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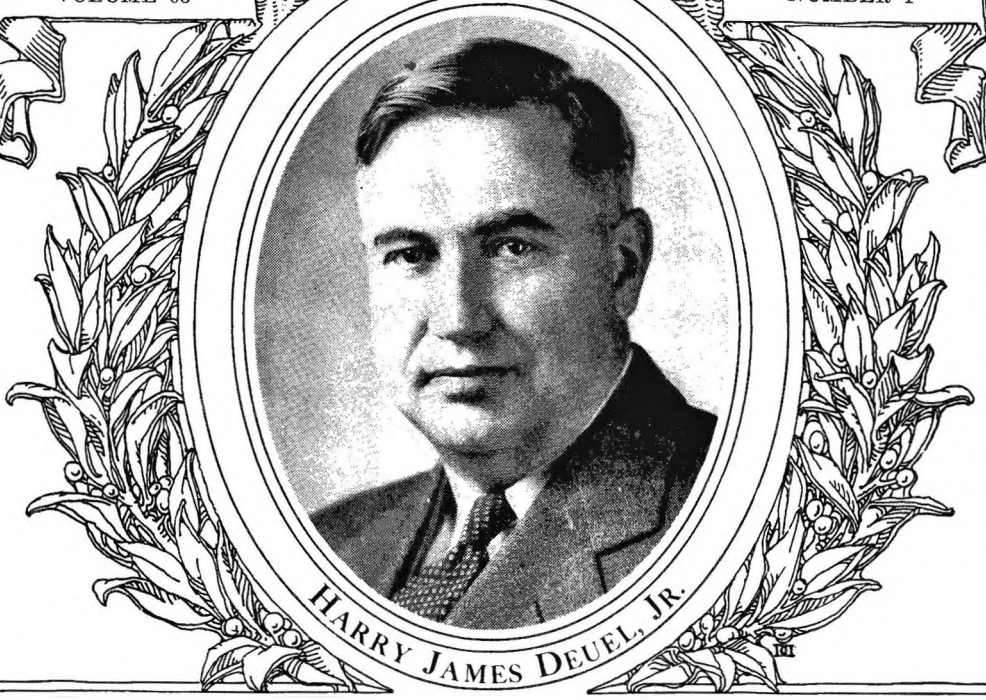

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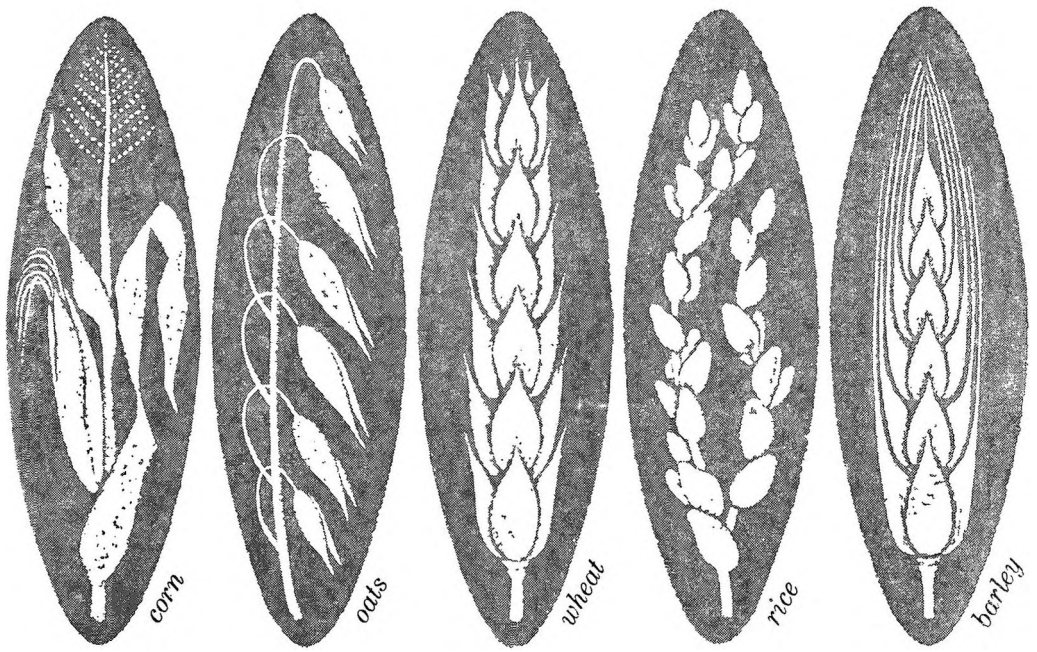

the calories in the cereal and milk serving are well balanced and low in fat

In the light of the modern trend toward less fat in the diet and for quick and lasting energy foods, the calories in the cereal and milk serving merit consideration. Both the cereal and the milk contribute well-balanced nourishment.

This serving provides quick and lasting energy, is *low in fat*, and is a good source of many nutrients as shown in the table below. It furnishes about 10 per cent of the daily needs of protein, important B vitamins, and essential minerals. Served with nonfat milk, the fat content is very low.*

nutritive composition of average cereal serving

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

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

Bowes, A. deP., and Church, C.F.: Food Values of Portions Commonly Used. 8th ed. Philadelphia: A. deP. Bowes, 1956.
Cereal Institute, Inc.: The Nutritional Contribution of Breakfast Cereals. Chicago: Cereal Institute, Inc., 1956.
Hayes, O. B., and Rose, G. K.: Supplementary Food Composition Table. J. Am. Dietet. A. 33:26, 1957.

