THE RELATION OF A CHELATING AGENT TO SMOOTH-SURFACE LESIONS IN THE WHITE RAT[1]

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Since the time of Miller (1890), acid decalcification of the enamel and dentin has been implicated by many investigators as the chief factor in the etiology of dental caries. However, since hereditary factors, the composition of the teeth and various systemic influences do affect the initiation and rate of progression of carious lesions significantly, the rate of acid decalcification may be determined or controlled by undefined agents or, indeed, other mechanisms than acid decalcification may be involved in the carious process. Various studies on the consumption of inorganic or organic acids in the drinking water, or of beverages with high titratable acidity have shown that extensive decalcification of the enamel occurred in rats but that typical carious lesions were not produced (McClure, '43; Restarski et al., '45; Wynn and Haldi, '48). On the other hand, in recent experiments in which decalcification was produced by chelating agents, lesions were produced on the smooth surfaces of rat molars that were similar in numerous regards to smooth-surface lesions in man (Zipkin, '51, '53; Stephan and Harris, '55). These chelating agents have been shown *in vitro* to be capable of decalcifying enamel and dentin and other hard tissues for histologic, histochemical and enzymatic examinations (Hahn and Reygadas,

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'51; Sreebny and Nikiforuk, '51; Nikiforuk and Sreebny, '53; and Hunter and Nikiforuk, '54). In this present series of three experiments with white rats, we have studied the effect of supplements of ethylene diamine tetraacetic acid (EDTA) to various diets under different circumstances as a means to define the nature of these influences *in vivo*.

TABLE 1

Composition of diets

INGREDIENTS	RATION 700	RATION 770	RATION 700 + 0.2% EDTA	RATION 700 + 0.4% EDTA	RATION 770 + 0.2% EDTA	RATION 770 + 0.4% EDTA
	gm	*gm*	*gm*	*gm*	*gm*	*gm*
Sucrose	670	...	670	670	...	...
Lard	...	120	...	...	120	120
Casein [1] with added B-complex vitamins [2]	240	240	240	240	240	240
Casein [1]	...	400	...	...	400	400
Corn oil with added vitamins A, D, E, K [2]	50	50	50	50	50	50
Salt mixture [2]	40	40	40	40	40	40
Whole liver extract (1:20)	...	20	...	...	20	20
Desiccated liver	40	20	40	40	20	20
Ethylene diamine tetraacetic acid (EDTA) [3]	...	...	2	4	2	4

[1] Borden's crude casein.

[2] J. Dent. Res., *26:* 47 (1947)

[3] Purchased under trade name Sequestrene, Alrose Chemical Co.

EXPERIMENTAL

The composition of the 6 diets used in these experiments is given in table 1. The experimental plans are listed in the first three columns of table 2. In all cases, weanling littermates were distributed with respect to weight and sex as evenly as possible among the groups of an experiment. The rats were caged in individual wire-bottom cages with their own food

TABLE 2

The effect of EDTA-containing diets on the incidence of occlusal caries and of smooth-surface lesions in the teeth of white rats

Values in parentheses refer to the standard error of the mean

GROUP	RATION	DURATION	NO. OF RATS	NO. OF TEETH WITH OCCLUSAL CARIES	NO. OF OCCLUSAL CARIOUS LESIONS	EXTENT OF OCCLUSAL CARIOUS LESIONS	NO. OF MOLARS WITH SMOOTH-SURFACE LESIONS	NO. OF SMOOTH-SURFACE LESIONS	EXTENT OF SMOOTH-SURFACE LESIONS
		wks.							
			Experiment 1. Relatively caries-susceptible rats						
1	700	12	9	4.6 (0.7)	7.4 (1.5)	23.4 + (5.4 +)	0	0	0
2	700 + 0.2% EDTA	12	11	6.4 (0.8)	11.9 (1.5)	41.7 + (5.1 +)	2.9 (0.9)	4.6 (1.7)	9.9 + (3.5 +)
3	700 + 0.4% EDTA	12	8	5.5 (0.8)	8.6 (1.2)	32.0 + (4.5 +)	4.6 (1.4)	6.4 (2.3)	15.9 + (6.3 +)
4	770	19	7	0	0	0	0	0	0
5	770 + 0.2% EDTA	19	7	0	0	0	0	0	0
6	770 + 0.4% EDTA	19	8	0	0	0	0	0	0
			Experiment 2. Sialoadenectomy in relatively caries-susceptible rats						
	Intact rats								
1	700	11	2	3.0 (2.1)	5.0 (3.5)	9.5 + (6.7 +)	0	0	0
2	700 + 0.2% EDTA	11	2	2.5 (1.1)	3.0 (1.4)	7.0 + (2.1 +)	0.5 (0.4)	1.0 (0.7)	1.0 + (0.7 +)
3	700 + 0.4% EDTA	11	2	2.5 (0.4)	3.0 (0.7)	4.5 + (1.8 +)	0	0	0
	Desalivated rats								
1	700	11	2	10.0 (1.4)	12.5 (2.5)	34.5 + (8.8 +)	0	0	0
2	700 + 0.2% EDTA	11	2	11.0 (2.8)	16.0 (5.7)	62.5 + (23.0 +)	12.0 (0)	22.0 (2.8)	37.0 + (10.0 +)
3	700 + 0.4% EDTA	11	3	10.0 (1.6)	20.0 (4.1)	70.7 + (1.9 +)	12.0 (0)	25.0 (1.6)	41.3 + (5.8 +)
			Experiment 3. Relatively caries-resistant rats						
1	700	20	7	4.7 (0.6)	5.9 (0.6)	11.1 + (1.8 +)	0	0	0
2	700 + 0.4% EDTA	20	8	4.8 (0.8)	6.9 (1.4)	16.4 + (5.0 +)	2.5 (0.8)	3.5 (1.3)	4.1 + (1.4 +)
3	700 + tube fed EDTA	20	8	3.3 (0.8)	3.6 (1.1)	7.0 + (1.8 +)	0.9 (0.5)	1.4 (0.8)	1.4 + (0.8 +)

cups and water bottles. They were housed in air-conditioned, temperature- and humidity-controlled rooms. At the end of each experiment, the heads were fixed in 95% alcohol for 48 hours, and then skinned. The molar teeth were examined and the lesions in the occlusal sulci evaluated under a binocular microscope ($\times$ 30) by grinding successive planes of the teeth with the help of a running grinding stone, by the method of Shaw et al. ('44).

The first experiment was designed to determine the influence of two levels of EDTA on the initiation and progression of carious lesions in the occlusal fissures and also upon the initiation of any lesions on the smooth surfaces. A comparison of the influence of a high-carbohydrate diet with that of a carbohydrate-free diet was included. The second experiment was conducted to test the influence of sialoadenectomy upon the initiation of EDTA-induced lesions. In both of these experiments, the subjects used were representatives of a relatively highly caries-susceptible strain. The objectives of the third experiment were two-fold: first, to determine the influence of EDTA in a highly caries-resistant strain of rodents and second, to test its influence when introduced into the stomach by a tube to prevent contact with the oral tissues. The basal diet for all groups in this experiment was no. 700. The subjects in the first group received no supplement while those in the second group were given 0.4% EDTA in the diet. The amount of ration consumed by each rat in the second group was determined every 24 hours and the amount of EDTA consumed by each rat calculated. This amount of EDTA in suspension was given by stomach tube to the respective littermates in the third group by the procedure described by Kite et al. ('50).

RESULTS

In addition to the carious lesions that are normally found in the occlusal sulci of our caries-susceptible strain of rats, the EDTA-fed rats maintained on high-carbohydrate diet 700 had a different type of lesion on the smooth surfaces. The

differentiation between carious lesions in the occlusal sulci and the lesions on the smooth surfaces was based upon their position and also upon the observation that the previously described occlusal carious lesions in our strain of rats were circumscribed slightly brown or dark pigmented areas, initiated in almost all cases at the base of the fissures, while the smooth-surface lesions were diffuse, whitish, rapidly progressing, soft areas.

After observation of the various stages of the lesions on the smooth surfaces, it appeared that the process was initiated as a small, whitish, diffuse patch or streak with a partial loss of the enamel's translucency. Later both the intensity and the extent of the areas increased over wide portions of the smooth surfaces. Extensive areas were involved before any detectable influence on the hardness of the enamel was observed. Later the lesions were soft and exploration of the affected area of the tooth with a fine probe caused a sloughing of the enamel in powdery particles. In the most advanced lesions, there was a conspicuous pitting of the surfaces and the areas were extensively softened. Typical examples of these lesions are shown in figures 1 to 4. The various stages in the development of these smooth surface lesions were arbitrarily assigned the following gradations: 0, normal tooth structure; 1 +, slight, whitish, diffuse, hard streaks or patches with partial loss of translucency; 2 +, white, diffuse, opaque, hard streaks or patches; 3 +, large, dull white, streaks which were soft on exploration with a dental broach; 4 +, extensive, white, soft streaks with pits.

In the evaluation process, lesions on the smooth surfaces were recorded on the usual caries scoring chart in terms of the number of molars with lesions, the number of lesions and the extent of lesions. The scores of the individual lesions were totalled per head as a means of expressing the amount of smooth surface involvement; this is a comparable procedure to that used in this laboratory for recording the degree of occlusal caries involvement.

In many instances among the EDTA-fed rats, diffuse, whitish borders were observed around the margin of the carious lesions in the occlusal fissures. In many ways these borders strongly resembled the smooth-surface lesions and were distinctly dissimilar to the borders of the occlusal lesions in rats fed the basal ration 700. Yet at the same time these borders were sufficiently small and in such early stages of involvement that they did not seem indicative of more rapid progression in the occlusal lesions.

The average number of molars with carious lesions in the occlusal sulci, the average number of these carious lesions and the average extent of these carious lesions and the frequency and extent of decalcified lesions on the smooth surfaces among the animals in the three experiments are presented in table 2. The rats in group 1, of the first experiment which were on ration 700, had an incidence of dental caries that was typical of intact representatives of this strain of caries-susceptible rats. The values for occlusal caries in the EDTA-fed rats of groups 2 and 3 had a tendency to be slightly higher than for the controls in group 1; however, there was no striking statistical significance to these minor increases. As would be expected from previous experiments in this laboratory with carbohydrate-free diets, none of the rats in groups 4 through 6 developed any carious lesions on the occlusal surfaces.

No lesions were observed on the smooth surfaces of the teeth of the rats in group 1. The molars of the rats in group 2 had a relatively high incidence of smooth surface lesions that were in various early stages of development. These lesions were relatively early ones that consisted of white, opaque, diffuse streaks on the buccal side of the teeth along the gingiva. Some of the streaks were soft, others were still hard. The molars of the rats in group 3 had a greater num-

DESCRIPTION OF FIGURES

Figs. 1 to 4 Photographs of fixed preparations of white rat mandibles that were selected to illustrate the types and stages of smooth-surface lesions observed in EDTA-fed subjects. Mag. × 18. Immediately above each figure are the individual scores for the lesions.

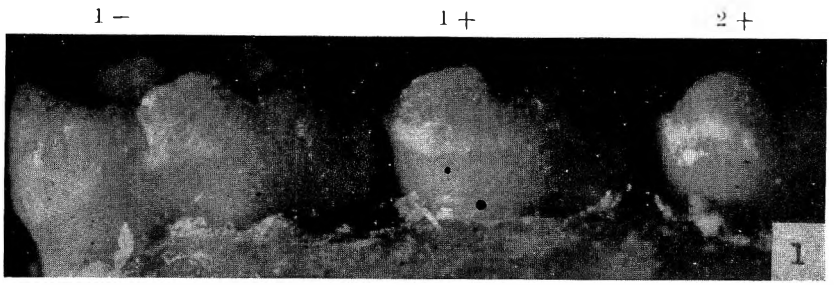

Fig. 1 Buccal aspect of the left mandibular molars to demonstrate mild smooth surface lesions.

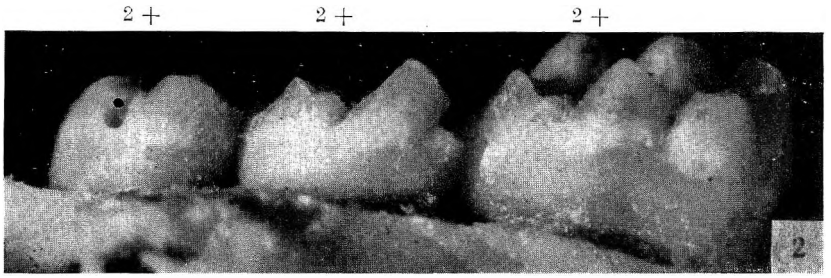

Fig. 2 Buccal view of the right mandibular molars of a rat with mild and moderate smooth surface lesions.

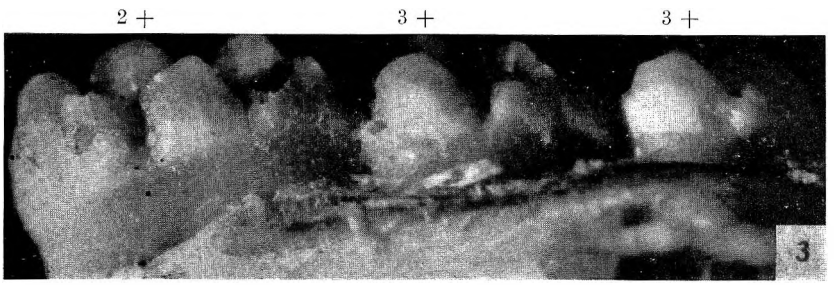

Fig. 3 Buccal view of the left mandibular molars of a rat with moderate to severe smooth surface lesions.

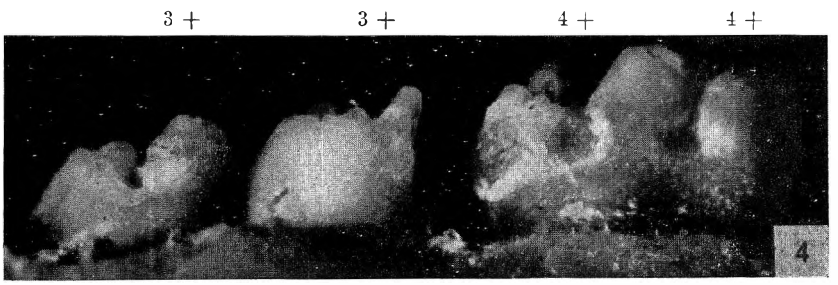

Fig. 4 Buccal aspect of the right mandibular molars of a rat with severely advanced smooth surface lesions.

ber of smooth surface lesions that were of more advanced stages of development. The extent and the frequency of lesions on the smooth surfaces had increased definitely with the increased concentration of EDTA. However, where EDTA supplements to carbohydrate-free diet 770 were fed to the rats in groups 5 and 6, no smooth surface lesions were caused. In other words, the carbohydrate-free diet, in addition to preventing carious lesions in the occlusal sulci, inhibited the formation of these EDTA-induced lesions on the smooth surfaces.

The molars of the sialoadenectomized rats in groups 4, 5 and 6 of experiment 2 had a much higher incidence of tooth decay than their intact littermates. No lesions were observed on the smooth surfaces of the molars of the intact or sialoadenectomized rats in groups 1 and 4 that were fed only the basal ration. The incidence of smooth surface lesions in the intact rats with EDTA supplements was observed to be very low; however, major and statistically significant increases in smooth surface lesions were observed in the molars of the sialoadenectomized animals.

In experiment 3, the rats in group 1 had an incidence of dental caries that was typical of this strain of caries-resistant rats, when maintained on cariogenic ration 700 for this time interval. In addition, no smooth surface lesions were observed. The rats in group 2 which were on diet 700 plus 0.4% EDTA had a comparable incidence of tooth decay and had a moderate incidence of smooth surface lesions in various stages of development. The three categories of dental decay for the rats in group 3, which were fed diet 700 with supplements of EDTA by stomach tube were slightly but not significantly lower than the scores for the rats in groups 1 and 2. Minor lesions on the smooth surfaces were observed in a few molars as white, opaque, diffuse streaks. These streaks were faint and hard unlike the ones found on the teeth of animals in group 2. Both the incidence and extent of occlusal caries and of lesions on the smooth surfaces were much less frequent in

the rats in group 2 than was observed for the rats from the caries-susceptible strain employed in experiments 1 and 2.

DISCUSSION

From the results of the above experiments, it is clear that the addition of EDTA to the high sucrose cariogenic diet 700 did not significantly increase the incidence of occlusal caries, although there was a trend toward an increase among the EDTA-fed rats. Moreover, EDTA, caused the production of lesions on the smooth surfaces of the molars when added to high sucrose cariogenic diet 700, but failed to produce any lesions of this type when added to high protein, high fat diet 770. The production of decalcified lesions in the molars of white rats appears to be primarily due to a local influence since a very low incidence of smooth surface lesions occurred where EDTA did not come in contact with the teeth, while definite decalcification was observed when EDTA was fed along with the food.

The inclusion of EDTA in the diet of desalivated rats caused much more damage to the smooth surfaces of the molars than did the same level of supplementation in intact littermates. In contrast, the same levels of EDTA were less effective in the production of smooth-surface lesions among caries-resistant rats than among caries-susceptible rats. Thus in both of these test cases, changes in the rate of initiation and progression of EDTA-induced smooth surface lesions closely paralleled concurrent alterations in the manifestations of occlusal caries. Yet at the same time, carious lesions on the occlusal surfaces were not influenced to a significant degree by EDTA supplements.

Stephan and Harris ('55) have described a significantly increased occurrence of smooth surface lesions on rat molars when 0.2% EDTA was incorporated into their caries-producing diets. The gross description of the lesions in their studies closely parallels that of the smooth-surface lesions in our studies. Histologic studies of teeth from their rats indicated that microorganisms were associated with the smooth-surface

lesions. In addition, supplements of penicillin, terramycim or bacitracin to the diet almost completely prevented the lesions induced by the feeding of EDTA in the diet (Stephan et al., '52; Fitzgerald, '55).

These observations suggest that the production of lesions by EDTA was not a simple decalcification of tooth surfaces. In all cases the production or the prevention of these lesions closely paralleled circumstances that led to the production or the prevention of carious lesions. From the studies with our strains of caries-susceptible and caries-resistant rats, it appeared that the EDTA has acted as an additional factor in the initiation and progression of caries-like lesions in areas where our normal procedures, including the extreme penalizing influence of sialoadenectomy, were to a large extent ineffective. At the present time, it would seem that the EDTA-induced lesions on the smoth surfaces of rat molars had a high similarity to true carious lesions. One of the perplexing facets of our studies has been the lack of any striking influence of the EDTA supplements on occlusal caries. Martin et al. ('54) have postulated that chelation may be an important part of the decalcification of tooth substance. If this postulate has any validity, it would be expected to hold for occlusal caries as well as for smooth-surface caries. Further definition of this comparison will require more exacting experiments than have been conducted.

SUMMARY

1. Ethylene diamine tetraacetic acid (EDTA), when added to a high sucrose cariogenic diet, produced lesions on the smooth surfaces of the molar teeth of white rats, which grossly resembled simple decalcification. The incidence and severity of these lesions increased in proportion to the concentration of EDTA in the diet.

2. EDTA failed to produce this type of lesion when added to a carbohydrate-free, high-protein, high-fat, non-cariogenic diet.

3. EDTA seemed to produce minor, but not statistically significant, increases in the rate of initiation and progression of carious lesions in the occlusal fissures.

4. Removal of the salivary glands caused major increases in the influence of EDTA in the production of lesions on the smooth surfaces of the molar teeth.

5. When the rats were fed cariogenic ration 700 ad libitum and EDTA by stomach tube only questionable or minor decalcified lesions developed on the smooth surfaces of the molar teeth.

6. In caries-resistant rats, EDTA produced much less frequent and less severe lesions on the buccal surfaces of the molars than in caries-susceptible rats.

These findings suggest that the smooth surface lesions observed in these experiments were more likely to be closely related or identical to smooth surface caries than to be the result of the simple process of decalcification of tooth substance by chelation.

LITERATURE CITED

FITZGERALD, R. J. 1955 Influence of antibiotics on experimental rat caries. In "Advances in Experimental Caries Research," ed. by R. F. Sognnaes, American Association for the Advancement of Science, Washington, p. 187.

HAHN, F. L., AND F. REYGADAS 1951 Demineralization of hard tissues. Science, *114:* 462.

HUNTER, T. A., AND G. NIKIFORUK 1954 Staining reactions following demineralization of hard tissues by chelating and other decalcifying agents. J. Dent. Res., *33:* 136.

KITE, O. W., J. H. SHAW AND R. F. SOGNNAES 1950 The prevention of experimental tooth decay by tube-feeding. J. Nutrition, *42:* 89.

MARTIN, J. J., H. D. ISENBERG, V. SCHATZ, G. S. TRELAWNY AND A. SCHATZ 1954 Chelation, or metal-binding, as a new approach to the problem of dental caries. Euclides, *14:* 311.

MCCLURE, F. J. 1943 The destructive action, in vivo, of dilute acids and acid drinks and beverages on the rat's molar teeth. J. Nutrition, *26:* 251.

MILLER, W. D. 1890 The micro-organisms of the human mouth. S. S. White Dent. Manf. Co., Philadelphia, 364 pp.

NIKIFORUK, G., AND L. SREEBNY 1953 Demineralization of hard tissues by organic chelating agents at neutral pH. J. Dent. Res., *32:* 859.

RESTARSKI, J. S., R. A. GORTNER, JR. AND C. M. MOCAY 1945 Effect of acid beverages containing fluorides upon the teeth of rats and puppies. J. Am. Dent. Assn., *32:* 668.

SHAW, J. H., B. S. SCHWEIGERT, J. M. MCINTIRE, C. A. ELVEHJEM AND P. H. PHILLIPS 1944 Dental caries in the cotton rat. I. Methods of study and preliminary nutritional experiments. J. Nutrition, *28:* 333.

SREEBNY, L. M., AND G. NIKIFORUK 1951 Demineralization of hard tissues by organic chelating agents. Science, *113:* 560.

STEPHAN, R. M., AND M. R. HARRIS 1955 Location of experimental caries on different tooth surfaces in the Norway rat. In "Advances in Experimental Caries Research," ed. by R. F. Sognnaes, American Association for the Advancement of Science, Washington, p. 47.

STEPHAN, R. M., M. R. HARRIS AND R. J. FITZGERALD 1952 The effect of penicillin and of terramycin on the development of buccal, labial, proximal, and occlusal surface dental caries in rats and on quantitative counts of oral microorganisms. J. Dent. Res., *31:* 475 (abst. no. 41).

WYNN, W., AND J. HALDI 1948 The erosive action of various fruit juices on the lower molar teeth of the albino rat. J. Nutrition, *35:* 489.

ZIPKIN, I. 1951 Further studies on in vivo decalcifying agents, versene (ethylene diamine tetra acetic acid) various polycarboxylic acids and various polyphosphates at pH 6.5. J. Dent. Res., *30:* 484 (abst. 67).

———— 1953 Caries potentiating effect of ethylene diamine tetraacetic acid in the rat. Proc. Soc. Exp. Biol. Med., *82:* 80.

QUANTITATIVE STUDIES ON THE URINARY EXCRETION OF TRYPTOPHAN METABOLITES BY HUMANS INGESTING A CONSTANT DIET [1]

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The studies of Heidelberger et al. ('49) demonstrated that tryptophan was a metabolic precursor of nicotinic acid in the rat. It has also been shown that the excretion of metabolites of nicotinic acid increased after the ingestion of tryptophan by man (Perlzweig et al., '47; Sarett and Goldsmith, '47; Holman and de Lange, '50; Reddi and Kodicek, '53; Holman, '54).

A large number of other metabolites of tryptophan have been identified in mammalian urine and the nature of these has been reviewed by Dalgliesh ('51) and Mehler ('55). As the result of the development of a convenient method for the determination of xanthurenic acid by Miller and Baumann ('45) and later by Rosen et al. ('51) this tryptophan metabolite has been studied extensively (Miller and Baumann, '45; Sprince et al., '51; Vilter et al., '53; Chiancone et al., '55; Henderson et al., '55).

The development of analytical methods for kynurenine, N^{α}-acetylkynurenine, *o*-aminohippuric acid, anthranilic acid

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[2] Scholar in Cancer Research of the American Cancer Society.

glucuronide, kynurenic acid (Brown and Price, '56) and *N*-methyl-2-pyridone-5-carboxamide (pyridone) (Price, '54) has now made it possible to obtain quantitative data concerning several other major metabolites of tryptophan. The use of these procedures (Brown and Price, '56) indicated that administration of a supplemental dose of L-tryptophan to man was followed by an increased urinary excretion of several of these metabolites.

In an effort to learn more about the metabolic fate of dietary tryptophan, studies have now been done on human subjects ingesting a constant amount of tryptophan and nicotinic acid. The use of a constant diet has also made it possible to determine the daily variation in excretion of the major known metabolites of tryptophan and to determine the relative importance of nicotinic acid and these other metabolites. In addition, the effect of a single oral dose of 2.0 gm of L-tryptophan on the excretion of these metabolites was determined. These results have been compared with similar studies on subjects ingesting self-selected diets (Brown and Price, '56).

EXPERIMENTAL

Subjects. The 4 subjects used in these experiments were male laboratory personnel ranging in age from 25 to 35 years. One subject was Japanese, one was from India, while the others were white Americans. None of the subjects had any history of neoplastic, renal, gastrointestinal or metabolic disease, and all were in apparent good health. During the studies each subject engaged in his usual activities. One subject was unable to complete the last two days of the experiment because the amount of food was excessive for him.

Diet. The diet consisted of natural foods listed in table 1. The food was prepared in the special diet kitchen by the hospital dietitians. All the food was taken from the same individual sources, with the exception of milk, lettuce, butter and eggs. Thus the canned goods were from the same case lot, the meat was from one beef round, the bread was from

the same formula from one baker, etc. The food was weighed on a torsion balance, except for the milk and lettuce which were weighed on a trip scale. The vegetables and fruits were drained, and the potatoes were peeled after boiling. The eggs were broken, beaten and weighed raw. The meat was prepared in individual frying pans, using part of the subject's daily allotment of butter. The frying pans were thoroughly scraped to obtain all the meat particles and gravy. The subjects consumed all the food on their trays at each

TABLE 1

The composition of the diet [1]

BREAKFAST		LUNCH		DINNER	
Food	Amount	Food	Amount	Food	Amount
	gm		*gm*		*gm*
Orange juice, unsweetened canned	100	Beef steak (round)	100	Beef steak (round)	100
		Potato, boiled	100	Potato, boiled	100
Eggs,		Peas, canned	80	Beets, canned	85
2 scrambled	103	Bread, 1 slice white	23	Butter [2]	...
Toast,		Butter [2]	...	Pears, canned	100
1 slice white	23	Applesauce, canned	125	Lettuce	30
Butter [2]	...	Lettuce	30	Milk, whole	244
Milk, whole	244	Milk, whole	244	Coffee, 1 cup black	...
Coffee, 1 cup black	...	Coffee, 1 cup black	...	Bread, 1 slice white	23

[1] The diet was calculated to contain the following: niacin, 15 mg; tryptophan, 900 mg; calories, 2,423; carbohydrate, 189 gm; protein, 94 gm; fat, 146 gm; calcium, 1.4 gm.; phosphorus, 1.6 gm; iron, 16 mg; vitamin A, 7,260 I.U.; thiamine, 1.1 mg; riboflavin, 2.2 mg; ascorbic acid, 106 mg.

[2] A total of 100 gm of butter was used for the three meals.

meal, with the exception of the butter which was used throughout one day and finished with the evening meal. Water was the only liquid allowed ad libitum. The diet was estimated to contain 125 micromoles (15 mg) of niacin and 4,420 micromoles (900 mg) of tryptophan.

Methods. The 24-hour urines were collected in amber bottles under toluene. The supplement of L-tryptophan was given with breakfast of the 7th day. The tryptophan metabolites were determined according to the procedures of Brown and Price ('56), except for xanthurenic acid which was deter-

mined by the method of Rosen et al. ('51), and *N*-methyl-2-pyridone-5-carboxamide (pyridone) which was determined according to Price ('54) as modified slightly by Walters et al. ('55).

RESULTS

The results are given in table 2. The average pyridone excretion for the 4 subjects increased somewhat the second day on the diet, which suggests that more nicotinic acid was being ingested. From the second to the 6th days on the constant diet the average pyridone excretion was quite constant, ranging from 101 to 110 micromoles per day. The 4 subjects stabilized at different basal levels of excretion; for example, the average basal excretions of pyridone with standard deviations for each of the 4 subjects were 80 ± 10, 106 ± 8, 112 ± 11 and 127 ± 5 micromoles per day. For this reason the standard deviations shown in table 2 were much larger than the standard deviations of each individual's average basal excretion. After ingestion of the single supplement of 9.8 millimoles (2.0 gm) of L-tryptophan the pyridone excretion of each subject increased, and remained elevated for three days. One subject had an unexplained increase in pyridone excretion on the last two days of the experiment.

Kynurenic acid excretion varied little from day to day. The individual average basal excretions of this metabolite by the 4 subjects were 13 ± 1, 13 ± 2, 18 ± 1, and 19 ± 1 micromoles per day.

The daily excretion of xanthurenic acid appeared to be quite variable on the basal diet, and the average values for the 4 subjects were 27 ± 3, 32 ± 7, 38 ± 9, and 51 ± 5 micromoles per day. However, with low levels of xanthurenic acid this method of analysis gives somewhat variable results (Rosen et al., '51). There did appear to be a slight increase in xanthurenic acid excretion following the supplementation with tryptophan.

In agreement with the results of Brown and Price ('56) aromatic amine "Fraction A" was not affected by ingestion

TABLE 2

Average micromoles of metabolite (± standard deviation) excreted in the urine per 24 hours by 4 subjects on the constant diet containing 125 micromoles of niacin and 4,420 micromoles of tryptophan per day. A single supplement of 9,800 micromoles of L-*tryptophan was given at the end of the 6th day*

METABOLITE [1]	DAYS ON CONSTANT DIET											
	1	2	3	4	5	6	7	8	9	10	11	12
MPCA	93 ± 34	110 ± 26	109 ± 20	103 ± 27	101 ± 21	108 ± 18	141 ± 22	131 ± 17	114 ± 29	114 ± 21	133 ± 34	117 ± 25
KA	16 ± 2.5	17 ± 3.7	16 ± 4.3	16 ± 3.6	16 ± 3.2	16 ± 2.7	67 ± 7.4	17 ± 2.6	18 ± 2.8	17 ± 1	18 ± 2.1	16 ± 2.1
XA	46 ± 16	34 ± 13	37 ± 13	41 ± 11	37 ± 11	37 ± 15	50 ± 16	33 ± 5	39 ± 6	35 ± 7	39 ± 4	49 ± 27
AAG	4.4 ± 1.3	3.8 ± 0.6	4.2 ± 1.9	4.4 ± 1.4	4.2 ± 1.2	4.4 ± 1.2	6.4 ± 1.0	3.8 ± 0.6	4.2 ± 1.2	3.7 ± 1.1	3.5 ± 1.2	2.8 ± 0.5
*o*AH	26 ± 7	26 ± 7	27 ± 8	26 ± 6	26 ± 7	27 ± 7	57 ± 26	26 ± 6	26 ± 6	27 ± 7	23 ± 3	24 ± 4
AcKyn	14 ± 2.1	12 ± 0.6	15 ± 4.4	16 ± 4.4	16 ± 3.2	16 ± 2.8	19 ± 5.3	14 ± 2.8	16 ± 5.6	13 ± 3.0	14 ± 3.3	13 ± 2.5
Kyn	13 ± 2.6	11 ± 3.2	13 ± 5.2	14 ± 3.0	12 ± 3.5	13 ± 3.7	43 ± 20.4	14 ± 3.0	13 ± 2.5	15 ± 1.0	11 ± 1.0	10 ± 2.0
A	56 ± 25	54 ± 29	56 ± 32	54 ± 32	50 ± 28	51 ± 26	52 ± 32	48 ± 37	50 ± 37	31 ± 13	40 ± 14	39 ± 13

[1] The following abbreviations are used; MPCA, *N*-methyl-2-pyridone-5-carboxamide; KA, kynurenic acid; XA, xanthurenic acid; AAG, anthranilic acid glucuronide; oAH, *o*-aminohippuric acid; AcKyn, *N*$^{\alpha}$-acetylkynurenine; Kyn, kynurenine; A, unknown diazotizable amine fraction "A".

of single doses of L-tryptophan. The nature of the diazotizable aromatic amine in this fraction is unknown (Brown and Price, '56).

The average daily basal excretion of anthranilic acid glucuronide was 3.0 ± 0.6, 3.3 ± 0.1, 5.2 ± 0.7, and 5.3 ± 0.4 micromoles; of *o*-aminohippuric acid 17 ± 1, 26 ± 4, 31 ± 2, and 31 ± 2 micromoles; of N^a-acetylkynurenine 12 ± 1, 12 ± 2, 17 ± 3, and 17 ± 3 micromoles; and of kynurenine 9 ± 1, 17 ± 2, 16 ± 3, and 17 ± 2 micromoles for each of the 4 subjects. There was a slight increase in the excretion of each of these aromatic amines following the administration of the single dose of tryptophan, except that one subject failed to show an increased excretion of acetylkynurenine. This subject excreted more kynurenine than any of the other subjects, and may not have been as efficient in acetylation of this metabolite.

