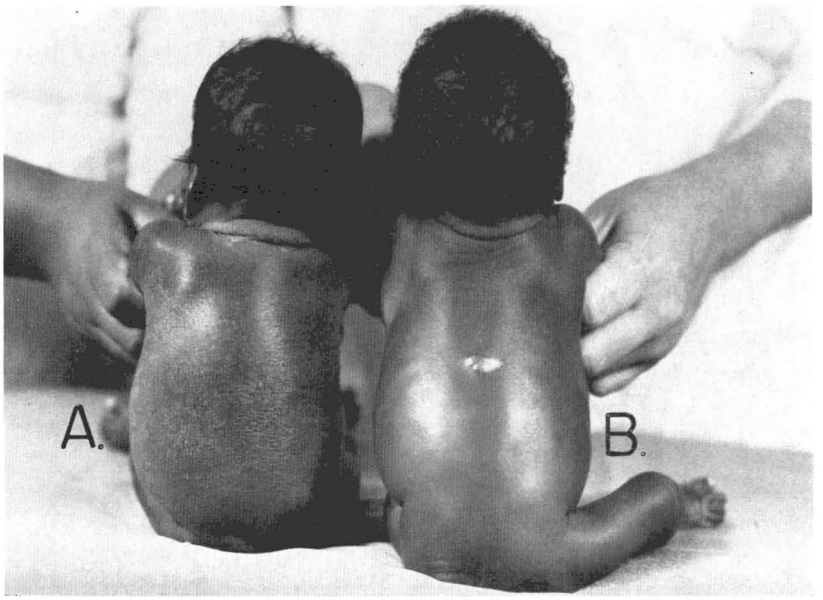

Fig. 2 Appearance of skin of 6-week-old twins. A, Given a skim milk mixture lacking linoleic acid. B, Given a milk mixture containing linoleic acid.

Case 22: This female negro infant weighed 3000 gm at birth. On the 7th day of life she was started on the milk mixture very low in fat content and at three weeks of age developed the typical skin features of fat deficiency. Changes in the character of the stools were only moderately severe. The opportunity arose to ascertain whether liberal amounts of topically applied olive oil would effect a therapeutic response or a change in the serum 2-, 3- and 4-double-bond fatty acids. The mother used the oil for three weeks with no alteration either clinically or chemically. Since no improvement had occurred, the infant's diet was changed to a milk mixture

which provided moderate amounts of linoleic acid (1.3% of the Calories) and within one week improvement in the skin was noted. Three weeks after the diet was changed the skin appeared normal. The values for the serum lipids are presented in table 1.

DISCUSSION

From the data presented it has been demonstrated quite definitively that young healthy infants within a relatively short time may develop symptoms when given diets extremely low in fat. Previous studies by von Gröer ('19) and von Chwalibogowski ('37) with infants on low-fat diets disclosed no changes in the skin. On the other hand, in short term studies using diets low in fat, Holt and coworkers ('35) found that one of three infants developed skin changes. Of special interest have been the observations of Hansen and Wiese ('44) on an infant with chylous ascites. This subject remained on a low-fat diet for almost two years and developed eczematous patches intermittently, a chronic dermatitis following prickly heat and a refractory impetiginous eruption. Infants with steatorrhea when maintained on relatively low fat intakes for long periods of time also have developed skin eruptions (Luzzatti and Hansen, '44). Indirect evidence that dietary fat may be of importance in the maintenance of a healthy skin has been supplied by observations on patients with eczema (Hansen and coworkers, '47).

It is not surprising that skin changes may develop in young infants inasmuch as one of the first outstanding evidences of fat deficiency in experimental animals was an abnormality of the skin (Burr and Burr, '29). It should be pointed out that only in young animals deprived of fat for relatively long periods of time are distinct skin changes demonstrable. In more mature animals evidences of fat deficiency are slower to develop and are not clear-cut. The likelihood of maintaining infants on a diet low in linoleic acid becomes less as the child grows older because cereal grains which are fed at an early age contain appreciable quantities of linoleic acid.

That there are individual variations in the susceptibility of the skin to changes in the kind and amount of fat in the diet is a distinct possibility. This is illustrated in our study by the larger proportion of negro infants who developed demonstrable skin changes compared with those of Latin-American and Anglo-Saxon extraction. Nevertheless, an abnormality of the skin as evidence of the lack of a specific fatty acid is indicated from the results of feeding various fats of known composition. For example, tripalmitin fed as 2% of the Caloric intake as well as saturated fats to equal 42% of the Calories did not improve the condition of the skin. In contrast, supplementation with trilinolein at a 2% Caloric intake of linoleic acid brought about striking improvement in the skin. On the basis of studies of the unsaturated fatty acids in blood serum, Wiese and coworkers ('58) found minimal normal levels were associated with diets which provided 1 to 2% of the Calories as linoleic acid. Also, optimum levels of intake of linoleic acid seemed to be in the range of those found in breast milk, namely 4 to 5% of the Caloric intake. This level of intake of linoleic acid resulted in optimum Caloric efficiency (Adam and coworkers, '58).

SUMMARY AND CONCLUSIONS

In an infant feeding study it was found that young infants fed on a skim milk diet extremely low in fat and linoleic acid, though otherwise nutritionally adequate, showed certain signs and symptoms. Within a short time, most of the 27 infants developed frequent large stools. Perianal irritation was a disconcerting feature in most instances. Within a matter of weeks alterations in the skin were discernible in the majority of infants. The first sign observed was dryness, then thickening and later desquamation with oozing in the intertriginous folds. These changes were particularly marked in the negro infants. Addition of saturated fatty acids to the diet did not improve the skin. On the other hand, the addition of linoleic acid as trilinolein to constitute 2% of the daily Caloric intake

restored the skin to a normal soft moist texture and appearance within one to two weeks. If the milk mixture was changed to 2 to 5% of the Calories with total fat constituting 42% of the Calories, restitution of the skin was equally as prompt. When a milk mixture containing 1.3% of the Calories as linoleic acid was given, the skin returned to normal in two to 4 weeks. In one instance, arachidonic acid given as the ethyl ester at a 2% Caloric level required about 5 weeks for the skin to return to normal. The serum of all the infants on the low-fat diet had extremely low values for the di- and tetraenoic acids and high values for trienoic acid which values changed with the addition of linoleic acid to the diet. The dienoic acid values reflected the dietary intake most markedly. Arachidonic acid administration did not change the dienoic acid level. It is concluded that young infants require linoleic acid in their diet.

ACKNOWLEDGMENT

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THE INFLUENCE OF ORALLY-ADMINISTERED PENICILLIN UPON GROWTH AND LIVER THIAMINE OF GROWING GERMFREE AND NORMAL STOCK RATS FED A THIAMINE-DEFICIENT DIET [1]

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Oral administration of antibiotics has been found to counteract in part the results of limited vitamin intakes (Lih and Baumann, '51; Sauberlich, '52; Guggenheim et al., '53; Schendel and Johnson, '54; Jones and Baumann, '55 and Scott and Griffith, '57). Weanling rats fed a diet deficient in thiamine but otherwise complete stop growing after about two weeks (Scott and Griffith, '57). Loss of weight follows and finally death occurs. Addition of antibiotics, especially penicillin, to the deficient diet makes sub-optimum growth possible and increases to a certain extent the thiamine level in the liver (Guggenheim et al., '53; Jones and Baumann, '55). As the phenomenon of the intestinal synthesis of thiamine seems well established, these observations have been explained by assuming an influence on the intestinal micro-flora resulting in a greater net synthesis of the vitamin (Guggenheim et al., '53; Jones and Baumann, '55). Another possibility is an increased absorption of available thiamine brought about (directly or indirectly) by the antibiotic. This possible mode of action is suggested by the work of Draper ('58) who found

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an increased uptake of C^{14} L-lysine from the gut track of penicillin-treated chicks. A direct systemic effect of the antibiotic on the host animal, resulting in a smaller net requirement for thiamine, could be another explanation.

In the framework of this laboratory's interest in the role of the intestinal flora in nutrition, the availability of flora-synthesized thiamine to the host animal is currently under investigation. It was felt that repeating the experiments on the thiamine-sparing action of penicillin with germfree rats would give valuable information as to the mechanism of this action and add to our knowledge about the role of intestinal flora.

METHODS

Weanling germfree albino rats (Lobund strain of Wistar origin) were housed in a Reyniers' germfree system (Reyniers, '56). They were divided into three experimental groups, each group normally consisting of three males and three females. Three animals (males or females) were housed in a ½ sq. foot screen-bottom cage. The diets used were L-464, which is a complete semi-synthetic diet (table 1) and L-465, which is essentially L-464 from which added thiamine has been omitted.

One group was fed the complete L-464 diet, another the deficient L-465 diet and the last group diet L-465 to which 50 mg/kg of radiation-sterilized procaine penicillin G had been added in the germfree unit (L-465PP). All diets were analyzed for thiamine by the thiochrome method. Sterilized L-464 is a complete diet containing 9.4 mg thiamine/kg, while sterilized L-465 is the same except that it contains only a limited amount of thiamine, 0.4 mg/kg. All food and water were available ad libitum. Control normal stock animals were fed the same sterilized diets and housed in the same manner in the animal house.

All animals were weighed twice a week. Most of them were sacrificed at the end of the 4-week experimental period. At that time the livers were removed, weighed and the thiamine

content determined by the thiochrome method and expressed as thiamine·HCl. Stomach contents were also checked for signs of coprophagia.