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THE AMINO ACID ADEQUACY OF MILO (GRAIN SORGHUM) FOR THE GROWTH OF RATS

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INTRODUCTION

The poor amino acid balance of the cereal grains as a group has been demonstrated many times. Corn has been known for some time to be deficient in lysine and tryptophan (Osborne and Mendel, '14). More recent work by Eggert et al. ('53) again demonstrated these deficiencies when corn was fed to growing pigs. Benton et al. ('55) showed that isoleucine would produce a marked increase in growth of rats fed a corn diet supplemented with lysine, tryptophan, threonine and valine.

Pecora and Hundley ('51) obtained a large improvement in growth rate of rats fed rice by supplementing the diet with lysine and threonine. Sure ('54) demonstrated that wheat and rye are deficient in lysine and threonine and further, that rye is deficient in valine.

Published work involving the amino acid adequacy of milo (grain sorghum) for growth is very limited. Shelton et al. ('51) reported that lysine is the first limiting amino acid in milo. This was also indicated in work reported by Hillier et al. ('54).

The large annual production of milo in the Southwest at present, and continuing agronomic improvements, point toward greater future production. Therefore, it seemed desirable to study the amino acid adequacy of milo for growth.

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EXPERIMENTAL

Male weanling albino rats of the Holtzman strain weighing from 50 to 60 gm were used in all experiments. In each experiment rats were divided equally among the experimental groups according to body weight. Each group contained 8 rats, except where otherwise indicated. The rats were kept in individual screen-bottom cages and were offered food and water ad libitum. Each rat was weighed on the 7th and 14th days of the experiment. All experiments were terminated after 14 days.

The basal diet used in all experiments appears in table 1. The low-protein purified control ration was composed of 81.85% of sucrose, 11.00% of casein and 1.00% of corn oil in addition to the mineral and vitamin mixtures used in the basal ration. The positive control ration contained 71.85% of sucrose, 21.00% of casein and 1.00% of corn oil in addition to the mineral and vitamin supplements.

In the experiment where determinations of liver fat were made, the livers were removed, blotted free of blood, and frozen at —4°C until analysis. They were prepared for analysis by macerating in a Potter-Elvehjem homogenizer. They were then dried at 110°C for 14 hours and ground for weighing. The liver-fat content of each sample was expressed as a percentage of the dry matter.

RESULTS

The results of each experiment have been summarized separately in tables 2 through 5. Statistical analyses of the data were performed according to the methods of Snedecor ('56).

In experiment 1, the addition of 0.1% of L-lysine [2] to the basal diet produced a highly significant increase ($P < 0.01$) in growth. A level of 0.2% of supplemental DL-threonine or 0.2% each of DL-threonine and DL-isoleucine failed to give a further increase in growth rate.

[2] Supplied as 95% L-lysine·HCl by E. I. duPont de Nemours and Co., Wilmington, Delaware.

Supplementing the basal diet with the same levels of lysine and threonine or lysine, isoleucine and threonine in experiment 2 (table 3) again produced a large increase in growth rate above that obtained with the basal ration. However, the addition to this ration of 0.1% of DL-methionine, 0.1% of DL-tryptophan and 0.2% of DL-valine alone or in all possible combinations did not promote any additional stimulation of growth rate. The growth obtained on the 11% casein ration was

TABLE 1

Basal ration used in rat growth studies

RATION COMPONENTS [1]	AMOUNT
	%
Milo (Kafir 44–14)	93.85
Salt mix[2]	4.00
B-vitamin mix[3]	2.00
Vitamin A and D supplement[4]	0.15
	100.00

[1] The Kafir 44–14 contained 9.13% crude protein, 0.20% lysine and 0.30% threonine. The mixed ration, therefore, contained 8.57% crude protein, 0.19% lysine, and 0.28% threonine. The amounts of other amino acids were not determined.

[2] Salts 4, Hegsted et al. ('41).

[3] Composed of sucrose and the following vitamins to provide, in milligrams per 100 gm of ration: riboflavin, 3.0; calcium pantothenate, 5.0; thiamine HCl, 0.2; niacin, 1.0; pyridoxine, 0.5; inositol, 10.0; para amino benzoic acid, 20.0; choline chloride, 50.0; folic acid, 0.2 vitamin B_{12}, 0.004; biotin, 0.10; and 2-methyl-1, 4-naphthoquinone, 0.50.

[4] Contains vitamins A and D to supply 1500 I.U. and 180 I.U. per gram of ration, respectively. Supplied by Nopco Chemical Co., Harrison, New Jersey.

approximately equal to that obtained with the basal ration supplemented with 0.1% of L-lysine and 0.2% of DL-threonine (12.2 and 12.9 gm gain per week, respectively). The need for more protein was demonstrated by the fact that the rats receiving 21% of casein gained an average of 19.8 gm per week.

When the amount of supplemental lysine was doubled (from 0.1 to 0.2%) in the diet of half of the rats retained on test for an additional two weeks (table 3, experiment 2A), the growth

rate was significantly increased in each case. The rats receiving lysine and threonine, those receiving lysine, isoleucine and threonine, and those receiving lysine, isoleucine, threonine, methionine, tryptophan and valine each responded similarly in growth rate when the level of added L-lysine was increased from 0.1 to 0.2%.

In experiment 3 (table 4) the addition of L-lysine to the basal ration produced an increase in growth rate above that obtained with the basal ration at all levels of lysine added. It is noteworthy that the effect on growth obtained from supplemental isoleucine and threonine was related to the level of lysine

TABLE 2

The effect of adding threonine or threonine and isoleucine to the basal ration supplemented with lysine on growth of rats

Experiment 1

TREATMENT	NO. OF RATS	AV. WEEKLY GAIN
		gm
Basal	8	3.3 ± 1.0[1]
Basal + 0.1% L-lysine	8	10.2 ± 1.3
Basal + 0.1% L-lysine + 0.2% DL-threonine	8	10.5 ± 1.4
Basal + 0.1% L-lysine + 0.2% DL-threonine 0.2% DL-isoleucine	8	11.1 ± 2.3

[1] Standard deviation of the mean.

present. At the 0.1% level of added L-lysine, growth rate was not significantly affected by the addition of 0.2% of DL-isoleucine and 0.2% of DL-threonine (9.5 versus 7.8 gm gain per rat per week). However, with higher increments of added L-lysine, the growth response steadily increased up to 0.5% of added L-lysine. The continuous increase in growth response with increased levels of L-lysine in the presence of isoleucine and threonine did not occur when lysine was added alone. A plateau was reached at the 0.3% level of added lysine in the latter case so that at the 0.5% level, the average growth rate was 11.0 gm per week in the absence of supplemental isoleucine and threonine versus 24.9 when both were present.