TABLE 3

A comparison of the urinary excretion of tryptophan metabolites by subjects on the constant diet and on self-selected diet [1,2]

METABOLITE	AVERAGE MICROMOLES EXCRETED PER SUBJECT PER DAY		AVERAGE MICROMOLE INCREASE IN EXCRETION AFTER 9.8 MILLIMOLE DOSE OF L-TRYPTOPHAN	
	Constant diet	Self-selected diet	Constant diet	Self-selected diet
Pyridone	106	136	76	103
Kynurenic acid	16	16	51	51
Xanthurenic acid	37	75	13	11
Anthranilic acid glucuronide	4	6	2	3
o-Aminohippuric acid	26	27	31	23
Acetylkynurenine	15	9	4	4
Kynurenine	13	16	30	18
Total	217	285	207	213

[1] Brown and Price ('56).

[2] The average basal excretion of the metabolites includes days two to 6 on the constant diet and two days before tryptophan supplementation for the subjects on self-selected diets. The increased excretion of pyridone was calculated for a 4- or two-day period after administration of the supplemental tryptophan on the constant and self-selected diets, respectively. The increased excretion of the other metabolites included only the first 24 hours in both experiments.

Table 3 shows a comparison of the results of the present study with the data of Brown and Price ('56). The subjects on the self-selected diets excreted more pyridone and about twice as much xanthurenic acid as did the subjects on the constant diet. Similar amounts of the other metabolites were excreted on both diets. On the constant diet which contained 125 micromoles of niacin the subjects excreted an average of 106 micromoles of the pyridone. The 111 micromoles of other tryptophan metabolites would account for 2.5% of the dietary tryptophan.

When the 9.8 millimole dose of supplemental tryptophan was administered, the subjects on the constant diet excreted somewhat less pyridone and more kynurenine and *o*-aminohippuric acid than did the subjects on the self-selected diets. The total yield of metabolites was similar in each study, and would account for about 2% of the dose of tryptophan. On the constant diet the pyridone was apparently the chief metabolite of the supplemental tryptophan, and it accounted for about 0.8% of the dose. Kynurenic acid was the second most important metabolite, followed closely in this respect by *o*-aminohippuric acid and kynurenine.

DISCUSSION

The interpretation of the urinary excretion of the pyridone was complicated because of its metabolic origin from both nicotinic acid and tryptophan. The studies of Walters et al. ('55) indicated that a single oral supplement of 164 micromoles of nicotinic acid or its amide could be accounted for as increased urinary pyridone to the extent of about 80%. Larger doses yielded more total pyridone but a lower percentage conversion to this metabolite. It would appear, therefore, that the pyridone was relatively more important as a metabolite of nicotinic acid on lower levels of intake. In the present experiments the diet was estimated to contain 125 micromoles of niacin. The average daily pyridone excretion by the subjects on the constant diet was 106 micromoles, which, neglecting tryptophan as a source, was suffi-

cient to account for about 85% of the estimated dietary niacin. On the basis of the studies by Walters et al. ('55) one might predict a conversion rate of this magnitude. Frazier, Prather and Hoene ('55) studied niacin metabolism in human subjects on a diet similar in niacin and tryptophan content to the present diet, and found that considerable amounts of *N*-methyl-nicotinamide were excreted. If the present subjects excreted similar amounts of this metabolite, over 100% of the dietary niacin would have been accounted for. This suggests that some tryptophan may be converted to niacin on a diet such as that used in the present studies. The urinary excretion of appreciable amounts of a number of the apparent by-products of the conversion of tryptophan to nicotinic acid also suggests that the subjects were using this metabolic pathway to some extent. The extent of the conversion of tryptophan to niacin on a natural diet presumably adequate in each might be difficult to obtain without the use of isotopes.

The pyridone excretion by the subjects studied by Frazier et al. ('55) would account for less of the dietary niacin than that which was accounted for in the present experiments. On a diet containing 16.3 mg of nicotinic acid and 1.12 gm of tryptophan the average pyridone excretion of their subjects was only 9.59 mg, which was enough to account for only about 48% of the dietary vitamin. However, Frazier et al. ('55) used a diet which was otherwise different from that used here. They also used female subjects and a different method for the determination of the pyridone.

From the data in table 3 it would appear that the pyridone was the chief urinary metabolite of tryptophan when a single supplemental dose of 2.0 gm of the amino acid was administered. From the increased urinary excretion of the other tryptophan metabolites, however, it would appear that considerable tryptophan was lost in side reactions along this pathway. Kynurenic acid was of most quantitative significance in this respect, followed closely by *o*-aminohippuric acid and kynurenine. That these other metabolites of tryptophan are by-products of the pathway leading to niacin

would be suggested from the studies of Brown and Price ('56) on the cat. It has been found that tryptophan cannot replace nicotinic acid in the diet of the cat (Carvalho da Silva, Fried and de Angelis, '52) and only traces of these metabolites of tryptophan could be found in cat urine after supplementation with the amino acid (Brown and Price, '56).

The basal excretion and the increased excretion of the metabolites by human subjects after supplemental tryptophan were similar on constant and self-selected diets (table 3). This may be a reflection of the relatively low yield of these metabolites from tryptophan, so that ordinary dietary variations in the intake of the amino acid have little effect on the daily excretion of these compounds. Since the pyridone was the major metabolite of nicotinic acid (Walters et al., '55), however, slight variations in the intake of the vitamin would readily be detectable in the excretion of this metabolite. Furthermore, the pyridone excretion did not return to normal levels for two or three days after the ingestion of tryptophan or nicotinic acid (Walters et al., '55). Even very large doses of tryptophan failed to alter the urinary excretion of the other metabolites of the amino acid for more than 24 hours (Brown and Price, '56).

In certain pathological conditions the levels of urinary excretion of many of these metabolites of tryptophan were so abnormal (Brown et al., '55) that the use of self-selected diets might be considered adequate for most quantitative studies of tryptophan metabolism.

SUMMARY

The urinary excretion of *N*-methyl-2-pyridone-5-carboxamide (pyridone), kynurenic acid, xanthurenic acid, kynurenine, N^{α}-acetylkynurenine, *o*-aminohippuric acid, anthranilic acid glucuronide and an unknown diazotizable aromatic amine was determined on 4 normal males ingesting a constant diet containing an estimated 15 mg of nicotinic acid and 900 mg of tryptophan. After 6 days on the constant diet 2.0 gm of

L-tryptophan was given as a single oral dose and the excretion of the metabolites was determined for 6 more days.

On the constant diet about 16 mg of pyridone were excreted per day. The other metabolites of tryptophan accounted for 2.5% of the amino acid.

Following the ingestion of a single 2.0 gm supplement of tryptophan the pyridone excretion increased to an extent which would account for about 0.8% of the dose. The increase in the excretion of the other metabolites accounted for 1.3% of the supplemental tryptophan. Kynurenic acid, *o*-aminohippuric acid, and kynurenine were important urinary metabolites of the oral supplement of amino acid. Xanthurenic acid, acetylkynurenine, and anthranilic acid glucuronide were minor metabolites by comparison.

A comparison of these results with data of a similar nature obtained with human subjects on a self-selected diet suggested that a constant diet was not necessary for quantitative studies on the metabolism of supplemental doses of tryptophan, unless the conversion to nicotinic acid and its metabolites was of particular interest.

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LITERATURE CITED

BROWN, R. R., AND J. M. PRICE 1956 Quantitative studies on metabolites of tryptophan in the urine of the dog, cat, rat and man. J. Biol. Chem., *219:* 985.

BROWN, R. R., J. M. PRICE AND J. B. WEAR 1955 The metabolism of tryptophan in bladder tumor patients. Proc. Am. Assoc. Cancer Research, *2:* 7.

CARVALHO DA SILVA, A., R. FRIED AND R. C. DE ANGELIS 1952 The domestic cat as a laboratory animal for experimental nutrition studies. III. Niacin requirements and tryptophan metabolism. J. Nutrition, *46:* 399.

CHIANCONE, F. M., E. GINOULHIAC, L. MAINARDI AND L. T. TENCONI 1955 The elimination of some tryptophan metabolites after removal of endocrine glands. Arch. Biochem. Biophys., *54:* 15.

DALGLIESH, C. E. 1951 Biological degradation of tryptophan. Quart. Revs. (London), *5:* 227.

FRAZIER, E. I., M. E. PRATHER AND E. HOENE 1955 Nicotinic acid metabolism in humans. I. The urinary excretion of nicotinic acid and its metabolic derivatives on four levels of dietary intake. J. Nutrition, *56:* 501.

HEIDELBERGER, C., E. P. ABRAHAM AND S. LEPKOVSKY 1949 Tryptophan metabolism. II. Concerning the mechanism of the mammalian conversion of tryptophan into nicotinic acid. J. Biol. Chem., *179:* 151.

HENDERSON, L. M., R. E. KOSKI AND F. D'ANGELI 1955 The role of riboflavin and vitamin B_6 in tryptophan metabolism. Ibid., *215:* 369.

HOLMAN, W. I. M. 1954 A colorimetric method for the determination of the principal metabolites of nicotinic acid in human urine. Biochem. J., *56:* 513.

HOLMAN, W. I. M., AND D. J. DE LANGE 1950 Role of tryptophane and other amino acids in the metabolism of nicotinic acid by humans. Nature, *166:* 468.

MEHLER, A. H. 1955 A symposium on amino acid metabolism, edited by W. D. McElroy and H. B. Glass. The Johns Hopkins Press, Baltimore, 882.

MILLER, E. C., AND C. A. BAUMANN 1945 Relative effects of casein and tryptophane on the health and xanthurenic acid excretion of pyridoxine-deficient mice. J. Biol. Chem., *157:* 551.

PERLZWEIG, W. A., F. ROSEN, N. LEVITAS AND J. ROBINSON 1947 The excretion of nicotinic acid derivatives after ingestion of tryptophane by man. Ibid., *167:* 511.

PRICE, J. M. 1954 The determination of *N*-methyl-2-pyridone-5-carboxamide in human urine. Ibid., *211:* 117.

REDDI, K. K., AND E. KODICEK 1953 Metabolism of nicotinic acid and related compounds in man and rat. Biochem. J., *53:* 286.

ROSEN, F., R. S. LOWY AND H. SPRINCE 1951 A rapid assay for xanthurenic acid in urine. Proc. Soc. Exp. Biol. Med., *77:* 399.

SARETT, H. P., AND G. A. GOLDSMITH 1947 The effect of tryptophane on excretion of nicotinic acid derivatives in humans. J. Biol. Chem., *167:* 293.

SPRINCE, H., R. S. LOWY, C. E. FOLSOME AND J. S. BEHRMAN 1951 Studies on the urinary excretion of "xanthurenic acid" during normal and abnormal pregnancy: A survey of the excretion of "xanthurenic acid" in normal nonpregnant, normal pregnant, pre-eclamptic and eclamptic women. Am. J. Obstet. Gynecol., *62:* 84.

VILTER, R. W., J. F. MUELLER, H. S. GLAZER, T. JARROLD, J. ABRAHAM, C. THOMPSON AND V. R. HAWKINS 1953 The effect of vitamin B_6 deficiency induced by desoxpyridoxine in human beings. J. Lab. Clin. Med., *42:* 335.

WALTERS, C. J., R. R. BROWN, M. KAIHARA AND J. M. PRICE 1955 The excretion of *N*-methyl-2-pyridone-5-carboxamide by man following ingestion of several known or potential precursors. J. Biol. Chem., *217:* 489.

NUTRITIONAL INTAKE OF CHILDREN

IV. VITAMINS A AND D AND ASCORBIC ACID [1]

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INTRODUCTION

Marked changes in the nutritional intake of infants and children over the past several decades may be attributed at least in part to changes in pediatric recommendations. The earlier introduction of solid foods, the trend toward replacement of orange juice by ascorbic acid preparations, and the differences in content of the most recently popular vitamin mixtures have had an obvious effect on the nutrient content of the diets of young children. These factors are superimposed on and modified by the voluntary intake and appetite of the children.

The present series of papers has been reporting the results of a study conducted over the past 10 years of the intake of a group of healthy children living at home. Intakes of 10 nutrients in the first 5 years of life have already been published (Beal, '53, '54, '55). This paper, the last of the series, will be concerned with the intakes of vitamins A and D and of ascorbic acid from both foods and vitamin preparations.

METHODS OF STUDY

A detailed description of the techniques of this study may be found in a previous publication (Beal, '53). Nutritional investigations were added in 1946 to the program of the Child

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

Research Council, which for more than 25 years has been engaged in research on the physical, physiological and psychological growth and development of a group of children in the Denver area. These children, who come from "upper middle class" familes, have been voluntarily enrolled in the study by their parents and are under the care of pediatricians in private practice. Since the purpose of this organization is research rather than therapy, no attempt has been made by the Council staff to influence the food intake of the children.

The nutrition data are based on a series of histories obtained by interview during home visits, with 4 consecutive 24-hour intakes recorded by the nutritionist and the mother. Histories are taken at monthy intervals during the first 6 months of life and thereafter at intervals of three months. Nutrients are calculated from food value tables (Bowes and Church, '56; U. S. Department of Agriculture, '48 and '50).

The data in this paper represent 1008 histories on 64 children (30 boys and 34 girls) who now range in age from 6 months to 12 years. Only the first 5 years of life are included. Intakes of breast-fed infants during the period of such feeding and two single histories on older children who had illnesses of sufficient severity to decrease markedly their food consumption during an entire three-month period have been excluded; all other histories taken during this age span on these 64 children have been included.

RESULTS AND DISCUSSION

Because the intakes of this group show a skewed rather than a normal distribution, the data are presented as 25th, 50th and 75th percentiles rather than as means and standard deviations. The percentile curves have been smoothed visually. The highest and lowest intakes observed are also indicated to give a picture of the very wide range in intake among children who are healthy and whose growth rates are satisfactory.

The dietary intake of vitamin A is presented as total intake, with separate figures for the contribution of animal and plant

sources; no attempt has yet been made to calculate the carotene content of the animal foods. In addition, the amount of vitamin A obtained from concentrates has been added to the dietary level to show the total consumption of vitamin A from all sources by these children. These data are presented in tables 1 and 2.

Animal food sources show little variation from one age to another and a relatively small range from minimum to maximum intakes as compared with the other vitamin A values presented here. The percentile curves of intake of animal sources rise to a peak at 15 to 18 months, when the median reaches a value of approximately 2300 I.U. daily, then decrease somewhat, due primarily to a decrease in milk, rising again after three years when milk consumption increases. We have found no significant correlation between the intake of animal food sources alone and the level of vitamin A in the plasma (Szymanski and Longwell, '51).

Plant sources, on the other hand, show a high degree of variability, both in the group percentiles and in individual intakes. The rise is rapid following the introduction of fruits and vegetables into the diet of the infants; of the 7 vegetables now available in canned puréed form, 4 are of the yellow or green leafy varieties high in carotene. A peak in intake of vitamin A from plant sources is reached between 9 months and one year of age, when the median is 3600 I.U. daily, after which there is a sharp decline as the canned puréed vegetables and fruit are replaced by a wider variety of home-prepared foods; this decline is accentuated during the preschool period when many of the children eat only small quantities of vegetables. At the lowest point between two and three years of age the median intake is approximately 1400 I.U. daily. After three years, intake rises slowly. A significant correlation was found between the intake of plant sources of vitamin A and the plasma carotene level, the peak in intake at the end of the first year being reflected in a peak in plasma carotene.

TABLE 1

Vitamin A intake of children from birth to 5 years of age

AGE	NO. OF CASES	TOTAL DIET AND CONCENTRATE, I.U.					TOTAL DIET ONLY, I.U.				
		Percentile					Percentile				
		Lowest	25	50	75	Highest	Lowest	25	50	75	Highest
years months											
0 0 to 0–1	32	700	1000	1600	3400	10,400	200	800	950	1100	2400
0–1 to 0–2	38	1500	3500	4700	6700	12,380	900	1150	1400	1500	2100
0–2 to 0–3	39	1700	4300	6000	8500	15,715	800	1400	1600	1900	3600
0–3 to 0–4	42	2650	5000	7000	9600	14,720	800	1600	2000	2700	3900
0–4 to 0–5	44	3365	5800	7900	10,600	15,900	600	2100	3000	3700	6100
0–5 to 0–6	46	3230	6300	8600	11,800	16,300	550	2800	3700	5200	7500
0–6 to 0–9	48	5360	7300	9800	13,300	18,035	3000	4000	5100	6400	11,700
0–9 to 1–0	50	4000	7300	10,100	12,600	22,515	2800	4750	5900	7300	11,900
1–0 to 1–3	51	3600	6800	9000	11,000	21,900	1700	4600	6000	7500	11,200
1–3 to 1–6	51	3200	5700	7900	10,000	24,500	2000	3500	5100	6800	10,600
1–6 to 1–9	49	2285	5000	6900	9500	23,500	1600	2900	4200	5900	11,000
1–9 to 2–0	49	1900	4500	6200	9000	20,300	1100	2700	3800	5400	10,400
2–0 to 2–3	46	2500	4100	5800	8700	15,120	1400	2500	3500	5100	13,200
2–3 to 2–6	45	1900	3900	5500	8400	16,600	1500	2400	3400	4900	8300
2–6 to 2–9	43	1900	3600	5400	8300	16,100	1600	2400	3400	4800	8000
2–9 to 3–0	43	1800	3500	5300	8200	11,600	1100	2400	3400	4700	8200
3–0 to 3–3	41	1300	3400	5400	8200	14,100	1300	2400	3500	4650	11,100
3–3 to 3–6	38	1500	3500	5600	8200	16,450	1500	2500	3700	4650	7600
3–6 to 3–9	38	1300	3700	5750	8400	17,550	1100	2600	3800	4700	8800
3–9 to 4–0	34	2500	3900	5900	8600	14,860	1100	2750	3900	4800	6500
4–0 to 4–3	35	1900	4100	6000	8700	24,455	1900	2900	4000	4900	6800
4–3 to 4–6	35	1800	4300	6100	8800	24,455	1500	3000	4100	5050	6200
4–6 to 4–9	35	2100	4400	6200	8900	17,100	1100	3100	4200	5150	6600
4–9 to 5–0	34	2400	4500	6300	8900	17,100	1500	3200	4250	5300	7100

TABLE 2

Vitamin A intake of children from birth to 5 years of age

AGE	NO. OF CASES	ANIMAL SOURCES, I.U.					PLANT SOURCES, I.U.				
		Percentiles					Percentiles				
		Lowest	25	50	75	Highest	Lowest	25	50	75	Highest
years months											
0–0 to 0–1	32	200	800	950	1100	2400	0	0	0	0	0
0–1 to 0–2	38	900	1125	1300	1500	2000	0	0	0	35	800
0–2 to 0–3	39	700	1200	1400	1600	2000	0	0	50	300	2300
0–3 to 0–4	42	600	1300	1500	1725	2300	0	25	350	1300	2600
0–4 to 0–5	44	50	1400	1600	1800	2500	20	400	1300	2100	4900
0–5 to 0–6	46	50	1450	1750	2000	3400	200	1200	1800	3200	6100
0–6 to 0–9	48	300	1575	2000	2800	3900	1100	1900	2700	3900	8900
0–9 to 1–0	50	500	1700	2200	3000	4800	500	2400	3600	4700	9600
1–0 to 1–3	51	1100	1750	2300	3000	4500	300	1900	3300	5100	9000
1–3 to 1–6	51	1000	1775	2275	2900	6900	400	1300	2600	4200	7200
1–6 to 1–9	49	900	1700	2175	2725	5300	300	1000	2000	3300	7500
1–9 to 2–0	49	800	1625	2100	2600	4700	300	800	1700	2800	8200
2–0 to 2–3	46	1000	1575	2025	2525	3800	200	700	1500	2550	10,800
2–3 to 2–6	45	800	1550	1950	2475	4400	200	700	1400	2400	5600
2–6 to 2–9	43	700	1525	1925	2450	3700	200	700	1350	2350	5100
2–9 to 3–0	43	900	1525	1900	2400	4200	200	700	1350	2300	4800
3–0 to 3–3	41	1000	1525	1900	2400	3300	200	700	1400	2350	8400
3–3 to 3–6	38	900	1550	1900	2400	3500	200	750	1450	2400	4800
3–6 to 3–9	38	800	1575	1925	2425	3500	100	800	1500	2450	5600
3–9 to 4–0	34	800	1600	1950	2450	3800	300	900	1600	2550	4000
4–0 to 4–3	35	1300	1625	1975	2500	3200	400	1000	1700	2600	3700
4 3 to 4 6	35	1200	1675	2000	2550	3400	300	1100	1800	2700	3400
4–6 to 4–9	35	1100	1725	2050	2600	4000	600	1150	1850	2800	4500
4–9 to 5–0	34	1200	1800	2100	2675	4100	300	1200	1900	2850	4500

In figure 1, in which the medians of these vitamin A sources are shown for comparison with each other, it may be seen that the curve representing total dietary vitamin A reflects the marked peak in plant sources. In considering the proportion of total dietary vitamin A which is supplied by plant sources, one finds that the level, which is at zero during the first two

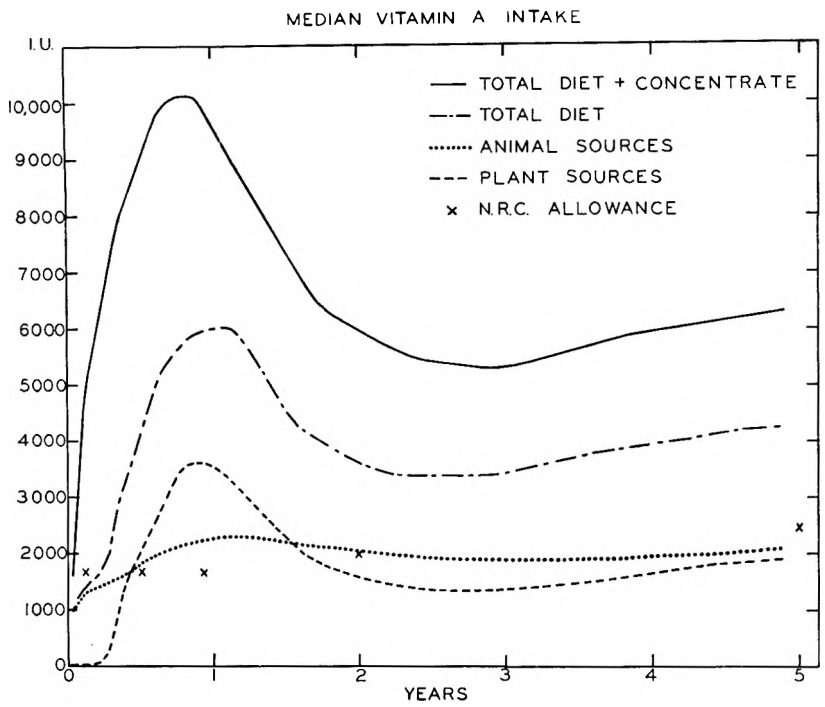

Fig. 1 Median intake of vitamin A from various sources contrasted with the Recommended Dietary Allowance in the first 5 years of life.

months, rises rapidly to 60% by one year, decreases to 40% by two and one-half to three years, then rises to nearly 50% by 5 years. However, at each age level there is a very wide range from minimum to maximum; for example, at two and one-half years the range is from 10 to 80% and at 5 years from 20 to 68%. Even though some additional carotene is supplied by animal foods, it seems unlikely that as much as two-thirds of the vitamin A in the diets of these children is

consumed as carotene throughout this age range. Such a finding would serve to accentuate the high level of intake of vitamin A from food sources alone by this group of children as compared with the Recommended Allowance of the National Research Council ('53), which is based on an estimation that "two-thirds of the vitamin A activity is derived from carotene

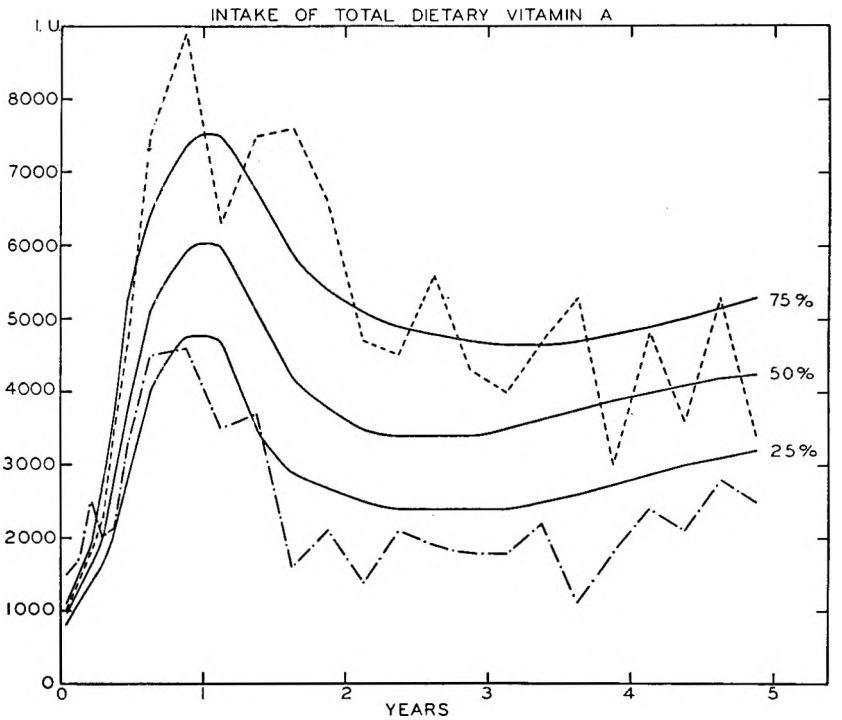

Fig. 2 Examples of individual variation in total dietary vitamin A intake compared to the 25th, 50th and 75th percentiles of the group from birth to 5 years of age.

and related compounds, while one-third is ingested as the preformed vitamin." Except at one to three months, the Allowance is below the 25th percentile of total dietary vitamin A of this group.

Individual patterns of two boys contrasted with the quartiles of total dietary vitamin A intake of the group are shown in figure 2. While they represent markedly different levels

of intake, they are typical of the group in that there is a tendency for each individual child to maintain a fairly constant position in the group The only marked sex differences in this age span occur between 6 months and three years. The boys show a higher intake of plant sources at the end of the first year and a greater drop in both animal and plant sources during the early preschool period than do the girls.

Concentrates of vitamin A, with at least one other vitamin, are given to these children approximately two-thirds of the time during the first 5 years of life. The average child in our series who has reached his 5th birthday has received a vitamin concentrate 64% of the time, although the range is from 17 to 98%. It should be noted, however, that only in a few cases is the concentrate given daily; the average frequency is 4 to 5 times weekly. The frequency has been determined at each history so that the proper adjustment of actual intake could be made. When the vitamin A supplied by concentrates is added to the dietary intake, the total is far in excess of the Recommended Allowance. At one year, for example, the median total intake is 6 times greater than the Allowance, and by 5 years it is still two and one-half times greater. Of more concern is the fact that approximately 10% of these children have received intakes 10 to 15 times higher than the Allowance throughout the first year. Although no symptoms of hypervitaminosis A have been observed, the intake seems far in excess of need. Indeed, dietary sources alone supply adequate amounts of vitamin A for the majority of these children. In the past few years there has been a tendency toward use of vitamin preparations lower in vitamin A content than previously. Between 1946 and 1949, 62% of these children were given concentrates containing more than 10,000 I.U. of vitamin A per 10-drop dose, with additional vitamin D; since 1950 this has dropped to 8%, with the remainder of the children receiving concentrates with 5000 I.U. of vitamin A per 10 drops, most commonly multi-vitamin preparations.

Intake of vitamin D is presented in table 3. The level is high during the first year, due both to concentrates and to the

common use of irradiated milk. The median reaches a peak of 1000 I.U. daily between 4 and 6 months, then decreases so that by 5 years the level is slightly below 400 I.U. daily. As previously noted in the discussion of vitamin A, a vitamin D

TABLE 3

Vitamin D intake of children from birth to 5 years of age

AGE	NO. OF CASES	VITAMIN D, I.U.				
		Percentile				
		Lowest	25	50	75	Highest
years months						
0–0 to 0–1	32	35	175	225	425	1290
0–1 to 0–2	38	300	600	750	1000	1820
0–2 to 0–3	39	325	700	975	1300	2290
0–3 to 0–4	42	90	725	990	1325	2080
0–4 to 0–5	44	0	720	1000	1330	2125
0–5 to 0–6	46	0	705	1000	1325	2125
0–6 to 0–9	48	0	635	965	1200	2185
0–9 to 1–0	50	0	470	840	1070	2110
1–0 to 1–3	51	0	350	700	985	2285
1–3 to 1–6	51	0	275	625	925	2215
1–6 to 1–9	49	0	210	560	875	2065
1–9 to 2–0	49	0	160	510	840	2520
2–0 to 2–3	46	0	125	475	820	1100
2–3 to 2–6	45	0	100	450	800	1930
2–6 to 2–9	43	0	75	440	780	1800
2–9 to 3–0	43	0	55	425	770	1150
3–0 to 3–3	41	0	40	415	755	1045
3–3 to 3–6	38	0	35	405	740	1435
3–6 to 3–9	38	0	30	400	730	1705
3–9 to 4–0	34	0	25	400	725	1620
4–0 to 4–3	35	0	20	395	720	2725
4–3 to 4–6	35	0	15	390	715	2725
4–6 to 4–9	35	0	10	385	710	1800
4–9 to 5–0	34	0	5	380	700	1800

concentrate was given an average of 64% of the time during the first 5 years.

Dietary intake of ascorbic acid (table 4) during the first 6 to 9 months is relatively low in this group since pediatricians in this area are placing an increasing dependence on ascorbic acid preparations and tend to delay the introduction of citrus

TABLE 4

Ascorbic acid intake of children from birth to 5 years of age

AGE	NO. OF CASES	DIET AND SUPPLEMENT, *mg*					DIET ONLY, *mg*				
		Percentile					Percentile				
		Lowest	25	50	75	Highest	Lowest	25	50	75	Highest
years months											
0–0 to 0–1	32	0	0	0	16	86	0	0	0	0	2
0–1 to 0–2	38	0	12	20	32	71	0	0	0	4	19
0–2 to 0–3	39	4	19	26	44	88	0	0	0	7	32
0–3 to 0–4	42	0	22	31	50	97	0	0	2	12	29
0–4 to 0–5	44	4	26	35	55	97	0	3	5	20	46
0–5 to 0–6	46	6	29	39	60	99	0	5	8	26	56
0–6 to 0–9	48	6	33	46	66	101	4	9	19	36	71
0–9 to 1–0	50	6	38	53	72	141	3	15	31	49	91
1–0 to 1–3	51	11	42	58	77	123	5	22	40	58	102
1–3 to 1–6	51	6	45	62	81	131	6	27	46	64	105
1–6 to 1–9	49	6	47	65	84	131	6	31	49	69	111
1–9 to 2–0	49	13	48	66	87	164	13	33	51	72	114
2–0 to 2–3	46	12	49	67	89	149	12	34	52	73	149
2–3 to 2–6	45	25	49	68	91	159	11	35	53	74	159
2–6 to 2–9	43	11	50	69	93	205	11	36	54	75	205
2–9 to 3–0	43	19	50	70	95	196	15	37	54	76	196
3–0 to 3–3	41	12	51	71	97	118	12	38	55	77	84
3–3 to 3–6	38	22	52	72	99	130	13	39	55	78	95
3–6 to 3–9	38	15	52	73	101	135	5	41	56	80	130
3–9 to 4–0	34	32	53	74	103	153	15	42	56	82	105
4–0 to 4–3	35	14	54	75	105	164	12	44	57	83	126
4–3 to 4–6	35	22	54	75	107	141	21	45	58	84	141
4–6 to 4–9	35	23	55	75	109	189	17	46	59	85	189
4–9 to 5–0	34	15	55	75	110	167	15	47	60	85	167

juices until near the end of the first year. Figure 3 shows that the rapid rise in ascorbic acid intake during the first year is due largely to such preparations. It should be noted that no value was given for vitamin C content of milk because of the unreliability of this value after processing (Holmes, '52), particularly with the sterilization of infant

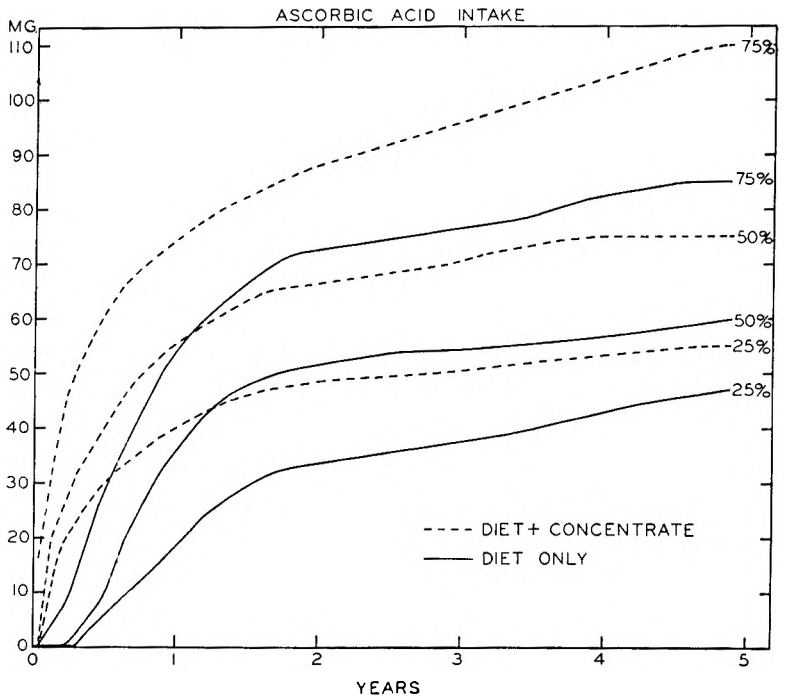

Fig. 3 25th, 50th and 75th percentiles of intake of ascorbic acid showing dietary levels with and without ascorbic acid preparations.

formulas. After the first year an increasing amount of ascorbic acid is derived from foods; ascorbic acid preparations are given an average of 40% of the time during the first 5 years to these children. The Recommended Allowance of the National Research Council approximates the 25th percentile of intake of ascorbic acid from diet alone after the first year.