TABLE 1

Composition of diet L-464 [1]

CONSTITUENT	AMOUNT	
Casein [2], gm	200	
Corn oil, gm	50	
Rice starch, gm	585	
Cellophane spangles, gm	50	
Salts L-II, gm	50	
Albimi yeast extract, gm	20	
Desiccated liver, gm	20	
Ascorbic acid, gm	2	
i-Inositol, gm	1	
Vitamin A, I.U.	8000	[3]
Vitamin D, I.U.	1000	[3]
Vitamin E, (mixed tocopherols), mg	1500	[3]
Vitamin K, mg	100	[3]
Corn oil carrier, gm	16.0	[3]
Thiamine, mg	60	
Riboflavin, mg	30	
Nicotinamide, mg	50	
Nicotinic acid, mg	50	
Calcium pantothenate, mg	300	
Choline chloride, mg	2000	
Pyridoxine hydrochloride, mg	20	
Pyridoxamine dihydrochloride, mg	4.0	
Biotin, mg	1.0	
Folic acid, mg	10	
Paraminobenzoic acid, mg	50	
0.1% Trituration vitamin B_{12} in mannitol, mg	250	
Cornstarch carrier, mg	2175.0	

[1] The L-465 diet is the same as L-464 except that added thiamine was omitted and rice starch was substituted for the cornstarch carrier. Diets were autoclaved at 17 lbs. pressure for 25 minutes.

[2] Labco.

[3] Twenty grams of this mixture were employed.

The results were obtained from three experimental runs. In the first (I) there were sufficient germfree weanlings for only diet groups L-465 and L-465PP. In the second (II) experiment enough animals were available for all three experimental groups: L-464, L-465, and L-465PP. However, during the last stage of this series there was a possible contamination with a slow growing unidentified organism. A

third (III) experiment comprising all three groups remained germfree throughout the 4-week period.

RESULTS

The results in all three experimental series were essentially the same. While added penicillin alleviated the effects of the thiamine-deficient diet in the normal stock animals, no such effect was seen in the germfree series. At the end of the 28-day experimental period the germfree animals on deficient diets L-465 and L-465PP were in bad condition, and those not sacrificed died between the 4th and 5th week. The normal stock animals on diet L-465 were better physically although death of these animals (not sacrificed) occurred between the 6th and 10th week. In the aforementioned groups, the stomach contents indicated extensive coprophagia in spite of the fact that the rats were housed on screen wire floors. The normal stock animals on diet L-465PP generally had a healthy, lively appearance. A typical set of growth curves (series III) is depicted in figure 1. The curves represent only females because on the thiamine-deficient diet survival of germfree females was better than germfree males. No influence of the possible contamination in series II was noted. Therefore, the numerical results of all three series were compiled in tables 2 and 3. Besides the fact that no beneficial influence of the administration of penicillin to germfree rats on a deficient diet could be detected, it was obvious that in all experimental groups the normal stock animal was in a better condition than its comparable germfree counterpart. Weight gain during the experimental period was always better.

Furthermore, with the addition of penicillin the liver weights of the normal stock animals on the deficient diet approximated those on a complete diet, while under the same conditions, the liver weights of the germfree were lower. A comparison of the thiamine content of the livers showed that on a complete diet (L-464) the level in the germfree animal was surprisingly "below normal." In both germfree and normal stock rats on L-465 the levels were approximately the same and equalled

the concentration found in germfree animals on diet L-465PP. The level in the normal stock rats on diet L-465PP was definitely higher than in the germfree animals, although far below the thiamine level when the normal stock animals were on the complete diet.

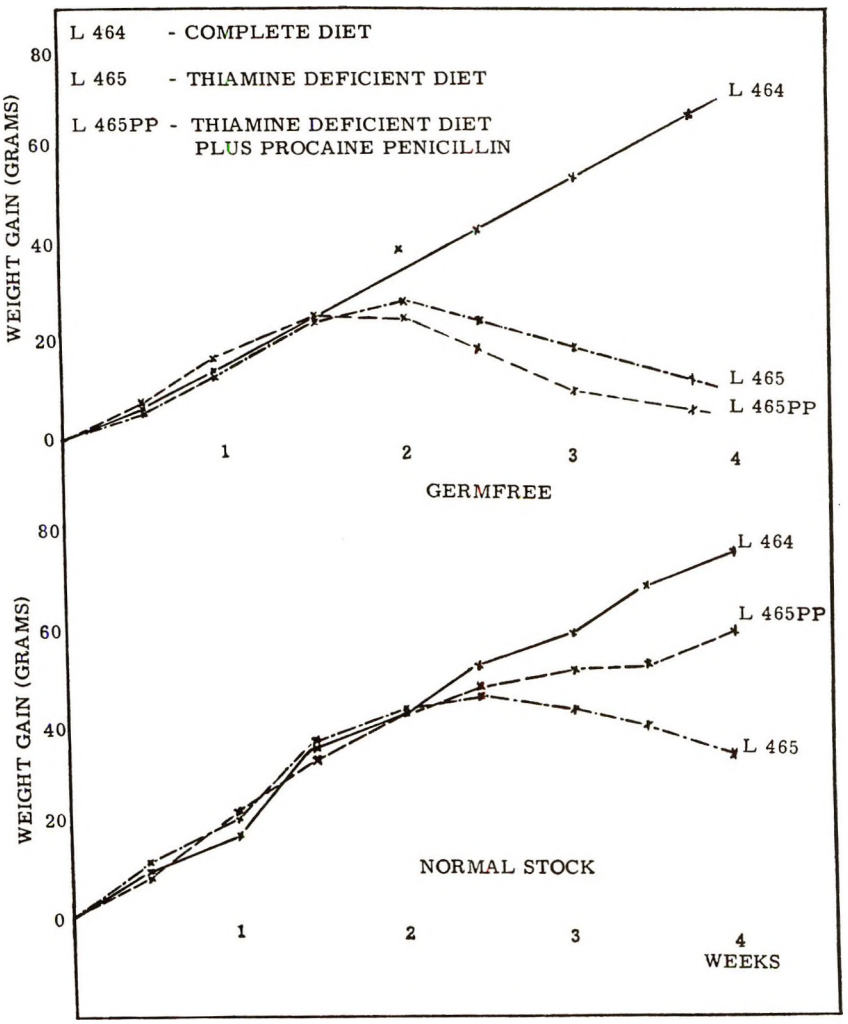

Fig. 1 Growth of germfree and normal stock female rats on complete diet (L-464), thiamine-deficient diet (L-465) and thiamine-deficient diet plus procaine penicillin (L-465PP).

TABLE 2

Weight change of germfree and normal stock rats surviving after 28-day period on a complete (L-464) or thiamine-deficient (L-465) diet with or without added procaine penicillin G

DIET	GERMFREE						NORMAL STOCK					
	MALES			FEMALES			MALES			FEMALES		
	Wt. change	S.D.M.	No. of animals	Wt. change	S.D.M.	No. of animals	Wt. change	S.D.M.	No. of animals	Wt. change	S.D.M.	No. of animals
	gm			*gm*			*gm*			*gm*		
L-464	100.4	± 3.40	6	68.8	± 3.86	5	119.0	± 5.52	6	96.1	± 6.36	6
L-465	— 1.6	± 1.76	4	— 0.7	± 1.76	8	53.2	± 10.88	8	34.4	± 8.89	9
L-465PP	— 1.9	± 2.99	9	— 3.0	± 3.35	8	92.0	± 10.48	9	70.8	± 5.79	9

TABLE 3

Thiamine content of livers of germfree and normal stock rats fed a complete L-464 or a thiamine-deficient L-465 diet with or without added procaine penicillin G. Males and females combined. Experimental period: 28 days. Numbers within parentheses indicate numbers of animals used. S.D.M. values given.

DIET	LIVER WEIGHTS		THIAMINE		TOTAL THIAMINE IN LIVER	
	Germfree	Normal stock	Germfree	Normal stock	Germfree	Normal stock
	gm/100 gm body weight		*μg/gm liver*		*μg/100 gm body weight*	
L-464	4.60 ± 0.20(9)	4.97 ± 0.22(5)	3.74 ± 0.32	8.57 ± 1.28	17.2 ± 1.37	42.0 ± 5.4
L-464PP	—	5.4 (2)	—	8.8	—	47.5
L-465	2.99 ± 0.10(9)	3.74 ± 0.27(12)	0.38 ± 0.21	0.50 ± 0.09	1.1 ± 0.13	1.9 ± 0.19
L-465PP	2.92 ± 0.14(11)	4.21 ± 0.22(10)	0.47 ± 0.07	1.16 ± 0.10	1.4 ± 0.26	4.9 ± 0.38

DISCUSSION

The failure of orally-administered penicillin G to counteract the detrimental effects of a thiamine-low diet in germfree rats is another strong indication that in the normal stock animals the effect of the antibiotic must be regarded as being caused by an action on the intestinal flora. A better utilization of available thiamine, caused by some direct effect of the antibiotic on the host animal, should have been evident also in the germfree environment. More efficient absorption from the gut, as seen by Draper ('58) in penicillin-treated normal stock chickens and apparently related to the influence of the antibiotic on the weight of the intestinal tract, should have manifested itself in both treated and untreated germfree groups. Data obtained in this laboratory have shown that the intestinal tract of the germfree rat is lighter than in the normal stock animal (Gordon, '59) while absorption of both glucose and 3-methyl glucose may be more efficient.[3] Moreover, Gordon et al. ('57–'58) showed that in normal stock chickens the weight reduction of the intestinal tract under the influence of antibiotics does not reach the reduction found in the germ-free animal.

The deficient L-465 diet contains approximately 25% of the thiamine needed for normal growth and development. Apparently the coprophagia seen in the untreated normal stock group on this diet (as judged from stomach contents) could not save the animals from thiamine starvation. This seems to indicate that on the deficient diet little additional thiamine of intestinal origin is available to the host even though the diet contains 58% of rice starch. In the penicillin-treated group, growth and thiamine data prove that more of the vitamin is available to the animal. But even the untreated normal stock group shows better characteristics on the deficient diet than the germfree groups as indicated by a longer maintained growth, more normal liver weights and better survival, though

[3] Csaky, T. Z., and H. A. Gordon, personal communication.

the thiamine concentration in the liver is about the same. However, these differences could be ascribed to the germfree state *per se,* as germfree rats show lower weight gain and possibly a somewhat reduced liver size.