When lysine was present at the 0.5% supplemental level, in experiment 4 (table 5), the addition of threonine produced significantly faster growth than when no threonine was added. This was true irrespective of what other amino acids were added.

TABLE 3

Effect of adding methionine, tryptophan and valine alone and in combination to the basal ration supplemented with isoleucine, lysine and threonine

Experiment 2

	TREATMENT	NO. OF RATS	AV. WEEKLY GAIN
			gm
1.	Basal	8	5.2 ± 1.4[1]
2.	Basal + 0.1% L-lysine + 0.2% DL-threonine	8	12.9 ± 2.1
3.	Basal + 0.1% L-lysine + 0.2% DL-threonine + 0.2% DL-isoleucine	8	12.5 ± 1.5
4.	3 + 0.1% DL-methionine	8	11.9 ± 1.7
5.	3 + 0.1% DL-tryptophan	8	11.8 ± 1.6
6.	3 + 0.2% DL-valine	8	13.3 ± 1.6
7.	3 + 0.1% DL-methionine + 0.1% DL-tryptophan	8	11.0 ± 1.1
8.	3 + 0.1% DL-methionine + 0.2% DL-valine	8	13.1 ± 1.8
9.	3 + 0.1% DL-tryptophan + 0.2% DL-valine	8	13.3 ± 2.1
10.	3 + 0.1% DL-methionine + 0.1% DL-tryptophan + 0.2% DL-valine	8	12.8 ± 1.8
11.	11% Casein	8	12.2 ± 1.4
12.	21% Casein	8	19.8 ± 1.7
	Experiment 2A		
1.	Basal	8	6.7 ± 1.2
2.	Basal + 0.1% L-lysine + 0.2% DL-threonine	4	18.0 ± 1.8
2a.	Basal + 0.2% L-lysine[2] + 0.2% DL-threonine	4	22.0 ± 3.2
3.	Basal + 0.1% L-lysine + 0.2% DL-isoleucine + 0.2% DL-threonine	4	18.8 ± 1.3
3a.	Basal + 0.2% L-lysine[2] + 0.2% DL-isoleucine + 0.2% DL-threonine	4	22.9 ± 1.7
4.	Basal + 0.1% L-lysine + 0.2% DL-isoleucine + 0.2% DL-threonine + 0.1% DL-methionine + 0.1% DL-tryptophan + 0.2% DL-valine	4	17.8 ± 3.3
4a.	Basal + 0.2% L-lysine[2] + 0.2% DL-isoleucine + 0.2% DL-threonine + 0.1% DL-methionine + 0.1% DL-tryptophan + 0.2% DL-valine	4	22.3 ± 1.0
5.	11% Casein	8	23.3 ± 2.6

[1] Standard deviation of the mean.

[2] Treatments 2a, 3a and 4a supplemental L-lysine was doubled (from 0.1 to 0.2%).

The addition of DL-isoleucine to the ration containing 0.5% of L-lysine did not improve growth rate significantly when added either at the 0.2 or 0.3% level. Similarly, the ration containing 0.5% of L-lysine was not improved by the addition of 0.05 or 0.10% of DL-methionine.

TABLE 4

Effect of supplemental lysine level on the growth of rats fed the basal ration supplemented with isoleucine and threonine

Experiment 3

	TREATMENT	NO. OF RATS	AV. WEEKLY GAIN
			gm
1.	Basal	8	3.5 ± 1.2[1]
2.	Basal + 0.1% L-lysine	8	9.5 ± 3.0
3.	Basal + 0.1% L-lysine + 0.2% DL-isoleucine + 0.2% DL-threonine	8	7.8 ± 2.8
4.	Basal + 0.2% L-lysine	8	9.6 ± 3.7
5.	Basal + 0.2% L-lysine + 0.2% DL-isoleucine + 0.2% DL-threonine	8	17.5 ± 4.3
6.	Basal + 0.3% L-lysine	8	13.7 ± 4.2
7.	Basal + 0.3% L-lysine + 0.2% DL-isoleucine + 0.2% DL-threonine	8	21.3 ± 2.6
8.	Basal + 0.4% L-lysine	8	11.3 ± 3.0
9.	Basal + 0.4% L-lysine + .02% DL-isoleucine + 0.2% DL-threonine	8	23.4 ± 3.6
10.	Basal + 0.5% L-lysine	8	11.0 ± 4.0
11.	Basal + 0.5% L-lysine + 0.2% DL-isoleucine + 0.2% DL-threonine	8	24.9 ± 3.4
12.	11% Casein	5	23.5 ± 2.4
13.	21% Casein	4	34.5 ± 1.3

[1] Standard deviation of the mean.

In all cases the addition of lysine and threonine to the basal ration produced growth rate approximately equal to that obtained with a purified diet containing 11% of casein, but inferior to that obtained when a 21% casein diet was used.