Unlike the pattern of most nutrients reported from this study, there is no decrease in ascorbic acid intake during the

preschool period. There is, instead, a plateau followed by a slight rise. The intakes of individual children maintain a remarkable constancy of position in the group. For example, a child whose intake at two years is at the 25th percentile of the group tends to maintain a similar level through 5 years.

SUMMARY

Data have been presented from 1008 nutrition histories on 64 children in the first 5 years of life. Intakes of vitamins A and D and of ascorbic acid have been computed in terms of quartiles and of maximum and minimum levels observed. Some individual patterns of intake of vitamin A are shown.

The intake of animal sources of vitamin A shows little variation from age to age and a relatively small range at any age. The intake of plant sources, however, shows much more variation; there is a marked peak at the end of the first year, a decline in the second and third years, and a rise following three years of age. After the first 4 months, plant sources supply an average of 40 to 60% of total dietary vitamin A. After the first three months more than three-fourths of these children exceed the N. R. C. Recommended Allowance in vitamin A intake from diet alone; in addition, vitamin concentrates are given an average of 64% of the time.

The median vitamin D intake increases to a peak of 1000 I.U. daily at 4 to 6 months, than decreases to a level just below 400 I.U. daily by 5 years.

During the first 6 to 9 months most of the ascorbic acid intake is from ascorbic acid preparations rather than from diet; thereafter diet supplies an increasingly larger amount. After two years the Recommended Allowance is approximately at the 25th percentile level of observed intake from diet alone.

LITERATURE CITED

BEAL, V. A. 1953 Nutritional intake of children. I. Calories, carbohydrate, fat and protein. J. Nutrition, *50:* 223.

———— 1954 Nutritional intake of children. II. Calcium, phosphorus and iron. Ibid., *53:* 499.

BEAL, V. A. 1955 Nutritional intake of children. III. Thiamine, riboflavin and niacin. Ibid., *57:* 183.

BOWES, A. DE P., AND C. F. CHURCH 1956 Food Values of Portions Commonly Used. College Offset Press, Philadelphia, Pa., 8th ed.

HOLMES, A. D. 1952 Stability of ascorbic acid at different stages of processing. J. Pediatrics, *40:* 91.

NATIONAL RESEARCH COUNCIL 1953 Recommended Dietary Allowances. Publication 302.

SZYMANSKI, B. B., AND B. B. LONGWELL 1951 Plasma vitamin A and carotene determinations in a group of normal children. J. Nutrition, *45:* 431.

UNITED STATES DEPARTMENT OF AGRICULTURE 1948 Vitamin and Mineral Content of Certain Foods as Affected by Home Preparation. Washington, D. C. Miscellaneous Publication No. 628.

———— 1950 Composition of Foods — Raw, Processed, Prepared. Agriculture Handbook No. 8.

AN ELECTROPHORETIC STUDY OF EXUDATIVE DIATHESIS IN CHICKS [1]

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Exudative diathesis is a vitamin E-deficiency disease which occurs in chickens during the first or second month of life. The exudate usually manifests itself as a subcutaneous edema found upon the breast and abdomen. Edema of muscles and connective tissue has also been reported (Dam and Glavind, '38, '39, '44; Bird and Culton, '40). Dam and Glavind ('40) with use of trypan blue presented evidence that increased capillary permeability is present in this condition. The exudate was said to have approximately the same composition as that of normal plasma, and it was considered unlikely that a decrease in serum proteins contributed toward its formation (Dam, '44).

The purpose of the experiment presented in this report was to study the protein composition of the plasma and exudate of diseased chicks in comparison with the plasma proteins of chicks which received a similar diet but high in vitamin E.

MATERIALS AND METHODS

One hundred White Plymouth Rock [2] male chicks were used. The experimental group received a vitamin E-free diet containing Torula yeast as the source of protein. This diet is

[1] Investigation supported in part by research grant no H-1654 from the National Heart Institute of the N. I. H., Public Health Service, and by Distillation Products Industries, Rochester, New York.

[2] Arbor Acre Strain, Arbor Acres Farms, Glastonbury, Conn.

known to produce a very high incidence of exudative diathesis (Scott et al., '55). The control group received this diet supplemented with a high level [3] of vitamin E.

Blood was obtained by cardiac puncture, 1 ml being removed into a hypodermic syringe which had been rinsed with a heparin sodium solution.[4] The blood was then transferred to a tube containing one drop of the same heparin solution and mixed gently by swirling. Initially, the exudate was collected (from the same chicks as the plasma) by killing and skinning the chicks and then aspirating the fluid into a heparin-rinsed syringe, the rest of the procedure being the same as for the blood. Later, in an effort to preserve the chicks for subsequent examination, the exudate was removed by perforating the skin with a 20-gauge hypodermic needle and expressing the fluid into the collecting tubes.

Total protein was determined by a semi-micro modification [5] of the biuret method of Gornall, Bardawill and David ('49). Hanging strip paper electrophoresis was employed using a "Durrum Type" cell [6] (Durrum, '50; Block et al., '55; Williams et al., '55). The buffer system was sodium barbital-barbituric acid of ionic strength 0.075 and pH 8.6. A sample of plasma or exudate (0.01 ml) was applied to each strip of Whatman no. 3MM filter paper. The separations were carried out for 16 hours at a constant current of 5 milliamperes for 8 strips in parallel, after which the strips were dried and stained with bromophenol blue as outlined in the technical manual pro-

[3] 20 mg of *d*-α-tocopheryl acetate per pound of diet.

[4] "Heparin Na for Injection." Lederle Laboratory Division of American Cyanamid Corp.

[5] The protein solution was mixed with enough water to make 1.5 ml and an equal volume of biuret reagent was added. After incubation at 38° for 15 minutes, the sample was read at 540 mμ in the Beckman Spectrophotometer against a reagent blank (1.5 ml of H_2O, 1.5 ml of biuret reagent) which had also been incubated. One milligram of protein = D540 of 0.095. The upper limit of determination is 2.0 mg of protein, which produces a D540 of 0.190. From the unpublished notes of D G. Goldman appearing in the Methods Manual, Enzyme Institute, University of Wisconsin.

[6] "Model B" paper electrophoresis apparatus manufactured by Specialized Instruments Corp., Belmont, Calif.

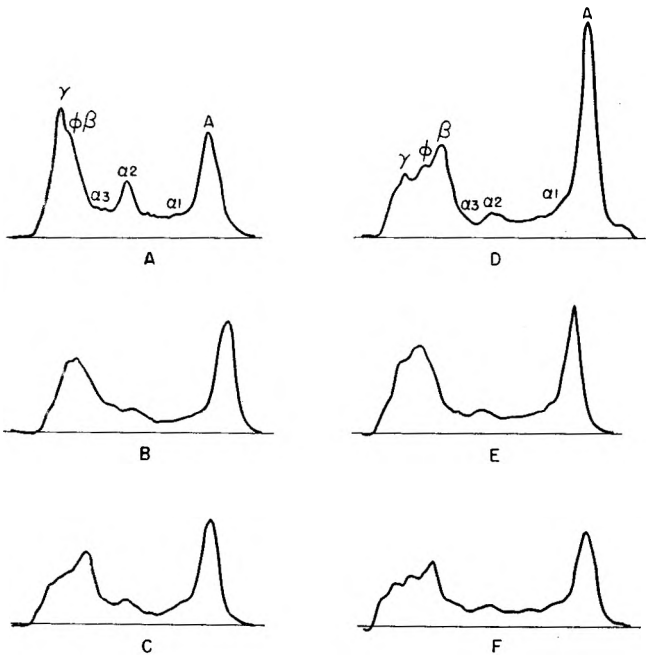

Fig. 1 Electrophoretic patterns of plasmas from chicks receiving the experimental diet supplemented with vitamin E.

A — One day (prior to receiving diet); B — 9; C — 14; D — 21; E — 28; and F — 35 days on diet.

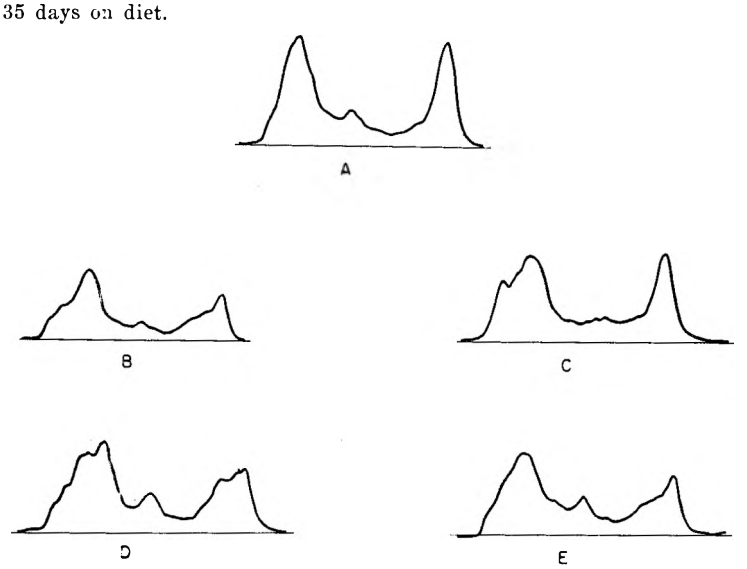

Fig. 2 Electrophoretic patterns of plasmas and exudates from chicks receiving the basal diet.

Plasmas: A — 9; B — 14; and C — 21 days.

Exudates: D — 14; E — 21 days on diet.

vided with the apparatus. After color equilibrium had been reached, the stained strips were scanned with a servo-type integrating scanner [7] (Block et al., '55).

EXPERIMENTAL

Part A. Electrophoretic patterns obtained from control and vitamin E-deficient chicks. Moving-boundary electrophoresis of normal adult chicken plasma in veronal buffer has shown the usual 6 Tiselius components (Sanders et al., '44), whereas in borate buffer an α3-globulin fraction appears to be present (Brandt et al., '52). McKinley et al. ('54) by the use of paper electrophoresis have shown the presence of an α 3-globulin component in chickens aged 7 to 15 weeks. Our results with male chicks also showed an α 3-globulin component.

Only pooled samples (from 4 or more chicks) both of plasmas and exudates were used. For the control chicks, patterns were obtained from the plasmas at the following ages: one day (prior to being placed on diet), 9 days, 14 days, 21 days, 28 days and 35 days (fig. 1).

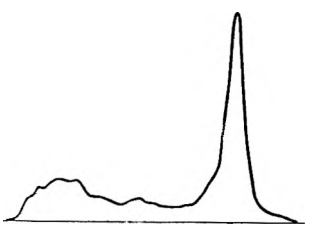

Fig. 3 Electrophoretic pattern of plasma from 28-day-old chicks receiving normal stock diet.

For the experimental chicks, patterns were obtained from the plasmas at the ages of 9, 14 and 21 days; exudate patterns, at 14 and 21 days (fig. 2).

Patterns were also obtained from the pooled plasma of 28-day-old chicks of the same sex and strain which had received a normal stock diet [8] (fig. 3).

Part B. Administration of a high dose of d-α-tocopheryl acetate to chicks suffering from exudative diathesis. Four 21-day-old birds suffering from severe exudates were given

[7] "Analytrol" scanner, manufactured by Specialized Instruments Corp., Belmont, Calif.

[8] Manufactured by Cooperative Grange League Federation Exchange, Inc., Ithaca, New York.

0.25 ml pure *d*-α-tocopheryl acetate directly into the crop. Prior to this, blood samples were taken from two of the birds in the usual manner. One of these birds subsequently died. Gross examinations were made and blood samples were taken from the three surviving birds two, three, 5, 7 and 9 days after receiving the dose. Electrophoretic patterns were obtained from all samples of plasma (fig. 4). No further vita-

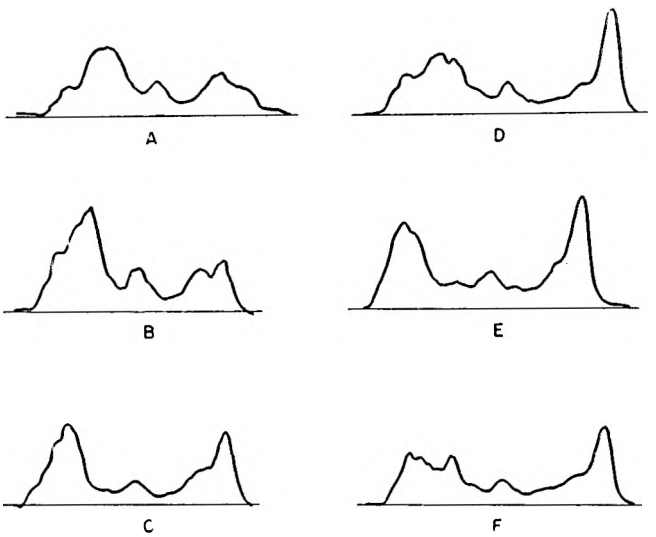

Fig. 4 Electrophoretic patterns of plasmas from a chick before and after administration of vitamin E.

A — prior to administration; B — 2; C — 3; D — 5; E — 7; and F — 9 days after receiving a single oral dose of 0.25 ml of *d*-alpha-tocopheryl acetate.

min E was administered, and the birds remained on the vitamin E-free diet throughout this period.

The concentration of various plasma components was determined by aligning the stained paper strips with the electrophoretic patterns, dropping perpendiculars at the regions of greatest separation and estimating the areas representing each component. In many cases, because of poor separation of the β-globulin and fibrinogen components, it was necessary to calculate these areas together.

DISCUSSION

Part A. The electrophoretic patterns of the plasmas from the control chicks all showed well-defined albumin peaks (fig. 1). The values for percentage of total protein in chicks receiving vitamin E, reported in table 1, were within the normal range reported by other workers (Brandt, et al., '51). The data presented in tables 1 and 2 show the percentage of total protein of the plasma to be lower for the vitamin E-deficient chicks than for the controls. A comparison of the electrophoretic patterns indicates that the exudates contained the same proteins as the blood plasma. At 14 days the vita-

TABLE 1

Total protein and relative distribution of plasma proteins in chicks fed the experimental diet supplemented with vitamin E

AGE WHEN BLOOD SAMPLE TAKEN	TOTAL PROTEIN	RELATIVE COMPOSITION						
		A/G [1]	α1/A	α2/A	α3/A	β/A	Φ/A	γ/A
days	%							
1	3.56	0.39	0.26	0.59	0.24	1.07		0.38
9	3.79	0.57	0.26	0.23	0.17	0.69		0.40
14	2.46	0.47	0.30	0.37	0.17	0.70	0.20	0.40
21	3.56	0.70	0.27	0.27	0.16	0.39	0.19	0.29
28	3.41	0.43	0.37	0.26	0.20	1.03		0.43
35	3.45	0.43	0.35	0.38	0.24	0.62	0.31	0.41
28 COM [2]	3.16	1.12	0.20	0.14	0.11	0.28		0.15

[1] A = albumin, G = globulins

[2] Twenty-eight-day-old chicks on stock diet.

TABLE 2

Total protein and relative distribution of plasma proteins in chicks fed the basal diet

AGE WHEN BLOOD OR EXUDATES TAKEN	TOTAL PROTEIN	RELATIVE COMPOSITION						
		A/G	α1/A	α2/A	α3/A	β/A	Φ/A	γ/A
days	%							
9 PL [1]	3.54	0.39	0.27	0.49	0.27	0.91		0.64
14 PL	2.30	0.17	1.10	0.80	0.50	2.30		0.90
14 EX	2.65	0.35	0.30	0.37	0.27	1.30		0.47
21 PL	2.44	0.20	1.00	0.95	0.50	1.10	0.65	0.95
21 EX	1.49	0.18	1.05	1.06	0.69	1.81		0.88

[1] PL = pooled plasmas, EX = pooled exudates.

min E-deficient or experimental chicks had a higher percentage of total protein in the exudate than in the plasma, whereas, at 21 days, the total percentage of plasma protein was greater than that of the exudate (table 2). The A/G ratio for the exudates of the 14-day-old chicks was significantly higher than for the corresponding plasmas, whereas the A/G ratios for the exudate and plasma of the 21-day-old chicks were approximately equal (table 2).

These results suggest either: (a) an increased capillary permeability which produces the lowering of the total proteins, especially albumin, by allowing them to escape into the tissues, or (b) a reduction in synthesis of plasma albumin which may result in a reduction in plasma osmotic pressure at 14 days with a resultant increase in exudate volume; or both. At 21 days, the albumin was almost equally distributed between plasma and exudate, thereby favoring the concept that the exudates were due to increased capillary permeability with resultant alteration in osmotic pressure during the early stages of the vitamin E deficiency.

The chicks on the stock diet were found to have a lower percentage of total protein, but a markedly higher A/G ratio (fig. 3 and table 1) than the control chicks of the same age on the vitamin E-supplemented experimental diet (fig. 1 E and table 1). This lower A/G ratio in chicks receiving the control diet may be a predisposing factor in producing the high incidence of exudative diathesis characteristically occurring when vitamin E is omitted from this diet.

Part B. The plasma pattern of one of the chicks with exudates before receiving 0.25 ml of *d*-α-tocopheryl acetate is shown in figure 4 A. The subsequent patterns (fig. 4 B, C, D, E, F) show the changes occurring after the administration of vitamin E. The results, as tabulated in table 3, show an initial percentage of total protein of 1.98 and an A/G ratio of 0.10 prior to administering the vitamin E. The percentage of total protein and the A/G ratio increased steadily thereafter for 5 days, and then leveled off. Gross examination of the chicks after two days showed their condition to be im-

proved, although they still exhibited exudates. After three days, all signs of exudates were absent, and the physical condition of the chicks appeared to be comparable to that in the control group. The chicks remained normal for the remainder of the experiment even though blood samples were taken from them regularly.

Since, in the vitamin E-deficient chicks, the electrophoretic pattern of the exudate fluid was similar to that of the plasma, it appears that the results presented here agree with those of Dam and Glavind ('40) in showing that increased capillary permeability is an important factor in the production of exu-

TABLE 3

Data obtained from chicks before and after administration of 0.25 ml d-α-tocopheryl acetate

CHICK 2171	TOTAL PROTEIN	RELATIVE COMPOSITION						
		A/G	α1/A	α2/A	α3/A	β/A	Φ/A	γ/A
days	%							
0 [1]	1.98	0.10	2.25	1.63	1.00	4.13		17.5
2	2.40	0.14	1.14	1.14	0.43	2.22	1.00	1.14
3	2.86	0.31	0.74	0.48	0.24	1.28		0.62
5	3.18	0.39	0.39	0.33	0.24	0.33	0.61	0.42
7	3.01	0.39	0.42	0.49	0.31	0.44	0.50	0.36
9	3.16	0.39	0.56	0.40	0.20	0.56	0.48	0.36

[1] Prior to the administration of *d*-α-tocopheryl acetate.

dative diathesis. However, reduced albumin concentration in the plasma because of failure of normal albumin synthesis during vitamin E deficiency may contribute to the edematous condition through lack of maintenance of normal osmotic relationships between the plasma and the tissue fluids. Dam ('44a), Hove ('46) and Moore ('49) have presented results with rats indicating that vitamin E is concerned in protein synthesis.

The failure of normal albumin synthesis during vitamin E deficiency in the chick is indicated by the low albumin concentrations in both the plasma and exudates, and by the fact that the plasma albumin level was rapidly restored upon administration of a single dose of *d*-α-tocopheryl acetate. Since

the level of total proteins in the plasma was reduced approximately 50% and the ratio of albumin to globulin was approximately 50% of normal, a 4-fold increase in extracellular fluid would have been necessary in order to dilute the albumin sufficiently to give the observed albumin values found in the exudates and plasma. If the albumin level had been reduced by dilution, the globulins should have been reduced equally, thereby maintaining a normal A/G ratio in both plasma and exudates. However the results showed that the A/G ratio did not remain normal, thereby showing a lack of synthesis of albumin or a marked increase in synthesis of globulins. Increased synthesis of globulins and marked dilution of tissue fluid proteins appears highly unlikely, since it was possible to obtain only 1 or 2 ml of exudate fluid from the most severely affected chicks. Furthermore, the concentration of total proteins, and the relationships between the various globulin fractions and the albumin quickly returned to levels equivalent to those of the control chicks upon administration of vitamin E. These results provide additional evidence, therefore, that vitamin E is concerned in plasma protein anabolism.

SUMMARY

Comparative paper electrophoretic analyses have been made of the proteins in the plasma of chicks which received a semi-purified diet with and without vitamin E. Similar analyses were conducted on exudates of the vitamin E-deficient chicks.

A decrease in the percentage of total protein, and more markedly in the albumin, was shown in chicks suffering from exudative diathesis. The exudates produced electrophoretic patterns which were qualitatively similar to those obtained from the plasmas of the same chicks. However, significant quantitative differences were found in the percentage of total protein and the A/G ratios.

Chicks on a stock ration showed a much higher A/G ratio than corresponding chicks of equivalent age on the basal experimental diet supplemented with a high level of vitamin E.

Oral administration of 0.25 ml of pure *d*-α-tocopheryl acetate produced complete recovery of chicks suffering from exudative diathesis.

The possible role of vitamin E in capillary permeability and protein synthesis has been discussed.

LITERATURE CITED

BIRD, H. R., AND T. G. CULTON 1940 Generalized edema in chicks prevented by *d,l*-alpha-tocopherol. Proc. Soc. Exp. Biol. Med., *44:* 543–547.

BLOCK, R. J., E. L. DURRUM AND G. ZWEIG 1955 A manual of paper chromatography and paper electrophoresis, New York, 401.

BRANDT, L. W., R. E. CLEGG AND A. C. ANDREWS 1951 The effect of age and degree of maturity on the serum proteins of the chicken. J. Biol. Chem., *191:* 105–111.

BRANDT, L. W., H. D. SMITH, A. C. ANDREWS AND R. E. CLEGG 1952 Electrophoretic investigation of the serum proteins of certain birds and their hybrids. Arch. Biochem. and Biophys., *36:* 11–17.

DAM, H. 1944 Studies on vitamin E deficiency in chicks. J. Nutrition, *27:* 193–211.

———— 1944a Vitamin E and length of life of rats fed a diet with fatally low protein content. Proc. Soc. Exp. Biol. Med., *55:* 55–56.

DAM, H., AND J. GLAVIND 1938 Alimentary exudative diathesis. Nature, *142:* 1077–1078.

———— 1939 Alimentary exudative diathesis, a consequence of E-avitaminosis. Ibid., *143:* 810–811.

———— 1940 Vitamin E and capillary permeability. Naturwiss., *28:* 207.

DURRUM, E. L. 1950 A microelectrophoretic and microionophoretic technique. J. Am. Chem. Soc., *72:* 2943–2948.

GORNALL, A. G., C. J. BARDAWILL AND M. M. DAVID 1949 Determination of serum proteins by means of the biuret reaction. J. Biol. Chem., *177:* 751–766.

HOVE, E. L. 1946 Interrelation between alpha-tocopherol and protein metabolism: body weight and tooth pigmentation in rats. Proc. Soc. Exp. Biol. Med., *63:* 508–511.

MCKINLEY, W. P., W. A. MAW, W. F. OLIVER AND R. H. COMMON 1954 Determination of serum protein fractions on filter paper electropherograms by the biuret reaction, and some observations on the serum proteins of the estrogenized immature pullet. Can. J. Biochem. and Physiol., *32:* 189–199.

MOORE, T. 1949 The significance of protein in vitamin E deficiency. Ann. N. Y. Acad. Sci., *52:* 206–216.

SANDERS, E., I. F. HUDDLESON AND P. J. SCHAIBLE 1944 An electrophoretic study of serum and plasma from normal and leucosis-affected chickens. J. Biol. Chem., *155:* 469–481.

SCOTT, M. L., F. W. HILL, L. C. NORRIS, D. C. DOBSON AND T. S. NELSON 1955 Studies on vitamin E in poultry nutrition. J. Nutrition, *56:* 387–402.

WILLIAMS, F. G. JR., E. G. PICKELS AND E. L. DURRUM 1955 Improved hanging-strip paper-electrophoresis technique. Science, *121:* 829–830.

EFFECT OF CARBOHYDRATE UPON RANCIDITY IN EXPERIMENTAL RATIONS [1]

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Development of rancidity in experimental diets has been a problem of continuing importance in this and other laboratories where nutritional studies have been conducted. Various dietary factors may be inactivated or destroyed in such diets (Burr and Barnes, '43; Holman, '54) and large amounts of rancid fat apparently have a toxic effect (Quackenbush, '45). It is well known that a variety of factors influence autoxidation of fats. Markley ('47) lists light, heat, concentration of oxygen and the presence of catalysts and inhibitors as affecting the reaction and points out that "it is sometimes difficult to evaluate the effect of a specific factor in the overall oxidation process because in most cases several of these factors are simultaneously active." Earlier studies in this laboratory (White et al., '53) indicated that a copper-choline complex salt which formed in the diet was a primary factor in the development of rancidity. This present study demonstrates that the kind of carbohydrate used in experimental diets is also a contributing factor in the onset of rancidity.

During a series of balance studies with dogs, we observed a loss of appetite rather frequently apparently as the result

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of a very slight change in the odor of the diets. It appeared on closer examination that change in odor, indicating the onset of rancidity, occurred more rapidly in diets made with dextrose than in those made with sucrose. As a result of these observations, the influence of the kind of carbohydrate in the diet upon development of rancidity was studied.

EXPERIMENTAL PROCEDURE

The diets used are shown in table 1. Small amounts of water soluble vitamins were added. In the first experiments the

TABLE 1

Composition of experimental diets

CONSTITUENT	AMOUNT
	%
Sucrose [1] or Hydrate Dextrose [2]	64
Crude casein	20
Celluflour	3
Salt mixture [3]	2.5
Corn oil	9
Cod liver oil	1
Choline chloride	0.2

[1] Revere Sugar Refining Co.
[2] Corn Products Refining Co.
[3] Jones and Foster ('42) mixture with calcium carbonate removed.

carbohydrates used were exactly as supplied by producers; in later experiments the carbohydrates were finely ground in a ball mill to insure uniformity in particle size. Other modifications will be indicated in the Results. The diets were thoroughly mixed and measured out in 5 gm aliquots in paper weighing cups. These cups were then placed in a 75°F. temperature-controlled room until analyzed. At appropriate times, fat from a sample was extracted with petroleum ether, the ether distilled off on a steam bath and the iodine number determined by the Wijs method (cited by A. O. A. C., '40). Duplicates were done until it was certain that the method was reproducible. The results of the iodine number assays are

reported in milliliters of 0.10 N thiosulfate since equal quantities of the fat were used in all the samples.

RESULTS AND DISCUSSION

As shown in table 2, diets made with dextrose developed rancidity much more rapidly than those made with sucrose.

TABLE 2

Effect of kind of carbohydrate on development of rancidity

TEST NO. 1 [1]			TEST NO. 2 [1]		
DAY	Sucrose	Dextrose	DAY	Sucrose	Dextrose
0	0.9	1.0	0	1.0	1.0
5	1.0	1.2	4	1.0	1.2
7	1.0	1.2	7	1.0	1.4
8	1.0	1.3	11	1.2	12.9
13	1.2	13.3	14	4.0	13.9

[1] Results reported as milliliters of 0.1 N thiosulfate/0.5 gm of fat.

TABLE 3

Effect of antioxidant on development of rancidity

DAY	CONTROL [1]		0.1% ANTIOXIDANT [1]	
	Sucrose [2]	Dextrose [2]	Sucrose [2]	Dextrose [2]
0	1.1	1.1	1.1	1.0
5	1.1	1.6	1.1	1.4
8	1.5	12.6	1.3	5.0
11	10.2	13.5	2.0	13.2
13			12.5	15.0

[1] Tenox, Eastman Chemical Products Co., Kingsport, Tenn.

[2] Both sugars powdered in the ball mill. Results reported as milliliters of 0.1 N thiosulfate/0.5 gm of fat.

Both carbohydrates were used in the manufactured state. In experiments performed at different times of the year, the number of days required for rancidity to develop was somewhat variable.

To eliminate the effect of physical size of crystals, the carbohydrates used in the remaining experiments were finely ground in the ball mill. Data in table 3 show that the carbo-

hydrate effect persisted after grinding. The addition of an antioxidant [2] retarded the development of rancidity in both diets, but rancidity developed sooner in the presence of dextrose.

Data shown in table 4 are the result of diets made without the salt mix as well as diets made with anhydrous dextrose. Diets without the salt mix were much more stable than those with the salt. Anhydrous dextrose, however, was inferior to the other sugars.

In other tests not shown here, the previous work of White et al. ('53) was confirmed whereby the removal of choline

TABLE 4

Effect of salt mixture and carbohydrate on development of rancidity

DAY	SUCROSE [1]		DEXTROSE [1]		ANHYDROUS DEXTROSE [1]	
	No salts	Plus salts	No salts	Plus salts	No salts	Plus salts
0	1.1	1.1	1.1	1.1	1.1	1.1
4	1.2	1.3	1.1	1.3	1.4	6.6
5	1.2	1.3	1.1	1.3	1.5	. .
7	1.2	1.3	1.1	6.6	6.5	13.2
8	1.2	1.3	1.1	11.3	10.6	13.2
11	1.2	3.4	1.3	14.4	14.0	15.0

[1] Results reported as milliliters of 0.1 N thiosulfate/0.5 gm of fat.

from the diets markedly improved stability. Although a few studies have been done, no effect of carbohydrate has been observed in the absence of choline.

SUMMARY

These studies have shown that the kind of carbohydrate used in a purified diet has an important effect upon the rate at which rancidity develops. Diets made with glucose were much more susceptible to auto-oxidation than those made with sucrose. The mechanism of the effect is unknown.

[2] A combination of butylated hydroxy-anisole, propyl gallate and citric acid. Supplied by Eastman Chemical Products Co., Kingsport, Tenn.

LITERATURE CITED

ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS 1940 Official and Tentative Methods of Analysis. Washington, D. C.

BURR, G. O., AND R. H. BARNES 1943 Non-caloric functions of dietary fats. Physiol. Rev., *23:* 256.

HOLMAN, R. T. 1954 Autoxidation of Fats and Related Substances. Pergamon Press, Ltd., London, p. 51.

JONES, J. H., AND C. FOSTER 1942 A salt mixture for use with basal diets either low or high in phosphorus. J. Nutrition, *24:* 245.

MARKLEY, K. S. 1947 Fatty Acids. Interscience Publishers, New York, New York.

QUACKENBUSH, F. W. 1945 Toxicity of rancid fat. Oil and Soap, *21:* 25.

WHITE, P. L., D. M. HEGSTED AND J. MAYER 1953 Two complex salts of choline and copper chloride and their activity as catalysts in fat oxidation. J. Am. Chem. Soc., *75:* 2352.

NUTRITIONAL STUDIES ON RATS ON DIETS CONTAINING HIGH LEVELS OF PARTIAL ESTER EMULSIFIERS [1]

I. GENERAL PLAN AND PROCEDURES; GROWTH AND FOOD UTILIZATION

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The partial fatty acid esters of the sugar alcohol-anhydrides, polyoxyethylene derivatives thereof, and polyoxyethylene derivatives of fatty acids have received wide acceptance as surface-active agents and emulsifiers. Recognizing possible applications of certain of these compounds which would involve their ingestion, Krantz and co-workers ('48, '49, '51, '52) carried out extensive pharmacologic studies with them. The apparent innocuousness of these substances encouraged the gradual extension of their use over the last 15 years in food and pharmaceutical products (Pratt '52). Numerous pharmacological and clinical studies, some quite extensive, were undertaken to establish the safety of these uses.

One of the most important of this class of compounds is Myrj 45 [polyoxyethylene (8) monostearate] employed for nearly a decade to retard the firming of bread. In applying for the inclusion of Myrj 45 as a permissive ingredient in bread, for which a definition and standard of identity was to be promulgated by the Food and Drug Administration, it was necessary to present evidence of its safety. The volu-

[1] This investigation was supported by a grant from the Atlas Powder Company, Wilmington, Delaware.

minous testimony placed in the record of the "bread hearings" (Federal Security Agency, '48–'49) was judged by the Administrator to be inadequate for the purpose of establishing the safety of Myrj 45. A substantial part of this toxicological evidence had been obtained in support of the use of various polyol esters of fatty acids in products other than bread, principally of a pharmaceutical nature. Much of the data had been accumulated prior to the publication by the Food and Drug Administration of its recommendations concerning the design of toxicological experiments to support the use of chemical additives in foods (Lehman et al., '49). However, the opinion of experts as expressed at these hearings was far from unanimous on the question of the inadequacy of the evidence particularly with respect to the chronic feeding studies. This fact, together with the cumulative experience of apparently safe pharmaceutical and food use of the polyol emulsifiers, prompted renewed investigation on the effect of chronic ingestion of the fatty acid partial esters.