Between the groups fed a complete diet (L-464) there is an obvious difference in the thiamine content of the liver, the level being decidedly lower in the germfree animals. Apparently even on the complete diet more thiamine is available to the flora-harboring animal, possibly because of a generous supply of thiamine synthesized by flora under these circumstances. Whether this additional supply was available in "first passage" or through recirculation of intestinal contents could not be determined. Although in the case of the complete diets, coprophagia was not obvious from the stomach contents, the fact remains that Barnes et al. ('57) found that even on screen bottoms at least 50 to 65% of the feces was eaten by rats fed a complete diet.

The few normal stock animals which were fed the complete diet plus added penicillin confirmed the observation that in this case the antibiotic has no effect. Such a group was not included in the germfree experiments because of lack of space and also because the germfree intestine was considered to have reached a maximum absorptive capacity. However, in the light of the differences found between germfree and normal stock rats fed a complete diet, the inclusion of such a group might have been worth while.

In conclusion, it can be said that for the normal stock rat on a thiamine-deficient diet (L-465), not enough flora-synthesized thiamine becomes available to make growth and survival possible. In a rat harboring a normal intestinal flora the addition of penicillin to the diet changes this situation indicating a direct flora effect as the responsible factor. However, it is not clear whether the extra amount is taken up on "first passage" possibly aided by an increased absorptive capacity of the gut or via limited coprophagia.

SUMMARY

Procaine penicillin G (50 mg/kg diet) was administered to young germfree and normal stock rats on a thiamine-deficient diet. Normal stock rats showed continued, though retarded growth, while the untreated controls started to lose weight after two weeks. In rats sacrificed after 4 weeks the thiamine content of the liver was 0.50 μg/gm in the untreated group, 1.16 in the treated group and 8.57 in animals receiving a complete diet. All untreated rats not sacrificed at that time died between the 6th and 10th weeks.

Germfree animals showed no influence of the feeding of penicillin. In both groups weight loss began after two weeks, while death occurred (if not sacrificed) between 4 and 5 weeks. Animals sacrificed after 4 weeks were invariably in bad condition. The thiamine content of the liver of the untreated averaged 0.38 μg/gm while the treated group showed approximately the same, 0.47. The germfree control group, receiving a complete diet, showed a value of 3.74.

Obviously, the action of penicillin is (caused by an action) on the intestinal flora, and thiamine synthesized under influence of the antibiotic is available to the host.

ACKNOWLEDGMENT

We acknowledge with thanks, the technical assistance of Mrs. Margaret Beaver and Mrs. Louise Lowney. The authors are also indebted to Dr. John G. Trump of the Massachusetts Institute of Technology for radiation-sterilization of procaine penicillin.

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SOME ASPECTS OF TRYPTOPHAN METABOLISM IN HUMAN SUBJECTS [1]

I. NITROGEN BALANCES, BLOOD PYRIDINE NUCLEOTIDES AND URINARY EXCRETION OF N^1-METHYLNICOTINAMIDE AND N^1-METHYL-2-PYRIDONE-5-CARBOXAMIDE ON A LOW-NIACIN DIET

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INTRODUCTION

Following the demonstration by Heidelberger, Abraham and Lepkovsky ('49) of the metabolic conversion of tryptophan to niacin in the rat evidence has been obtained to indicate that tryptophan may be used to furnish a portion of the niacin requirement of man (Sarett and Goldsmith, '49; Reddi and Kodicek, '53; Holman and deLange, '50). Recent evidence suggests that the tryptophan-niacin requirements vary with the existing experimental conditions. Koeppe and Henderson ('55) found that tryptophan served more effectively as a niacin source when a relative lack of some other amino acid limited its use for protein formation. Chaloupka, Williams, Reynolds and Elvehjem ('57) observed a certain sequence of events in their studies which suggested a preferential use of limited amounts of tryptophan in the young adult rat fed a niacin-tryptophan-deficient ration. When tryptophan levels in this diet were gradually increased the animals first established and maintained nitrogen equilibrium, secondly demonstrated

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

Data in this paper are taken from a thesis submitted by Virginia M. Vivian in partial fulfillment of the requirement for the degree of Doctor of Philosophy.

growth, and finally there was a restoration of the blood pyridine nucleotides.

This paper presents the results from an investigation of the metabolic use of tryptophan in human subjects on a low-niacin, low-tryptophan, semi-synthetic diet supplemented with gradually increasing levels of tryptophan. The data obtained suggest that the human subjects used tryptophan first to maintain nitrogen balance and then to produce blood pyridine nucleotides. Further increase in the tryptophan intake was associated with an increased urinary excretion of niacin metabolities.

EXPERIMENTAL

Subjects. Four college women in apparent normal health and ranging in age from 20 to 34 years volunteered as subjects. One student (subject 1) was from India, one (subject 4) was from Egypt and the other two were white Americans. Each subject engaged in her normal activities while on the experiment.

TABLE 1

The control diet [1]

BREAKFAST		LUNCH		DINNER	
Food	Amount	Food	Amount	Food	Amount
	gm		*gm*		*gm*
Applesauce, canned	100	Apricots, canned	50	Beef pattie (raw weight)	100
Bread, white	40	Banana	50	Potatoes, boiled	100
Butter	20	Pineapple, canned	50	Broccoli, frozen	100
Jelly, grape	20	Lettuce	20	Orange juice, frozen	100
Milk, whole	180	Salad dressing	20	Pears, canned	100
Sugar [2]		Cheese, cheddar	35	Milk, whole	180
		Bread, white	40		
		Butter	20		
		Ice cream, vanilla	100		
		Milk, whole	180		

[1] The control diet was calculated to provide the following nutrients: Calories, 2000; protein, 62 gm; fat, 100 gm; Carbohydrate, 210 gm; calcium, 1.3 gm; phosphorus, 1.2 gm; iron, 9 mg; vitamin A, 7,800 U; thiamine, 0.9 mg; riboflavin, 1.9 mg; ascorbic acid, 145 mg.

[2] Amount of sugar varied with caloric needs of each subject. Calories from added sugar are not included in the calorie total given in footnote 1.

Diets. The general plan of administration of the diet was that described by Jones, Baumann and Reynolds ('56). A diet of ordinary foods, as listed in table 1, was given for 6 days to establish control values for comparison with the values obtained in the experimental periods. Food in the control period supplied daily approximately 10 mg of niacin, 680 mg of tryptophan and 10 gm of nitrogen as well as generous amounts of all other known dietary essentials. A three-day transition

TABLE 2

Foods in the semi-synthetic diet [1]

FOOD	AMOUNT
	gm
Applesauce, canned	200
Apricots, canned	100
Banana	25
Jelly, grape	45
Lemon juice	75
Lettuce	25
Orange juice, frozen	300
Peaches, canned	100
Pineapple, canned	25
Tomatoes, canned	100
Sucrose	180
Butter oil	60
Cornstarch pudding	1 recipe
Cornstarch wafers	1 recipe

[1] The foods were calculated to provide the following nutrients: Calories, 2500; protein, 6 gm; fat, 95 gm; carbohydrate 440 gm. Extra calories to meet the needs of each subject were provided by sugar, candy and beverage.

period was provided for adjustment to the semi-synthetic regimen during which time foods were gradually withdrawn and the synthetic components were introduced.

For the duration of the experiment a semi-synthetic basal diet was fed in which ordinary foods such as a few low-protein fruits, butter oil, cornstarch, sucrose and a vegetable oil[2] contributed about 2.5 mg of niacin and 25 mg of tryptophan (table 2). The synthetic portion of the diet included all of

[2] Wesson oil.

the essential amino acids (except tryptophan) plus arginine, histidine, cystine and tyrosine, each of which was supplied in amounts equivalent to those found in 20 gm of egg protein. As in all of our metabolic studies, the natural isomers of all amino acids, with the exception of DL-isoleucine, were used. The remainder of the 10 gm of nitrogen was provided by glycine and diammonium citrate. All of the remaining known mineral and vitamin requirements (except niacin) were furnished by a mineral suppliment [3] and a vitamin supplement.[4] Sugar, cornstarch pudding and wafers (Leverton et al., '56), a lemon flavored sugar candy [5] and some carbonated beverages [6] supplied extra calories in amounts adjusted to maintain a constant weight for each subject. A purified hemicellulose compound [7] was used to provide bulk in the diet. Tea was allowed according to each subject's personal desires, but was kept constant for each individual throughout the experiment. Water, salt and various spices [8] were allowed ad libitum.

The semi-synthetic diet was administered for 5 periods (III through VII), each of which was 6 days in length except for period VII which was 5 days long. These periods differed from one another only in that the daily tryptophan intake was gradually increased from 25 mg in period III to 810 mg in period VII. As the tryptophan supplement was increased in quantity, glycine was withdrawn in appropriate amounts to maintain an isonitrogenous intake.

Methods: Daily urine samples were collected under toluene, diluted to a convenient volume and frozen until ready for analyses. To evaluate the completeness of urinary collections,

[3] Prepared by Nutritional Biochemicals, Inc., following a formula furnished to them by Leverton et al. ('56).

[4] The vitamin supplement as provided and assayed by Hoffman-LaRoche, Inc., furnished per capsule 5,300 U vitamin A, 4.2 U vitamin E, 153 mg methionine, 198 mg choline, 3.5 mg pyridoxine, 0.164 mg biotin, 0.79 mg folic acid, 8.3 mg panthenol, 0.80 μg vitamin B_{12}, 5.3 mg vitamin B_1 and 3.2 mg vitamin B_2.

[5] Life Savers.

[6] Ginger ale and 7–up.

[7] Mucilose Flakes, Winthrop-Stearns, Inc.

[8] Celery salt, garlic salt, pepper, salt, sage, thyme, cinnamon, celery seed, oregano, and curry.

daily creatinine excretion was determined by means of an alkaline picrate reagent. Fecal collections were separated into periods with carmine markers and prepared for analyses as described by Jones, Baumann, and Reynolds ('56).