The livers of rats fed the unsupplemented basal ration in experiment 4 contained significantly more ($P < 0.01$) fat than the livers of rats fed the basal ration supplemented with 0.5% of L-lysine and 0.2% of DL-threonine. The livers of rats fed the basal ration contained 19.41 ± 2.44% of fat on a dry-matter

TABLE 5

A comparison of various levels and combinations of isoleucine, methionine and threonine when added to the basal ration supplemented with 0.5% L-lysine

Experiment 4

	TREATMENT	NO. OF RATS	AV. WEEKLY GAIN
			gm
1.	Basal[1]	6	2.3 ± 1.9[2]
2.	Basal + 0.5 L-lysine	8	9.7 ± 2.6
3.	Basal + 0.5% L-lysine + 0.2% DL-isoleucine	8	10.3 ± 3.9
4.	Basal + 0.5% L-lysine + 0.2% DL-threonine[1]	8	27.2 ± 3.6
5.	Basal + 0.5% L-lysine + 0.2% DL-isoleucine + 0.2% DL-threonine	8	26.5 ± 2.0
6.	Basal + 0.5% L-lysine + 0.2% DL-isoleucine + 0.2% DL-threonine + 0.05% DL-methionine	8	28.6 ± 7.1
7.	Basal + 0.5% L-lysine + 0.2% DL-isoleucine + 0.2% DL-threonine + 0.10% DL-methionine	8	28.3 ± 5.5
8.	Basal + 0.5% L-lysine + 0.3% DL-isoleucine	8	8.8 ± 3.5
9.	Basal + 0.5% L-lysine + 0.3% DL-threonine	8	28.2 ± 6.4
10.	Basal + 0.5% L-lysine + 0.3% DL-isoleucine + 0.3% DL-threonine	8	30.8 ± 3.9
11.	Basal + 0.5% L-lysine + 0.05% DL-methionine	5	8.1 ± 3.5
12.	Basal + 0.5% L-lysine + 0.10% DL-methionine	5	9.3 ± 6.9
13.	11% Casein	4	26.2 ± 1.0
14.	21% Casein	4	40.8 ± 1.7

[1] The livers of these rats were removed at the close of the experiment for liver fat determination. The livers of the rats receiving the basal diet contained 19.41 ± 2.44% fat on a dry-weight basis versus 9.54 ± 3.54% for those receiving basal plus lysine and threonine. This difference was highly significant ($P < 0.01$).

[2] Standard deviation of the mean.

basis, versus 9.54 ± 3.54% for the livers of those fed the lysine and threonine supplemented ration.

DISCUSSION

The fact that supplemental lysine was in all cases effective in improving the growth rate of rats fed milo as the sole source of protein verifies the results reported by Shelton et al. ('51) which indicated that lysine is the first limiting amino acid in milo protein.

It is significant to note the importance of a sufficiently high level of the first limiting amino acid in allowing a growth

response from a less limiting amino acid. This was indicated in experiment 1 and further supported in subsequent studies in which the level of supplemental lysine was increased to as high as 0.5% of the ration.

Microbiological assay [3] of the milo for lysine and threonine agreed quite closely with published values. The milo contained 0.20 % of lysine and 0.30 % of threonine. Since the protein content of the variety tested (Kafir 44–14) was 9.13%, the milo protein contained 2.19 and 3.29 gm % of lysine and threonine, respectively. These values are considerably below the requirements estimated for the rat by Rose ('37) and for the growing pig by Mertz et al. ('52).

On the basis of these analyses the milo ration supplemented with 0.5% of L-lysine and 0.2% of DL-threonine contained a total of 0.69% of lysine and 0.38% of threonine when expressed as the biologically active L-form. This corresponds to 7.52 gm of lysine and 4.14 gm of threonine per 100 gm of protein. Since both of these values exceed the estimated requirements, on a percentage of protein basis, it is not surprising that the largest growth response was obtained when lysine and threonine were added at those levels.

The lack of growth response from supplemental methionine in these studies is not explained, in view of the fact that calculations from average analyses of milo indicate that methionine is present in insufficient amounts for normal growth. In this regard, Benton et al. ('55) found that little if any growth response could be obtained when methionine was added to a corn diet fed to rats, although a rapid rate of growth was obtained by adding other amino acids. It is possible that the cystine level of the milo used in these studies was sufficiently high to meet the sulfur amino acid requirement.

The average isoleucine and leucine content of milo indicates that isoleucine is present in an inadequate amount for normal growth while leucine is present far in excess of the requirement for growth. Since Harper et al. ('55) demonstrated an

[3] Done under the direction of Dr. R. J. Sirny, Agricultural Chemistry Department.

anatagonistic effect of large amounts of leucine on the availability of isoleucine, a beneficial effect on growth rate seemed likely by supplementation with isoleucine in these studies. The failure of added isoleucine to stimulate growth rate in any of these studies is not readily explained. It is possible that another amino acid not yet considered is more limiting than isoleucine, thereby preventing any response from isoleucine in these studies. The lipotropic effect of added amino acids obtained in experiment 4 supports the findings of Harper et al. ('53) and Winje et al. ('54), who reported that threonine reduced the liver fat of rats fed low-protein diets.

No growth response was obtained from added valine or tryptophan in experiment 2, and good growth was obtained without their addition in experiments 3 and 4. Average amino acid analyses for milo indicate that both are present somewhat in excess of the requirement for growth. The dissimilarity between milo protein and corn protein with respect to tryptophan (Osborne and Mendel, '14) and between milo and rye with respect to valine (Sure, '54) is indicated here.

The reason for the difference among experiments in growth response of rats fed rations containing 11 and 21% of casein is not clear. It is possible that the inefficient temperature control of the room in which the rats were housed, especially during the summer months, had some influence in this regard.

It appears that lysine is without question the first limiting amino acid and that threonine is probably the second limiting amino acid in milo grain.