Experiments were undertaken to determine the effects of these esters on growth, food utilization and metabolism, reproduction and lactation, physiological behavior, mortality, and post-mortem pathology, when included in the diet of rats throughout their lifetime, at levels far exceeding any conceivable use-concentration in the human diet. In the evolution of the plan for these experiments, careful consideration was given by all parties concerned to the magnitude and scope of the studies. In general the plan followed the pattern suggested for chronic feeding studies by the Division of Pharmacology of the Food and Drug Administration. The experiments were designed to cover successive generations of rats as well as the life cycle of the parent generation. The diets consisted exclusively of a nutritionally adequate basal ration modified only to the extent necessary by graded additions of the emulsifiers or of fat.

The studies are to be reported in a series of papers, this introductory report covering the general plan of the investigation and procedures which apply throughout, as well as

the responses in respect to growth and food utilization. Subsequent papers will be devoted to reproduction and lactation studies, clinical and metabolic observations, survival data, and gross and microscopic pathology.

GENERAL PROCEDURE

Test materials. The emulsifiers included in the investigation were the 6 different products listed below and a single mixture of these.

Trade name [2]	*Chemical name*
Myrj 45	Polyoxyethylene (8) stearate
Myrj 52	Polyoxyethylene (40) stearate
Span 60	Sorbitan monostearate
Tween 60	Polyoxyethylene (20) sorbitan monostearate
Tween 65	Polyoxyethylene (20) sorbitan tristerate
Tween 80	Polyoxyethylene (20) sorbitan monooleate

During the two-year period of these experiments, beginning in March, 1952, 8 lots of each of the Atlas products were delivered in 5-gallon containers each identified by a "C.E.L." production number. Intermittent shipments of each product were made in order to avoid possible rancidification of the fatty acid moiety of these materials during storage over the two-year period. In addition to these individual products, a mixture of all 6 was fed in proportions designed to approximate the ratios in which they might appear in the human diet, namely, 25% each of Myrj 45 and Span 60, and 12.5% each of the other 4 Atlas emulsifiers.

Samples of each shipment were taken upon receipt and analyzed chemically to confirm their identity. Determinations were made of the hydroxyl number, saponification number, and acid value. In all cases the values found were in substantial agreement with the manufacturer's specifications. Diets furnishing several levels of a triglyceride fat were included in this study as a basis for observing the effect of variations in organoleptic qualities, fatty acid content, caloric density, and physical consistency of the diets on food con-

[2] Trade marks registered by Atlas Powder Company.

sumption and growth. However, it is not to be inferred that any of the emulsifiers, though they might have nutritional value, were regarded as equivalent to or substitutes for triglycerides. The neutral fat employed was a "pure vegetable shortening made from hydrogenated vegetable oil." [3]

Experimental diets. Since the ultimate objective of these experiments was to provide a basis for determining the safety of the use of the fatty acid partial ester emulsifiers in the human diet, special consideration was given to the choice of a basal diet for the chronic feeding studies.

Three types of basal ration are employed in chronic toxicological studies, the so-called synthetic or purified diets, commercially prepared feeding mixtures (mashes or chows), and laboratory-prepared diets composed principally of natural foodstuffs. The synthetic or purified type of diet was considered unsuitable for these experiments because of the more or less arbitrary selection of protein, carbohydrate, and fat components and the lack of variety as compared with the mixture of foods comprising the human diet. Furthermore the possibility of inducing effects due to the deficiency of unrecognized essential nutrients could lead to adventitious results in these chronic studies. Commercial mixes or chows were rejected on the ground that no direct or immediate control could be maintained over the composition or uniformity of such mixtures during the course of a prolonged experiment. Moreover the necessity for replacing high proportions of a whole prepared ration with test materials could lead to wide variations in the proportions of essential nutrients in the test and control diets.

For the foregoing reasons it was decided to employ a basal diet prepared in the laboratory primarily from natural foods such as wheat, corn, milk, meat, etc., supplemented with various micronutrients. It was possible not only to insure the uniformity of composition but, even more important, to incorporate replacements of as much as 20% of esters or fat

[3] Primex, purchased in 50 lb. cartons at intervals, as required, from the Procter and Gamble Distributing Company, Kew Gardens, New York.

in such a way as to minimize variations in the balance of essential nutrients. Except for the reduction in fat level, the composition of the basal diet adopted for these studies bears a reasonable resemblance to the food consumption pattern of the U. S. population (U.S.D.A. Miscellaneous Publication 691, '50) with respect to meat, milk, and grain components. Addition of 15% fat yields a composition in which the caloric distribution among protein, fat and carbohydrate likewise conforms to that of the average American diet.

TABLE 1

Composition of rations [1]

INGREDIENT	LEVEL, PER CENT			
	0 (Basal)	5	10	20
Emulsion or fat	0	5	10	20
Whole wheat	42	39	35	28
Yellow corn	21	19	18	15
Non-fat milk solids	20	20	20	20
Meat meal	10	10	10	10
Alfalfa meal	2	2	2	2
Dried yeast	1	1	1	1
Dried pork liver	0.48	0.48	0.48	0.48
Celluflour	1	1	1	1
Sodium chloride	0.5	0.5	0.5	0.5
Vitamin B-complex mixture [2]	1	1	1	1
$MnSO_4 \cdot H_2O$	0.02	0.02	0.02	0.02
Vitamins in oil [3]	1	1	1	1

[1] To facilitate preparation of the test and control diets, large batches were made up of a mixture comprising all ingredients in the basal diet except that the wheat was reduced to 28%, the yellow corn to 15%, and the vitamin oil was omitted. The final diets were prepared at intervals of not more than two weeks by the addition of the fat or ester components and of the vitamin oil mixture. In diets containing test supplement at levels of less than 20% the balance was made up with wheat and corn in the ratio of 2: 1.

[2] Providing, per 100 gm of ration: Thiamine, 600 μg; riboflavin, 1200 μg; pyridoxine, 400 μg; Ca pantothenate, 4 mg; niacin, 5 mg; choline chloride, 200 mg; inositol, 100 mg; *p*-aminobenzoic acid, 2.5 mg; biotin, 1 μg; vitamin B_{12}, 1 μg; folic acid, 1 μg; liver concentrate, 25 mg. (Celluflour carrier.)

[3] Providing, per 100 gm of ration: Vitamin A, 200 units; vitamin D, 20 units; α-tocopherol, 12 mg; menadione, 100 μg; cottonseed oil, 1 gm (furnishing 450 mg linoleic acid).

The composition of the basal and supplemented diets is shown in table 1. Supplementation of the diet with esters or fat was accomplished by replacing equivalent amounts of the wheat-corn fraction which is present in the proportion of 2:1 in the basal diet, the other constituents of the diet remaining unchanged. Thus when an emulsifier was included to the extent of 20%, the protein content of the ration was only slightly affected, being reduced from 22 to approximately 20%, while the energy value varied within the narrow limits of 2.99 to 3.42 Cal. per gram (table 2).

TABLE 2

Caloric density of basal and test diets [1]

(All values in calories per 100 gm of diet)

SUPPLEMENT TO BASAL DIET	LEVEL, PER CENT			
	0	5	10	20
None	341.8			
Myrj 45		341.4	341.0	340.2
Myrj 52		331.1	320.4	299.0
Span 60		342.3	342.8	343.8
Tween 60		335.3	328.8	315.8
Tween 65		342.0	342.1	342.4
Tween 80		335.9	329.9	318.0
Mixture		339.0	336.1	330.4
Primex		369.0	396.2	450.6

[1] The caloric values of the emulsifiers are based on the assumption of 9.4 Cal. per gram for their fatty acid moieties, corrected for the observed coefficients of digestibility, viz. Myrj 45–3.32, Myrj 52–1.26, Span 60 3.50, Tween 60–2.09, Tween 65–3.42, Tween 80–2.21, Mixture — 2.83 calories per gram, the last mentioned being the weighted average for the component emulsifiers.

Thiamine assays conducted on batches of the diet containing 0, 5, 10, and 20% of Myrj 45 showed no appreciable destruction of this vitamin after 6 weeks' storage at room temperature, in contrast with the finding of Nelson ('53). In any case completion of the diet batches by the addition of the emulsifiers, fat and vitamin mixtures was accomplished about once a week so that no diets were more than 10 days old when fed.

Calculations based on the fatty acid moiety of the various esters, assuming a mean value of 9.4 Cal. per gram of fatty acid, indicated the caloric value of these emulsifiers to range from 1.32 (Myrj 52) to 6.54 (Span 60) Cal. per gram. In computing the data in table 2, however, account was taken of the coefficients of digestibility of the fatty acid moieties of the partial esters, as determined experimentally. This was necessary since certain of the emulsifiers are composed of high proportions of stearic acid, generally recognized to be incompletely absorbed at high feeding levels.

Pratt ('52) showed that the combined amount of the various Atlas emulsifiers required to perform their technological functions in foods, if used at maximum practical levels (as distinguished from customary desired levels) would seldom exceed 0.3% of any food product. The total consumption of these emulsifiers, assuming their use at normal levels in all food products where they may have a function, would be 0.48 gm per person per day or 0.08% of the average diet, dry basis. By similar calculations based on maximum practical levels of use, the total intake would be 0.75 gm per day or 0.125% of the diet. Thus the lowest feeding level adopted for these investigations, 5%, represent 40 times the maximum human dietary level. Previously reported studies suggested that adverse physiological responses might be expected at the 20% level; hence this was selected as the highest feeding level. The intermediate level, 10%, is the logarithmic mean.

Test animals. The rats used in this study were obtained from Food Research Laboratories' stock colony originated in 1928 with breeders of the Wistar strain. While the colony has not been genetically inbred through brother-sister matings, cross strains have not been introduced.

Litters were weaned at 21 days and were maintained on the breeder diet until 28 days old when the individual weights ranged between 50 and 70 gm. At this time rats weighing within 5 gm of the average weight of each litter were divided according to sex and litter among 25 groups consisting of 12 males and 20 females each. With the aid of tables of

random numbers (Cochrane and Cox, '50) litter-mates were assigned randomly to diets having the same level of supplementation. The groups consisted of more females than males because it was anticipated that the stresses of pregnancy and lactation might result in higher mortality, thereby leaving an insufficient number of females to establish statistically sound longevity data.

Housing and maintenance. For the entire duration of the investigation the rats were housed in suspended wire-mesh cages in an air-conditioned room maintained at 76 ± 2°F. and 50 ± 10% relative humidity. For the first 12 weeks on the test diets they were housed individually but when matings were started they were transferred to larger cages, one male being housed with one or two females. When litters were about to be cast the does were transferred to separate cages containing shredded paper wads for nesting where they remained until their litters were weaned. During rest periods between matings small groups of rats of the same sex were housed together.

Diets were furnished ad libitum in non-scatter food cups. Water was supplied through glass delivery tubes from bottles suspended on the outside of the cages. Cages were washed once a week or more frequently if they became soiled by soft stools.

Observations. During the first 12 weeks, the body weight of each rat was recorded at weekly intervals. This practice was followed not only in the initial generation but in each succeeding generation throughout the study. For the remainder of the experiment the animals were weighed biweekly. Records were made of food consumption of 5 rats of each sex, randomly chosen from each group, for the first 12 weeks. Additional checks on food consumption were made on these rats for a two-week period at the 0.5, 1, 1.5, and 2-year stages in the initial generation and for 12 weeks after weaning in succeeding generations.

At the termination of the 12-week period, 6 rats from each group (three males and three females) were randomly selected

for the following examinations: erythrocyte and leukocyte counts, differential leukocyte count, blood sugar and non-protein nitrogen determinations, microscopic and chemical examination of the urine. Additional observations were made at 6-month intervals in the case of the F_0 generation on similar representative rats from each group, and terminally on all survivors.

During the course of the study observations were made of physical appearance, behavior, laxation and water consumption.

Reproduction and lactation responses were based on the results of matings throughout the fertile life span of the parent generation and for the first two litters of each succeeding generation.

At death or sacrifice of moribund rats, and at the termination of the two-year period, each animal was autopsied. The kidneys, liver, spleen, heart, and adrenals of at least 6 rats per sex and group were weighed and all major organs were placed in formalin for subsequent histopathological study. Most of the animals were sacrificed by asphyxiation with illuminating gas except for three males and three females in each group in which cases spinal fracture was used prior to collection of blood for cholesterol determination.

GROWTH

The rats were weighed weekly during the first 12 weeks on the experimental diets and biweekly thereafter. Growth responses expressed as average body weight by sexes within groups at logarithmically equal intervals are presented in table 3; the weights for the even one- and two-year periods are also shown.

Inspection of the data reveals that in general the growth responses were not markedly affected by variations in the levels of either fat or emulsifiers within the 5 to 20% range employed. In the females especially, the curves for the several levels of each emulsifier are practically superimposable. However, the trend among the males at the 20% emulsifier levels

Summary of growth responses of F_0 generation of rats during two-year feeding experiment

FAT OR EMULSIFIER	NUMBER OF RATS AND SEX	AVERAGE BODY WEIGHTS, 0 TO 104 WEEKS								
		0	3	6	12	24	48	52	96	104
		gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
None	12M	57	175	260	326	371	420	417	446[7]*	431[7]
	20F	56	139	176	209	262	307	301	329[13]	315[12]
5% Level										
Myrj 45	12M	57	175	264	334	385[11]	415[10]	420[10]	441[6]	435[5]
	20F	53	133	171	205	268[13]	304[15]	303[15]	327[5]	319[5]
Myrj 52	12M	60	180	257	341	389	430	434	426[7]	421[7]
	20F	54	134	171	202	281	312[19]	302[18]	313[10]	316[7]
Span 60	11M	52	167	249	322	341	404	412	431[8]	434[5]
	20F	54	130	165	195	267[19]	287[16]	288[16]	292[13]	282[10]
Tween 60	12M	57	162	243	320	373[11]	424[11]	424[10]	450[3]	455[3]
	21F	55	133	169	200	256[20]	290[13]	288[13]	317[10]	292[9]
Tween 65	12M	58	165	252	320	368	393	397	448[5]	446[5]
	20F	53	132	170	206	266[17]	297[16]	302[16]	313[10]	296[8]
Tween 80	12M	56	170	246	310	354[11]	393[11]	383[11]	412[6]	423[5]
	20F	54	140	179	210	260[18]	303[17]	311[17]	317[14]	311[8]
Mixture	12M	55	171	257	327	378	421	416	428	416[12]
	20F	57	137	177	209	264[16]	303[15]	307[15]	315[9]	326[8]
Primex	12M	56	175	264	350	402	451[11]	472[11]	465[6]	454[4]
	20F	57	136	176	212	290	319[19]	320[19]	351[12]	371[10]
10% Level										
Myrj 45	12M	56	166	238	299	355	392[13]	398[11]	419[5]	414[5]
	20F	60	142	178	208	274[19]	313[18]	312[18]	343[13]	330[7]
Myrj 52	12M	57	172	234	302	321[13]	408[11]	406[11]	478[5]	489[4]
	20F	57	136	173	205[19]	270[18]	307[18]	296[16]	302[12]	310[10]
Span 60	10M	57	157	231	296	364	415	412	400[7]	401[5]
	20F	58	128	164	198	270	297[17]	297[17]	329[11]	326[9]
Tween 60	12M	58	170	238	308	373	423	419[11]	444[9]	438[8]
	20F	57	135	174	206	272	302[18]	300[18]	337[9]	323[8]
Tween 65	12M	57	170	239	300	356	406	407	412[5]	387[4]
	20F	57	135	174	206	283[19]	303[17]	305[17]	323[12]	319[10]
Tween 80	12M	58	159	242	305[10]	375[10]	409[10]	414[9]	438[3]	387[3]
	20F	57	137	177	209	270[19]	325[18]	306[18]	332[12]	315[10]
Mixture	12M	56	160	235	301	360	413	410	445[8]	450[5]
	20F	55	132	172	203	272[18]	314[16]	310[15]	338[13]	334[10]
Primex	12M	58	177	252	331	395	437	447	485[6]	493[4]
	20F	55	131	174	207	269[19]	305[17]	323[17]	343[14]	322[11]
20% Level										
Myrj 45	12M	57	155	239	310	353[11]	409[10]	410[10]	450[6]	449[6]
	20F	56	129	173	203	262[19]	306[15]	312[15]	353[13]	341[11]
Myrj 52	12M	56	145	203	277	318[10]	377[10]	374[10]	396[8]	380[6]
	21F	58	118	166	206	267[20]	305[17]	309[17]	321[10]	327[6]
Span 60	12M	56	137	215	275	305[10]	373[8]	375[8]	383[8]	385[6]
	20F	58	120	161	194	253[19]	288[17]	295[17]	293[13]	289[13]
Tween 60	12M	58	133	212	280[11]	334[11]	388[10]	397[10]	407[6]	404[6]
	20F	58	118	172	202	274[19]	300[17]	310[17]	316[8]	333[5]
Tween 65	12M	57	129	206	269	338[11]	385[10]	398[9]	441[5]	414[4]
	21F	57	124	167	204	255	310[19]	302[19]	329[11]	357[7]
Tween 80	12M	58	141	205	277	351[10]	388[8]	388[8]	426[6]	429[6]
	20F	57	117	166	200	270[19]	300[14]	307[14]	332[8]	340[6]
Mixture	12M	58	150	218	281	339[10]	395[10]	387[10]	440[8]	409[6]
	20F	59	129	172	202	256[19]	305[16]	311[16]	332[12]	320[10]
Primex	12M	62	181	267	349	412				
	20F	56	135	181	218	295		discontinued		

* Superscripts indicate number of survivors.

was toward somewhat lesser gains than at the 5 or 10% levels. In the case of the Primex groups, the higher responses at the 20% level may be explained by the increased caloric density of the diet. It may be recalled that Deuel et al. ('47) showed better growth to result when the fat level of the diet of rats was increased from 10 to 50% although the gains per 100 Cal. ingested remained constant.

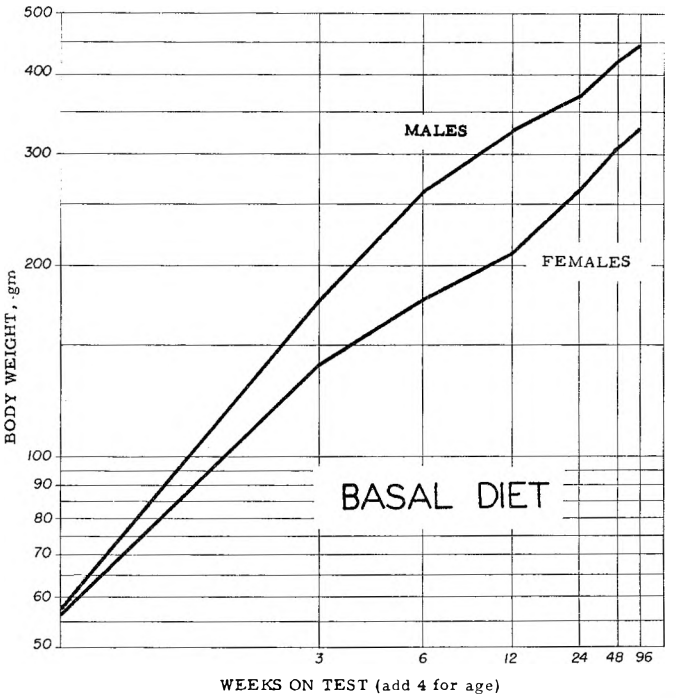

Fig. 1 Average growth curves of control rats.
Coordinates are log of body weight vs. reciprocal of age in weeks (i.e. 4 + weeks on test).

In the interest of conserving space, all the curves have not been included; however, when plotted on the log weight: reciprocal age basis according to the method of Zucker and Zucker ('42) an essentially linear transformation is obtained. This is illustrated for the groups on the basal and Myrj 45 diets in figures 1 to 3. Since these curves are linear and the groups assembled for this study had approximately the same

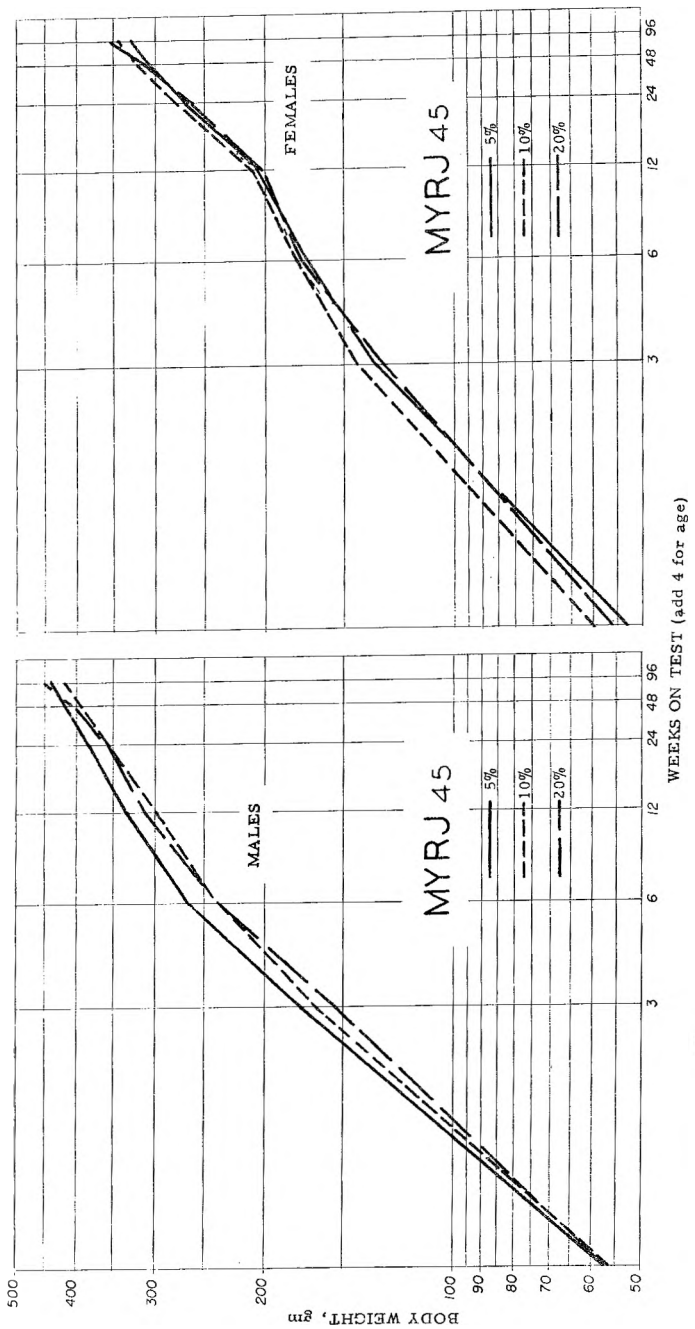

Figure 2

Figure 3

Fig. 2 and Fig. 3 Average growth curves of male and female rats, respectively, fed graded levels of Myrj 45.

initial weights, their weights at the actively-growing period of 12 weeks on test can be used as an index of relative growth response. Rank order tabulation of the growth responses to the various emulsifiers at comparable feeding levels indicated essentially the same relationship among the groups at 48 weeks as at 12 weeks. Hence it is justifiable to assess the voluminous growth data both within and between generations

TABLE 4

Average gains at 12 weeks of rats fed graded levels of emulsifiers (F_0 generation)

FAT OR EMULSIFIER	LEVEL, PER CENT			
	0	5	10	20
	gm	*gm*	*gm*	*gm*
			Males	
None	269			
Myrj 45		277	243	253
Myrj 52		281	245	221
Span 60		270	239	219
Tween 60		263	250	222
Tween 65		262	243	212
Tween 80		254	249	219
Mixture		272	245	223
Primex		294	273	287
			Females	
None	153			
Myrj 45		152	148	147
Myrj 52		148	148	148
Span 60		141	140	136
Tween 60		145	149	144
Tween 65		153	149	147
Tween 80		156	152	143
Mixture		152	148	143
Primex		155	152	167

by comparing the responses at a fixed period as has been done in table 4 for the F_0 generation at 12 weeks, the period when rats normally reach approximately two-thirds of their maximum weight. Inasmuch as food consumption data were collected during the initial 12 weeks of more active growth, it was possible to evaluate the weight responses for this period in relation to food and caloric intake.

Reference to table 4 shows that the male and female rats on the basal diet (containing no added fat or emulsifier) gained 269 and 153 gm, respectively, during the initial 12 weeks.

The average weight gains of the 5% emulsifier groups in 12 weeks ranged from 254 to 281 gm for the males and from 141 to 156 gm for the females. Statistical analysis according to the multiple range test of Duncan ('53) shows that none of these gains was significantly different (at the $p = 0.05$ level) from those of the basal control group. The only animals in the 5% emulsifier groups whose 12-week gain was significantly lower than the corresponding Primex animals, were the male rats on Tween 80.

The 12-week gains for the males at the 10% level of emulsifiers ranged from 239 to 250 gm, whereas the 10% Primex males gained 273 gm; the average gains for the corresponding females were 140 to 152 gm in the emulsifier groups and 152 gm in the Primex group. The differences between these gains in the emulsifier groups and either the control or Primex groups were likewise found not to be statistically significant according to the same criterion.

Increasing the dietary level of supplementation to 20% resulted in some growth depression in the emulsifier groups. The gain of 253 gm for the males on the 20% Myrj 45 diet was not significantly less than that of the basal controls. However the 12-week gains of the males in the remaining emulsifier groups ranged between 212 and 223 gm and were significantly lower. Among the females in the 20% groups, the range of weight gains on the emulsifier diets was 136 to 148 gm, the differences from the controls being not significant. The weight gains of the 20% Primex animals, both male and female, were statistically higher than those of the comparable control rats.

To sum up, it may be stated that at the 5 and 10% dose levels the differences in 12-week average net gain between the emulsifier groups and the controls were not statistically significant; and at the 20% level the gains in the emulsifier

groups were significantly lower than the basal control group in the males (except for those on Myrj 45) but not in the females.

It should be observed at this point that the caloric density of the basal diet approximated that of the emulsifier diets (table 2) irrespective of dosage level or identity of the emulsifier, whereas that of the Primex diets was considerably higher and increased with the dosage levels.

Despite the absence of significant differences in the gain in body weight at the 5 and 10% levels, it was of interest to list the responses of the emulsifier groups in order of magnitude. Such a ranking showed no consistent order either within each sex or within dosage levels. However one of the emulsifiers, Span 60, showed a uniform tendency to appear toward the bottom of the rankings. The relative magnitude of this lower response is indicated by the combined average 12-week gains for all three dose levels of Span 60, namely 243 and 139 gm for the males and females, respectively, versus 269 and 153 gm for the controls and 285 and 156 gm for the Primex rats.

FOOD UTILIZATION

Although the growth responses among the emulsifier and control groups varied only within narrow limits, it is pertinent to evaluate them in relation to the voluntary food consumption (as influenced, for example, by palatability) and to the caloric value of the diets. In the case of nutritionally equivalent diets, growth is normally proportional to the amount of food consumed; in other words, the efficiency of food utilization at given age periods should be constant from group to group. When diets vary only in respect to caloric density, diminution in efficiency of utilization expressed on a caloric equivalent basis may be regarded as indicative of some impairment of physiological function, either systemic or gastrointestinal.

In table 5, the efficiency of food utilization (EFU, the gain in body weight per 100 gm of diet consumed) and the efficiency of caloric utilization (ECU, the gain per 100 Cal. consumed)

TABLE 5

Comparison of responses of four generations of rats (five of each sex per group) during 12-week feeding periods on test diets

FAT OR EMULSIFIER	GENERATION	AVERAGE NET GAIN	AVERAGE FOOD INTAKE	EFU [1]	ECU [1]	AVERAGE NET GAIN	AVERAGE FOOD INTAKE	EFU [1]	ECU [1]	AVERAGE NET GAIN	AVERAGE FOOD INTAKE	EFU [1]	ECU [1]
		gm	*gm*	*gm/100 gm food*	*gm/100 Cal.*	*gm*	*gm*	*gm/100 gm food*	*gm/100 Cal.*	*gm*	*gm*	*gm/100 gm food*	*gm/100 Cal.*
None	F_0	201	1248	16.1	4.71								
	F_1	210	1333	15.8	4.62								
	F_2	202	1342	15.1	4.42								
	F_3	226	1394	16.2	4.74								
		5% Level				10% Level				20% Level			
Myrj 45	F_0	187	1176	15.9	4.66	193	1095	17.6	5.16	205	1224	16.7	4.91
	F_1	196	1307	15.0	4.39	202	1328	15.2	4.46	211	1385	15.2	4.47
	F_2	190	1305	14.6	4.28	213	1334	16.0	4.69	195	1414	13.8	4.05
	F_3	220	1353	16.3	4.77	214	1380	15.5	4.55	215	1519	14.2	4.18
Myrj 52	F_0	217	1289	16.8	5.13	189	1185	15.9	4.96	186	1350	13.8	4.61
	F_1	194	1268	15.3	4.62	205	1332	15.4	4.81	214	1492	14.2	4.75
	F_2	204	1322	15.4	4.65	196	1419	13.8	4.31	207	1501	13.8	4.61
	F_3	227	1438	15.8	4.77	206	1469	14.0	4.37	221	1675	13.2	4.41
Span 60	F_0	210	1189	17.7	5.17	188	1133	16.6	4.84	178	1116	15.9	4.63
	F_1	212	1406	15.1	4.41	195	1293	15.1	4.40	184	1372	13.4	3.90
	F_2	189	1248	15.1	4.41	205	1165	17.6	5.13	194	1302	14.9	4.33
	F_3	201	1403	14.3	4.18	208	1361	15.3	4.46	203	1252	16.3	4.74
Tween 60	F_0	210	1183	17.8	5.34	193	1152	16.8	5.11	188	1255	15.0	4.75
	F_1	198	1346	14.7	4.38	208	1416	14.7	4.47	189	1423	13.3	4.21
	F_2	212	1323	16.0	4.77	199	1477	13.5	4.10	181	1486	12.2	3.86
	F_3	233	1401	16.6	4.95	208	1411	14.7	4.47	202	1714	11.8	3.74
Tween 65	F_0	212	1187	17.9	5.23	180	1077	16.7	4.88	180	1123	16.0	4.59
	F_1	196	1249	15.7	4.59	208	1236	16.8	4.91	201	1396	14.4	4.21
	F_2	199	1346	14.8	4.33	209	1349	15.5	4.44	204	1380	14.8	4.33
	F_3	230	1416	16.2	4.74	242	1505	16.1	4.70	213	1594	13.4	3.92
Tween 80	F_0	218	1271	17.2	5.12	196	1130	17.3	5.25	190	1243	15.3	4.79
	F_1	198	1256	15.8	4.70	214	1391	15.4	4.67	229	1535	14.9	4.69
	F_2	203	1398	14.5	4.32	202	1379	14.6	4.43	211	1429	14.8	4.65
	F_3	204	1462	14.0	4.17	224	1426	15.7	4.76	235	1681	14.0	4.40
Mixture	F_0	206	1190	17.3	5.10	196	1119	17.5	5.21	187	1200	15.6	4.72
	F_1	218	1389	15.7	4.63	201	1379	14.6	4.34	207	1422	14.6	4.42
	F_2	206	1355	15.2	4.48	224	1442	15.5	4.61	226	1420	15.9	4.81
	F_3	212	1268	16.7	4.93	211	1413	14.9	4.43	201	1542	13.0	3.94
Primex	F_0	226	1182	19.1	5.18	217	1044	20.8	5.25	234	1031	22.7	5.04
	F_1	219	1239	17.7	4.80	216	1284	16.8	4.24	231	1254	18.4	4.08
	F_2	185	1162	15.9	4.31	192	1098	17.5	4.42				
	F_3	188	1217	15.4	4.17	203	1208	16.8	4.24		discontinued		

the efficiency of food utilization ECU efficiency of caloric utilization

are shown not only for the parent or F_0 generation, with which the preceding discussion of weight gains has been concerned, but for the three succeeding generations (F_1, F_2 and F_3) as well. In order to condense the table, weight gains and food consumption for the initial 12-week period are shown for combined sexes. The groups consisted of 5 males and 5 females initially selected from as many different litters as possible within each generation.

Inspection of the data is sufficient to reveal that the mean consumption of practically all diets was somewhat higher in the descendent generations than in the initial generation; however no trend either upward or downward was noted in respect to weight gains. It follows therefore that the EFU for the F_0 rats was higher than for the succeeding generations.

The data in table 5 also reflect the tendency for certain of the emulsifiers (e.g. Myrj 52, Tween 60) to yield lower EFU values with increasing dietary level, in contrast with the Primex groups where the EFU rose as the level of added fat increased from 0 to 20%.

More significant than the EFU is the ECU which adjusts for the differences in caloric density of the diets. Here too, slightly higher values were noted for the F_0 generation than for their progeny but, with the possible exception of one emulsifier (Tween 60), no consistent diminution of ECU with increasing dietary level was evident (fig. 4).

To clarify the interpretation of these gross observations, the mean values for EFU and ECU of the male rats have been examined statistically using the factorial analysis of variance and Duncan's multiple range and multiple F tests. From table 6 it can be seen that whereas the variance in EFU due to the emulsifiers was significant, this was not the case when adjustment was made for the differences in caloric density of the diets. An interaction between emulsifiers and levels also appears to affect the EFU significantly but not the ECU. In other words the variance in EFU due to the level of emulsifier in the diet was more prominent in certain emulsifiers than in others but this interaction was not sig-

nificant when the differences in caloric density of the diets were taken into account. However even after such adjustment was made, the variances due both to levels of the emulsifiers and generations proved to be significant.