A food composite equivalent to one day's diet was prepared from aliquots of all the foods eaten in each period except for the butter oil, beverages and candy. The nitrogen content of the amino acids, foods, urine and fecal samples was determined by a modification of the Kjeldahl method of Scales and Harrison ('20). The tryptophan content of the foods and tryptophan supplement was determined by a microbiological assay.[9] The amounts of other dietary components were calculated from data in Agriculture Handbook No. 8 (Watt and Merrill, '50).

The method of Huff and Perlzweig ('47) was used to measure the daily excretion of N^1-methylnicotinamide (N–Me) in the urine. The daily urinary excretion of N^1-methyl-2-pyridone-5-carboxamide (pyridone) was determined by the method of Price ('54) with the larger diameter columns as used by Walters et al. ('55). For each batch of resins prepared, distilled water was passed through two columns in the same manner as that used for the urine samples and the ultraviolet light absorption of the effluent was used as a blank in the determinations on the urine samples. The correction was negligible when the pyridone concentration was high but as the pyridone excretion decreased this correction was of increasing importance.

On the last day of each period the pyridine nucleotide (PN) level in the blood was determined by a modification of the method of Kring and Williams ('54). In these analyses the sum of the triphosphopyridine nucleotide, diphosphopyridine nucleotide and N^1-methylnicotinamide was calculated using diphosphopyridine nucleotide as a standard and is expressed as micromoles of PN per milliliter of whole blood.

RESULTS AND DISCUSSION

The mean values of the data for each subject are summarized in table 3. It may be noted that three of the 4 subjects were in

[9] Tryptophan assays were made by the Wisconsin Alumni Research Foundation.

positive nitrogen balance during the control period. During period III, when the tryptophan intake on the semi-synthetic regimen was only 25 mg daily, three of the 4 subjects showed a marked nitrogen loss. All of the subjects, however, showed nitrogen retention during period IV with a tryptophan intake of 170 mg. The data show considerable individual variation. With the exception of subject 3, who was in negative nitrogen balance during the control period on an intake of 680 mg of tryptophan, the subjects generally showed nitrogen storage when 170 mg or more of tryptophan were supplied daily.

When the subjects were transferred from a diet of ordinary foods to the semi-synthetic diet containing 25 mg of tryptophan and 2.5 mg of niacin there was a sharp decrease in the pyridone excretion (table 3). Although the tryptophan intake was increased in the following two periods (IV and V) the excretion of pyridone continued to decrease. Averaging the data per subject per period obscured the fact that the pyridone values for two of the subjects were zero on several days. There was no appreciable change in excretion when the tryptophan intake was increased from 220 to 315 mg in period VI. The pyridone excretion by subjects 1, 2 and 3 increased sharply in period VII on an intake of 810 mg of tryptophan. Subject 4 excreted increased amounts of pyridone on days one and 5 of this period but maintained an average excretion for the period only slightly higher than that in the preceding period (VI). The tryptophan intake in period VII was approximately two and one half times that of the preceding period and the average pyridone excretion exhibited an increase of similar magnitude. It is likely that if this period had been continued for a longer time the excretion values would have returned to or surpassed the control values. On the last day of period VII the urinary pyridone excretion of subject 1 had returned to the original level of the control period. It is possible that the more rapid return to this level of pyridone excretion by this subject may be related to the fact that she did not demonstrate a nitrogen loss at any time during the experiment.

TABLE 3

Summary of data per subject per period

SUBJECT	MEAN DAILY N BALANCE	PN PER ML BLOOD	MEAN DAILY URINARY EXCRETION		RATIO PYRIDONE *N*-Me
			Pyridone	*N*-Me	
	gm	*μM*	*μM*	*μM*	
	Control (680 mg tryptophan, 10 mg niacin)				
1	0.94	0.098	45.9	31.0	1.48
2	0.10	0.084	48.0	30.2	1.59
3	— 0.61	0.074	81.9	62.2	1.32
4	0.33	0.055	63.7	49.6	1.28
mean	0.19	0.078	59.9	43.3	1.42
	Period III (25 mg tryptophan, 2.5 mg niacin)				
1	1.14	0.064	13.1	13.1	1.00
2	— 0.80	0.050	14.4	14.6	0.99
3	— 0.67	0.060	15.3	21.8	0.70
4	— 0.98	0.062	18.9	19.9	0.95
mean	— 0.33	0.059	15.4	17.4	0.91
	Period IV (170 mg tryptophan, 2.5 mg niacin)				
1	0.89	0.057	8.7	15.2	0.57
2	0.48	0.039	6.9	17.4	0.40
3	0.17	0.035	7.8	16.3	0.48
4	0.15	0.057	9.4	15.0	0.63
mean	0.42	0.047	8.2	16.0	0.52
	Period V (220 mg tryptophan, 2.5 mg niacin)				
1	1.13	0.056	6.4	18.9	0.34
2	0.18	0.038	3.5	11.7	0.30
3	0.01	0.058	6.0	13.5	0.44
4	0.08	0.057	5.2	13.4	0.39
mean	0.35	0.052	5.3	14.4	0.37
	Period VI (315 mg tryptophan, 2.5 mg niacin)				
1	0.88	0.092	8.4	14.6	0.58
2	0.81	0.073	3.2	14.9	0.21
3	0.57	0.046	6.1	12.9	0.47
4	0.07	0.049	4.4	16.4	0.27
mean	0.58	0.065	5.5	14.7	0.38
	Period VII (810 mg tryptophan, 2.5 mg niacin)				
1	1.15	0.100	25.2	48.2	0.52
2	0.64	0.063	8.7	15.8	0.55
3	0.41	0.075	17.6	23.0	0.76
4	0.25	0.074	8.7	17.8	0.49
mean	0.61	0.078	15.1	26.2	0.58

The pattern of excretion of *N*–Me generally followed that of the pyridone. The daily excretion of *N*–Me showed a greater individual variation than that of the pyridone excretion. The level of excretion of *N*-Me on the molar basis did not reach as low values as that of the pyridone. The *N*–Me excretion of subject 1 during period VII exceeded that of the control period. The other subjects excreted from one-third to one-half as much *N*–Me in period VII as in the control period.

The range of values for the metabolites shows fairly wide individual variation in periods I and VII, but a striking uniformity of values in periods III through VI. Thus it appears that when tryptophan is supplied in abundance (680 to 810 mg) individual variability in the metabolic utilization of tryptophan occurs, while a similar pattern of utilization in all subjects is seen when tryptophan is limited (25 to 315 mg).

The ratio of pyridone to *N*–Me found in the urines collected during the control period ranged from 1.28 to 1.59 (table 3). These ratios were in agreement with the ratios (recalculated on a molar basis) reported by Perlzweig, Rosen and Pearson ('50) for 10 healthy adults on self-selected diets. The ratio decreased to an average of 0.39 in period VI and increased to an average of 0.58 in period VII. Rosenthal, Goldsmith and Sarett ('53) suggested that the ingestion of nicotinic acid in excess of that required for normal metabolism is disposed of as the pyridone. If this suggestion is correct, the low pyridone to *N*–Me ratios found in urine samples collected during the time on the semi-synthetic diet would indicate that little excess niacin was available either in the diet or from body sources.

When the subjects were placed on the semi-synthetic diet the blood PN levels decreased gradually until the end of period IV. However, unlike the urinary metabolites of niacin, the blood PN started to increase during period V on a tryptophan intake of 220 mg. The increase in blood PN continued through the remaining periods until at the end of period VII the values equalled those of the control period. It seems reasonable to suggest that the increase in blood PN values was a direct response to the increased dietary intake of tryptophan. The

individual variations observed in these data might be expected since Duncan and Sarett ('51) reported that the concentration of PN in the red blood cells of their subjects who were given test doses of nicotinic acid or tryptophan appeared to be related to body weight, total blood or red cell volume, the state of nutriture, or sex.

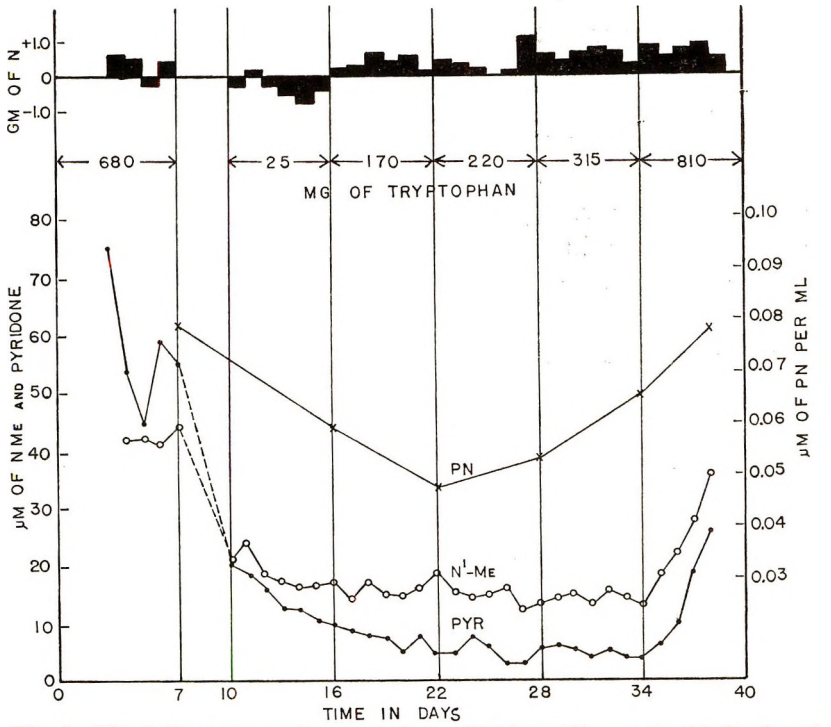

Fig. 1 The daily average nitrogen balance, blood pyridine nucleotide level, and urinary *N*-¹methylnicotinamide and *N*¹-methyl-2-pyridone-5-carboxamide excretion of 4 human subjects on varied tryptophan intakes.