SUMMARY

Weanling male rats were used to study the effects of supplementing milo with amino acids. The addition of 0.5% of L-lysine and 0.2% of DL-threonine to the basal ration produced growth approximately equal to that obtained with a purified diet containing 11% of casein, but inferior to that obtained with a 21% casein purified diet. The addition of 0.2 or 0.3% of DL-isoleucine, 0.05 or 0.10% of DL-methionine, 0.1% of DL-

tryptophan or 0.2% of DL-valine had no effect on growth rate in any of the combinations used.

The liver fat content of rats receiving the basal ration was significantly reduced by the addition of 0.5% of L-lysine and 0.2% of DL-threonine.

Lysine and threonine are probably the most limiting amino acids in milo for growth.

LITERATURE CITED

BENTON, D. A., A. E. HARPER AND C. A. ELVEHJEM 1955 Effect of isoleucine supplementation on the growth of rats fed zein or corn diets. Arch. Biochem. Biophys., *57:* 13.

EGGERT, R. G., M. J. BRINEGAR AND C. R. ANDERSON 1953 The quality of protein of normal and high protein corn for growing swine. J. Animal Sci., *12:* 282.

HARPER, A. E., D. A. BENTON AND C. A. ELVEHJEM 1955 L-leucine, an isoleucine antagonist in the rat. Arch. Biochem. Biophys., *57:* 1.

HARPER, A. E., W. J. MONSON, D. A. BENTON AND C. A. ELVEHJEM 1953 The influence of protein and certain amino acids, particularly threonine, on the deposition of fat in the liver of the rat. J. Nutrition, *50:* 383.

HEGSTED, D. M., R. C. MILLS, C. A. ELVEHJEM AND E. B. HART 1941 Choline in the nutrition of chicks. J. Biol. Chem., *138:* 459.

HILLIER, J. C., R. MACVICAR AND W. G. POND 1954 Grain sorghum as a feed for swine. Okla. Agr. Exp. Sta. Misc. Pub. MP–34, p. 94.

MERTZ, E. T., W. M. BEESON AND H. D. JACKSON 1952 Quantitative threonine requirement of the weanling pig. J. Animal Sci., *11:* 771.

OSBORNE, T. B., AND L. B. MENDEL 1914 Nutritive properties of proteins of the maize kernel. J. Biol. Chem., *18:* 1.

PECORA, L. J., AND J. M. HUNDLEY 1951 Nutritional improvement of white polished rice by the addition of lysine and threonine. J. Nutrition, *44:* 101.

ROSE, W. C. 1937 The nutritive significance of the amino acids and certain related compounds. Science, *86:* 298.

SHELTON, M., J. R. COUCH, F. HALE, J. H. JONES, R. E. LEIGHTON, C. M. LYMAN AND J. K. BRIGGS 1951 Grain sorghum by-product feeds for farm animals. Texas Agr. Exp. Sta. Bul. 743.

SNEDECOR, G. W. 1956 Statistical Methods. 5th Edition, The Iowa State College Press.

SURE, B. 1954 Protein supplementation-relative nutritive values of proteins in whole wheat and whole rye and effect of amino acid supplements. J. Agr. Food Chem., *2:* 1108.

WINJE, M. E., A. E. HARPER, D. A. BENTON, R. E. BOLDT AND C. A. ELVEHJEM 1954 Effect of dietary amino acid balance on fat deposition in the liver of rats fed low protein diets. J. Nutrition, *54:* 155.

SIGNIFICANCE OF DIETARY ZINC FOR THE GROWING CHICKEN[1]

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The indispensability of zinc in the diet of animals has been recognized for many years, but the symptoms of a deficiency in the chick have not been described. Since the zinc requirement of the growing rat is so low that extreme care must be exercised in order to produce a deficiency (Stirn et al., '35), the significance of zinc in practical nutrition was almost dismissed until it was observed (Tucker and Salmon, '55) that zinc will cure parakeratosis in swine. In view of the high levels of zinc now added to swine rations and reports of zinc toxicity in poultry, Mehring, Brumbaugh and Titus ('56) studied the effect of graded levels, 15 to 778 p.p.m., of zinc added to a practical broiler ration that contained about 40 p.p.m. of zinc. Under these conditions zinc had no effect on growth or feed efficiency.

Dannenburg et al. ('55), and Morrison et al. ('55) have reported the existence of an unidentified mineral required by the chick fed a simplified diet that contained a purified soybean protein. Subsequently, Camp et al. ('56) and Menge et al. ('56) observed a growth stimulation from the addition of the ash of natural products to practical type corn-soya diets. Morrison et al. ('56) have extended their observations on the unidentified mineral required by the chick and reported

[1] Approved by the Director of the Missouri Agricultural Experiment Station, Journal Series no. 1856. A preliminary report of this work has been presented, Federation Proc., *16:* 394, 1957.

that it is involved in bone formation. O'Dell and Savage ('57) found that, for optimum growth, the basal diet of Dannenburg et al. ('55) is deficient in both potassium and zinc and they suggested that zinc is an important constituent of the ash of distillers' dried solubles.

This report is concerned with the significance of zinc in purified diets as used in assays for unidentified factors and with the pathology of a zinc deficiency in the growing chick. It was found that with respect to zinc some commonly used purified diets are on the borderline of adequacy.