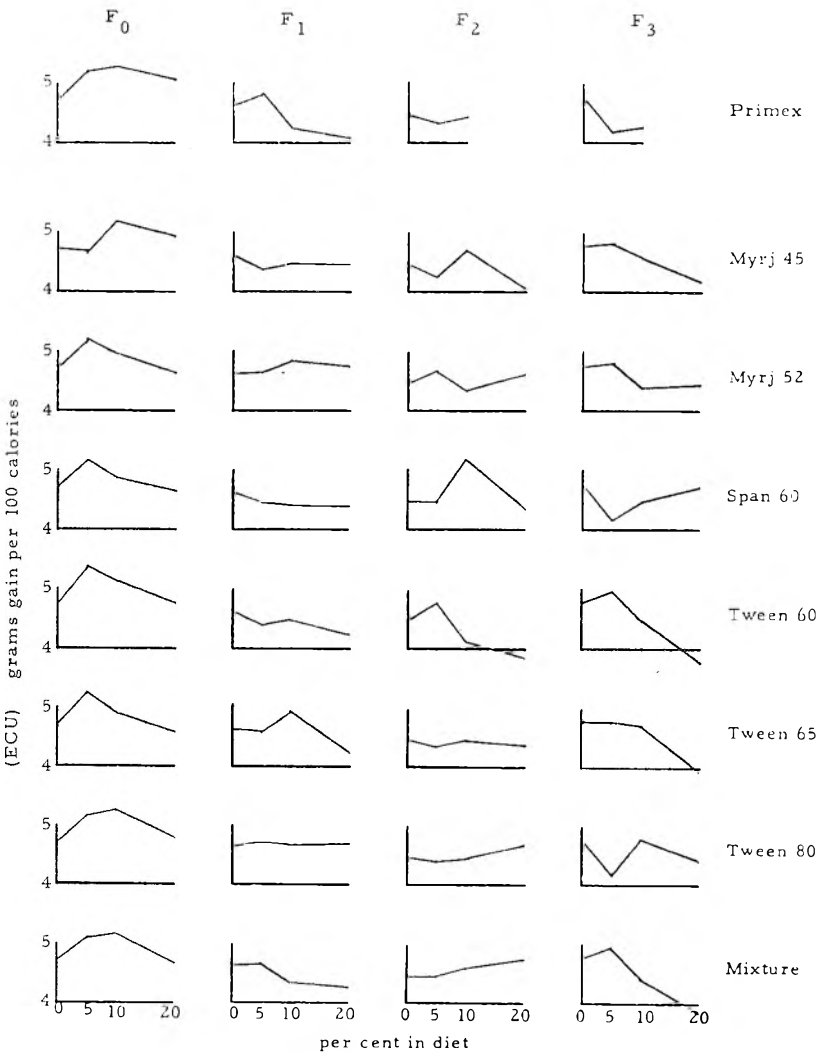

Fig. 4 Changes in efficiency of caloric utilization (ECU) with increasing dietary level of emulsifiers or Primex in 4 successive generations of rats.

At this point it may be mentioned that the analysis in table 6 is based not only on the groups receiving the 7 emulsifiers (i.e. 6 products and the mixture) but also on three additional groups, those receiving the basal diet alone and those supplemented with the two lower levels (5 and 10%) of Primex. These diets were selected to provide orthogonality in the analysis of variance; they resembled the test diets most closely in respect to both caloric density and total fatty acid content, whereas the 20% Primex group (which incidentally was terminated at one year in the F_0 generation) deviated to the greatest extent in both respects.

TABLE 6

Factorial analysis of variance of efficiency of food and caloric utilization in four generations of rats receiving diets containing three levels of emulsifiers [1]

VARIANT	DEGREES OF FREEDOM	EFU [2]		ECU [3]	
		Sums of squares	Mean square	Sums of squares	Mean square
Emulsifiers (E)	7	3790	541.4 [4]	4180	597.1
Levels (L)	2	1979	989.5 [4]	12045	6022.5 [4]
Generations (G)	3	5077	1692.3 [4]	49634	16544.7 [4]
E × L	14	3957	282.6 [4]	11265	804.6
E × G	21	1038	49.4	6510	310.0
L × G	6	370	61.7	4667	777.8
Error	42	3335	79.4	24935	593.7
Total	95	19546		113236	

[1] Including the groups receiving basal diet, 5 and 10% Primex, respectively.
[2] Grams gain in body weight per 100 gm food consumed.
[3] Grams gain in body weight per 100 Cal. consumed.
[4] Significant at $p = 0.001$.

The variance due to generations was further studied by the multiple ranking procedure. As seemed apparent by inspection, this was due entirely to the difference in ECU between the F_0 on the one hand and the F_1, F_2 and F_3 generations on the other. In table 7 the horizontal lines connect groups (i.e. generations or levels) among which the differences in ECU were not significant. The tendency of the F_0 generation to show higher ECU values than the following generations was least marked in the Myrj 52 groups. This is

indicated by the dual lines for this emulsifier in table 6 showing that the ECU values for the F_0 generation, while not significantly higher than for F_1, were higher than for F_2 and F_3.

In the case of the Span 60 series it is interesting to recall that the growth response in the F_0 generation was somewhat

TABLE 7

Grouping of ECU values according to statistical significance of differences among generations and dietary levels [1]

	GENERATIONS				DIETARY LEVELS		
	F_0	F_1	F_2	F_3	5%	10%	20%
Myrj 45	——	——	——	——	——	——	——
Myrj 52	——	——	——	——	——	——	——
Span 60	——	——	——	——	——	——	——
Tween 60	——	——	——	——	——	——	——
Tween 65	——	——	——	——	——	——	——
Tween 80	——	——	——	——	——	——	——
Mixture	——	——	——	——	——	——	——
Primex [2]	——	——	——	——	——	——	—— [2]

[1] Generations or levels not included in the continuous lines are significantly different ($p = 0.05$) from those so included, according to Duncan's multiple range and multiple F tests.

[2] 0, 5 and 10% Primex groups, the latter two being included to provide orthogonality.

lower than that in the other emulsifier groups; however this inferior performance was not seen in the subsequent generations nor was it evident in the data for ECU.

The most probable explanation for the generally higher ECU values in the first generation rats is that when placed on the test diets the F_0 animals' prenatal and preweaning nutrition was qualitatively, if not quantitatively, different

from that of the succeeding generations, whereas in the latter the nutritional reserves at weaning were uniform.

The factorial analysis of variance reveals a significant effect of the dosage level of the emulsifiers on the ECU values (table 6). When the influence of dosage level is examined on the basis of individual emulsifiers, as represented graphically in figure 4, it is seen to have been most marked in the response of the Tween 60 groups. Only in this series was a consistent downward trend in ECU noted in all generations as the dosage level increased. This was confirmed by the multiple ranking test (table 7) which revealed that the ECU value for the 20% group was significantly different from the 5, but not from the 10% group receiving this emulsifier.

It should also be noted that with Tween 65 the ECU at the 20% level group tended to be somewhat reduced but the diminution was not statistically significant at the $p = 0.05$ level.

The 5 males and 5 females representing each test group for purposes of studying food utilization during the initial 12-week period were reexamined at subsequent half-yearly periods for changes in the food intake pattern. (In the latter part of the two-year study alternate rats were substituted to replace losses by death). A trend toward increased intake was noted as the animals reached the terminal stages of the two-year test. The longer-lived rats were presumably hardier and may have been inclined to eat more than those which succumbed earlier. However no marked or consistent differences were observed in the amounts of food consumed by the various emulsifier groups when comparison was made for the same periods.

SUMMARY AND CONCLUSIONS

The plan of a chronic feeding study is described in which rats were maintained for two years on nutritionally adequate diets composed principally of natural ingredients, supplemented with 0, 5, 10 and 20% levels of partial ester emulsifiers or of hydrogenated vegetable oil. The emulsifiers were

Myrj 45, Myrj 52, Span 60, Tween 60, Tween 65, Tween 80 and a mixture thereof. The parent generation consisted of 810 rats. Three successive generations comprising 1440 additional rats were likewise observed for growth, food efficiency and reproductive performance.

Observations were made of body weight and food consumption permitting estimates of the efficiency of food utilization; hemocytology and hemochemistry; physical appearance and behavior; water consumption; laxation; metabolic utilization of the partial esters; reproduction and lactation mortality rates; and gross and microscopic pathology. The findings and conclusions are to be reported in a series of papers of which this is the first.

The growth responses of the emulsifier groups at the 5 or 10% levels were not significantly different from the controls. The only emulsifier group at these levels showing a significantly lower gain than the corresponding Primex group were the males on 5% Tween 80. At the 20% level the males (but not the females) in all emulsifier groups except Myrj 45 showed a moderate but statistically significant reduction in weight gain as compared with the controls; the 20% Primex group gained even more than the controls.

Since food consumption records were maintained for the first 12 weeks of the test and the growth curves plotted on a log weight: reciprocal age basis were essentially linear, extensive statistical analyses were made for this initial period. The data reveal a trend in the direction of higher food intake with increasing emulsifier level whereas the opposite was noted in the Primex groups whose diets, in contrast with the emulsifier diets, increased substantially in caloric density as the fat level increased. Regardless of the test supplement fed, the efficiency of food utilization (EFU) as well as the efficiency of caloric utilization (ECU) were somewhat higher in the initial (F_0) generation than in the succeeding generations. This may be explained by the difference in nutritional reserves at weaning between the first generation and all the descendent generations.

Analyses of the data for efficiency of utilization of the diets after adjustment to an isocaloric basis showed no statistically significant ($p = 0.05$) difference either among the groups receiving the various emulsifiers or between them and the basal control group with one exception, namely a small but significant diminution in efficiency of the diet containing 20% Tween 60.

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LITERATURE CITED

COCHRANE, W. G., AND G. M. COX 1950 Experimental Designs. Wiley, New York.

DEUEL, H. J., JR., E. R. MESERVE, E. STRAUB, C. HENDRICK AND B. T. SCHEER 1947 The effect of fat level of the diet on general nutrition. J. Nutrition, *33:* 569.

DUNCAN, D. B. 1953 Multiple Range and Multiple F Tests. Technical Report no. 6a. Department of Statistics and Statistical Laboratory, Virginia Agricultural Experiment Station, Virginia Polytechnic Institute, Blacksburg, Va. (Sponsored by the Office of Ordnance Research, U. S. Army.)

FEDERAL SECURITY AGENCY, Washington, D. C. 1948–49 Bread Hearings before the Administrator.

KRANTZ, J. C., JR., J. F. CRAR, J. G. BIRD AND S. COOK 1948 Sugar alcohols XXVI — Pharmacodynamic studies of polyoxyalkylene derivatives of hexitol anhydride partial fatty acid esters. J. Pharm. Exp. Ther., *93:* 2.

KRANTZ, J. C., JR., J. F. CARR, H. M. BUBERT AND J. G. BIRD 1949 Sugar alcohols XXVII — Drug allergy in the canine family. Ibid., *97:* 2.

KRANTZ, J. C., JR., P. J. CULVER, C. J. CARR AND C. M. JONES 1951 Sugar alcohols XXVIII. Toxicologic, pharmacodynamic and clinical observations on Tween 80. Bull. School Med., Univ. of Maryland, *36:* 48.

KRANTZ, J. C. JR., R. D. MUSSER AND N. J. KNAPP 1952 Sugar alcohols XXIX. Sorbitan polyoxyethylene fatty acid esters and polyoxyethylene monostearate and respiration of kidney. Proc. Soc. Exp. Biol. Med., *81:* 3.

LEHMAN, A. J., E. P. LAUG, G. WOODARD, J. H. DRAIZE, O. G. FITZHUGH AND A. A. NELSON 1949 Procedures for the Appraisal of the Toxicity of Chemicals in Foods. Food Drug Cosmetics Law Quarterly, *4:* 412.

NELSON, E. M. 1953 Cited in The Safety of Polyoxyethylene Stearates for use as Intentional Additives in Foods. National Research Council, Pub. no. 280.

PRATT, C. D. 1952 Certain partial ester emulsifier levels in food. Food Technology, *6:* 425.

U. S. Dept. Agr. Misc. Pub. no. 691 1950 Supplement for 1949 to Consumption of Food in the U. S. 1909–48.

ZUCKER, T. F., AND L. M. ZUCKER 1942 A simple time:weight relation observed in well nourished rats. J. Gen. Physiol., *25:* 445.

METABOLIC PATTERNS OF A GROUP OF OVERWEIGHT, UNDERWEIGHT AND AVERAGE WEIGHT WOMEN [1]

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The possibility that metabolic differences may exist between overweight and underweight individuals has challenged nutritionists from the early work of Strouse et al. ('24–'25) and Strang and McClugage ('31) to the present time (Keys and coworkers, '50; Johnston and Bernstein, '55; Mayer, '55; Clayton and Randall, '55). The existence of such metabolic differences does not vitiate the concept of balance between intake and outgo of energy but recognizes that the rate and pathways of catabolism of food may be influenced by metabolic differences.

It has been observed that the total basal energy expenditure of an overweight man tends to be higher than that of an average or underweight man of similar build. Lower fasting respiratory quotients have been associated with obesity (Hagedorn et al., '27; Strang and McClugage, '31; Lyon et al., '32), and the evidence for a decreased capacity to metabolize carbohydrate associated with obesity in middle-age is extensive (Beaudoin et al., '53). Specific dynamic effects and blood lipids which have been reported do not follow consistent patterns (DuBois, '36; Hetényi, '36).

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In recent years advances in biochemical techniques have made it possible to increase the number of metabolic responses that can be observed simultaneously. This investigation was planned to explore possible biochemical differences in the metabolism of fat and carbohydrate among women of average, above and less than average body fat through simultaneous respiration studies and analyses of various blood and urinary constituents before and following test meals high in fat or carbohydrate.

TABLE 1

Physical description of subjects

	SUBJECTS		
	Underweight	Average weight	Overweight
No.	7	7	7
Age, years			
Range	25 to 51	25 to 52	25 to 57
Mean	36	36	40
Height, cm			
Range	154.5 to 168.0	157.0 to 173.0	161.5 to 167.8
Mean	163.4	164.8	164.5
Weight, kg			
Range	46.0 to 52.5	53.5 to 69.9	76.2 to 112.0
Mean	49.8	60.9	87.5
Percentage deviation from desirable weight			
Range	− 12 to − 21	− 10 to + 13	+ 21 to + 66
Mean	− 15.5	− 0.5	+ 35.5

EXPERIMENTAL PLAN

Twenty-one women, from 25 to 57 years of age, were subjects (table 1). All were in good health and physically active; 7 were married and 6 had had children. The subjects were grouped into three classes: overweight, greater than 15% above; average weight, within + 15 to — 10% of; and underweight, more than 10% below desirable weight. The desirable weight for each subject was estimated from tables of the

Metropolitan Life Insurance Co. ('51), anthropometric measurements and clinical appraisal of muscular development and subcutaneous fat deposits. None of the subjects was on a gaining or a reducing regimen.

There were two test days for each subject scheduled at least a week apart. On the first day a high-fat test meal was given, on the second, a high-carbohydrate meal. In the high-fat test meal 70% of the calories were supplied by fat, 25.0% by carbohydrate, and 5.0% by protein; this meal consisted of 50 gm white bread, toasted, 38 gm butter, 100 gm water and one cup of coffee. Relative proportions of calories in the high carbohydrate meal were 94.5% from carbohydrate, 0.5% from fat and 5.0% from protein; this meal consisted of 50 gm white bread, 86 gm apple jelly, 100 gm reconstituted frozen orange juice and one cup of coffee. Fluid intake was constant for both meals.

Each subject was requested to eat a dinner low in fat and no food after 7 P.M. on the day preceding the test, to obtain at least 8 hours of sleep and rest during the night and to rise in time to be leisurely about necessary activity. It was requested that the bladder be emptied on rising and that one glass of water be drunk. The subject came to the laboratory in a post-absorptive state.

After a 30-minute rest period, the volume of expired air was measured and sampled for two consecutive 6-minute periods using a Kofranyi-Michaelis respirometer,[3] adjusted to provide a continuous sampling of 0.6% of the expired air. Following this, a 30 ml blood sample was taken from the antecubital vein. After 30 minutes, the serum was separated by centrifugation and sampled for analyses of total lipids, chylomicron counts, lipoproteins,[4] and cholesterol.

[3] Max Planck Institut für Arbeitsphysiologie, Dortmund, Germany. These instruments and the net micrometer used for chylomicron counts were provided by contributions of the alumni to the Michigan State University Fund. The assistance is gratefully acknowledged.

[4] Appreciation is expressed to Dr. George V. Mann, Harvard School of Public Health, for the lipoprotein fraction analyses.

A fasting capillary blood sample was taken from the finger and sampled immediately for glucose and pyruvic acid analyses; fasting capillary serum was obtained for chylomicron counts and analysis of total lipids. A fasting urine collection was made preceding the administration of the meal.

Capillary blood samples were obtained one-half, one, two, three, 4, and 5 hours after the subject had finished the test meal. Blood glucose concentrations and serum chylomicron counts were determined for each blood sample; pyruvic acid and serum total lipid concentrations were determined for the one-, three- and 5-hour samples only. The volume of expired air was measured for single 8-minute periods at one, three and 5 hours after the test meals and sampled for gas analyses. The subject was at bed rest preceding each measurement of expired air. Urine samples were obtained at one, three and 5 hours after completion of the test meals. The subjects were in the laboratory during the entire test period. They were allowed to converse, read, write or do hand sewing except for the periods of bed rest. No food other than the test meal was consumed and water was permitted only in small amounts at the end of each expiratory air sampling period; there was no smoking.

Analytical methods. The metering device of the Kofranyi-Michaelis respirometer was calibrated and correction factors for absorption and diffusion of carbon dioxide and oxygen in the rubber collection bag were established. Samples of expired air were analyzed for carbon dioxide and oxygen by the conventional Haldane-Henderson-Bailey method (Peters and Van Slyke, '32). Overall respiratory quotients, total oxygen consumption, non-protein respiratory quotients and total heat production were calculated according to Brody ('45). The Zuntz non-protein respiratory quotient factors were used for converting oxygen consumption to calories per liter. When the determined non-protein respiratory quotients were greater than 1.00 or less than 0.70, the Zuntz factors for 1.00 and 0.70, respectively, were used. DuBois ('36) has shown that the error of such a procedure is negligible. Energy

expenditures were evaluated in terms of total calories per hour.

The following methods were used for analyses of blood constituents: serum total lipids, Bragdon, '51; serum total cholesterol, Sperry and Webb, '50; blood glucose, Nelson, '44; and blood pyruvic acid, Tsao and Brown, '50. Certain modifications in the handling of samples and analyses were made; details have been described previously (Hawthorne, '54).

Chylomicron counts were made on serum samples under oil immersion with dark field illumination; the microscope was fitted with a net micrometer (squared 10×10) placed into the eyepiece. The method of handling samples of serum was similar to that reported by Marder et al. ('52). For samples of low concentrations, the entire ruled area of 100 squares was counted; for samples of higher concentrations 50, 20 or 10 small squares were counted and the average results were converted to the entire ruled area by the appropriate factors.

Urine aliquots were analyzed qualitatively for albumin, glucose and acetone bodies by conventional methods (Simmons and Gentzkow, '44). Urinary nitrogen was determined by micro-Kjeldahl digestion and subsequent nesslerization.

RESULTS

The mean data for all determinations at fasting and at intervals following both test meals for the three groups are presented in figure 1. Analysis of variance was applied to the data. When the differences among the means of the fasting values were significant, the influence of the fasting values on the values observed after the test meals was removed by analysis of covariance (Snedecor, '46). When the differences among the means of fasting values were not significant, covariance analysis was not used.

Energy expenditure. The mean basal energy expenditures, including both test days, were: underweight, 47.0, average weight, 52.2 and overweight, 65.0 Cal. per hour. The differ-

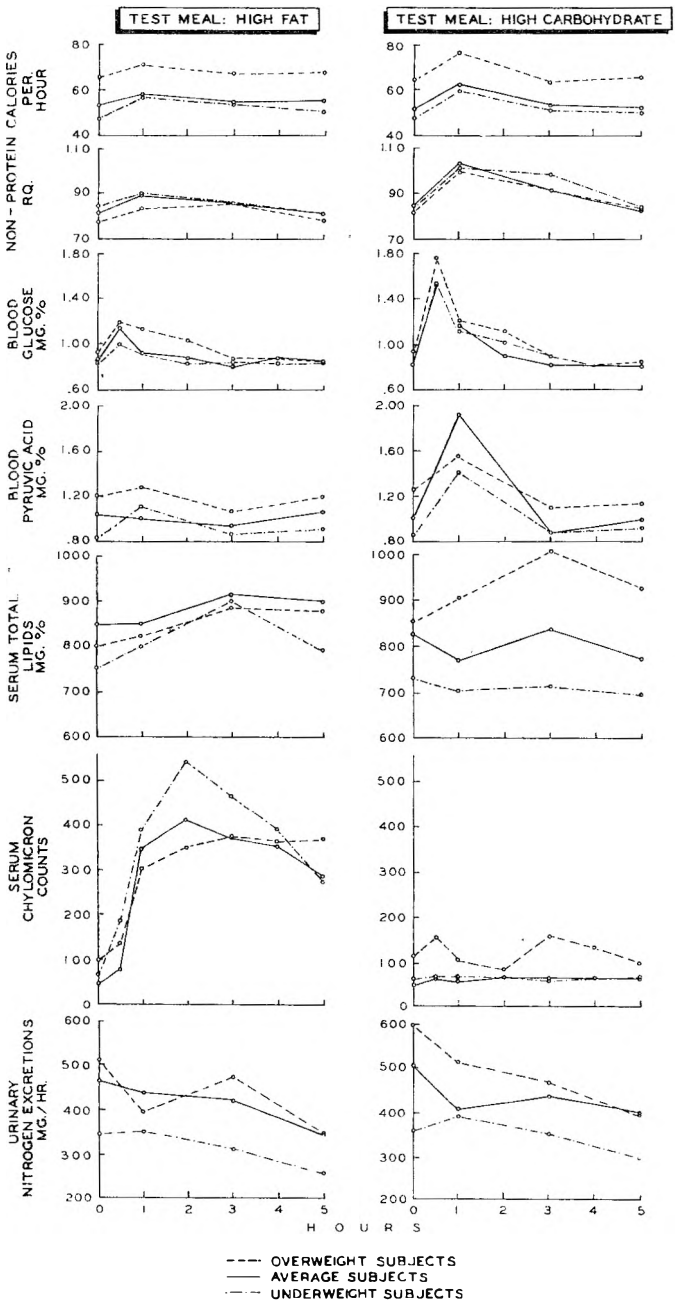

Fig. 1 Summary of mean metabolic patterns for underweight, average weigh and overweight subjects.

ences among groups were highly significant and individual subjects showed a significantly positive relationship with percentage deviations from desirable weight, $r = 0.911$ ($P \leq 0.01$). These observations confirmed reports of various previous investigations that the total basal metabolism of overweight individuals exceeded that of individuals of lesser body weight (DuBois, '36; Keys and Brozek, '53; and Johnston and Bernstein, '55).

Total calories per hour expended at rest at intervals after the test meals also were higher for the overweight subjects

TABLE 2

Mean calorie expenditures above basal during five-hour periods following two test meals

SUBJECTS	TOTAL CUMULATIVE CALORIES ABOVE BASAL					
	Following high-fat test meal			Following high-carbohydrate test meal		
	1st Hour	3rd Hour	5th Hour	1st Hour	3rd Hour	5th Hour
Underweight	4.7	20.6	29.9 ± 3.6 [1]	5.8	20.4	24.9 ± 4.2 [1]
Average weight	2.8	10.3	15.4 ± 4.8	5.4	18.0	20.4 ± 1.8
Overweight	2.6	8.4	11.0 ± 4.8	5.6	16.6	17.3 ± 3.6
F-value [2] (Analysis of variance)			5.31*			1.27

[1] Standard error of the mean.

[2] * = $P \leq 0.05$. (F-values: $P \leq 0.05$, 3.55; $P \leq 0.01$, 6.01.)

than for the average or underweight subjects at each hour. Differences between the overweight and average weight subjects were highly significant. Differences between average weight and underweight subjects were significant only at the 5th hour following the high-fat test meal.

Calorie increments after the test meals, however, were greater for the underweight subjects than for the average weight or overweight subjects (table 2). The increments were measured according to the method of Glickman et al. ('48) in which the hourly energy expenditures above the basal energy expenditures were plotted and the total expenditure for a given time period was derived. The incre-

ments in energy expenditures after the high-fat test meal were significantly higher for the underweight subjects than for the average and overweight subjects; differences among groups following the high-carbohydrate test meal were not significant. Further, the mean calorie expenditure for the underweights was less after the high-carbohydrate test meal than after the high-fat test meal, whereas the majority of the overweight and average weight subjects showed the greater calorie expenditure following the high-carbohydrate test meal.

The observed dynamic effect of food may be affected by the nutrient combination in which it is metabolized, the method of disposition of absorbed nutrients by the individual and by the technique of measurement. The differences in energy expense following the test meals in this study suggest that the underweight subjects may have had a metabolic pattern different from that of the average or overweight subjects, although the calorie differences were quantitatively small.

The mean fasting non-protein respiratory quotients for the underweight, average weight and overweight subjects were 0.83, 0.83 and 0.79, respectively; the differences were not statistically significant. Following the high-carbohydrate test meal, the changes in mean respiratory quotients of the overweight and average weight groups were similar; the underweights had a delayed decrease in respiratory quotient from peak values. In contrast, peak responses were reached less quickly for the overweight subjects than for the others after the high-fat test meal.

Blood glucose and pyruvic acid. There was a highly significant relationship between the fasting glucose concentrations in capillary blood and the percentage deviations from desirable weight (fig. 2). Eleven of the 14 fasting values for the 7 overweight subjects were between 90 and 101 mg/100 ml of blood, whereas only three fasting values for the average weight and one for the underweight subjects equalled or exceeded 90 mg/100 ml. Previous studies indicating that fasting blood glucose concentrations were higher among overweight than among average weight subjects have been clinical

investigations concerned with diabetics or potential diabetics. In this study all of the fasting blood glucose concentrations for the overweight subjects were well within the range of values reported for healthy adults (Peters and Van Slyke, '46). All overweight subjects were aglycosuric. The mean fasting blood glucose concentrations for the overweight, average weight and underweight subjects, including determinations of both experimental days, were 93, 83 and 81 mg/100 ml, respectively.

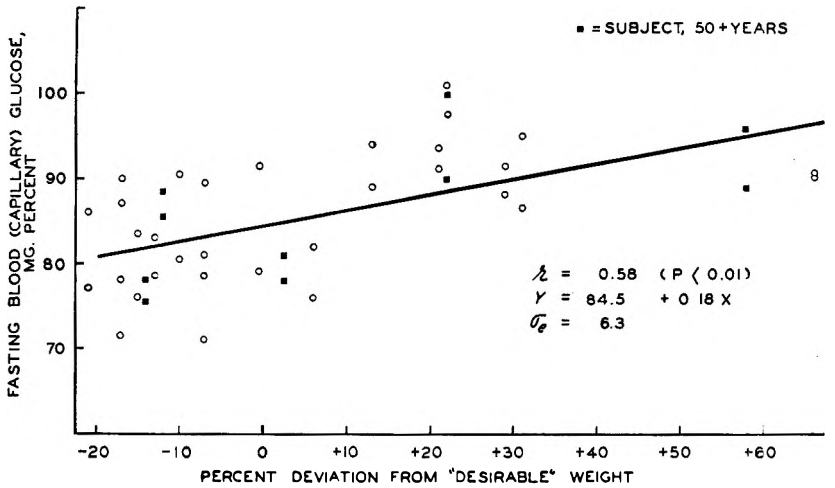

Fig. 2 Regression of fasting blood glucose on percentage deviations from desirable weight.

The blood glucose concentrations at the various time intervals following the test meals displayed greater individual variations within groups than did the fasting concentrations. Following the high-fat test meal all subjects, except one overweight subject, showed increased blood glucose concentrations apparently in response to the 26 gm of carbohydrate which was included in the meal. At the one-half, first and second hours, the mean concentrations of the overweight subjects were significantly higher than those of the average and underweight subjects. The higher concentrations at these hours in the overweight subjects were shown to be related in part

to the higher mean fasting values in this group; the increments of elevation above fasting concentrations were higher, but not significantly higher, among the overweight subjects than among the average or underweight subjects. On the other hand, the mean blood glucose concentrations of the overweight subjects were significantly lower than fasting concentrations at the 4th and 5th hours; this response was not observed among the other two groups.

Following the high-carbohydrate test meal, there were no significant group differences in blood glucose concentration until the second hour, although the mean values for the overweight group were consistently higher than those of the other groups. At the second hour the mean blood glucose concentration of the overweight subjects was significantly higher than that of the average weight subjects but not of the underweight subjects. This higher concentration of the overweight group again was related in part to their higher fasting concentrations. And again at the 4th and 5th hours the blood glucose concentrations of the overweight subjects were below their fasting concentrations. The decrease in blood sugar was particularly rapid in view of the higher concentration for this group at the one-half hour period. Thus a distinct pattern of response was observed among the overweight subjects in the decrease of blood glucose concentrations below fasting values at the 4th and 5th hours following both test meals. The occurrence of greater post-absorptive hypoglycemias following carbohydrate feeding among obese than among "normal" subjects has been observed previously (Ogilvie, '35).

Mean fasting concentrations of blood pyruvic acid were 0.85, 1.02 and 1.22 mg/100 ml of blood for the underweight, average weight and overweight subjects, respectively. The mean fasting concentration reported by Meyer and Winkler ('52) for underweights was 0.89 and for overweights, 1.08 mg/100 ml.

Following the high-fat test meal, there was little change in blood pyruvic acid concentrations for any subject from

hour to hour, although the underweight subjects displayed the most marked elevation from fasting concentrations at the first hour. Following the high-carbohydrate test meal, the mean blood pyruvic acid concentrations of all three groups showed an increase at the first hour, a decrease toward fasting values or below at the third hour, and a slight increase at the 5th hour. The elevations from fasting concentrations were significantly different among the three groups at the first hour; the increase in mean concentration from fasting for the overweight group, 0.31 mg/100 ml, was significantly less than was that of the average weight group, 0.92 mg/100 ml of blood.

Blood lipids. Group mean fasting values for the various lipid fractions determined are given in table 3. There were no significant differences among the fasting means of the underweight, average weight or overweight subjects for any of the individual lipid constituents measured and no constituent showed a significant correlation with the subjects' percentage deviations from desirable weight. Fasting capillary serum chylomicron counts were 67, 48 and 105 per standard dark ground field for the underweight, average weight and overweight groups, respectively. Mean venous serum chylomicron counts were lower than those of capillary serum for each group, but individual deviations were not consistent in direction.

The greatest group differences among the fasting lipoprotein S_f 12 to 100 fractions measured were in the S_f 12 to 20 fraction. Fasting venous serum total cholesterol concentrations ranged from 151 to 396 mg/100 ml; the two subjects with concentrations above 300 mg/100 ml were of the average weight group. Fasting total lipid concentrations determined in this study were higher than mean values frequently reported (Peters and Van Slyke, '46) and were higher than total lipid values calculated from determined constituents (Wilmot and Swank, '52). Values in this study were comparable to those reported by Herzstein et al. ('53), who determined total serum lipids by the Bloor method, and to those of Goldbloom ('52) and Kornerup ('50), who determined

TABLE 3

Mean fasting serum lipid constituents

SERUM LIPID CONSTITUENT	SUBJECTS			F-VALUE [1] Analysis of variance Total
	Underweight	Average weight	Overweight	
Total lipids, venous,[2] mg/100 ml	795 ± 46.3 [3]	685 ± 62.6 [3]	796 ± 50.0 [3]	0.25
Total lipids, capillary,[4] mg/100 ml	750 ± 42.5	808 ± 76.5	826 ± 35.3	0.41
Total cholesterol, venous, mg/100 ml	214 ± 12.7	253 ± 19.7	228 ± 9.6	0.90
Lipoprotein fractions, venous:[5]				
S_f12–20, mg/100 ml	34 ± 6.8	18 ± 3.3	38 ± 13.1	1.37
S_f21–35, mg/100 ml	14 ± 3.8	10 ± 3.0	14 ± 6.8	0.21
S_f35–100, mg/100 ml	29 ± 9.1	22 ± 6.9	31 ± 14.0	0.22
S_f12–100, mg/100 ml	78 ± 17.5	51 ± 12.0	83 ± 33.7	0.57
Chylomicron counts, venous [2]	60 ± 13	35 ± 7	91 ± 15	3.50
Chylomicron counts, capillary	67 ± 11	48 ± 7	105 ± 17	3.52

[1] F-values: $P \leq 0.05$, 3.55; $P \leq 0.01$, 6.01.

[2] Venous total lipid and chylomicron count data include those of six underweight and six average weight subjects only.

[3] Standard error of the mean.

[4] Values for 7 underweight, 6 average weight and 6 overweight subjects.

[5] Values for 6 underweight, 6 average weight and 6 overweight subjects on single samples. Chylomicron counts, particles per standard dark ground field.

total lipids by a gravimetric method. The position of the average weight group with respect to the lipoprotein fractions of venous blood and chylomicron counts is of some interest since low values in these blood constituents might be interpreted as measuring an effective mechanism for the metabolism of fats.

Following the test meals, capillary serum chylomicrons and capillary serum total lipids were the only lipid constituents measured. Serum total lipid concentrations were not obtained for all subjects at all hours following both test meals. There were 6 underweight, 5 average weight and 6 overweight subjects following the high-fat test meal and 5 underweight, 4 average weight and 6 overweight subjects following the high-carbohydrate test meal for whom data were complete. Mean values for these subjects were graphed in figure 1.