The mean values of the results for all subjects have been expressed graphically in figure 1. A study of this figure reveals a pattern of tryptophan utilization. On an intake of 2.5 mg of niacin and 25 mg of tryptophan in period III there was a loss of nitrogen, a decrease in blood PN levels and a sharp decrease in the urinary excretion of niacin metabolites. On an intake of 170 mg of tryptophan the blood PN continued to

decrease, there was no change or a slight decrease in urinary niacin metabolites, but nitrogen retention was observed. When the tryptophan intake was increased to 220 or 315 mg daily the blood PN levels increased toward normal without any significant change in urinary niacin metabolite excretion. When the tryptophan intake was 810 mg daily, the blood PN levels returned to normal, the excretion of the urinary niacin metabolites increased sharply toward normal and nitrogen retention was continued. These results indicate that under these experimental conditions human subjects on a low-niacin, low-tryptophan diet utilized a gradually increasing level of tryptophan intake in the following sequence: first to establish nitrogen equilibrium, secondly to synthesize blood pyridine nucleotides and finally there was an increased excretion of the urinary metabolites of niacin. These data are in accord with the preferential use of tryptophan in niacin-tryptophan deficient rats as reported by Chaloupka and co-workers ('57).

SUMMARY

Nitrogen balance, blood pyridine nucleotide levels and the urinary excretion of the metabolites of niacin, N^1-methylnicotinamide and N^1-methyl-2-pyridone-5-carboxamide, were studied in 4 college women who were transferred from a nutritionally adequate diet of ordinary foods to a semi-synthetic diet which was low in niacin and tryptophan. A daily intake of 10 gm of nitrogen was maintained throughout the entire experiment. During the control period the diet of ordinary foods supplied 680 mg of tryptophan and 10 mg of niacin. The niacin level in the semi-synthetic diet was kept constant at 2.5 mg daily. The tryptophan content of the semi-synthetic diet was increased stepwise, providing daily intakes of 25, 170, 220, 315 and 810 mg in consecutive periods.

Nitrogen loss occurred on the 25 mg intake of tryptophan; nitrogen storage occurred when the tryptophan intake was increased to 170 mg or above. The urinary excretion of the niacin metabolites decreased sharply when the subjects were

placed on the semi-synthetic regimen and remained at low levels until the tryptophan intake was increased to 810 mg daily. The blood pyridine nucleotide levels decreased in a similar manner until the end of the period in which the tryptophan intake was 220 mg daily, following which they gradually increased toward normal.

Under these experimental conditions it appears that tryptophan was used first to establish and maintain nitrogen equilibrium, second for the synthesis of blood pyridine nucleotides and finally, when the blood pyridine nucleotides had reached nearly normal levels, there was an increase in the urinary excretion of the two measured niacin metabolites.

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SOME ASPECTS OF TRYPTOPHAN METABOLISM IN HUMAN SUBJECTS

II. URINARY TRYPTOPHAN METABOLITES ON A LOW-NIACIN DIET [1,2]

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The urinary excretion of tryptophan metabolites by human subjects on self-selected (Brown and Price, '56) and constant diets (Price, Brown and Ellis, '56) containing adequate amounts of niacin and tryptophan have been reported. The studies of Horwitt, Harvey, Rothwell, Cutler and Haffron ('56) indicated that about 60 mg of tryptophan give rise to 1 mg of niacin in human subjects. Whether tryptophan is converted to niacin to the same extent when dietary niacin is very low is difficult to ascertain. Goldsmith, Gibbens, Unglaub and Miller ('56) suggested, as a partial explanation for some of their data, that niacin-containing enzymes were required for the conversion of tryptophan to quinolinic acid and niacin compounds and that these enzymes were exhausted in severe niacin depletion. Niacin (as reduced triphosphopyri-

[1] Supported in part by grants from the National Institute of Arthritis and Metabolic Diseases (no. A-1499), United States Public Health Service; from the Wisconsin Division of American Cancer Society; and from the American Cancer Society.

[2] Published with approval of the Director of the Wisconsin Agricultural Experiment Station.

[3] American Cancer Society, Charles S. Hayden Foundation Professor of Surgery in Cancer Research.

dine nucleotide) is necessary for the formation of hydroxykynurenine from kynurenine (de Castro, Price and Brown, '56; Saito, Hayaishi and Rothberg, '57). Thus, it might be expected that the extent of the conversion of tryptophan to niacin would be dependent upon the pre-existing levels of triphosphopyridine nucleotide in the body.

Studies of Chaloupka, Reynolds, Williams and Elvehjem ('57) using rats and of Vivian, Chaloupka and Reynolds ('58) with human subjects suggested that when a limited amount of tryptophan was provided in a diet low in niacin the amino acid was used preferentially to maintain nitrogen balance and used for niacin synthesis only after nitrogen balance was achieved. For these reasons it was of interest to measure the urinary excretion of intermediates between tryptophan and niacin by subjects receiving low levels of niacin and graded amounts of tryptophan added to a semi-synthetic diet. These studies also afforded the possibility of checking the specificity and sensitivity of the analytical methods used for urinary tryptophan metabolites since on the low-niacin low-tryptophan diet the urinary levels of these metabolites were expected to decrease significantly.

EXPERIMENTAL

The subjects, diets and urine samples were those described by Vivian, Chaloupka and Reynolds ('58). The urine samples were collected under toluene, measured and refrigerated and analyses were done as soon as possible thereafter, usually within two days. Samples of urine were then frozen for repeat analyses if needed.

The basic semi-synthetic diet provided 2.5 mg of niacin and 25 mg of tryptophan per day and to this was added graded amounts of L-tryptophan at the intervals and amounts indicated in figures 1 and 2. Thus, in period I the subjects ingested a controlled natural diet; in period II increasing amounts of the semi-synthetic diet were substituted for natural foods. In period III and all subsequent periods the subjects

were completely on the semi-synthetic diet containing 2.5 mg of niacin and the amounts of tryptophan indicated. At the start of the last day of the study (day 39) a single 2.0 gm dose of L-tryptophan was ingested.

Analytical Methods. The analytical methods used for aromatic amines and quinaldic acid were those described by Brown and Price ('56). The kynurenine and acetylkynurenine values were obtained by the volatile amine method (Brown and Price, '56). The less specific method of measuring the diazotizable amines in these two fractions gave values slightly higher and more variable than did the volatile amine method. The method for determination of 3-hydroxykynurenine was that of Brown ('57). Kynurenic acid and xanthurenic acid were measured by the spectrophotofluorometric method of Satoh and Price ('58) and xanthurenic acid-8-methyl ether (8-methoxy-4-hydroxyquinaldic acid) was measured by a fluorometric method.[4] *N*-Methyl-2-pyridone-5-carboxamide (pyridone) values were determined in our laboratories by the method of Price ('54) and were in good agreement with the values obtained by Vivian, Chaloupka and Reynolds ('58).

RESULTS

The results are shown graphically in figures 1 and 2. Each point is the average for the 4 subjects in the last two days of each diet period except in period VII in which only the one day averages are plotted. Aromatic amine fraction "A" did not change consistently although lowest values were found in diet period III. This fraction was previously shown not to vary with tryptophan intake (Brown and Price, '56) and is not plotted on the figures. Excretion of quinaldic acid was almost identical with the values for kynurenine (fig. 2), therefore, quinaldic acid was not plotted.

Pyridone excretion decreased sharply in period III but did not reach its lowest value until period V. All other metabolites were at a minimum in period III or IV and had started

[4] Satoh, K., and J. M. Price. Unpublished data.

to increase in period IV or V while pyridone excretion was still decreasing. The excretion of all metabolites except quinaldic acid increased sharply in period VII and approached or surpassed the control levels found in period I.

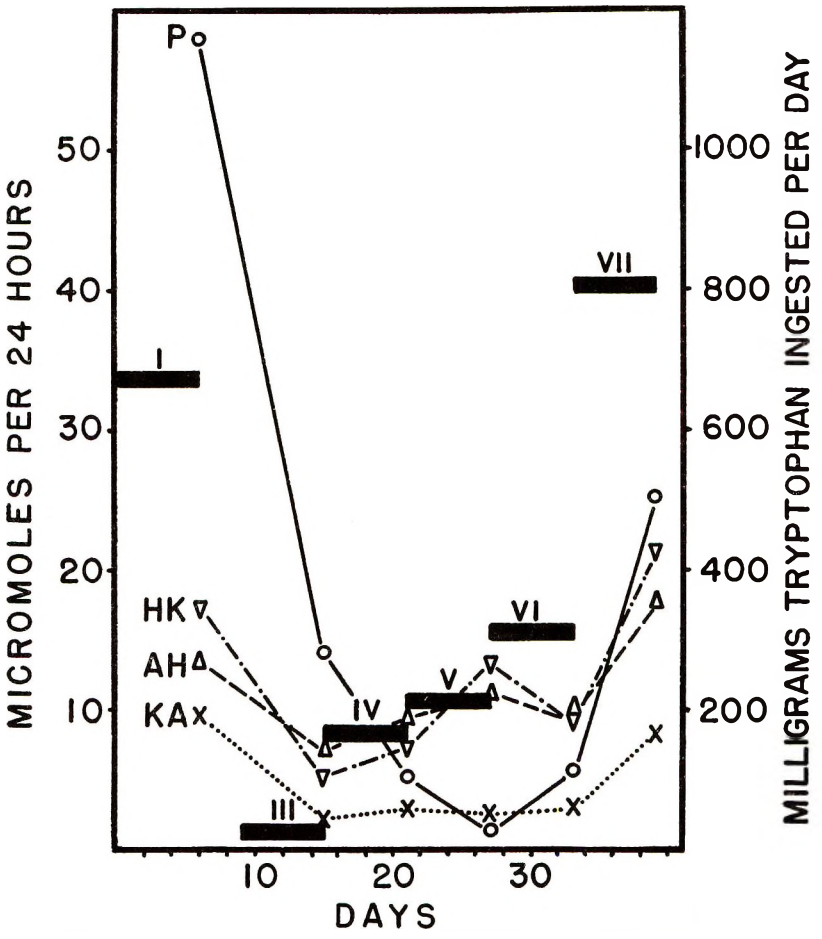

Fig. 1 The points indicated on the chart are the average urinary excretion values of *N*-methyl-2-pyridone-5-carboxamide (P), hydroxykynurenine (HK), *o*-aminohippuric acid (AH) and kynurenic acid (KA) for the 4 subjects at the end of each diet period. The tryptophan intake during each diet period is indicated by the heavy horizontal bars and the scale on the right side of the chart. Dietary niacin was constant at 2.5 mg per day throughout periods III to VII.