TABLE 1

Composition of basal ration

CONSTITUENTS		COMPOSITION OF SALT MIXTURE	
	%		%
Washed soy protein	25.0	$CaHPO_4$ (U.S.P.)	56.8
Glucose hydrate	62.85	K_2HPO_4 (N.F.)	4.3
DL-Methionine	0.8	$CaCO_3$ (U.S.P.)	9.74
Glycine	0.4	$MgSO_4 \cdot 7H_2O$ (Reagent)	9.5
Salt mixture	6.0	NaCl (U.S.P.)	15.6
KCl (U.S.P.)	0.5	Fe Citrate$\cdot 5H_2O$ (U.S.P.)	2.6
$CaCO_3$ (U.S.P.)	1.25	$MnSO_4 \cdot H_2O$ (Reagent)	1.36
Soybean oil	3.0	KI (Reagent)	0.07
Choline Cl	0.2	$CuSO_4 \cdot 5H_2O$ (Reagent)	0.03
Vitamin supplement[1]	+		

[1] The vitamin supplement supplied per 100 gm of diet: vitamin A, 2000 I.U.; vitamin D, 430 I.C.U.; menadione, 2.5; alpha-tocopheryl acetate 2.5; thiamine·HCl, 1.0; riboflavin, 1.0; pyridoxine·HCl, 1.0; Ca pantothenate, 3.0; niacin, 5.0; inositol, 50.0; biotin, 0.04; folacin, 0.2, and cyanocobalamin, 0.003 mg. The fat-soluble vitamins were premixed with soybean oil and an antioxidant, so that the final diet contained 0.0125% Santoquin (Monsanto Chemical Co.). The other vitamins were premixed with glucose.

EXPERIMENTAL

The composition of the basal diet used in this study is shown in table 1.[2] The diet of Dannenburg et al. ('55) was modified to include 0.5% of KCl and 1.25% of $CaCO_3$ and the vitamin supplement was one commonly used in our laboratory. The soy protein[2] was washed three times with 5

[2] The C–1 assay protein was obtained from the Drackett Products Company, now owned by the Archer-Daniels-Midland Company, Cincinnati, Ohio.

volumes of tap water and once with distilled water. It was then dried at 60°C and ground. This procedure decreased the ash content to less than 1.5% but lowered the zinc content only slightly. The basal ration contained about 0.4% of potassium, 1.5% of calcium and 0.7% of phosphorus. Analysis by a spectrographic procedure indicated 15 p.p.m of zinc (average of 8 analyses) and 0.6 p.p.m. of molybdenum. Feed was supplied ad libitum and distilled water was available in glass and plastic fountains.

Male, day-old, White Rock or White Cornish-White Rock cross-bred chicks, produced by hens on a breeder-type ration, were used. They were kept in galvanized, electrically-heated batteries except that in the later trials the batteries were treated with lacquer to decrease the availability of zinc from the galvanized parts.

Distillers' dried solubles were added to the ration at the expense of protein and glucose so as to maintain a constant level of nitrogen. The ash of distillers' dried solubles was prepared by preliminary burning in a pyrex dish followed by ashing in a muffle furnace at 500°C for 8 hours. The yield of ash was, on the average, 10% of the air dry weight.

The bones used for measurements and ash determination were taken from the chicks immediately after sacrifice and were cleaned of most of the muscle and connective tissue. The bones were then placed in boiling water for one minute to aid in removal of remaining soft tissue. Measurements were made with calipers so as to give maximum length. The diameter was measured at the minimum point but across the flat portion of the bone. Ash determinations were made on dry, fat-free bones according to the AOAC procedure ('55).

RESULTS

A. Growth response and zinc requirement

The growth response to zinc and ash supplements under the various conditions employed is shown in table 2. The level of zinc commonly added to purified diets is 5 to 7 p.p.m.

TABLE 2

Effect of zinc and distillers' dried solubles on weights of chicks at 4 weeks of age

RATION NUMBER	SUPPLEMENTS		GALVANIZED BATTERY WHITE ROCKS		GALVANIZED BATTERY CORNISH × W. R.		LACQUERED BATTERY CORNISH × W. R.	
	Zinc	Other	No.	Weight	No.	Weight	No.	Weight
	p.p.m.			*gm*		*gm*		*gm*
4732	0	None	49	350 ± 8 [1]	40	431 ± 7	36	170 ± 7
4733	7	None	50	386 ± 7	20	457 ± 13	39	305 ± 9
4988	10	None			9	466 ± 18	19	346 ± 11
4779	13	None	19	405 ± 12	20	482 ± 11	20	399 ± 12
5070	23	None					19	476 ± 10
4734	63	None	66	423 ± 7	38	470 ± 16	19	471 ± 11
4787	119	None	20	437 ± 14	10	474 ± 30		
4985	7	Ash of DDS [2]					40	407 ± 8
4780	63	Ash of DDS [2]	19	440 ± 15	38	490 ± 12		
4735	63	DDS (5%)	48	457 ± 7	38	512 ± 7		

Significance of differences by "t" test

	UNTREATED BATTERY WHITE ROCKS		UNTREATED BATTERY CORNISH × W. R.	
	t	p	t	p
4733 vs. 4734	3.89	< 0.01	0.54	> 0.05
4734 vs. 4780	1.16	> 0.05	1.01	> 0.05
4734 vs. 4735	3.44	< 0.01	2.28	ca 0.02

[1] Standard error of mean.

[2] DDS = Distillers' dried solubles. The amount of ash fed was equivalent to 5% of distillers' dried solubles.

The addition of 7 p.p.m. of zinc (4733), always gave a growth response over the basal ration (4732) and the addition of 63 p.p.m. of zinc (4734) gave a response over that obtained on 4733. This response was highly significant ($p < 0.01$) in the first trials carried out in relatively new, galvanized batteries. In later trials, in which the crossbred chicks were used, the response to zinc decreased. It seems possible that more zinc became available from the galvanized metal with aging. When the batteries were lacquered the response to zinc supplementation was dramatic and the growth rate increased progressively with supplementation.