Following the high-fat test meal the serum chylomicron concentrations of the underweight subjects increased more quickly, reached higher peak concentrations and then decreased more rapidly than did those of the overweight subjects. The chylomicrographs determined in this study were in contrast to the results reported by Frazer and Stewart ('39), who stated that their obese subjects (absolute weights not reported) showed higher chylomicron concentrations than did their thin subjects taking an identical standard meal, and of Clayton and Randall ('55), who reported that average chylomicron counts increased and decreased more rapidly in their subjects of heaviest total body weight following fat feeding. Garcia, Roderuck and Swanson ('55) have reported no significant relationship between percentage of ideal weight and the maximum number of chylomicrons. Deviations from desirable weight among the subjects of Clayton and Randall and Garcia and co-workers were not as great as were those among the subjects of this investigation, however.

In contrast to the changes in chylomicron counts, total lipid concentrations following the high-fat test meal were erratic and were increased only slightly above fasting values for all subjects. The small increases in serum total lipids of the

three groups seemed inconsistent with the prevalent view that a significant rise in total serum lipids occurs following the ingestion of fat (Hetényi, '36; Peters and Van Slyke, '46; Frazer, '53). Three studies of fat tolerance following fat feeding have reported changes of total lipid concentrations of magnitudes similar to those found in this investigation (Rony and Levy, '29; Corcoran and Rabinowitch, '37; Herzstein et al., '53). Post-prandial blood lipid concentrations are known to be influenced by the amount of fat fed, by exercise, carbohydrate content of the test meal and the analysis of capillary rather than venous serum.

Following the high-carbohydrate test meal, fluctuations in serum chylomicron concentrations were observed among 4 of the overweight subjects. Although there were individual differences, the mean concentrations of the overweight group were significantly higher than those of the average weight or underweight groups at the one-half and third hours. Increases in concentrations of capillary serum total lipids also were observed following the high-carbohydrate test meal in 5 overweight subjects and two average weight subjects. Elevated serum total lipid concentrations occurred in three of the same subjects in which elevated chylomicron counts were observed, but 4 subjects showed elevated serum total lipid concentrations without measured increases in chylomicron concentrations following carbohydrate ingestion. Further, increased concentrations of the two did not necessarily occur concurrently even when both were observed in the same individual.

Urinary findings. Marked individual variation characterized the fasting hourly urinary nitrogen excretions; this variation existed both within groups and between days for the same individual. The differences among groups were not statistically significant. Both test meals had the effect of decreasing hourly nitrogen excretions among all groups. However, the mean total 5-hour nitrogen excretions of the underweight subjects were significantly less than those of the other two groups following both test meals. The data

indicated some better protein-sparing effect by the high-fat test meal than by the high-carbohydrate test meal.

Urinary albumin was not found. Sugar was found in the urine of two underweight subjects in the first hour following the high-carbohydrate test meal. Only one subject showed a sufficient excretion of acetone substances for a positive test and that occurred in the 5th hour following both test meals; marked depressions of non-protein respiratory quotients occurred concurrently.

DISCUSSION

Certain interrelationships among the various metabolites measured at fasting were observed. There was a significant negative correlation between fasting non-protein respiratory quotients and fasting blood glucose concentrations ($r = -0.513$; $P \leq 0.05$). On the other hand there was no significant correlation between fasting non-protein respiratory quotients and the determined fasting serum total lipid concentrations, $r = -0.184$. There was a significant positive correlation between fasting blood pyruvic acid concentrations and fasting blood glucose concentrations, $r = 0.526$ ($P \leq 0.05$), although the relationship was skewed since fasting pyruvic acid values were concentrated between 0.90 and 1.00 mg/100 ml of blood for a wide range of fasting glucose concentrations. The fasting venous total cholesterol concentrations showed a significantly positive relationship with fasting venous serum total lipid concentrations, $r = 0.795$ ($P \leq 0.01$); no significant correlation was found between total cholesterol and chylomicron counts. Fasting chylomicron counts likewise were not significantly related to serum fasting total lipid concentrations.

Fasting basal energy expenditures were higher among the overweight and lower among the underweight than they were among the average weight subjects; mean non-protein respiratory quotients were lower, but not significantly so, among the overweight subjects than among the other two groups; blood glucose concentrations were significantly higher

among the overweight than among the average or underweight subjects; blood pyruvic acid concentrations were significantly higher among the overweight than among the underweight subjects; the ratio of blood pyruvic acid to glucose was higher among the overweight and lower among the underweight than it was among the average weight subjects; and, whereas there were no significant group differences among the various blood lipid constituents measured, fasting chylomicron counts were higher among the overweight subjects than among the other two groups.

Age has been reported as a factor influencing the fasting or basal metabolic pattern of individuals. The mean age and range of ages for subjects on the three groups in this investigation were comparable (table 1). Further, the data for the 5 subjects of 50 or more years were considered in relation to the younger subjects for all metabolites measured. In this study the rate of basal energy expenditure was not consistently decreased with age. There was no correlation of respiratory quotients, fasting glucose concentrations, fasting pyruvic acid concentrations or fasting hourly urinary nitrogen excretions with age. Although lower serum lipid concentrations occurred generally in younger subjects, the relationship between age and serum lipid concentrations was not consistent.

Interpretation of metabolic patterns following test meals is limited since individual differences may exist in both time and intensity of metabolic reactions and since statistical methods relating factors of time and degree of change are lacking. Variations of individual subjects within the overweight, underweight and average weight groups in this investigation corroborated findings of previous studies which have indicated that no single metabolic pattern is associated with the condition of overweight or underweight. Nevertheless, certain differences in metabolic patterns associated with the overweight and underweight subjects were significantly greater than variations among individuals within the groups.

The overweight women as a group appeared to exhibit a delay in utilization of carbohydrate, although not of the degree reported for obese subjects with typical "diabetic glucose tolerance curves" (Newburgh, '42). There was no question of the ability of the overweight subjects to utilize carbohydrate; however, the existence of a different stimulus to glucose removal following both test meals was indicated by the early delays in glucose removal, the significant decreases in blood glucose concentrations below fasting values by the 4th or 5th hours, and the significantly higher post-absorptive blood glucose concentrations associated with the overweight group.

The significantly lower elevation of blood pyruvic acid concentrations together with the mobilization of blood lipids among the overweight subjects following the high-carbohydrate test meal likewise indicated the possibility of a different mechanism for the utilization of carbohydrate. Three overweight subjects showed no increase in blood pyruvic acid concentrations at the times measured following the high-carbohydrate test meal; the other overweight subjects showed blood pyruvic acid increases lower than those of the average weight subjects and subsequent decreases of concentrations below fasting values. These results would not support the hypothesis of Stadie ('40) and Pennington ('53) that a metabolic defect occurs in utilization of pyruvic acid by the obese; the results are more nearly in accord with the findings of Guggenheim and Mayer ('52) who reported that depressed pyruvic acid responses occurred for obese mice of the hereditary obesity-diabetes-syndrome strain.

The slower rate of removal of chylomicrons and total lipids following fat feeding among the overweight compared to the underweight subjects might further indicate a difference in the utilization of nutrients. The non-protein respiratory quotients provided no real evidence toward clarifying the mechanisms involved; however, the respiratory quotients of the overweight subjects reached peak values more slowly

following the high fat test meal than those of the average or underweight subjects.

The overweight subjects had significantly higher energy expenditures at all hours following both test meals. Calorie increments above basal energy expenditures were not significantly higher than those of the other groups, however.

The underweight subjects as a group displayed a metabolic pattern which differed less from that of the average weight subjects than did the pattern of the overweight group. However, their metabolic pattern might be interpreted as indicating some greater preference for carbohydrate in metabolism. Following the high fat test meal, blood glucose concentrations showed lower elevations and decreased to fasting values more rapidly than did those of the average weight subjects; concurrently, blood pyruvic acid concentrations showed the most marked elevation above fasting values of any group and the most rapid decrease to fasting values. The resulting high pyruvic acid to glucose ratio was in contrast to the low pyruvic acid to glucose ratio at fasting among the underweight subjects. Chylomicron and total lipid concentrations early increased to the greatest extent of any group following the fat meal, but the subsequent rate of removal of these lipids from the blood was most rapid. Non-protein respiratory quotients were elevated longer than those of the other groups following the high-carbohydrate test meal.

In addition, significantly higher cumulative calorie increments following the high-fat test meal and significantly lower cumulative nitrogen excretions for 5 hours following both meals were characteristic of the underweight group.

SUMMARY

Metabolic patterns of a group of 7 overweight, 7 underweight and 7 average weight women were investigated at fasting and following two test meals of varying carbohydrate and fat composition. Respiratory quotients, hourly energy expenditures and hourly urinary nitrogen excretions were determined simultaneously with blood glucose, blood pyruvic

acid, serum total lipids and serum chylomicron concentrations at fasting and at intervals for 5 hours. Although individual differences in responses were observed among all groups, certain metabolic patterns associated with the overweight and underweight subjects in this investigation were significantly greater than variations among individuals within the groups. The overweight women as a group appeared to exhibit a delay in utilization of carbohydrate in the test meals compared to the average weight and underweight women. There were some indications of a greater preference for carbohydrate in metabolism by the underweight than by the average weight women.

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LITERATURE CITED

BEAUDOIN, R., T. B. VAN ITALLIE AND J. MAYER 1953 Carbohydrate metabolism in "active" and "static" human obesity. J. Clin. Nutrition, *1:* 91–99.

BRAGDON, J. H. 1951 Colorimetric determination of blood lipids. J. Biol. Chem., *190:* 513–517.

BRODY, SAMUEL 1945 Bioenergetics and Growth. Reinhold Publishing Corporation, New York, Chap. 4, pp. 59–75.

CLAYTON, M. M., AND S. W. RANDALL 1955 Blood changes following breakfasts of different types. Blood sugar and chylomicrons in young women of different weights. J. Am. Diet. Assn., *31:* 876–882.

CORCORAN, A. C., AND I. M. RABINOWITCH 1937 A study of the blood lipids and blood protein in Canadian eastern arctic Eskimos. Biochem. J., *31:* 343–348.

DUBOIS, E. F. 1936 Basal Metabolism and Health and Disease, 3rd edition. Lea and Febiger, Philadelphia, 494 pp.

FRAZER, A. C. 1953 Chap. 7. Lipid Metabolism. G. H. Bourne and G. E. Kidder, Editors. Biochemistry and Physiology of Nutrition, Vol. 1. Academic Press, Inc., New York, pp. 212–264.

FRAZER, A. C., AND H. C. STEWART 1939 The interpretation of the normal chylomicrograph. J. Physiol., *95:* 23P–24P.

GARCIA, P., C. RODERUCK AND P. SWANSON 1955 The relation of age to fat absorption in adult women together with observations on concentrations of serum cholesterol. J. Nutrition, *55:* 601–610.

GLICKMAN, N., H. H. MITCHELL, E. H. LAMBERT AND R. W. KEETON 1948 The total specific dynamic action of high protein and high carbohydrate diets on human subjects. Ibid., *36:* 41–57.

GOLDBLOOM, A. A. 1952 Clinical studies of blood lipid metabolism. II. Blood serum variations of cholesterol, phospholipids, neutral fats, total lipids and blood lipid fraction percentages in peptic ulcer patients. Gastroenterology, *20:* 79–89.

GUGGENHEIM, K., AND J. MAYER 1952 Studies of pyruvate and acetate metabolism in hereditary obesity-diabetes syndrome of mice. J. Biol. Chem., *198:* 259–264.

HAGEDORN, H. C., C. HOLTEN AND A. H. JOHANSEN 1927 The pathology of metabolism in obesity. Arch. Int. Med., *40:* 30–37.

HAWTHORNE, B. E. 1954 Metabolic patterns of a group of overweight, underweight and average weight women. Unpublished Ph.D. thesis, Michigan State University, East Lansing, Michigan.

HERZSTEIN, J., C. WANG AND D. ADLERSBERG 1953 Fat-loading studies in relation to age. Arch. Int. Med., *92:* 265–272.

HETÉNYI, G. 1936 Untersuchungen über die Entstehung der Fettsucht. Deutsches Arch. klin. Med., *179:* 134–141.

JOHNSTON, L. C., AND L. M. BERNSTEIN 1955 Body composition and oxygen consumption of overweight, normal and underweight women. J. Lab. Clin. Med., *45:* 109–118.

KEYS, A., AND J. BROZEK 1953 Body fat in adult man. Physiol. Rev., *33:* 245–325.

KEYS, ANCEL, J. BROZEK, A. HENSCHEL, O. MICKELSEN, H. L. TAYLOR AND OTHERS 1950 Human Starvation. University of Minnesota Press, Minneapolis.

KORNERUP, V. 1950 Concentrations of cholesterol, total fat and phospholipid in serum of normal man. Arch. Int. Med., *85:* 398–415.

LYON, D. M., D. M. DUNLOP AND C. P. STEWART 1932 Respiratory quotient in obese subjects. Biochem. J., *26:* 1107–1117.

MARDER, L., G. H. BECKER, B. MAIZEL AND H. NECHELES 1952 Fat absorption and chylomicronemia. Gastroenterology, *20:* 43–59.

MAYER, JEAN 1955 The physiological basis of obesity and leanness. Part I. Nutr. Abst. Rev., *25:* 597–611.

METROPOLITAN LIFE INSURANCE CO. 1951 Desirable weights for women. Metrop. Life Insur. Bulletin T 5043, New York.

MEYER, W. C., AND A. WINKLER 1952 Uber des Verhalten der Brenztraubensaure bei verschiedenen endokrinen Regulations-krankheiten. Deutsches Arch. klin. Med., *199:* 359–368.

NELSON, NORTON 1944 A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem., *153:* 375–380.

NEWBURGH, L. H. 1942 Control of the hyperglycemia of obese "diabetics" by weight reduction. Ann. Int. Med., *17:* 935–942.

OGILVIE, R. F. 1935 Sugar tolerance in obese subjects; review of 65 cases. Quart. J. Med., *4:* 345–358.

PENNINGTON, A. W. 1953 An alternate approach to the problem of obesity J. Clin. Nutrition, *1:* 100–106.

PETERS, J. P., AND D. D. VAN SLYKE 1932 Quantitative Clinical Chemistry, Vol. II. Methods. The Williams and Wilkins Co., Baltimore, pp. 84–127; pp. 495–515.

———— 1946 Quantitative Clinical Chemistry, Vol. I. Interpretations. The Williams and Wilkins Co., Baltimore, pp. 385–589.

RONY, H. R., AND A. J. LEVY 1929 Studies on fat metabolism. I. Fat tolerance in obesity. A preliminary study. J. Lab. Clin. Med., *15:* 221–228.

SIMMONS, J. S., AND C. J. GENTZKOW 1944 Laboratory Methods of the United States Army. Lea and Febiger, Philadelphia, pp. 98–119.

SNEDECOR, G. W. 1946 Statistical Methods Applied to Experiments in Agriculture and Biology. Iowa State College Press, Ames, pp. 103–168; 214–252.

SPERRY, W. M., AND M. WEBB 1950 A revision of the Schoenheimer-Sperry method for cholesterol determination. J. Biol. Chem., *187:* 97–106.

STADIE, W. C. 1940 Fat metabolism in diabetes mellitus. J. Clin. Invest., *19:* 843–861.

STRANG, J. M., AND H. B. MCCLUGAGE 1931 The specific dynamic action of food in abnormal states of nutrition. Am. J. M. Sc., *192:* 49–80.

STROUSE, S. AND CO-WORKERS 1924–25 The metabolism of obesity, I–IV. Arch. Int. Med., *34:* 267–274; 275–281; 573–583; *36:* 297–417.

TSAO, M., AND S. BROWN 1950 Pyruvic acid determination: A micro-method. J. Lab. Clin. Med., *35:* 320–323.

WILMOT, V. C., AND R. L. SWANK 1952 The influence of low-fat diet on blood lipid levels in health and in multiple sclerosis. Am. J. Med. Sci., *223:* 25–34.

NITROGEN UTILIZATION BY LAMBS FED PURIFIED RATIONS CONTAINING UREA, GELATIN, CASEIN, BLOOD FIBRIN, AND SOYBEAN PROTEIN [1]

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It is often assumed that ruminants utilize different proteins with equal efficiency regardless of their quality. Johnson and co-workers ('42, '44) presented evidence from their own investigations and a literature review suggesting that all crude proteins fed at the level of 10 to 12% have biological values approximating 60 for ruminants. They therefore assumed that bacterial protein has a biological value of 60 for sheep and that most feed nitrogen is converted into bacterial protein.

In contrast to the findings of Johnson et al. ('42, '44), several workers have reported biological values greater than 60 and significant variations in the biological values of different nitrogenous supplements when fed to sheep. Lofgreen et al. ('47) supplemented a timothy hay and corn ration with urea, urea plus methionine, linseed oil meal, and whole egg protein. This ration contained 10% of crude protein, 40% of which was supplied by the individual supplement. Biological values of 71, 74, 76 and 80 were obtained for rations containing urea, urea plus methionine, linseed oil meal and whole egg proteins respectively. Williams and Moir ('51) have confirmed and extended the work of Lofgreen et al. Supplements of

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urea, urea plus methionine, linseed oil meal, subterranean clover seed, casein, and whole egg protein supplied 40% of the ration's total nitrogen and had biological values of 68.7, 75.2, 79.7, 83.0, 82.0 and 86.7 respectively for the sheep. Slen and Whiting ('55) have shown that lactalbumin is superior to linseed oil meal, peas, urea, or alfalfa for pregnant ewes. Ewes receiving lactalbumin made larger gains during pregnancy and bore heavier lambs.

With few exceptions, nitrogen balance trials with ruminants have been conducted with practical type rations containing two or more sources of nitrogen. The results from such trials cannot be attributed to any single protein but must be considered as the result of feeding a mixture of proteins.

The object of this investigation was to determine the relative efficiency of nitrogen utilization by lambs fed purified rations in which either a purified protein or urea supplied nearly 100% of the total nitrogen. Concurrent objectives were to determine metabolic and endogenous nitrogen values for lambs and to characterize each nitrogen source as to its solubility within the rumen.

EXPERIMENTAL

Wether lambs of Texas origin, weighing 63 to 80 lbs., were used in this experiment. A ruminal cannula was fitted in each lamb. The composition of the purified ration used in this experiment is shown in table 1. The ration was fed in two separate portions: the basal or nitrogen-free ration and the nitrogen-vitamin supplement. Preliminary tests with other lambs indicated that the ration was not palatable to most lambs over an extended feeding period. To improve palatability the basal ration was mixed with water to give it a 23% moisture content, autoclaved at 15 p.s.i. for 25 minutes, and dried over steam coils. The nitrogen and vitamin supplement was added to the basal ration at feeding. The individual nitrogen sources used were urea,[2] gelatin, casein, bovine blood

[2] Courtesy E. I. duPont Company, Wilmington, Delaware.

fibrin,[3] and a purified soybean protein.[4] Each nitrogen source was diluted with cerelose to contain approximately an equal percentage of nitrogen. A vitamin A and D supplement [5] was mixed with each individual nitrogen source to supply 3,600 I. U. of vitamin A and 640 I. U. of vitamin D per sheep per day.

TABLE 1

Composition of experimental ration

INGREDIENT		NITROGEN CONTENT	
		Ingredient	Amount
	%		%
Basal ration		Basal ration	0.004
Roughage [1]	40.3		
Starch	27.7	Nitrogen-vitamin supplement	
Cerelose	15.0	Gelatin	10.70
Lard	2.0	Casein	11.24
Minerals [2]	5.0	Blood fibrin	11.33
Nitrogen-vitamin supplement [3]		Soybean protein	10.60
Nitrogen source	9.8	Urea	11.67
Vitamins A and D	0.2		
	100.0		

[1] Solka Floc, The Brown Company, Berlin, New Hampshire.

[2] Composition of mineral mixture (gm): $CaHPO_4$, 817.2; NaCl, 454; KCl, 544.8; MgO, 90.8; Na_2SO_4, 431.1; $MnSO_4 \cdot 4H_2O$, 5.6; $Fe(C_6H_5O_7) \cdot 3H_2O$, 23.8; $ZnCl_2$, 0.4; $CoSO_4 \cdot H_2O$, 0.135; CaF_2, 0.090; $Na_2MoO_4 \cdot 2H_2O$, 0.300; $Na_2B_4O_7$, 0.900; 90% KI in calcium stearate, 0.070.

[3] Protein or urea diluted with cerelose.

A daily feeding of 720 gm of basal ration and 80 gm of an individual nitrogen source was given in two equal portions. These rations contained approximately 7% of crude protein ($N \times 6.25$) and supplied 3.6 therms of calculated gross energy per day. Cerelose replaced the nitrogen source, weight

[3] Obtained from Armour Incorporated, Chicago, Illinois.

[4] Trade name: Drackett. Obtained from the Drackett Products Company, Cincinnati 32, Ohio.

[5] Stabilized Vitamin A and D guaranteed to contain 2250 I.U. Vitamin A and 400 I.U. Vitamin D per gram. Obtained from Thompson Hayward Company, Kansas City, Missouri.

for weight, during the nitrogen-free feeding period. The nitrogen-free ration contained 0.004% of nitrogen by analysis. Lambs were force fed via ruminal cannula when feed refusals occurred on the nitrogen-free ration.

An 8-day preliminary feeding period and a 6-day collection period were used in this work. Nitrogen balance trials using the various nitrogen supplements were followed by a nitrogen-free feeding period. Fecal and urinary collections were made using metabolism stalls designed and built at this station. Total fecal and urinary output were measured daily and an aliquot of each frozen for analysis. Feces and urine were analyzed for nitrogen by the method of the Association of Official Agricultural Chemists ('45) with the exception that a 2% boric acid solution was used as the receiving fluid during distillation. Ruminal ammonia was determined by the method outlined by Hawk ('54) using Conway micro-diffusion cups.

The original experimental design was a replicated 5 by 5 Latin square with 10 randomly assigned lambs. Two sheep, one in each replicate, refused feed at sometime during the test. Therefore, the results were considered as being from randomly assigned individuals and analyzed by single classification analysis of variance as outlined by Snedecor ('46).

RESULTS

Endogenous and metabolic nitrogen values. Lambs transferred to the nitrogen-free ration began refusing feed on the 5th to the 7th day following the ration change. Force feeding began when feed refusals were first noted. Two sheep scoured on the 8th day and were removed from the nitrogen-free ration. Fecal and urine collections were started on the 9th day and continued until the onset of scouring made separation of feces and urine impossible. Values obtained from the nitrogen-free period are shown in table 2.

Metabolic nitrogen was expressed two ways: milligrams per gram of dry matter intake (method 1) and milligrams per gram of fecal dry matter excreted (method 2). Metabolic ni-

trogen expressed on the basis of excreted dry matter was less variable (coefficient of variation, 6.86) than when expresed on the basis of dry matter intake (coefficient of variation, 9.21). These results for the sheep are in accordance with observations on man and on cattle (Heupke, '33, and Murkerjee, '46 as cited by Blaxter and Mitchell, '48). Both of these investigators found a closer relationship between metabolic nitrogen and excreted dry matter than between metabolic nitrogen and dry matter intake.

TABLE 2

Minimum nitrogen excretion of lambs on a nitrogen free ration

WEIGHT OF LAMB	ENDOGENOUS NITROGEN	METABOLIC NITROGEN	
kg	*mg N per kg body weight*	*mg N per gm dry matter intake*	*mg N per gm excreted dry matter*
36.7	24.6	2.18	7.65
30.4	21.3 [1]	0.91 [1]	9.06 [1]
29.5	37.5	1.67	7.46
35.4	28.5	2.16	5.50
35.4	34.0	2.92	7.58
34.0	35.2	2.83	5.24
25.8	30.2	1.81	8.71
35.8	33.8	3.19	8.06
Mean	31.9	2.39	7.17
Standard error of mean	1.7	0.22	0.49
Coeff. of variation	5.3	9.21	6.86

[1] These values excluded from mean due to low fecal and urinary excretion of individual (less than one-third of the average of the other 7 lambs).

The metabolic nitrogen level determined in this trial (2.39, calculated by method 1) is approximately 50% lower than values reported by Sotola ('30), Turk et al. ('34), and Harris and Mitchell ('41). This may be explained, in part by the completely purified nitrogen-free ration used in this experiment whereas the reported values were for sheep fed "nitrogen-free" rations containing wheat straw. The nitrogen-free ration used in this study contained only 0.004% nitrogen, much less than was contained in the reported nitrogen-free rations. A ration composed of purified ingredients such as

used in this study might also be less abrasive to the alimentary tract than one containing wheat straw. The metabolic nitrogen level reported here is almost identical to the value (2.5 mg N per gram of dry matter intake) reported by Lofgreen and Kleiber ('53) for calves receiving a liquid diet.

The endogenous nitrogen level reported here (31.9) is only slightly lower than those reported in the literature. An endogenous nitrogen level of 33.3 has been obtained by Sotola ('30), Turk et al. ('34), and Harris and Mitchell ('41).

TABLE 3

The true digestibilities, daily nitrogen balances, and biological values of urea, gelatin, casein, blood fibrin, and soybean protein for the lamb

NITROGEN SOURCE	TRUE DIGESTIBILITY OF NITROGEN		NITROGEN BALANCE	BIOLOGICAL VALUE OF NITROGEN	
	% [1]	% [2]	*gm N/day*	[1]	[2]
Urea	78.5	82.0	0.78	51.2	53.7
Gelatin	83.9	82.2	1.66	61.2	57.4
Casein	84.6	86.5	2.16	69.2	72.7
Blood fibrin	80.5	82.5	2.78	81.6	83.1
Soybean protein	85.6	85.4	2.86	82.0	82.4
L.S.D.[3]					
0.05	N.S.D.[4]	N.S.D.	0.59	10.1	8.9
0.01	N.S.D.	N.S.D.	0.79	13.6	11.9

[1] Calculated from metabolic nitrogen expressed on the ingested dry matter basis.
[2] Calculated from metabolic nitrogen expressed on the excreted dry matter basis.
[3] L.S.D. least difference between means for significance.
[4] N.S.D. no significant difference between means.

Nitrogen balance. The results obtained for the different nitrogen sources are compiled in table 3. Each value in the table is the mean of 8 separate balances on 8 different lambs except for blood fibrin. Results were obtained from only 7 different lambs fed blood fibrin.

True digestibility. True digestibility was calculated as the percentage of nitrogen intake which was absorbed. There was no significant difference in the true digestibility of nitrogen from any of the nitrogen sources. Smaller differences between true digestibilities were obtained when metabolic nitro-

gen was expressed on excreted dry matter rather than on ingested dry matter.

Harris and Mitchell ('41) reported casein and urea to be, respectively, 86.9 and 88.8% truly digestible when fed in a semi-synthetic ration to lambs. These values are only slighlty larger than those obtained in this trial (86.5 and 82.0).

Daily nitrogen balances. Daily nitrogen balances were calculated as the difference between the total fecal and urinary nitrogen and the nitrogen intake for any one collection period, divided by the days of that collection period. Marked differences were obtained between the daily nitrogen balances for lambs fed nitrogen from various sources. Lambs fed urea stored less nitrogen ($P < 0.01$) than those fed any of the true proteins; those fed either soybean protein or blood fibrin stored more nitrogen ($P < 0.05$) than those fed casein. In turn, the casein-fed lambs stored more nitrogen ($P = 0.07$) than those fed gelatin.

Biological values. Biological values of the various nitrogen sources were calculated as the percentage of absorbed nitrogen which was retained. Absorbed nitrogen was computed using metabolic nitrogen expressed by method 1 (dry matter intake basis) and method 2 (dry matter excreted basis); therefore, two biological values are recorded for each nitrogen source. The use of method 2, as compared to method 1, resulted in a larger value for casein and a smaller value for gelatin; otherwise, the two methods of expressing metabolic nitrogen gave comparable results. These data are shown in table 3. Calculation of biological values using method 2 resulted in less variation within the values obtained for any one nitrogen source. This is indicated by the smaller necessary difference between means for statistical significance for method 2 as compared to method 1. Therefore, biological values based upon method 2 are considered a more accurate reflection of the true value of the various nitrogen sources.

Biological values of the 5 nitrogen sources may be grouped into three categories with respect to statistical significance. These categories include different nitrogen supplements de-

pending upon the method of expressing metabolic nitrogen. When metabolic nitrogen was based on dry matter intake the values for blood fibrin and soybean protein were larger ($P < 0.05$) than those for casein and gelatin which were larger ($P < 0.05$) than for urea. When metabolic nitrogen was based on excreted dry matter the biological values for blood fibrin and soybean protein were larger ($P < 0.05$) than that of casein which was larger ($P < 0.01$) than those for gelatin or urea.

TABLE 4

Ammonia concentrations in rumen ingesta of lambs fed nitrogen from different sources

Values expressed as milligrams ammonia nitrogen per 100 ml of rumen ingesta

NITROGEN SOURCE	TIME AFTER FEEDING, HOURS			
	0	3	6	9
Urea	2.0[1] ± 0.3[2]	26.0 ± 3.3	12.0 ± 4.2	4.6 ± 1.2
Gelatin	2.8 ± 0.7	4.2 ± 1.2	7.0 ± 1.7	8.9 ± 2.4
Casein	3.8 ± 1.3	6.8 ± 2.6	8.1 ± 1.6	3.0 ± 0.6
Blood fibrin	9.4 ± 3.9	3.3 ± 1.2	4.6 ± 0.8	5.8 ± 1.2
Soybean protein	4.0 ± 2.2	1.4 ± 0.3	4.2 ± 1.6	6.6 ± 3.0

[1] Each value is a mean of duplicate determinations on 5 different lambs.
[2] Standard error of the mean.

Ruminal ammonia formation. Ruminal ammonia concentrations exhibited by lambs fed the different nitrogen sources used in this experiment are shown in table 4.

The extent of ruminal ammonia formation varied more between animals than between nitrogen supplements. This is indicated by the large standard errors of the means in table 4. Statistically, the ruminal ammonia concentrations varied only slightly with different nitrogen sources, with the exception of urea. The ruminal ammonia concentration three hours after feeding urea was larger ($P = 0.01$) than ammonia concentrations associated with any other supplement. Ruminal concentrations of 30 and 55 mg of ammonia nitrogen per 100 ml of rumen fluid three hours after feeding casein were reported by McDonald ('52) and by Annison et al. ('54).

Ruminal concentrations of 30 mg of ammonia nitrogen per 100 ml of rumen fluid three hours after feeding gelatin were reported by McDonald ('52). The ammonia concentrations recorded in this paper are much smaller than those reported in the literature.

DISCUSSION

Chalmers and Synge ('54) have shown that the formation of excessive ruminal ammonia lowers the value of a protein for sheep. McDonald ('52) and Annison et al. ('54) reported that proteins differ in the extent to which they are deaminated in the rumen. The different values (as measured

TABLE 5

Biological values of nitrogen sources determined for the growing lamb compared to literature values for the growing rat

NITROGEN SOURCE	BIOLOGICAL VALUE FOR LAMBS (THIS REPORT)	BIOLOGICAL VALUE FOR RAT	REFERENCE FOR RAT BIOLOGICAL VALUE
Urea	53.7	..	
Gelatin	57.4	23	Block and Mitchell ('46)
Casein	72.7	69	Block and Mitchell ('46)
Soybean protein	82.4	75 (soy flour)	Mitchell et al. ('45)
Blood fibrin	83.1	77 [1]	Forbes and Yohe ('55)

[1] Net nitrogen utilization value (coefficient of true digestibility × biological value).

by biological values and daily nitrogen balances) for the lambs fed the nitrogen sources used in this trial cannot be ascribed to variations in ruminal ammonia formation. There was no significant difference between ammonia production for the true proteins, yet they differed markedly in their ability to promote nitrogen retention by lambs.

The biological values of the true proteins reported here for lambs have the same rank that they possess for the growing rat (i.e. blood fibrin > soybean protein > casein > gelatin). This is shown in table 5 with a comparison of biological values reported for the two species.

The biological values for casein, soybean protein, and blood fibrin are quite similar for the two species. The value of 77 for the biological value of blood fibrin for the rat is net nitrogen utilization (coefficient of true digestibility × biological value). Assuming that 93% of the nitrogen of blood fibrin was digested by the rat, then blood fibrin has a biological value of 83 for the rat the same as reported here for the lamb. The biological values for urea and gelatin demonstrated the upgrading of poor quality ration nitrogen by rumen microorganisms and approach the value of 60 commonly reported for ruminants. Loosli et al. ('49) have reported a biological value of 56 for urea when it was fed as the sole source of nitrogen to lambs. This value is very similar to those (51.2 and 53.7) determined in this investigation. The large biological values of proteins superior to gelatin in essential amino acid distribution may indicate that the rumen flora is benefited by one or more essential amino acids. This supposition is based on reports that a large percentage (40 to 80) of the protein fed to ruminants is ultimately converted into bacterial protein (McDonald, '52 and Richardson, '55). A similar beneficial role of an amino acid for ruminants was found when methionine was added to urea rations (Williams and Moir, '51) or natural rations composed of alfalfa or field peas (Klosterman et al., '51). Regardless of the intermediate fate of essential amino acids in the rumen it seems obvious that the growing lamb, like the growing monogastric animal, responds to quality differences of proteins.

SUMMARY

Lambs were fed purified rations supplemented with either urea, gelatin, casein, soybean protein, or bovine blood fibrin to supply nearly 100% of the total nitrogen of the ration.