The response of these subjects to the test dose of 2.0 gm of L-tryptophan expressed as μmoles increase above the excretion of the previous day was for pyridone 50.4; kynurenic acid 56.3; xanthurenic acid 8.6; anthranilic acid glucuronide 3.9; *o*-aminohippuric acid 39.6; acetylkynurenine 12.1; kynurenine 36.3; xanthurenic acid-8-methyl ether 1.0; quinaldic acid 1.8; hydroxykynurenine 27.8.

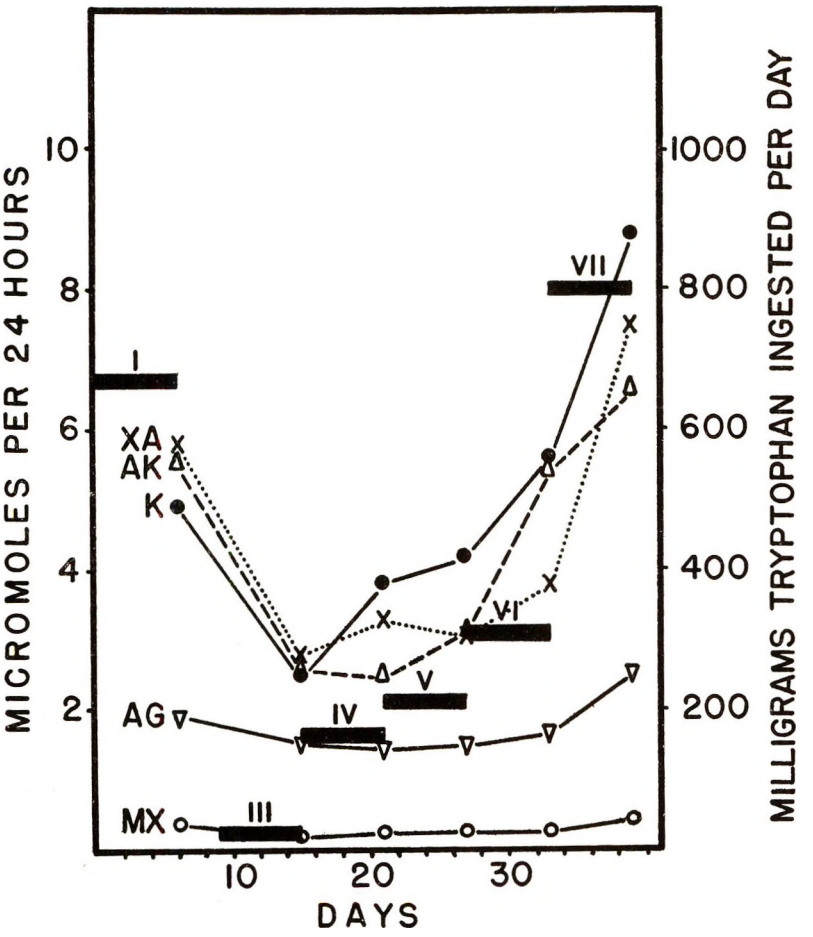

Fig. 2 The same as figure 1 for xanthurenic acid (XA), acetylkynurenine (AK), kynurenine (K), anthranilic acid glucuronide (AG) and the 8-methyl ether of xanthurenic acid (MX).

DISCUSSION

The results of Vivian, Chaloupka and Reynolds ('58) indicated that these subjects were in negative nitrogen balance during period III. The stepwise addition of L-tryptophan to the diet was associated with the occurrence of nitrogen balance, increased blood levels of pyridine nucleotides, and increased urinary excretion of niacin metabolites, in that order.

Pyridone excretion remained at very low levels until the last period and thus did not parallel the tryptophan intake. This suggests that pyridone was excreted in significant amounts only when excess niacin or tryptophan was present and when the pyridine nucleotide pools were repleted. The excretion of all the other metabolites more closely paralleled the tryptophan intake throughout the various diet periods except that kynurenic acid, xanthurenic acid, and xanthurenic acid-8-methyl ether tended to increase more slowly. Hydroxykynurenine excretion increased in period IV suggesting that the depletion of pyridine nucleotides in period III was not sufficient to inhibit detectably the reduced triphosphopyridine nucleotide catalyzed hydroxylation of kynurenine.

Normal male subjects on constant and self-selected diets of ordinary foods excreted a total of 207 and 213 μmoles respectively of certain tryptophan metabolites in response to a 2.0 gm dose of L-tryptophan (Price, Brown and Ellis, '56). The female subjects in this study excreted a total of 207 μmoles of the same metabolites following ingestion of a single dose of 2.0 gm of L-tryptophan. Acetylkynurenine, kynurenine and *o*-aminohippuric acid were excreted in slightly larger amounts in the present study and pyridone excretion was somewhat less in response to the 2.0 gm supplement. However, in the present study the pyridone excretion was determined for only one day following the 2.0 gm supplement of L-tryptophan which would not be sufficient time for a return to normal urinary levels of pyridone (Walters et al., '55; Price, Brown and Ellis, '56).

Evaluation of the specificity and sensitivity of the various analytical methods used was difficult because of the possibility of variable amounts of tryptophan liberated from body proteins when the subjects were in negative nitrogen balance and the unknown amount of this tryptophan which might have entered the kynurenine-niacin pathway. However, the fact that on several days the pyridone values for several of the subjects were zero suggests that on this semi-synthetic diet, at least, there were no other urinary chemicals which interfered with the determination of the pyridone. None of the values for other tryptophan metabolites reached zero, but they did decrease considerably during period III suggesting that the analytical methods were reasonably specific and may be considered reliable in studies of this nature.

The fact that the urinary excretion of kynurenine and hydroxykynurenine did not cease even with the lowest level of tryptophan intake suggests that the conversion of tryptophan to niacin continued at all times during the study. Since the curve of the excretions of the intermediary metabolites on the pathway from tryptophan to niacin more nearly resembled that of the blood pyridine nucleotides than that of the urinary pyridone and *N'*-methylnicotinamide (Vivian, Chaloupka and Reynolds, '58), it would appear that on a low-niacin diet there may be considerable conversion of tryptophan to niacin without a significant rise in the urinary excretion of niacin metabolites.

SUMMARY

The excretion of 10 tryptophan metabolites was measured in the urine of 4 college women maintained on a semi-synthetic diet containing 2.5 mg of niacin per day and varying in tryptophan content from 25 to 810 mg per day. The excretion of all metabolites was very low when the tryptophan intake was 25 mg per day. After stepwise additions of tryptophan to the diet, *N*-methyl-2-pyridone-5-carboxamide excretion remained at low levels until blood pyridine nucleotide levels were restored. The excretion of the other tryptophan metab-

olites more closely followed the tryptophan intake. When given a 2.0 gm loading dose of L-tryptophan these subjects excreted essentially the same amounts of these metabolites as did previous subjects ingesting ordinary foods.

ACKNOWLEDGMENTS

The authors wish to acknowledge the assistance of Mrs. Marian Prahl and Mrs. Judith Linnane with some of the analyses.

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THE REPLACEMENT BY THIAZOLIDINECARBOXYLIC ACID OF EXOGENOUS CYSTINE AND CYSTEINE [1]

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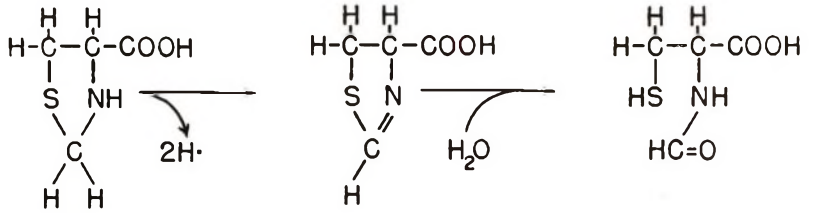
Several years ago it was observed in this laboratory that L-thiazolidinecarboxylic acid is oxidized by liver mitochondria with the uptake of one oxygen atom per molecule of substrate (Mackenzie, '55). Subsequently the end product of the reaction was isolated in high yields and identified as *N*-formylcysteine (Harris and Mackenzie, '55; Mackenzie and Harris, '57).



The initial oxidation is catalyzed by a specific thiazolidinecarboxylic acid dehydrogenase, as shown by the fact that related compounds are not oxidized, or only at a very slow rate, when added to the mitochondrial system. These observations

[1] These investigations were made possible by grants from the National Institutes of Health, United States Public Health Service (No. A969–C5) and from the Association for the Aid of Crippled Children, and constituted a part of the thesis submitted by Harold J. DeBey in fulfillment of the requirement for the degree of Doctor of Philosophy.

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prompted us to explore the possibility that L-thiazolidinecarboxylic acid could replace exogenous cystine and cysteine in the intact animal.

In 1915, Osborne and Mendel had observed that the addition of cystine to a low-casein diet produced a growth response in young rats. The apparent indispensability of cystine was confirmed by many other investigators. However, some 15 years later, Jackson and Block ('31, '32) discovered that methionine could replace cystine as a growth factor in animals fed a low-cystine diet. Finally, William C. Rose ('38), in his monumental studies with amino acid diets, provided convincing evidence that methionine is the indispensable sulfur amino acid and that cystine elicits a growth response only when the methionine content of the diet is suboptimal (Womack and Rose, '41).