From the data obtained with chicks kept in lacquered batteries, it is possible to estimate the zinc requirement under the conditions imposed. The basal ration assayed 15 p.p.m. of zinc and this value, added to the supplemental levels given in table 2 is the assumed zinc content of the diets. When the weights were plotted on the y axis and the logarithm of the zinc content on the x axis a straight line relationship existed up to about 30 p.p.m. of zinc. The equation for this line as determined by the method of least squares is $y = -760 + 802x$. The weights at 38 and 77 p.p.m. were essentially equal and the point of intersection of a line through these points with the first line gives an estimate of the zinc requirement. The value obtained by this procedure is 35 p.p.m. This value is of the same order of magnitude as the zinc requirement of swine, (Lewis et al., '57).

The zinc content of the distillers' dried solubles, as determined by chemical and spectrographic analysis, was about 70 p.p.m. Thus the ash equivalent to 5% should have supplied about 3.5 p.p.m. Biological assay based on the curve described above and the weight obtained on ration 4985 indicated that the ash equivalent to 5% supplied 6 to 7 p.p.m. of zinc. Although this value is higher than that obtained by chemical assay and might be interpreted to indicate an additional nutrient in the ash, the difference may be within the experimental error. This interpretation seems more likely in view of the failure to obtain a significant growth response from

the addition of ash (4780) to an adequate level of zinc. Supplements of the unashed distillers' dried solubles consistently gave a slight growth response when added to a diet that contained sufficient zinc.

B. Bone and feather development

In addition to the decreased rate of growth, the most obvious symptom of a mild zinc deficiency was the shortening and thickening of the long bones and a tendency for the hock joint to enlarge. Other obvious defects were poor feather development and a rapid and labored respiration. The feathers tended to be frizzly and stand out from the body. These characteristics are illustrated in the 6-week old chick in figure 2. The results of bone measurements and ash determinations are shown in table 3.

The bones from deficient chicks were shorter than the control bones but the greater effect was on bone diameter. The diameters of the tibiae and humeri were related to body weight and the average ratio of diameter to body weight was significantly greater in the deficient chicks. The ratio of length to diameter of the bones was significantly less in the zinc-deficient animals. Ash determinations made on tibiae from 10 mildly deficient chicks reared in galvanized batteries and on bones from equal numbers of control chicks showed no significant difference at either 4 or 6 weeks of age. The difference in the case of the more severely depleted birds kept in lacquered batteries, approached statistical significance at the 5% level.

C. Effect of dietary calcium on zinc requirement

The calcium content (1.6%) of the basal diet was higher than is commonly used in chick diets. This experimental design was used on the assumption that high calcium would accentuate zinc deficiency symptoms. In order to test this hypothesis three levels of calcium, 1.1, 1.6, and 2.1% were fed, with and without added zinc. The lowest level of calcium was

TABLE 3

Effect of zinc deficiency on bone development

ZINC ADDED	NUMBER ANALYZED	AVERAGE BODY WEIGHT	TIBIA			METATARSUS	
			Ash [1]	Diam./B.W.[2]	L/D [3]	Diam./B.W.	L/D
p.p.m.		*gm*	%	$\times 10^{-3}$		$\times 10^{-3}$	
			A. Chicks in galvanized battery; killed at 4 weeks				
0	28	359	45.45	1.62 [5] ± 0.04 [4]	11.4 [5] ± 0.3	1.86 [5] ± 0.04	7.3 [5] ± 0.2
63	28	413	45.40	1.43 ± 0.03	12.4 ± 0.2	1.54 ± 0.03	8.5 ± 0.1
			B. Chicks in galvanized battery; killed at 6 weeks				
0	13	593	46.96	1.28 [5] ± 0.03	10.2 [5] ± 0.4	1.33 [6] ± 0.03	7.1 [5] ± 0.6
63	7	875	47.46	0.90 ± 0.01	12.5 ± 1.4	0.93 ± 0.03	9.0 ± 0.8
			C. Chicks in lacquered battery; killed at 4 weeks				
0	16	164	45.83	2.8 [5] ± 0.1	11.2 [5] ± 0.5	3.3 [6] ± 0.1	6.6 [5] ± 0.4
63	7	452	47.35	1.3 ± 0.5	13.1 ± 0.3	1.5 ± 0.5	8.7 ± 0.3

[1] Ash of dry, fat-free bones.

[2] Ratio of diameter of bone in centimeters to body weight in grams.

[3] Ratio of length to diameter of bone.

[4] Significance of difference of means determined by the "t" test.: [5] signifies $p < 0.01$; [6] signifies $p < 0.05$.

provided by the substitution of 1.25% of glucose for an equal amount of calcium carbonate in the basal diet and the highest level by the substitution of 1.25% of calcium carbonate for glucose. The results are presented in table 4. In this comparison the chicks were kept in lacquered batteries and at the end of the trial were scored as to feathering, incidence of keratosis and of an abnormal gait. The gross appearance of the keratosis is shown in figure 8. Nearly all of the deficient chicks in this trial exhibited an unsteadiness of

TABLE 4

Effect of dietary calcium level on zinc deficiency

(Chicks in lacquered battery)

CALCIUM CONTENT	ZINC ADDED	NUMBER OF CHICKS	WEIGHT AT 4 WEEKS	FEATHER SCORE [1]	INCIDENCE OF KERATOSIS [2]	ABNORMAL GAIT [3]
%	*p.p.m.*		*gm*		%	%
1.1	0	30	208 ± 10 [4]	< 1	17	67
1.1	63	27	468 ± 12	3.5	0	0
1.6	0	36	170 ± 7 [5]	< 1	44	83
1.6	63	49	462 ± 7	3.5	0	0
2.1	0	30	162 ± 8 [5]	< 1	50	83
2.1	63	29	478 ± 10	3.5	0	0

[1] Excellent feathering was assigned a score of 4.

[2] Chicks with cracked and scaly feet were classed as having keratosis.

[3] Abnormal gait implies unsteadiness of gait or tendency to squat and raise feet abnormally high or both.