A metabolic nitrogen level of 2.39 mg of nitrogen per gram of dry matter intake or 7.17 mg of nitrogen per gram of dry matter excreted was determined for lambs fed a nitrogen-free ration.

Metabolic nitrogen expressed on the basis of excreted dry matter rather than on dry matter intake gave more consistent results.

An endogenous nitrogen level of 31.9 mg of nitrogen per kilogram of body weight was determined for lambs.

Ruminal ammonia concentrations were significantly higher following the feeding of urea than they were following the feeding of any other nitrogen source. There was no significant difference between ruminal ammonia concentrations resulting from the feeding of gelatin, casein, soybean protein, or bovine blood fibrin.

The mean biological values (calculated using metabolic nitrogen expressed on the basis of excreted dry matter) of the nitrogen sources were: urea, 53.7; gelatin, 57.4; casein, 72.7; soybean protein, 82.4; and bovine blood fibrin, 83.1. The biological values of bovine blood fibrin and soybean protein were significantly larger ($P < 0.05$) than that of casein, however, the casein values were significantly larger ($P < 0.01$) than those of gelatin and urea.

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LITERATURE CITED

ANNISON, E. F., M. I. CHALMERS, S. D. M. MARSHALL AND R. L. M. SYNGE 1954 Ruminal ammonia formation in relation to the protein requirement of sheep. III. Ruminal ammonia formation with various diets. J. Agr. Sci., *44:* 270–273.

ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS 1945 Official and Tentative Methods of Analysis. 6th Ed., Washington, D. C.

BLAXTER, K. L., AND H. H. MITCHELL 1948 The factorization of the protein requirements of ruminants and of the protein values of feeds, with particular reference to the significance of the metabolic fecal nitrogen. J. Animal Sci., *7:* 351–372.

BLOCK, R. J., AND H. H. MITCHELL 1946 The correlation of the amino acids composition of proteins with their nutritive value (a review). Nutrition Abst. and Rev., *16:* 249–278.

CHALMERS, M. I., AND R. L. M. SYNGE 1954 Ruminal ammonia formation in relation to the protein requirement of sheep. II. Comparison of casein and herring-meal supplements. J. Agr. Sci., *44:* 263–269.

FORBES, R. M., AND M. YOHE 1955 Net protein value of blood fibrin for the albino rat: Evaluation of nitrogen balance and carcass analysis methods. J. Nutrition, *55:* 439–498.

HARRIS, L. E., AND H. H. MITCHELL 1941 The value of urea in the synthesis of protein in the paunch of the ruminant. I. In maintenance. Ibid., *22:* 167–182.

HAWK, P. B., B. L. OSER AND W. H. SUMMERSON 1954 Practical Physiological Chemistry. 13th Ed. The Blakiston Co., Inc., New York.

JOHNSON, B. C., T. S. HAMILTON, H. H. MITCHELL AND W. B. ROBINSON 1942 The relative efficiency of urea as a protein substitute in the ration of ruminants. J. Animal Sci., *1:* 236–245.

JOHNSON, B. C., T. S. HAMILTON, W. B. ROBINSON AND J. C. GAREY 1944 On the mechanism of non-protein-nitrogen utilization by ruminants. Ibid., *3:* 287–298.

KLOSTERMAN, E. W., D. W. BOLIN, E. L. LASLEY AND W. E. DINUSSON 1951 The effect of methionine supplementation upon the utilization of pea and alfalfa proteins by sheep. Ibid., *10:* 439–446.

LOFGREEN, G. P., AND M. KLEIBER 1953 The metabolic fecal nitrogen excretion of the young calf and the true digestibility of casein. J. Nutrition, *49:* 183–190.

LOFGREEN, G. P., J. K. LOOSLI AND L. A. MAYNARD 1947 The influence of protein source upon nitrogen retention by sheep. J. Animal Sci., *6:* 343–347.

LOOSLI, J. K., H. H. WILLIAMS, W. E. THOMAS, F. H. FERRIS AND L. A. MAYNARD 1949 Synthesis of amino acid in the rumen. Science, *110:* 144.

MCDONALD, I. W. 1952 The role of ammonia in ruminal digestion of protein. Biochem. J., *51:* 86–90.

MITCHELL, H. H., T. S. HAMILTON AND J. R. BEADLES 1945 The importance of commercial processing for the protein value of food products. J. Nutrition, *29:* 13–25.

RICHARDSON, E. J. 1955 The amino acid composition of rumen and intestinal liquor fractions from sheep on purified and natural rations. Ph.D. Thesis, Cornell University, Ithaca, New York.

SLEN, S. B., AND F. WHITING 1955 Wool and lamb production as affected by the source of proetin in the ration of the mature ewe. J. Animal Sci., *14:* 844–858.

SNEDECOR, G. W. 1946 Statistical Methods. 4th Ed. The Iowa State College Press, Ames, Iowa.

SOTOLA, J. 1930 Biological values and supplementary relations of the proteins in alfalfa hay and in corn and alfalfa silage. J. Agr. Res., *40:* 79–96.

TURK, K. L., F. B. MORRISON AND L. A. MAYNARD 1934 The nutritive value of the proteins of alfalfa hay and clover hay when fed alone and in combinations with the proteins of corn. Ibid., *48:* 555–570.

WILLIAMS, V. J., AND R. J. MOIR 1951 Ruminal flora studies in the sheep. III. The influence of different sources of nitrogen upon nitrogen retention and upon the total number of free microorganisms in the rumen. Australian J. of Scientific Res., Series B, Biol. Sciences, *4:* 377–390.

DENTAL CARIES IN RELATION TO pH ON TOOTH SURFACES

I. pH AND LACTATE CONCENTRATION IN RELATION TO THE EXTENT OF THE LESIONS IN RATS' TEETH [1]

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A direct relationship between acid production from food in the mouth and the initiation and progress of dental caries has been postulated by a number of investigators (Kesel, '56; National Research Council, '52). In previous studies, we have found that pronounced differences occur in the extent of carious lesions of the teeth of animals on two different cariogenic diets that we have employed in our laboratories in several investigations. The present investigation was undertaken to determine whether or not the spread of the carious lesions that develop when rats are fed these two diets is associated with an increase in the acidity of the lesions.

EXPERIMENTAL

As our interest was in the intrinsic acidity of carious lesions and since this would be overshadowed in the presence of saliva, the experiments were conducted on desalivated animals.

Experiment 1. This experiment was designed to determine the pH on the tooth surfaces of rats with no caries, moderate caries, and severe caries.

[1] A preliminary report of these investigations was presented at the meeting of the I.A.D.R. in Chicago, March 1955.

Twenty-one weanling rats of the Wistar strain were desalivated by the block dissection method in which the ducts to the major salivary glands are ligated and cut and the glands excised. This method is described in detail elsewhere (Haldi, Wynn, Shaw and Sognnaes, '53). The animals were divided into three groups. Two groups were fed diets that have been designated in a previous publication (Wynn, Haldi, Shaw and Sognnaes, '53) as the Emory and the Harvard diets. The basic composition of these diets is given in table 1. The former has been found to be slightly cariogenic whereas the latter produces severe caries in a relatively short time.

TABLE 1

Composition of the Emory and Harvard high sucrose diets [1]

CONSTITUENTS	EMORY DIET	HARVARD DIET
	%	%
Sucrose	64	64
Casein	20	23
Hydrogenated vegetable oil	8	..
Corn oil	..	5
Yeast and liver extract	4	..
Liver concentrate	..	4
Salt mixture [2]	4	4

[1] Vitamin supplements were added to each of these diets. For composition see Wynn et al. ('53).

[2] The composition of the salt mixture is given in Wynn et al. ('53).

The third group was fed a commercial laboratory chow [2] in order to have, for comparative purposes, animals with little or no caries. We had found in previous experiments that this stock diet is practically non-cariogenic.

When the animals had been on the experiment 70 days, at which time no caries had developed in the rats on the stock diet, only a moderate amount of caries on the Emory diet, and extensive caries on the Harvard diet, pH readings were obtained on all the animals on the three diets 4 different times during the day. Eight readings (two in each quadrant) were taken each time. This gave a total of 224 readings on

[2] Purina.

the teeth of the animals on each diet. The animals were then sacrificed and the teeth removed and scored for caries.

The pH determinations were made with a specially constructed antimony electrode somewhat similar to the one which has been described by Stephan ('40). Small cylinders were made by drawing molten antimony up to about 5 mm in a glass tube with a diameter of approximately 1 mm. The antimony was allowed to solidify and the glass tube was removed by heating. The cylindrically molded antimony was soldered to a copper wire and inserted and sealed in a small glass tube. The antimony tip which projected 2 to 3 mm from the tube was then polished down until it was 0.4 mm or less in diameter. The free end of the copper wire, which had been passed through the glass tube, was brought into communication with a Leeds-Northrup pH meter. This electrode gave readings within 0.03 pH units of those obtained with the glass electrode on the two different cariogenic diets that had been moistened with water.

Experiment 2. A second experiment was designed to determine whether there is a relationship between the extent of the carious lesions, the pH of the lesion and the lactate concentration on the surface of the tooth.

In this experiment 18 albino rats from the same colony as in the previous experiment were desalivated at weaning and thenceforth fed the highly cariogenic Harvard diet. Two animals of this group were sacrificed [3] after they had been on the diet for two weeks. Thereafter, two more were sacrificed each week. The purpose of this procedure was to obtain teeth with a progressive increase in the number and extent of carious lesions. Immediately before the animals were sacrificed, pH readings were taken on the first and second upper and lower molars. Ten minutes later the teeth were carefully extracted. This period was allowed to elapse before removal of the teeth in order to minimize bleeding. The extracted teeth were scored for caries under a dissecting microscope as described elsewhere (Haldi and Wynn, '52).

[3] An overdose of nembutal was used.

The caries score ranged from 1 to 5 depending on the extent of the lesion. All adherent tissue was then carefully removed, the roots cut off and each tooth dropped into a separate vial containing 10 ml of 1% $CuSO_4$ solution, preparatory for the determination of lactate. The procedure for lactate analysis was that described by Barker and Summerson ('41) as modified by Moore ('52) for determining such extremely small amounts of lactate as are present in the rat's tooth. According to Barker and Summerson ('41) this method is highly specific for lactate in biological materials. This specificity was further demonstrated by Moore ('52). We found this method very satisfactory. In a preliminary lactate determination on the noncarious teeth of an animal selected at random, there was an average difference of only 0.3 μg between the lactate on the first molars in the right quadrant and on the corresponding teeth in the left quadrant.

RESULTS

Experiment 1. A striking feature of the 224 pH readings taken on each group of animals the day they were sacrificed was the large number (82%) of the readings above 7.0 on the teeth of the animals on the stock diet and a progressively smaller number of these higher readings (35 and 22% respectively) on those of rats fed the Emory and Harvard diets. On the other hand, there were no readings below pH 6 on the teeth of rats fed the stock diet, whereas there were 3 and 25% below pH 6.0 on the Emory diet and Harvard diet, respectively. The distribution of pH readings is shown in the nomogram in figure 1. Examination of the teeth revealed, as had been anticipated, that there was no caries in the teeth of the animals on the stock diet, whereas on the Emory diet there was a moderate amount of caries with the score ranging from 0 to 2 and on the Harvard diet extensive caries with a score of 1 to 5.

The relationship between the pH readings on the teeth and the caries score is shown in figure 2. It will be noted that there was only a slight drop in the average pH as the caries

score advanced from 1 to 3, which was not statistically significant ($P > 0.3$). As the score increased from 3 through 4 to 5, the drop in pH was much more pronounced and was statistically significant ($P < 0.01$). The data for this graph

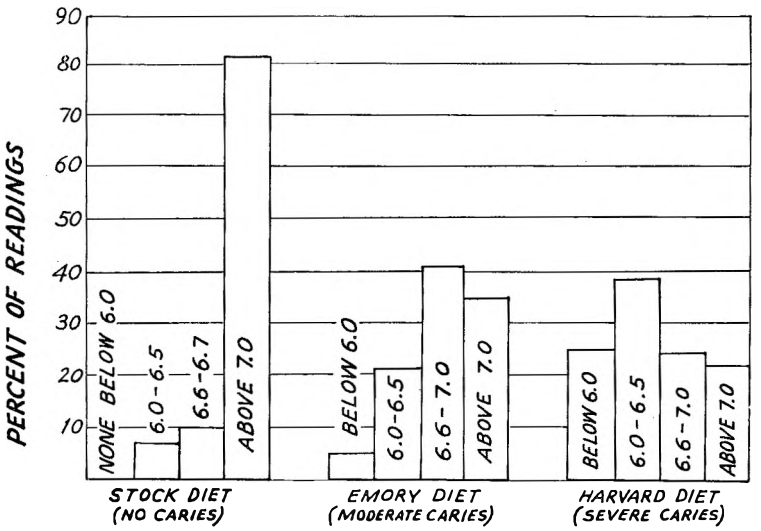

Fig. 1 Range of pH readings on the teeth of rats fed different diets.

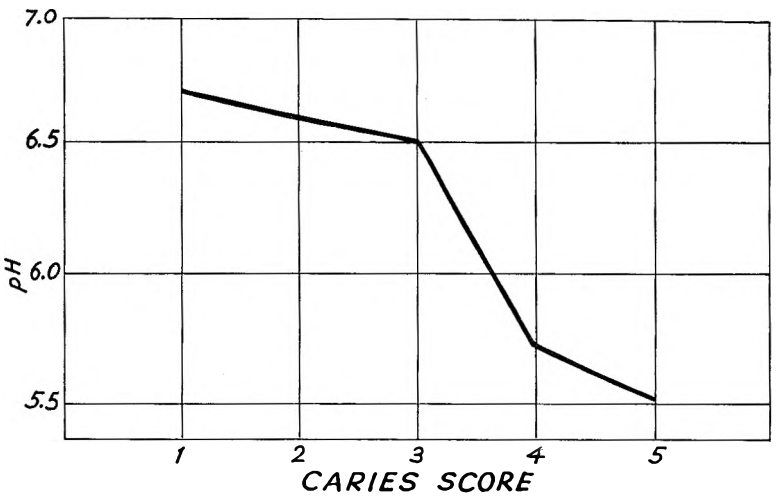

Fig. 2 Relation of pH and caries scores of the teeth of rats fed a highly cariogenic diet.

were obtained from the teeth of the animals that had been fed the Harvard diet as it was only this group of animals that had carious lesions with scores greater than 2. The pH readings, as stated previously, were taken on the day the animals were sacrificed. Four readings were made on each tooth at different times of the day and the average of these 4 readings taken as the representative pH reading for the tooth.

Experiment 2. The results obtained in the second experiment are presented in table 2. The relationship between the caries score and the pH on the surface of the teeth was the same as in the first experiment. In both experiments there

TABLE 2

pH, lactate and caries scores on the teeth of rats fed the Harvard diet for varying lengths of time[1]

Caries score	0	1	2	3	4	5
Average pH	6.3 ± 0.4	6.1 ± 0.4	6.2 ± 0.2	6.1 ± 0.3	5.6 ± 0.2	5.3 ± 0.3
Readings below 6.0, %	16	25	33	67	83	100
Total lactate, μg	10 ± 3	13 ± 2	14 ± 4	15 ± 6	38 ± 11	50 ± 15

[1] Values in the table were obtained on the first and second upper and lower molars of 18 rats (144 teeth) with varying degrees of caries.

was very little change in the pH as the score rose from 0 through 3 whereas an appreciable drop occurred with progression of the caries score from 3 through 4 and 5. Likewise, with an increase in the caries score from 0 to 2, there was a very moderate increase (from 16 to 33%) in the number of readings below 6.0. When the carious lesions had advanced to the point where they gave a score of 4, 83% of the pH readings were below 6.0. With further spread of the lesion, 100% of the readings were below this level.

A direct relationship was observed between the caries score and the amount of lactate recovered from the tooth. As shown in table 2, there was very little difference in the pH and the lactate on the teeth with a caries score of 1 through 3;

but as the severity of the lesion increased to give caries scores of 4 and 5, there was a pronounced increase in the lactate on the teeth, which in turn was accompanied by a clear-cut drop in the pH on the tooth surface.

DISCUSSION

It is apparent that in these experiments the intrinsic processes involved in the spread of carious lesions in rats' teeth were associated with a lowering of the pH (increase in acidity) and an increase in the amount of lactate in and on the teeth. In seeking an interpretation of these phenomena, two possibilities should be taken into consideration: (1) that the production of acid may have been the primary event which led to furtherance of the decay process, in which event the spread of the lesion would have been due to acid dissolution of the tooth substance; (2) as the carious lesion progressed, it may have provided a more favorable medium for the growth of acidogenic bacteria. If this were the case, acid production may have been incidental to and not the cause of the spread of the lesion.

The available data do not provide conclusive evidence for the solution of this problem. As there was no significant fall in the pH with the progress of the carious lesion until the caries score advanced from 3 to 4 it would appear that the significant lowering of the pH with advanced lesions was the result and not the cause of the spread of the lesions. If the reverse were true, one would expect to find a more pronounced progressive drop than was obtained in these experiments as the caries score advanced from 1 through 3.

This study, as stated previously, was conducted on desalivated animals in order to ascertain the intrinsic processes that occur with the advance of carious lesions. It is possible that if saliva were present, acid might be neutralized as fast as it is produced. It would be of interest to know whether under these conditions an advance of the carious lesions would occur without a lowering of the pH on the tooth surface. Further experimentation will be required to settle this question.

SUMMARY AND CONCLUSIONS

In one experiment three groups of weanling albino rats were desalivated and fed from weaning diets that were non-cariogenic, moderately and severely cariogenic. Determinations of the pH on the tooth surfaces were made at the end of 70 days, the animals sacrificed and teeth scored for caries.

In the animals with no caries there were no pH readings below 6.0. As the caries score advanced from 1 to 3, there was a slight but insignificant drop in the average pH. The drop in pH was much more pronounced and was statistically significant as the caries score went from 3 through 4 to 5.

In another experiment desalivated albino rats of the same colony as in the previous experiment, were fed a highly cariogenic diet from weaning. Two animals were sacrificed after two weeks on the diet and then two more each successive week. Readings of the pH on the molar teeth were taken immediately before sacrifice. The teeth were then scored for caries and analyzed for lactate.

A direct relationship was observed between the pH, the amount of lactate recovered from the tooth and the caries score. Pronounced changes in pH and lactate on the tooth were obtained only when there was severe caries.

It is apparent that the intrinsic processes involved in the spread of carious lesions in rats' teeth in these experiments were associated with a lowering of pH and an increase in the lactate in and on the teeth.

ACKNOWLEDGMENT

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LITERATURE CITED

BARKER, S. B., AND W. H. SUMMERSON 1941 The colorimetric determination of lactic acid in biological material. J. Biol. Chem., *138:* 535.

HALDI, J., AND W. WYNN 1952 Cariogenicity of sugar containing diets. J. Nutrition, *46:* 425.

HALDI, J., W. WYNN, J. H. SHAW AND R. F. SOGNNAES 1953 The relative cariogenicity of sucrose when ingested in the solid form and in solution by the albino rat. Ibid., *49:* 295.

KESEL, R. G. 1956 Methods of prevention and control of dental caries. J. Am. Dent. Assn., *52:* 455.

MOORE, B. W. 1952 An investigation of some biochemical properties of dental plaque. Thesis, Northwestern University.

NATIONAL RESEARCH COUNCIL 1952 Survey of the literature of dental caries. Washington, D. C.

STEPHAN, R. M. 1940 Changes in hydrogen-ion concentration on tooth surfaces and in carious lesions. J. Am. Dent. Assn., *27:* 718.

WYNN, W., J. HALDI, J. H. SHAW AND R. F. SOGNNAES 1953 Difference in the caries-producing effects of two purified diets containing the same amount of sugar. J. Nutrition, *50:* 267.

THE RELATION BETWEEN THE NATURE OF DIETARY PROTEIN AND THE PRODUCTION OF EXPERIMENTAL ALCAPTONURIA IN THE RAT[1]

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Papageorge and Lewis ('38) were the first to demonstrate that experimental alcaptonuria could be produced in rats which had ingested large amounts of L-phenylalanine over a long period of time. Since then experimental alcaptonuria has been successfully produced in normal animals by the feeding of both phenylalanine (Butts et al., '38; Closs and Braaten, '41; Lanyar, '43) and tyrosine (Abbott and Salmon, '43; Butts et al., '41; Lanyar, '43). More recently, however, it was shown that a dietary deficiency of both cystine and methionine might be responsible for the appearance of homogentisic acid (HA[3]) in the rat urine (Glynn et al., '45). Neuberger and Webster ('47) found that a dietary deficiency of lysine or tryptophan also interfered with the metabolism of the aromatic amino acids.

In the present work, the influence of the content and nature of dietary protein on the production of alcaptonuria was studied. Synthetic diets containing proteins of different bio-

[1] This material was taken in part from a dissertation submitted to the Rackham School of Graduate Studies by Helen C. Wu in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the University of Michigan, 1953.

[2] Professor Howard B. Lewis died on March 7, 1954.

[3] This abbreviation is used for homogentisie acid throughout this paper.

logical values were employed and alcaptonuria was produced by supplementing these diets with either phenylalanine or tyrosine. A comparison of the effectiveness of the DL and L forms of these aromatic amino acids in producing alcaptonuria was also made.

EXPERIMENTAL

Care of the animals. Male white rats of approximately 150 gm weight from litter groups of 4 were kept in separate metabolism cages. They were given a diet of bread, milk and lettuce for three to 4 days prior to the administration of the synthetic diet. After a control period of 6 to 8 days the animals were fed extra doses of the aromatic amino acid for three successive days. Four to 6 days after the last feeding of the aromatic amino acid, the urinary excretion of HA had again reached that of the control level. Hence it took about 15 days to complete one experiment. The rats were weighed weekly, and also before and after each dosage period.

Urine collection. Urine collection was started three to 4 days after the synthetic diet was given. Twenty-four-hour urine samples were collected in the presence of 1.5 ml of glacial acetic acid as the preservative. The urine and the washings were combined, filtered and diluted to 100 ml. Two milliliters of the diluted alcaptonuric urine samples or 10 ml of the diluted control urine samples was used for the analysis of HA.

Diets. The synthetic diets used varied only in the kind and amount of the protein used. The general composition was as follows: vitamin mixture 1%, salt mixture (Osborne and Mendel, '19) 4%, sucrose 24%, corn oil 4%, cod liver oil 2% and protein plus starch 65%. The vitamin mixture contained per 98 gm starch: 37 mg thiamine, 37 mg pyridoxine, 75 mg riboflavin, 300 mg calcium pantothenate, 300 mg *p*-aminobonzoic acid and 375 mg nicotinic acid. The protein of the diet was increased at the expense of starch. The protein and amino acid supplements of the diets are listed in the first columns of tables 1 and 2.

The food consumption was restricted to 10 gm per rat per day regardless of the diet employed. In experiments with diets containing zein or gliadin, the rats were pair-fed with those kept on a casein diet of the same nitrogen content. The aromatic amino acid supplements (see tables 1 and 2) were

TABLE 1

The excretion of homogentisic acid (HA) after feeding DL-*phenylalanine to rats receiving casein, gliadin and zein diets*

DIET	NO. OF RATS [1]	DL-PHENYLALANINE FED PER 100 gm BODY WEIGHT	EXTRA HA EXCRETED PER gm DL-PHENYLALANINE FED
		gm	*mg*
Casein,[2] 18.4%	4[a]	0.78 ± 0.08 [3]	2.6 ± 0.3
Gliadin,[4] 18.3%	4[a]	1.08 ± 0.20	33.9 ± 15.2
Casein, 18.4%	2[b]	0.32 ± 0.19	1.0 ± 1.0
Gliadin, 17.9%			
+ L-lysine,[5] 0.37%	2[b]	0.49 ± 0.33	1.2 ± 0.8
Gliadin, 17.9%			
+ L-lysine, 0.37%	4	0.75 ± 0.09	3.2 ± 0.9
Casein, 20%	2[c]	0.86 ± 0.03	6.8 ± 1.6
Zein,[6] 21.2%	2[c]	0.86 ± 0.02	141.4 ± 10.4
Casein, 20%	2[d]	0.71 ± 0.01	3.6 ± 0.4
Zein, 20.6%			
+ L-tryptophan,[5] 0.64%	2[d]	0.96 ± 0.01	46.6 ± 27.5
Casein, 20%	2[e]	0.93 ± 0.01	4.3 ± 0.3
Zein, 19.9%			
+ L-lysine, 0.64%			
+ L-tryptophan, 0.64%	2[e]	1.03 ± 0.01	23.0 ± 11.7

[1] The numbers bearing the same superscript represent pair-fed litter mates.
[2] The Borden Company (Labco brand).
[3] The mean and its average deviation.
[4] Huron Milling Company, Harbor Beach, Michigan.
[5] Merck and Company.
[6] Corn Products Sales Company (Argo brand).

thoroughly incorporated into the daily ration during dosage periods and their intake was obtained by the difference between the amount given and the amount calculated from the food remaining in the cup. Generally the rats ate all the daily ration within a short time and there was very little, if any spillage.

Chemical methods. Ammoniacal silver nitrate, freshly prepared each day, was used at room temperature as a qualitative test for the onset of alcaptonuria. The iodometric titration method as described by Neuberger ('47) was used for the quantitative estimation of HA. The method was standardized with a sample of synthetic HA generously supplied by Dr. Lynn D. Abbott Jr.

TABLE 2

The effect of the administration of L *and* DL *forms of tyrosine and phenylalanine on the excretion of homogentisic acid (HA)*

DIET	NO. OF RATS	ISOMER	AMINO ACID FED PER 100 gm BODY WEIGHT	EXTRA HA EXCRETED PER gm AMINO ACID FED
			gm	*mg*
			Phenylalanine	
Casein, 5%	2	L [1]	1.10 ± 0.19 [2]	0.46 ± 0.12
Casein, 5%	6	DL [3]	1.03 ± 0.04	4.09 ± 0.42
Casein, 20%	5	L	1.00 ± 0.11	0.22 ± 0.11
Casein, 20%	10	DL	1.04 ± 0.05	2.84 ± 0.03
Casein, 50%	8	L	0.86 ± 0.11	0.03 ± 0.01
Casein, 50%	6	DL	0.79 ± 0.08	3.51 ± 1.00
			Tyrosine	
Casein, 20%	13	L [4]	1.31 ± 0.08	17.60 ± 8.36
Casein, 20%	11	DL [4]	1.41 ± 0.06	0.09 ± 0.03
Zein, 21.2%	6	L	1.08 ± 0.20	140.7 ± 43.3
Zein, 21.2%	7	DL	1.41 ± 0.30	2.78 ± 1.18

[1] This amino acid was supplied to Professor Lewis by Dr. Seiichi Izume. A description of this preparation appeared in footnote 2 of Chandler and Lewis ('32).

[2] The mean and its standard error.

[3] Winthrop-Stearns Incorporated.

[4] Merck and Company.

RESULTS

Effect of the nature of the dietary protein on the production of alcaptonuria. Table 1 summarizes the effect of various diets on the excretion of HA. It is noted in this table that the increased excretion of HA was 15 times as great in rats receiving the 18.3% gliadin diet as in their pair-fed litter mates on the 18.4% casein diet. However, administra-

tion of DL-phenylalanine to pair-fed rats on the casein diet and the 17.9% gliadin plus 0.37% L-lysine diet resulted in no difference in the excretion of HA. It was observed that the gliadin plus lysine diet caused rats to lose appetite and consume only a part of the daily ration. The increased excretion of HA by the casein-fed rats of this experiment was less than that of the previous one. This difference is probably due to the smaller intake of DL-phenylalanine per 100 gm body weight by the casein-fed rats which were pair-fed with those on the gliadin plus lysine diet.

In order to make a fairer comparison of the gliadin diet with the gliadin plus lysine diet on the production of experimental alcaptonuria, 4 more rats were put on the latter diet which was supplemented with twice as much DL-phenylalanine as previously incorporated in this diet. With an intake of 0.75 gm DL-phenylalanine per 100 gm body weight, the increase in the excretion of HA was 3.2 mg per gram of DL-phenylalanine fed. This level of excretion was not significantly greater than that of casein-fed rats with a similar intake of DL-phenylalanine, but it was considerably less than that of gliadin-fed rats. It appears that the deficiency of lysine in gliadin may be entirely responsible for the great increase in the HA excretion when DL-phenylalanine was incorporated in the gliadin diet.

The effect of zein diets on the excretion of HA following the feeding of DL phenylalanine was next studied. Three diets containing zein as the only protein were employed, namely 21.2% zein, 20.6% zein plus 0.64% L-tryptophan, and one containing 19.9% zein plus 0.64% each of L-lysine and L-tryptophan. Preliminary experiments showed that when the three zein diets were not supplemented with DL-phenylalanine, there was essentially no difference in the urinary level of HA between these rats and their casein-fed paired mates. However, with an intake of 0.86 to 1.03 gm DL-phenylalanine per 100 gm body weight, the zein diet caused the greatest increase in the excretion of HA, while the zein plus tryptophan diet produced a greater increase than did the zein plus lysine and tryptophan

diet. In the presence of added DL-phenylalanine in the diet, a deficiency in both lysine and tryptophan has thus induced a much greater increase in the excretion of HA than a deficiency in lysine alone. It may also be pointed out that while rats on the gliadin plus lysine diet excreted no greater amount of HA than their casein-fed paired mates, rats on the zein plus lysine and tryptophan diet excreted considerably more HA than their casein-fed controls.

Effects of the administration of L *and* DL *forms of phenylalanine and tyrosine on the excretion of HA.* In a preliminary experiment, 8 rats were fed a 20% casein diet for three days. The average daily excretion of HA on this diet was found to be 0.77 mg with a range from 0.62 to 0.91 mg per rat. Under similar conditions, the average daily output of HA was 0.74 (0.68 to 0.79) mg and 0.64 (0.57 to 0.70) mg per rat respectively on 50 and 5% casein diets. These results show that varying the casein content of the diet over a range of 5 to 50% did not affect the level of HA excretion. Furthermore, it can be seen in table 2 that the difference in the excretion of extra HA following the addition of L- or DL-phenylalanine to these three casein diets was very slight indeed.

When the effectiveness of L- and DL-phenylalanine in producing HA was compared, it was found that DL-phenylalanine always caused a greater increase in the HA excretion than did the same amount of the L isomer. On the other hand, administration of L-tyrosine to rats given the 20% casein diet resulted in remarkably higher excretion of HA than that of the DL isomer. The increase in the excretion of HA was greatly enhanced by substituting the casein diet with the zein diet. Again, L-tyrosine was found to be much more effective than DL-tyrosine. The extraordinary behavior of L-tyrosine in this respect deserves attention. It is interesting to point out that on the 20% casein diet, the aromatic amino acids, in the order of decreasing effectiveness in increasing the HA level of the rat urine, are L-tyrosine, DL-phenylalanine, L-phenylalanine, and DL-tyrosine.

DISCUSSION

The use of proteins deficient in lysine, or lysine and tryptophan, in the diet resulted in the excretion of large amounts of HA when additional quantities of DL-phenylalanine were given. Although the production of experimental alcaptonuria in gliadin-fed rats could be prevented by supplementing the diet with lysine, the excretion of HA was still high above the control level in zein-fed rats, even though both tryptophan and lysine were incorporated in the diet. Hence it may be stated that supplementation of an incomplete protein diet with the missing essential amino acids does reduce the excretion of HA, but this does not necessarily imply a complete prevention of the occurrence of the condition. Also, it is possible that the relative abundance of amino acids in the diet might play an important role in the production of experimental alcaptonuria. Comparison of the amino acid composition of casein and zein (Block and Bolling, '51) shows that zein is lower in threonine and valine as well as lysine and tryptophan, but its content of leucine is more than twice that of casein. Only by employing a diet containing a mixture of pure amino acids can one determine whether each essential amino acid plays a role in the normal metabolism of the aromatic amino acids.

It was observed in the present study that in all the experiments, DL-phenylalanine was more effective in producing alcaptonuria than the natural isomer. This is contrary to the results of other authors Closs and Braaten, '41; Lanyar, '42, '43; Neuberger and Webster, '47). Explanation of this effect is not available at present. However, it has been reported that the urinary excretion of phenylpyruvic acid was higher following the ingestion of D-phenylalanine than of L-phenylalanine (Jervis et al., '40; Kotake et al., '22). Perhaps a similar relation holds true in the excretion of HA. On the other hand, the observations made in this study on the L and DL forms of tyrosine are in good agreement with those of others (Butts et al., '38; Butts et al., '41; Lanyar, '42, '43). No selective absorption of the D and L forms of amino acids

has been found (Berg and Bauguess, '32; Chase and Lewis, '34; Dakin, '08; Wilson and Lewis, '29). However, there is evidence to indicate that D-tyrosine when ingested in the DL form is totally unavailable for normal use in man (Albanese et al., '46). By employing Millon's reaction (Folin and Ciocalteu, '27) as modified by Medes ('32), a much higher "tyrosine" value was found in the urine of rats fed DL-tyrosine than in that of those fed L-tyrosine. It may be assumed that a part of the DL-tyrosine fed is excreted in the urine and what remains in the body is insufficient to give rise to extra HA in a well-fed animal. It is also possible that the toxic effect of L-tyrosine (Martin, '42–'43; Schweizer, '47) may impair the normal ability of the body to metabolize large quantities of the aromatic amino acids, and consequently it favors the production of experimental alcaptonuria.