In attempting to assemble a low-methionine diet that would give a growth response upon the addition of cystine, we were confronted with the fact that the "protein free milk" (Osborne and Mendel, '15) and the milk vitamin concentrate (du Vigneaud, Dyer and Harmon, '33) used as vitamin sources by earlier investigators, were no longer available. We therefore employed synthetic B vitamins and soy bean protein, which is low in both methionine and cystine.[3] However, at a level of soy bean protein (12%) that limited growth, the response to added cystine and methionine was poor. Consequently, we turned to casein as a source of protein and found that when it was incorporated in the purified diet at a 7% level the addition of cystine produced a significant growth response.[4] The present paper reports the growth produced on this diet by the addition of cystine, thiazolidinecarboxylic acid, and related compounds. At the same time we have investigated the ability

[3] Drackett Assay Protein Cl containing 1% methionine and 0.6% cystine by microbiological assay.

[4] Mitchell ('31) has shown that cysteine promotes the growth of rats fed a "cystine"-deficient diet. Because of the ease with which cysteine is autooxidized, especially in the presence of metals, the disulfide form was used in the dietary experiments described in this paper.

of thiazolidinecarboxylic acid to replace cysteine in counteracting the acute toxicity of thiourea.

MATERIALS AND METHODS

The animals used in the nutritional experiments were male rats of the Sprague-Dawley strain. They were housed in individual cages with raised screen bottoms and given food and water ad libitum. The composition of the experimental

TABLE 1

Cystine and methionine test diets

CONSTITUENT	CYSTINE	METHIONINE
	gm	*gm*
Casein [1]	70	0
Amino acid mixture [2]	0	172
Sucrose	678	574
Crisco	190	190
Corn oil [3]	10	10
Salt mixture [4]	40	40
Choline chloride	2	4
Vitamin mixture [5]	10	10

[1] Nutritional Biochemicals Corp., Vitamin Free.

[2] Mackenzie et al. ('50) with 13 gm of L-leucine replacing the 26 gm of DL-leucine. This mixture does not contain cystine or methionine.

[3] Each 10 ml of corn oil contained 40 mg of α-tocopherol acetate, 1 mg of Menadione, and 7 drops of oleum percomorphum (Mead). The latter provided approximately 8,800 I.U. of vitamin A and 1,300 I.U. of vitamin D.

[4] Hubbell, Mendel and Wakeman ('37) prepared by Nutritional Biochem. Corp.

[5] The vitamin mixture was composed of 10 mg thiamine·HCl, 10 mg riboflavin, 10 mg pyridoxine·HCl, 10 mg nicotinamide, 50 mg calcium pantothenate, 0.1 mg biotin, 10 mg *p*-aminobenzoic acid, 100 mg *m*-inositol, and pulverized sucrose to 10 gm. When used in the methionine test diet, 2 mg folic acid and 0.2 mg vitamin B_{12} were added.

diets is shown in table 1. Rats fed the cystine test diet weighed approximately 60 gm at the beginning of the experiment, and those fed the methionine test diet weighed approximately 140 gm. The cystine-deficient rats were weighed twice a week, and the methionine-deficient animals were weighed daily.

L-Thiazolidine-4-carboxylic acid was synthesized by the procedure previously described from this laboratory (Mackenzie

and Harris, '57). DL-1,3-Thiazane-4-carboxylic acid was synthesized from DL-homocysteine and formaldehyde by the method of Wriston and Mackenzie ('57). L-*N*,*N*'Diformylcystine was synthesized by the procedure of du-Vigneaud, Dorfman and Loring ('32). Thiazolidine hydrochloride was prepared from formaldehyde and β-mercaptoethylamine [5] by the method of Ratner and Clarke ('37). The melting points of all of the foregoing compounds agreed with the values given in the literature.

L-2-Methylthiazolidine-4-carboxylic acid was synthesized by a modification of the procedure described by Cook and Heilbron ('49). Six grams of L-cysteine were dissolved in 15 ml of hot water and the pH of the solution was adjusted to 2.5 with hydrochloric acid. Five milliliters of freshly distilled acetaldehyde were added and the reaction mixture was allowed to stand at room temperature for several hours. The solution was then concentrated *in vacuo* to a heavy syrup. The product was precipitated by the addition of absolute ethanol and collected on a sintered glass funnel. The yield, after recrystallization from 95% ethanol, was 2.6 gm. The melting point was 162–163°, in agreement with the value reported by Cook and Heilbron ('49).

L-*N*-Formylcysteine was synthesized by the direct formylation of L-cysteine. Six grams of L-cysteine were suspended in 5 ml of 88% formic acid and 6 ml of acetic anhydride were added immediately. If crystals were not formed after two or three hours at room temperature, crystallization was initiated by scratching the wall of the reaction flask with a glass rod. The mixture was then placed in the refrigerator overnight, and on the following day the *N*-formylcysteine was collected at the water pump on a sintered glass filter. The crystals were washed with small quantities of cold absolute ethanol and with ethylether. The capillary melting point of the product was 132 to 134°, uncorrected. The melting point did not change following recrystallization from water or from aqueous

[5] Evans Chemetics, Inc., Waterloo, New York.

ethanol. The compound gave a strongly positive nitroprusside reaction and the following elementary analysis:

$C_4H_7O_3NS$.	Calculated	C 32.20,	H 4.74,	N 9.39
	Found	C 32.40,	H 4.81,	N 9.37

In the pulmonary edema experiments with adult rats fed laboratory chow [6] the test compounds were dissolved in distilled water to give the following concentrations: 0.9% of thiourea, 5.0% of L-cysteine, 5.0% of L-thiazolidinecarboxylic acid. The latter solutions was adjusted with sodium hydroxide to pH 7.

In the thyroid inhibition experiment with immature rats, 1% thiourea was mixed in pulverized laboratory chow and fed for two weeks. At the end of this time the animals were killed, and their thyroids were removed and weighed on a torsion balance.

EXPERIMENTAL

In the first experiments, cystine and thiazolidinecarboxylic acid were added to the casein diet at levels of from 0.1 to 0.5%. As shown in table 2 the lowest level of each compound produced a maximal growth response. Moreover, at all levels fed, thiazolidinecarboxylic acid was as effective as cystine. The effect of these compounds was not due simply to an increase in the nitrogen content of the ration, for alanine failed to increase growth when added to the basal diet at a 0.15% level (table 2). It appears, therefore, that thiazolidinecarboxylic acid was the source of a specific amino acid required for protein synthesis.

The experiments with 0.1% of thiazolidinecarboxylic acid and cystine were repeated in a longer feeding test (table 3) and once again the heterocyclic compound was found to be as active as cystine. A comparable growth response was also observed when an equivalent amount of methionine or homocystine was added to the basal diet. Furthermore, a combination of methionine and cystine was no more effective than cystine alone. Consequently, the casein diet did not distinguish be-

[6] Purina.

tween cystine and methionine or homocystine, and another type of diet was required to determine whether or not thiazolidinecarboxylic acid could be converted to homocysteine or methionine in the body.

For this purpose 4 rats were fed a purified diet containing the essential and nonessential amino acids with the exception of methionine and cystine (table 1). When 0.6% of methionine was added to this diet, the animals gained approximately 4 gm

TABLE 2

Replacement of dietary cystine by thiazolidinecarboxylic acid

Each supplement was added to the basal 7% casein diet and fed to 6 male rats.

SUPPLEMENT	TIME IN WEEKS	AVERAGE GAIN	T VALUE [1]
		gm	
None	3	19 ± 2.5 [2]	
0.5% L-cystine	3	37 ± 3.5	4.2
0.5% L-thiazolidine-COOH	3	38 ± 4.7	3.6
None	1	6 ± 1.2	
0.2% L-cystine	1	18 ± 2.3	4.6
0.2% L-thiazolidine-COOH	1	15 ± 1.9	4.0
None	1	7 ± 1.0	
0.1% L-cystine	1	15 ± 1.3	4.9
0.1% L-thiazolidine-COOH	1	16 ± 1.9	4.2
0.15% DL-alanine	1	7 ± 1.2	0

[1] In this and subsequent tables the value of T was calculated for each experimental group and the corresponding control group by the method of Fisher ('32).

[2] The standard error of the mean.

TABLE 3

Growth effect of sulfur amino acids

Each compound was incorporated in the 7% casein diet at a level equivalent to 0.1% cystine, on the basis of sulfur content, and was fed to 8 rats for 4 weeks.

SUPPLEMENT	AVERAGE GAIN	T VALUE
	gm	
None	24 ± 1.9	
L-Thiazolidine-COOH	38 ± 3.7	3.3
L-Cystine	39 ± 4.1	3.3
DL-Homocystine	38 ± 4.6	2.8
DL-Methionine	36 ± 3.8	2.6
DL-Methionine + L-cystine	40 ± 4.3	3.4

a day. A typical growth curve is shown in figure 1. Replacement of the methionine with 1.1% of thiazolidinecarboxylic acid resulted in a precipitous loss of weight. However, when 0.54% of homocystine was added in addition to thiazolidinecarboxylic acid there was an immediate growth response.

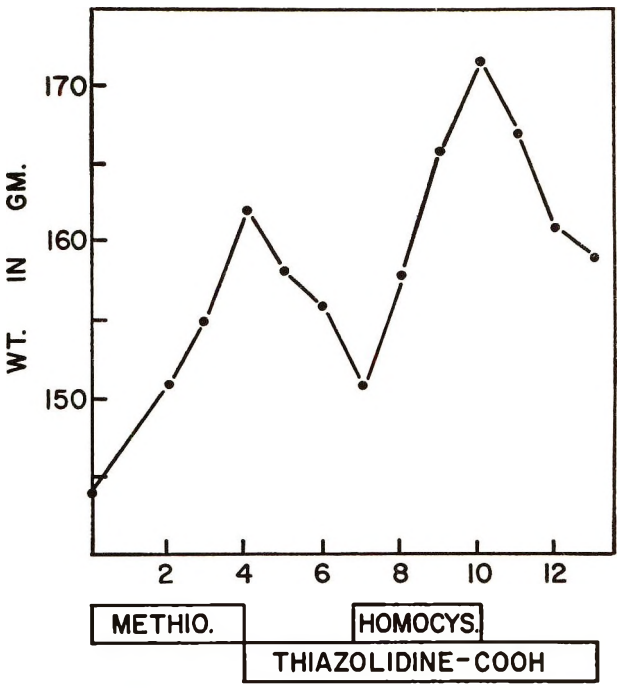

Fig. 1 A typical growth curve showing the response of a rat fed an amino acid diet to supplements of 0.6% DL-methionine, 1.1% L-thiazolidinecarboxylic acid, and 0.54% DL-homocystine. The number of days of the experiment are shown on the abscissa.

Withdrawal of the homocystine was followed by a prompt loss of weight. These results show that thiazolidinecarboxylic acid is not converted to methionine or homocystine to an appreciable extent in the body. Furthermore, they indicate that the growth effect of thiazolidinecarboxylic acid observed on the casein diet is due to its conversion in the animal to cysteine or cystine.

Modification of the structure of thiazolidinecarboxylic acid eliminated its ability to replace dietary cystine. Thus, as shown in table 4, removal of the carboxyl group to give thiazolidine actually inhibited the slow growth otherwise observed on the casein basal diet. Introduction of an extra carbon in the ring of thiazolidinecarboxylic acid to form the next higher homologue, thiazanecarboxylic acid, also abolished growth activity. The latter observation is in accord with our earlier discovery (Wriston and Mackenzie, '57) that L-thiazanecarboxylic acid-2-C^{14} is not oxidized by liver homogenates or in the intact

TABLE 4

Replacement of thiazolidinecarboxylic acid with structurally and metabolically related compounds

Each compound was incorporated in the 7% casein diet at a level equivalent to 0.1% thiazolidinecarboxylic acid, on the basis of sulfur content, and was fed to 10 rats for 4 weeks.

SUPPLEMENT	AVERAGE GAIN	T VALUE
	gm	
None	22 ± 2.0	
L-Thiazolidine-4-COOH	34 ± 3.0	3.3
Thiazolidine·HCl	0 [1]	
DL-1,3-Thiazane-4-COOH	25 ± 3.7	0.7
L-2-Methyl thiazolidine 4-COOH	38 ± 1.8	5.6
L-*N*-Formyl cysteine	35 ± 2.3	4.3
L-*N*,*N'*-Diformyl cystine	37 ± 2.9	4.2

[1] Discontinued after one week. In this experiment $NaHCO_3$ equivalent to the HCl was added to the basal diet.

animal. On the other hand, substitution of a methyl group for a hydrogen atom at position 2 of thiazolidinecarboxylic acid did not reduce the growth effect. It cannot be assumed, however, that the methyl derivative is metabolized through a pathway similar to that of the parent compound, for when 2-methyl-thiazolidinecarboxylic acid was incubated with liver mitochondria the oxygen uptake was both slower and less extensive, and cystine, not acetylcysteine, was the reaction product. It seems probable from these enzymatic experiments that the methyl derivative is first hydrolyzed to acetaldehyde

and cysteine, and that the cysteine is then oxidized to cystine (Mackenzie, '55; Mackenzie and Harris, '57).

As indicated in the introduction of this paper, thiazolidinecarboxylic acid is quantitatively oxidized by liver mitochondria to yield N-formylcysteine. We therefore tested the ability of the latter compound to replace thiazolidinecarboxylic acid and cystine in the diet. As shown in table 4, the growth response to *N*-formylcysteine was equivalent to that observed with its metabolic precursor, thiazolidinecarboxylic acid. *N,N'*-Diformylcystine, which is slowly formed by the auto-

TABLE 5

Prevention of thiourea-pulmonary edema by cysteine and thiazolidinecarbolxylic acid

Each group consisted of 6 male rats (325 to 400 gm) injected intraperitoneally with 10 mg of thiourea per kilogram body weight. Cysteine and thiazolidinecarboxylic acid were injected immediately after the thiourea in the opposite side of the peritoneal cavity at levels of 300 mg per kilogram body weight. Surviving animals were killed and autopsied at 24 hours.

THERAPY	% MORTALITY	FLUID IN PLEURAL CAVITY AT 24 HOURS
		ml
None	100	13.0 ± 0.87
L-Cysteine	0	4.7 ± 0.64
L-Thiazolidine-COOH	0	0.2 [1]

[1] Four rats had no fluid and two had 0.5 and 0.8 ml of fluid.

oxidation of *N*-formylcysteine in aqueous solutions at room temperature, was equally effective in promoting growth on the casein diet.

In view of the results of the feeding experiments, all of which showed that thiazolidinecarboxylic acid was as effective as cystine (cysteine) in promoting growth and protein synthesis, we were particularly interested in testing the effectiveness of thiazolidinecarboxylic acid as a substitute for cysteine (DuBois, Holm and Doyle, '46) in preventing the massive pleural effusion and death produced in adult rats by thiourea (Mackenzie and Mackenzie, '43). As shown in table 5, injection of a single dose of thiourea resulted in the accumulation of a

large volume of serous exudate in the pleural cavity and death within 24 hours. When cysteine was injected immediately after the thiourea, death was prevented in all of the test animals. However, respiratory embarrassment was still apparent and at the end of 24 hours an appreciable quantity of fluid, 2.5 to 7.0 ml, was present in the pleural cavity of each rat. The injection of thiazolidinecarboxylic acid completely prevented the accumulation of pleural fluid in 4 animals, and only minimal quantities of exudate were found in two additional cases. None of the thiazolidinecarboxylic acid-treated rats showed symptoms of respiratory distress.

Immature rats, as shown earlier by Mackenzie and Mackenzie ('43), are "immune" to the pulmonary edema effect of thiourea and develop hyperplastic goiters, when fed thiourea, as a result of the inhibition of thyroxine synthesis by this antithyroid drug (Mackenzie and Mackenzie, '43). Thiazolidinecarboxylic acid, when added at a 1% level to a 1% thiourea diet, did not reduce the size of the goiters that were produced in a two-week test period.

DISCUSSION

Although a number of cases exist in which amino acids can be replaced by their keto, hydroxy, acetyl and methyl derivatives, this is the first instance in which such a dissimilar compound as a heterocyclic ring has been shown to replace an aliphatic amino acid. Furthermore, the ring compound, L-thiazolidinecarboxylic acid, was as effective as cystine itself in promoting growth on the cystine-deficient diet. It appears from this equality in activity that thiazolidinecarboxylic acid undergoes no extensive side reactions in the animal body prior to its conversion to cysteine or cystine.

As previously shown, liver mitochondria contain a potent and specific dehydrogenase that results in the quantitative conversion of thiazolidinecarboxylic acid to *N*-formylcysteine (Mackenzie and Harris, '57). This probably explains the fact that thiazolidinecarboxylic acid is more effective than cysteine

in counteracting the acute toxicity of thiourea in the adult rat, a detoxification reaction believed to be due to the presence of sulfhydryl groups (DuBois, Holm and Doyle, '46). The higher potency of thiazolidinecarboxylic acid in such a reaction is not altogether surprising when one considers that the frozen sulfhydryl groups of thiazolidinecarboxylic acid are liberated only inside of the cell and hence are not subject to oxidation during transport in the extracellular fluids. Thus, thiazolidinecarboxylic acid and similar compounds provide the possibility for a new sulfhydryl therapy based on the administration of enzymatically generated sulfhydryl groups.

The experiments described in this paper do not eliminate the possibility that *N*-formylcysteine is oxidized to the disulfide form prior to the removal of the formyl group. However, we have recently found an enzyme in the supernatant fraction of liver which hydrolyzes *N*-formylcysteine without attacking *N,N'*-diformylcystine. Thus a pathway appears to exist for the conversion of thiazolidinecarboxylic acid to cysteine without cystine as an intermediate.

Thiazolidinecarboxylic acid → *N*-formylcysteine → cysteine + formate

Inasmuch as our present knowledge of protein structure indicates that cysteine, rather than cystine, is the amino acid incorporated into polypeptide chains, such a pathway from thiazolidinecarboxylic acid to cysteine assumes additional biochemical significance. Finally, at physiological pH the spontaneous condensation of formaldehyde and cysteine to form thiazolidinecarboxylic acid is so rapid (Ratner and Clarke, '37) that when these two compounds are added to mitochondria the metabolism of cysteine is for all practical purposes entirely by way of the thiazolidine (Mackenzie and Harris, '57). The formation of thiazolidinecarboxylic acid *in vivo* therefore provides a possible mechanism for the detoxification of exogenous and endogenous formaldehyde.

SUMMARY

The heterocyclic ring compound, L-thiazolidine-4-carboxylic acid, can replace dietary cystine (cysteine) for purposes of

growth and protein synthesis. On the basis of its sulfur content, thiazolidinecarboxylic acid is as effective as cystine as a growth factor. Thiazolidinecarboxylic acid is even more active than cysteine as a detoxifying agent in the pulmonary edema produced by thiourea. However, thiazolidinecarboxylic acid, like cystine, is unable to replace completely either dietary methionine or homocystine.

The ability of thiazolidinecarboxylic acid to substitute for cystine in the diet is eliminated by removing the carboxyl group or by inserting an extra methylene group in the ring.

N-Formylcysteine, the mitochondrial oxidation product of thiazolidinecarboxylic acid, is as effective as its metabolic precursor in promoting growth on a cystine deficient diet. *N,N'*-Diformylcystine is also active in growth experiments.

These experiments indicate that thiazolidinecarboxylic acid is converted quantitatively to cysteine and cystine, via *N*-formylcysteine, in the intact animal.

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