SUMMARY

Deficiency in lysine and in both lysine and tryptophan very greatly enhanced the homogentisic acid excretion following the ingestion of DL-phenylalanine. This effect was completely abolished by supplementing the gliadin diet with lysine, but only partially abolished by supplementing the zein diet with lysine and tryptophan.

Administration of L-phenylalanine to rats fed casein diets caused lower excretion of homogentisic acid than the same dose of the DL isomer. DL-Tyrosine, on the contrary, did not increase the urinary excretion of homogentisic acid in the casein-fed rats, whereas the same amount of the L-isomer produced a considerable output of homogentisic acid. When the zein diet was used, DL-tyrosine also caused an increased excretion of homogentisic acid, but L-tyrosine brought about very severe alcaptonuria.

LITERATURE CITED

ABBOTT, L. D., JR., AND C. L. SALMON JR. 1943 Experimental alkaptonuria in the white rat on high tyrosine diets. J. Biol. Chem., *150:* 339.

ALBANESE, A. A., V. IRBY AND M. LEIN 1946 The utilizaiton of *d*-amino acids by man. VI. Tyrosine. Ibid., *166:* 513.

BERG, C. P., AND L. C. BAUGUESS 1932 Tryptophane metabolism. III. The rate of absorption of *l*- and *dl*-tryptophane and tryptophane derivatives from the gastrointestinal tract of the rat. Ibid., *98:* 171.

BLOCK, R. J., AND D. BOLLING 1951 The amino acid composition of proteins and foods. Chas. C Thomas, Springfield, Illinois, 2nd edition.

BUTTS, J. S., M. S. DUNN AND L. F. HALLMAN 1938 Studies in amino acid metabolism. IV. Metabolism of *dl*-phenylalanine and *dl*-tyrosine in the normal rat. J. Biol. Chem., *123:* 711.

BUTTS, J. S., R. O. SINNHUBER AND M. S. DUNN 1941 The metabolism of *l*(—)-tyrosine. Ibid., *140:* XXII.

CHANDLER, J. P., AND H. B. LEWIS 1932 Comparative studies of the metabolism of the amino acids. V. The oxidation of phenylalanine and phenylpyruvic acid in the organism of the rabbit. Ibid., *96:* 619.

CHASE, B. W., AND H. B. LEWIS 1934 Compartive studies of the metabolism of amino acids. VI. The rate of absorption of leucine, valine, and their isomers from the gastrointestinal tract of the white rat. Ibid., *106:* 315.

CLOSS, K., AND K. BRAATEN 1941 Weitere Untersuchungen über den Stoffwechsel des Phenylalanins. Z. physiol. Chem., *271:* 221.

DAKIN, H. D. 1908 Note on the relative rate of absorption of optically isomeric substances from the intestine. J. Biol. Chem., *4:* 437.

FOLIN, O., AND V. CIOCALTEU 1927 On tyrosine and tryptophane determinations in prcteins. Ibid., *73:* 627.

GLYNN, L. E., H. P. HIMSWORTH AND A. NEUBERGER 1945 Pathological states due to deficiency of the sulfur-containing amino acids. Brit. J. Exp. Path., *26:* 326.

JERVIS, G. A., R. J. BLOCK, D. BOLLING AND E. KANZE 1940 Chemical and metabolic studies on phenylalanine. II. The phenylalanine content of the blood and spinal fluid in phenylpyruvic oligophrenia. J. Biol. Chem., *134:* 105.

KOTAKE, Y., Y. MASAI AND Y. MORI 1922 Über das Verhalten des Phenylalanins im tierischen Organismus. Z. physiol. Chem., *122:* 195.

LANYAR, F. 1942 Über experimentelle Alkaptonurie bei der weissen Maus. Ibid., *275:* 225.

———— 1943 Über experimentelle Alkaptonurie bei der weissen Ratte. Ibid., *278:* 155.

MARTIN, G. J. 1942–1943 The hypertensive effect of diets high in tyrosine. Arch. Biochem., *1:* 397.

MEDES, G. 1932 CXI. A new error of tyrosine metabolism: tyrosinosis. The intermediary metabolism of tyrosine and phenylalanine. Biochem. J., *26:* 917.

NEUBERGER, A. 1947 Studies on alcaptonuria. 1. The estimation of homogentisic acid. Ibid., *41:* 431.

NEUBERGER, A., AND T. A. WEBSTER 1947 Studies on alcaptonuria. 3. Experimental alcaptonuria in rats. Ibid., *41:* 449.

OSBORNE, T. B., AND L. B. MENDEL 1919 The nutritive value of the wheat kernel and its milling products. J. Biol. Chem., *37:* 557.

PAPAGEORGE, E., AND H. B. LEWIS 1938 Comparative studies of the metabolism of the amino acids. VII. Experimental alcaptonuria in the white rat. Ibid., *123:* 211.

SCHWEIZER, W. 1947 Studies on the effect of *l*-tyrosine on the white rat. J. Physiol., *106:* 167.

WILSON, R. H., AND H. B. LEWIS 1929 Comparative studies of the metabolism of amino acids. II. The rate of absorption of amino acids from the gastrointestinal tract of the white rat. J. Biol. Chem., *84:* 511.

THE EFFECT OF THE THYROID ON THE CONVERSION OF INTRAVENOUSLY ADMINISTERED AQUEOUS DISPERSIONS OF CAROTENE TO VITAMIN A IN THE RAT

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Numerous reports have appeared concerning the effect of the thyroid on the conversion of carotene to vitamin A. The earlier work has been reviewed by Drill ('43) who concluded: "All the evidence to date indicates that in the absence of the thyroid gland carotene is not metabolised to vitamin A." The results of later work, however, appear somewhat confusing. Thus Canadell and Valdecasas ('47) reported that they were unable to prevent ocular symptoms in hypothyroid rats following oral administration of carotene while Drill and Truant ('47) reported similar findings in thyroidectomised rats following subcutaneous administration of carotene in sesame oil. Remington, Harris and Smith ('42), on the other hand, noted that oral dosing with carotene was equally as effective as vitamin A in curing eye conditions in thyroidectomised rats.

Johnson and Baumann ('47) and Kelly and Day ('48), using liver storage of vitamin A as a criterion, found that after the same dose of carotene animals rendered hypothyroid stored less vitamin A than controls. In contrast, however, Wiese, Mehl and Deuel ('48), using growth as a criterion, showed that the same amount of carotene was required to elicit a fixed growth response in hypothyroid animals as in normal animals.

Recently in this laboratory the effect of the thyroid on the formation of vitamin A from carotene, administered intravenously as an aqueous dispersion in Tween 40 [1] (polyoxyethylene sorbitan monopalmitate), has been studied in rats. Parenteral administration was chosen in preference to the oral route in order to overcome any possible effects that variations in absorption might have on the results. Carotene was administered intravenously as an aqueous dispersion since it is now well recognized that under these conditions it is readily converted to vitamin A (Bieri and Pollard, '54; Kon, McGillivray and Thompson, '55; McGillivray, Thompson and Worker, '56), whereas when injected as an oily solution it appears to be inactive (Sexton, Mehl and Deuel, '46). In preliminary experiments (McGillivray et al., '56), using blood levels of vitamin A and liver storage as criteria, the thyroid was shown to be without effect on the conversion of carotene to vitamin A. The present paper covers subsequent work of a similar nature using blood levels and liver storage of vitamin A, and in addition the remission of xerophthalmia, as criteria.

EXPERIMENTAL

All rats used in these experiments were albinos of the Wistar strain inbred from stock introduced into New Zealand by Dr. I. J. Cunningham, Superintendent of the Wallaceville Research Station, Wellington. Those used in experiment I were maintained throughout life on a basal diet [2] of the following percentage composition: ground wheat, 42; ground barley, 10.5; ground oats, 4; dried skim milk, 34; wheat germ, 8; $CaCO_3$, 1; NaCl, 0.5. At slaughter (200 to 300 gm) they were almost deficient in vitamin A as evidenced by low levels of vitamin A in the blood and the absence of appreciable quantities in the liver. The rats used in experiment H were maintained on the U.S.P., vitamin A-free test diet (Hawk, Oser and Summerson, '47) from weaning until such time as xerophthalmia became apparent, a period usually of 4 to 6 weeks.

[1] Atlas Powder Company, Wilmington, Delaware.

[2] Prepared in pellet form by W. and R. Fletcher, Ltd., Wellington, New Zealand.

Rats were rendered hypothyroid by complete removal of the thyroids or by treatment with thiouracil, or rendered hyperthyroid by injections with the sodium salt of l-thyroxine. All animals not thyroidectomized, including controls, were subjected to sham operation. As a measure of the effect of these treatments the oxygen consumption of the rats was determined by a modification of the method of Morrison ('47). The determinations were made on groups of up to three rats in three consecutive 15-minute periods.

For injection crystalline carotene [3] was used. Aqueous dispersions of the pigment were prepared with a 20% (v/v) solution in water of Tween 40 [4] as described by Bieri and Pollard ('54). All injections were into the vena cava exposed by ventral mid-line incision. Diethyl ether anaesthesia was used throughout all operations.

Carotene and vitamin A were estimated in individual blood and liver samples essentially as described by Thompson, Ganguly and Kon ('49) and Kon et al. ('55).

RESULTS

The results presented in table 1 (experiment I) clearly show that thyroid activity has little effect on the conversion to vitamin A of carotene administered intravenously as an aqueous dispersion. Thus, 24 hours after injection of 400 μg of carotene in Tween 40, liver vitamin A levels in normal, hyperthyroid, hypothyroid and thyroidectomised rats increased from 1.1 μg per liver to 11.0, 10.6, 12.8 and 10.2 μg per liver, respectively, while blood vitamin A levels increased from 11 μg/100 ml plasma to 19, 17, 21 and 18 μg/100 ml plasma, respectively.

That the vitamin A appearing after injection was physiologically active and that its activity was in no way affected by thyroid activity were confirmed by the results presented in table 2. Thus after injection of carotene ocular symptoms were relieved equally effectively in all 4 experimental groups

[3] L. Light and Company, Ltd.

[4] See footnote 1.

TABLE 1

Experiment I

Effect of thyroid activity on the conversion of carotene to vitamin A in rats partially deficient in the vitamin. Vitamin A content of plasma and liver 24 hours after the administration of an aqueous dispersion of carotene in Tween 40

RATS		TREATMENT	OXYGEN CONSUMPTION	CAROTENE ADMINISTERED	BLOOD PLASMA		LIVER			
							Vitamin A			CAROTENE
No. used	Mean weight				Vitamin A alcohol	CAROTENE	Alcohol	Ester	Total	
	gm		*litres/kg/hour*	*μg*	*μg/100 ml*	*μg/100 ml*	*μg*	*μg*	*μg*	*μg*
2	230	Sham operated 28 days prior to injections	35	None	11	0	0.5	0.6	1.1	Trace
3	230	Sham operated 28 days prior to injection	35	400	19	Trace	4.4	6.6	11.0	15
5	200	Sham operated; 30 μg l-thyroxine subcutaneously daily for 28 days prior to injection	52	400	17	Trace	5.0	5.6	10.6	11
5	300	Sham operated; 250 mg thiouracil daily in food for 28 days prior to injection	20	400	21	Trace	5.2	7.6	12.8	17
5	300	Thyroidectomised; 100 mg thiouracil daily in food for 28 days prior to injection	23	400	18	Trace	3.8	6.4	10.2	14

TABLE 2

Experiment II

Effect of the thyroid on the remission of xerophthalmia and on weight increases in rats following intravenous injection of carotene as an aqueous dispersion in Tween 40

NO. RATS USED	MEAN WEIGHT	TREATMENT	CAROTENE ADMINISTERED	INTERVAL OVER WHICH OCULAR SYMPTOMS DISAPPEARED IN GROUP	WEIGHT INCREASE AFTER INJECTION 14-DAY PERIOD
	gm		*μg*	*days*	*gm*
2	100	Sham operated at first sign of xerophthalmia; injected 4 days later	400	6–10	21
5	108	Sham operated at first sign of xerophthalmia; 15 μg l-thyroxine daily for 4 days prior to injection	400	5–10	24
5	115	Sham operated at first sign of xerophthalmia; 150 mg thiouracil in food daily for 4 days prior to injection	400	5–11	14
6	118	Thyroidectomised at first sign of xerophthalmia; injected 4 days later	400	7–9	19

while the weight increases of all groups, with the exception of that treated with thiouracil, were all approximately the same.

DISCUSSION

The main aim of the present experiments was to determine whether the activity of the thyroid gland had any influence on the course of conversion of intravenously administered aqueous dispersions of carotene to vitamin A. Conflicting reports are still appearing in the literature concerning the effect of the thyroid on the conversion of carotene to vitamin A following oral administration, some workers claiming conversion to be impaired by hypothyroidism and enhanced by hyperthyroidism. Cama and Goodwin ('49) have suggested, however, that these differences may be associated more directly with the effect of the thyroid on intestinal absorption than with its effect on the mechanism of conversion. Intravenous administration of carotene, providing that the carotene is presented in a state suitable for utilization by the animal, offers a convenient means of studying any direct effect which the thyorid may have on the conversion mechanism without the complicating effects of intestinal absorption. It may be argued that the mechanism of conversion following intestinal absorption of carotene is not necessarily the same as that following intravenous injection of aqueously dispersed carotene but the results of recent experiments in this laboratory would dispute this. They tend to show, on the contrary, that the two mechanisms are in fact probably identical and that factors which affect the one would also necessarily affect the other.

From the results presented above it is clearly evident that thyroid activity has no direct effect on the conversion of carotene to vitamin A. This conclusion is based on increases of the vitamin A levels of the liver and blood after injection of a single dose of carotene into normal, hyperthyroid, hypothyroid, and thyroidectomised rats, and on remission of specific symptoms of vitamin A deficiency after injection of carotene into animals similarly treated. The results confirm,

therefore, the work of Remington et al. ('42), and Wiese et al. ('48). They contrast, however, with that of Canadell and Valdecasas ('47), Johnson and Baumann ('47), and Kelley and Day ('48), due probably to differences associated with intestinal absorption. They contrast also with the results of Drill and Truant ('47) but an explanation for this is difficult to offer.

It is of interest to note the significantly higher ($P < 0.05$) liver vitamin A storage in the group rendered hypothyroid with thiouracil (table 1), an irregularity which has also been reported in blood by Bieri ('49) and which would appear to be attributable to thiouracil *per se* rather than to the thyroid. The somewhat smaller weight increase in the hypothyroid group after injection would appear also to confirm the observations of Wiese et al. ('48) who have emphasised that when assessing the effect of drugs such as thiouracil on carotene metabolism by biological assays involving weight increases, allowance must be made for the growth-inhibiting action of the drugs themselves.

SUMMARY

The effect of the thyroid on the conversion of carotene to vitamin A has been studied in the rat, carotene being administered intravenously as an aqueous dispersion in Tween 40 (polyoxyethylene sorbitan monopalmitate).

Twenty-four hours after the injection of carotene into normal, hyperthyroid, hypothyroid, and thyroidectomised animals partially deficient in vitamin A, liver levels of vitamin A increased from 1.1 μg to 11.0, 10.6, 12.8 and 10.2 μg/liver, respectively, while blood levels of vitamin A increased from 11 μg to 19, 17, 21 and 18 μg/100 ml plasma, respectively.

Within 11 days of injection into completely deficient animals, ocular symptoms had disappeared from all 4 treated groups. The rate at which symptoms disappeared was approximately the same in all groups while weight increases, with the exception of the hypothyroid group which was lower, were the same also.

From these results it is concluded that the thyroid is without effect on the conversion of carotene to vitamin A.

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LITERATURE CITED

BIERI, J. G. 1949 Conversion of carotene to vitamin A by the rat. Fed. Proc., *8:* 184.

BIERI, J. G., AND C. J. POLLARD 1954 Studies on the site of conversion of β-carotene injected intravenously into rats. Brit. J. Nutrition, *8:* 32–44.

CAMA, H. R., AND T. W. GOODWIN 1949 The role of the thyroid in carotene and vitamin A metabolism. Biochem. J., *45:* 236–241.

CANADELL, J. M., AND F. G. VALDECASAS 1947 Inhibiting action of thiouracil on carotene. Experientia, *3:* 35–36.

DRILL, V. A. 1943 Interrelations between thyroid function and vitamin metabolism. Physiol. Rev., *23:* 355–379.

DRILL, V. A., AND A. P. TRUANT 1947 Effect of thyroidectomy on the conversion of carotene to vitamin A. Endocrinology, *40:* 259–264.

HAWK, P. B., B. L. OSER AND W. H. SUMMERSON 1947 Practical physiological chemistry. Maple Press Company, York, Pa., Publishers.

JOHNSON, R. M., AND C. A. BAUMANN 1947 The effect of the thyroid on the conversion of carotene into vitamin A. J. Biol. Chem., *171:* 513–521.

KELLEY, B., AND H. G. DAY 1948 Thiouracil and the conversion of carotene to vitamin A in the rat. Ibid., *175:* 863–866.

KON, S. K., W. A. MCGILLIVRAY AND S. Y. THOMPSON 1955 Metabolism of carotene and vitamin A given by mouth or vein in oily solution or aqueous dispersion to calves, rabbits and rats. Brit. J. Nutrition, *9:* 244–267.

MCGILLIVRAY, W. A., S. Y. THOMPSON AND N. A. WORKER 1956 Further studies on the metabolism of intravenously administered aqueous dispersions of carotenoid pigments. Ibid., *10:* 126–134.

MORRISON, P. R. 1947 An automatic apparatus for the determination of oxygen consumption. J. Biol. Chem., *169:* 667–679.

REMINGTON, R. E., P. L. HARRIS AND C. L. SMITH 1942 Relationship between vitamin A and iodine metabolism in the rat. J. Nutrition, *24:* 597–606.

SEXTON, E. L., J. W. MEHL AND H. J. DEUEL, JR. 1946 The relative provitamin A activity of carotene when introduced orally and parenterally in the rat. Ibid., *31:* 299–319.

THOMPSON, S. Y., J. GANGULY AND S. K. KON 1949 The conversion of β-carotene to vitamin A in the intestine. Brit. J. Nutrition, *3:* 50–78.

WIESE, C. E., J. W. MEHL AND H. J. DEUEL, JR. 1948 Conversion of carotene to vitamin A in the hypothyroid rat. J. Biol. Chem., *175:* 21–28.

PREVENTION OF INANITION IN VITAMIN B_6-DEPRIVED RATS BY INSULIN TREATMENT

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In any nutritional deficiency in experimental animals, decreased appetite is evident early in deprivation. The consequent decrease in food intake may be responsible for changes which have been ascribed, frequently, to the specific deficiency under study but which are actually unspecific. An example of such a change is a decreased storage of body fat. Pair feeding was recommended by Mitchell and Beadles ('30) to provide control animals differing from the deprived ones by the single factor of the deprivation under study. However, pair-fed controls are not normal animals. Force feeding of deprived animals has been used in an attempt to eliminate inanition but the procedure is tedious and it may not be possible to provide sufficient food. It is well known that appetite can be stimulated by administration of insulin and it seemed advantageous to utilize this effect to cause deprived animals to eat an increased amount of food. It was realized that this procedure could be criticized because the metabolism of the insulin-treated animals would be distorted by the hormone. Nevertheless, this procedure has been investigated with rats deprived of vitamin B_6. The results show that at least one of the unspecific effects of vitamin B_6 deficiency can be prevented and that one specific biochemical change is still evident despite insulin treatment.

MATERIALS AND METHODS

Two separate experiments were undertaken to investigate the effects of insulin administration on vitamin B_6-deficient rats. In each experiment, groups of 10 rats were employed, except that the initial groups in experiment II comprised only 8 rats each. Wistar strain rats [1] were used throughout. Average initial body weights were: experiment I, males 113 gm, females 110 gm; experiment II, males 134 gm, females 121 gm. Rats were housed in individual screen-bottom cages in a room maintained at 75 $\pm$ 2°F. The basal diet contained 20% of casein, 20% of corn oil, and was similar to that described previously (Beaton et al., '53), except that agar was replaced with a non-nutritive cellulose.[2] For one week prior to the experiments, all animals received the basal diet plus pyridoxine hydrochloride at a level of 5 mg per kilogram of diet. It was estimated that each animal had about 50 µg of pyridoxine hydrochloride per day. The same concentration of this vitamin was present in the diet fed to control groups throughout the experimental period.

In both experiments separate groups of male and female rats were used so that differences due to sex could be determined. In experiment I, the treatments were: initial controls, fasted for 18 hours and killed to determine initial composition; deprived; deprived treated with insulin; control; control treated with insulin. Insulin [3] administration of 4 units per day subcutaneously was begun on the 10th experimental day. All of the rats in experiment I were fed ad libitum. In experiment II, the treatments were similar except that a pair of control groups were pair fed with the untreated deprived groups; insulin was not given to either pair-fed or ad libitum-fed controls. Deprived animals given insulin [3] received the following dosage subcutaneously per rat per day: during the first 20 days, 4 units; 20 to 27 days, 6 units; 27 to 32 days, 4 units.

[1] Carworth Farms.

[2] Alphacel.

[3] Protamine zinc insulin, Toronto, 40 units per milliliter.

On day 32 in both experiments, rats were fasted for 18 hours and then killed by stunning and decapitation. Samples of the liver were removed and immediately homogenized for the determination of enzyme activities. Since it was not possible to carry out the large number of analyses required for the determination of carcass composition in individual animals, the carcasses were pooled by groups and quickly frozen; they were later passed through a power grinder and duplicate samples were taken for the determination of carcass composition. Data of carcass composition are averages for groups; statistical treatment of such data to determine significance of difference is not possible.

Aspartic-glutamic transaminase activity of liver homogenates was determined by the method of Tonhazy et al. ('50). Alanine-glutamic transaminase activity was determined by this method as modified by Caldwell and McHenry ('53). Transaminase activities are expressed as the microliters of pyruvate-CO_2 formed per milligram of wet tissue per hour. Catalase activities of liver homogenates were determined by the method of Feinstein ('49); activities are expressed as the milliequivalents of sodium perborate destroyed per milligram of wet tissue per hour. Carcass composition was determined by the following procedures: total crude fatty acids, Gavin and McHenry ('40); total nitrogen by the macro-Kjeldahl procedure, multiplying by 6.25 to obtain protein; moisture by the standard procedure of drying in open aluminum dishes at 105°C. for 5 hours.

RESULTS AND DISCUSSION

It was found that the administration of insulin to vitamin B_6-deprived rats resulted in an increased number of deaths; insulin treatment caused no deaths in the control groups. Increased sensitivity to insulin as the deficiency progressed was seen in both experiments. Beaton ('55a, b) reported that changes in carbohydrate metabolism were evident in vitamin B_6-deprived rats and that the blood sugar response to insulin injection was slightly greater in deprived than in control

rats. Because of the increasing sensitivity to insulin, the experiments were terminated before an acute deficiency had been attained. Incipient acrodynia was in evidence; insulin administration had no obvious effect on either the time of onset or degree of the acrodynia.

TABLE 1

Body composition in vitamin B_6-deprived and control rats

GROUP	AVERAGE FOOD INTAKE	AVERAGE WEIGHT GAIN	COMPOSITION OF THE WEIGHT GAIN		
			Water	Fat	Protein
	gm/rat/day	*gm/rat*	*gm*	*gm*	*gm*
Experiment I					
A. Males					
$-B_6$	13	61	37	6	15
$-B_6$ + insulin	14	97	45	28	16
$+B_6$	16	143	76	34	27
$+B_6$ + insulin	16	144	74	35	28
B. Females					
$-B_6$	11	32	14	7	11
$-B_6$ + insulin	13	58	18	26	13
$+B_6$	12	67	31	20	12
$+B_6$ + insulin	15	96	34	42	15
Experiment II					
A. Males					
$-B_6$	12	90	58	9	21
$-B_6$ + insulin	14	124	61	36	23
$+B_6$ pair fed	12	145	79	35	27
$+B_6$ fed ad libitum	15	149	87	31	30
B. Females					
$-B_6$	10	45	21	11	8
$-B_6$ + insulin	12	74	23	35	10
$+B_6$ pair fed	10	81	37	26	13
$+B_6$ fed ad libitum	13	86	42	25	15

Data regarding food intakes, body weight gains, and changes in carcass composition are shown in table 1. Since only group averages are available, it is impossible to calculate the significance of differences. Cautious interpretation is in order. The purpose of insulin administration was to

cause an increase in food consumption in an attempt to maintain the body weights of deprived animals and the amounts of fat in those rats closer to the values for control rats. The success of such an attempt is dependent on the insulin dosage to a considerable extent. The dosages used were determined in the light of previous work in this laboratory (Beaton and Curry, '56) and were selected to minimize mortality as much as possible. The results obtained are applicable only to the dosage used and might have been quite different with other dosages. The principal finding was that the deprived rats given insulin were able to store about as much fat as did control animals. In all deprived rats not given insulin, the failure in fat storage was evident. Similar trends were shown by both sexes. Vitamin B_6 deficiency *per se* did not prevent the deposition of body fat.

It has been reported that pyridoxal or pyridoxamine phosphate can activate the apoenzyme of the aspartic-glutamic transaminase (Meister et al., '54) and that some form of the vitamin probably acts as coenzyme for several other transaminases (Cammarata and Cohen, '50). In table 2, the effects of vitamin B_6 deprivation on two liver transaminases are shown. Examination of the individual results revealed no correlation between sex and response to treatment, hence the sexes were combined for statistical analysis. These results confirm previous observations of lowered activities of both transaminases in deprived rats as compared to controls (Beaton et al., '54). An analysis of variance was carried out to determine the significance of the results of experiment 1, allowing for the different sizes of groups. Deprivation of vitamin B_6 had a significant effect on the activity of both the alanine-glutamic and aspartic-glutamic transaminases (alanine-glutamic, $t = 7.00$; aspartic-glutamic, $t = 5.30$; both significant at the 1% level). There was a significant interaction between insulin and vitamin B_6 with reference to the aspartic-glutamic enzyme ($t = 3.92$, significant at the 1% level); that is, the effect of vitamin B_6 deprivation on the activity of this enzyme was accentuated by insulin administra-

tion. No such interaction was seen with respect to the alanine-glutamic transaminase. There was no general effect of insulin on the activities of either of these enzymes.

Although an analysis of variance was not possible in the second experiment due to its design, the "t-test" was used to ascertain the significance of the differences between means. In general, confirmation of the results of the first experiment

TABLE 2

Alterations in liver enzyme activities in vitamin B_6 deficiency

(mean ± standard error of the mean)

GROUP	NUMBER OF RATS	CATALASE [1]	ALANINE-GLUTAMIC TRANSAMINASE [2]	ASPARTIC-GLUTAMIC TRANSAMINASE [3]
Experiment I				
$-B_6$	20		41 ± 1.9	110 ± 3.0
$-B_6$ + insulin	14		38 ± 1.9	92 ± 3.0
$+B_6$	20		60 ± 2.8	115 ± 1.7
$+B_6$ + insulin	20		59 ± 3.8	120 ± 3.2
Experiment II				
$-B_6$	16	0.70 ± 0.026	37 ± 1.4	104 ± 2.6
$-B_6$ + insulin	12	0.64 ± 0.030	37 ± 1.6	99 ± 6.1
$+B_6$ pair fed	16	0.61 ± 0.033	51 ± 2.5	106 ± 2.7
$+B_6$ fed ad lib.	16	0.63 ± 0.025	50 ± 2.8	116 ± 2.7

[1] Activities expressed as milliequivalents of sodium perborate destroyed per milligram of wet tissue per hour. Method of Feinstein ('49).

[2] Activities expressed as microliters of pyruvate-CO_2 formed per milligram of wet tissue per hour. Method of Tonhazy et al. ('50) as modified by Caldwell and McHenry ('53).

[3] Activities expressed as microliters of pyruvate-CO_2 formed per milligram of wet tissue per hour. Method of Tonhazy et al. ('50).

was obtained. Deprivation of vitamin B_6 led to a significant depression of alanine-glutamic transaminase activity as compared to pair-fed or ad libitum-fed controls ($t = 4.83$ and 4.10, both significant at the 1% level); insulin administration to deprived rats had no effect on the activity of this enzyme nor did the restriction of the food intake of control rats have any effect. The deprived rats exhibited a lower aspartic-glutamic transaminase activity than did the ad libitum-fed

controls ($t = 3.21$, significant at the 1% level); however, food restriction also caused a significant lowering of the activity in control rats ($t = 3.11$, significant at the 1% level), thus the difference between deprived and pair-fed control rats was not significant. Previous studies have revealed that as the deficiency becomes more pronounced, there is a much greater difference in the activity of the aspartic-glutamic transaminase between deprived and pair-fed control rats (Beaton et al., '54). The lowering of aspartic-glutamic transaminase activity produced in this experiment by the administration of insulin to deprived rats was not significant. Liver catalase activity in the second experiment was not affected by either vitamin B_6 deprivation or by insulin administration; these determinations serve to indicate that the alterations seen in transaminase activities are not reflections of overall changes in liver enzymes. The lowering of alanine-glutamic transaminase activity, and probably also that of the aspartic-glutamic enzyme, are effects of vitamin B_6 deficiency *per se*. In this respect, they differ from the failure in fat storage which was nullified, partially at least, by insulin administration. In considering these results, it is interesting to remember that insulin administration had no visible effect upon the appearance of acrodynia, the commonly accepted external sign of vitamin B_6 deficiency.

SUMMARY

Insulin administration is a means whereby the food intake of vitamin B_6-deprived rats can be increased. This provides another method of separating the effects of inanition from those of the vitamin deficiency.

By this method it has been ascertained that vitamin B_6 deficiency *per se* does not impair the deposition of fat in the rat. The decrease in fat storage usually seen in this deficiency is an unspecific effect.

Changes in liver transaminase activities seem to be a specific effect of the deficiency.

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LITERATURE CITED

BEATON, J. R. 1955a Further studies on carbohydrate metabolism in the vitamin B_6-deprived rat. Canadian J. Biochem. Physiol., *33:* 161.

———— 1955b *In vitro* studies on carbohydrate metabolism in the vitamin B_6-deprived rat. Ibid., *33:* 562.

BEATON, J. R., J. L. BEARE, G. H. BEATON, E. F. CALDWELL, G. OZAWA AND E. W. MCHENRY 1954 Studies on vitamin B_6. V. Chronological sequence of biochemical defects in the vitamin B_6-deprived rat. J. Biol. Chem., *207:* 385.

BEATON, J. R., J. L. BEARE, J. M. WHITE AND E. W. MCHENRY 1953 Studies on vitamin B_6. I. Biochemical changes in vitamin B_6 deficiency in rats. Ibid., *200:* 715.

BEATON, G. H., AND D. M. CURRY 1956 A comparison of the effects of growth hormone and of insulin administration. Endocrinology, *58:* 797.

CALDWELL, E. F., AND E. W. MCHENRY 1953 Studies on vitamin B_6 and transamination in rat liver. Arch. Biochem. Biophys., *44:* 396.

CAMMARATA, P. S., AND P. P. COHEN 1950 The scope of the transamination reaction in animal tissues. J. Biol. Chem., *187:* 439.

FEINSTEIN, R. N. 1949 Perborate as substrate in a new assay of catalase. Ibid., *180:* 1197.

GAVIN, G., AND E. W. MCHENRY 1940 The B vitamins and fat metabolism. III. The effects of vitamin B_6 upon liver and body fat. Ibid., *132:* 41.

MEISTER, A., H. A. SOBER AND E. A. PETERSON 1954 Studies on the coenzyme activation of glutamic-aspartic apotransaminase. Ibid., *206:* 89.

MITCHELL, H. H., AND J. R. BEADLES 1930 The pair-feeding method in nutrition experiments. J. Nutrition, *2:* 225.

TONHAZY, N. E., N. G. WHITE AND W. W. UMBREIT 1950 A rapid method for the estimation of the glutamic aspartic transaminase in tissues and its application to radiation sickness. Arch. Biochem., *28:* 36.

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Nominations are invited for the Osborne and Mendel Award of $1000.00 established by the Nutrition Foundation, Inc., for the recognition of outstanding accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the most significant published contribution in the year preceding the annual meeting of the Institute, or who has published a series of contemporary papers of outstanding significance.

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Nominations may be made by anyone. The following information must be submitted: Name of the Award for which candidate is proposed and as convincing a statement as possible as to the basis of the nomination (this may include a pertinent bibliography but reprints are not required). *Five copies* of all documents, including seconding statements, must be sent to the Chairman of the Nominating Committee before January 1, 1957, to be considered for the 1957 Award.

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BORDEN AWARD IN NUTRITION

Nominations are solicited for the 1957 Award and a gold medal made available by the Borden Company Foundation, Inc. The American Institute of Nutrition will make this award in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of the components of milk or of dairy products. The award will be made primarily for the publication of specific papers during the previous calendar year, but the Jury of Award may recommend that it be given for important contributions made over a more extended period of time not necessarily including the previous calendar year. The award is usually given to one person, but if in their judgment circumstances and justice so dictate, the Jury of Award may recommend that it be divided between two or more collaborators in a given research. The Jury may also recommend that the award be omitted in any given year if in its opinion the work submitted does not warrant the award. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award. Employees of the Borden Company are not eligible for this honor.

Nominations may be made by anyone. The following information must be submitted: Name of the Award for which candidate is proposed and as convincing a statement as possible as to the basis of the nomination (this may include a pertinent bibliography but reprints are not required). *Five copies* of all documents, including seconding statements, must be sent to the Chairman of the Nominating Committee before January 1, 1957, to be considered for the 1957 Award

Chairman, Nominating Committee:
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