[4] Standard error of the mean.

[5] $p < 0.01$ when zinc deficiency rations that contained 1.6% or 2.1% of calcium were compared to the ration that contained 1.1% of calcium and no added zinc.

gait and a leg weakness or tendency to squat unless vigorously disturbed. Nearly all the chicks on the low-zinc diets in this trial showed severe deficiency symptoms, including poor growth, almost complete failure in feather development after one week, skin lesions, particularly on the feet and around the beak, leg weakness, and an abnormal and rapid respiration. The gross appearance and feather developments are shown in figures 4 and 6, respectively. The feathers that developed were extremely brittle and because of lack of barbules failed to lace together.

It is clear from the data in table 4 that the chick can tolerate rather high levels of calcium, if adequate zinc is supplied, inasmuch as the highest rate of gain occurred at the highest calcium level and there were no gross abnormalities. In the case of the low-zinc diets the increase of calcium from 1.1 to 1.6% depressed the rate of gain about 20%, a decrease that is statistically significant. With the number of chicks observed, 2.1% calcium was no more damaging than 1.6%. The higher levels of calcium tended to increase keratosis and the incidence of abnormal gait but none of the effects were striking. It would appear that high levels of calcium in the diet of a chick are not as detrimental as in the case of swine, (Leucke et al., '57; Lewis et al., '57).

D. Blood studies

The rate of respiration in the zinc-deficient chicks was abnormally high and averaged about 50 per minute as compared to about 40 in the controls. This difference was exaggerated by excitement. There was no pathology of the respiratory system that would explain the dyspnea. Since carbonic anhydrase contains zinc and is involved in carbon dioxide transport, the concentration of this enzyme in blood was determined by the use of an adaptation of the method of Philpot and Philpot ('36). The results, summarized in table 5, showed no significant difference, but the deficient birds tended to have a higher carbonic anhydrase activity. The explanation for this probably lies in the fact that there were actually more erythrocytes per volume of blood in the deficient chicks.

The volume of packed red cells was about 25% higher (table 5) in the deficient chicks than in the zinc-supplemented controls. Whether this is due to a stimulation of erythropoiesis or to hemoconcentration is not clear, but the latter seems more probable in view of decreased feed and probably decreased water consumption. The average hematocrit value for the zinc-supplemented chicks was lower than for the controls fed a practical diet and somewhat lower than the ac-

cepted normal value. Approximately 10% of the chicks had hematocrits low enough to be classed as anemic. The explanation for these low values is not obvious. Supplements of iron, copper and cobalt as well as of distillers' dried solubles did not improve the blood picture. Apparently a high percentage of red cells is typical of a severe zinc deficiency but it probably is unrelated to the abnormal respiration. The concentration of carbonic anhydrase in the red cells does not explain the respiratory difficulty and at present there is no obvious explanation.

TABLE 5

Effect of zinc on hematocrit values and on carbonic anhydrase content of blood

SUPPLEMENTS TO BASAL		HEMATOCRIT	CARBONIC ANHYDRASE [1]
Zinc	Other than zinc		
p.p.m.		*vol. %*	*sec*
0	None	35 (28) [2]	8.6 (4)
63	None	28 (39)	11.6 (3)
63	5% DDS [3]	28 (10)	—
63	6 mg % $CuSO_4 \cdot 5H_2O$ 80 mg % $FePO_4 \cdot 4H_2O$ 4 mg % $CoCl_2 \cdot 6H_2O$	28 (9)	—
Practical ration [4]		31 (19)	—

[1] Carbonic anhydrase was determined by the method of Philpot and Philpot ('36). The blank required 42 sec to reach the end point. One milliliter of blood (100 × dilution) was used for the comparisons.

[2] The numbers of animals studied are given within parentheses.

[3] DDS = Distillers' dried solubles.

[4] Ration 4428, Laerdal et al. ('57).

Histopathology

Long bones. The histology of the long bones from the zinc-supplemented chicks appeared normal in all respects (fig. 9), but sections of the tibia, femur and humerus from chicks fed the low-zinc diet showed rather marked pathology. The epiphyseal cartilage was reduced considerably in width and there was less cell division. The hypertrophied maturing cartilage cells were smaller and appeared less active (fig. 10). The bony collar of the shaft was thinner but the diameter

of the bone was greater in the deficient birds. Osteoblasts were fewer in number and the Haversian canals were larger (fig. 12). A striking observation was the presence of considerable hemopoietic activity in the Haversian canals of chicks that received the low-zinc diet.

Myeloid and lymphoid systems. There was no difference in the appearance of marrow of the long bones between the deficient and zinc-supplemented groups. The lymphoid tissues of the thymus and bursa of Fabricius were atrophic in the zinc-deficient chicks.

Circulatory and respiratory systems. Tissues examined from these two systems showed no difference between the groups on the low-zinc diet and the controls.

Skin. No abnormalities were observed in the skin of control birds (fig. 14). The skin of birds on the low-zinc diet showed hyperkeratinization and a mild thickening of the epidermis. These changes were most marked in the skin of the wings and that of the legs and feet (fig. 13). The epidermis was increased in thickness from the normal of three to 5 cells to layers of 7 or 8 cells, but there was no detectable increase in mitotic activity in the basal layer. On the surface of the epithelium the keratinized layers were increased in thickness. This was most pronounced on the shanks and feet where it is normally thickest. The hyperkeratinization of the skin extended into the feather follicle and resulted in atrophy of both follicle and feather or in failure of feather development. In many places the follicles were replaced by a fibrous tissue containing a few large mononuclear cells. The follicles in early stages of degeneration showed a milder degree of keratinization with an infiltration of polymorphonuclear leukocytes, large mononuclear phagocytes, lymphocytes and fibroblasts.

Nervous system. Sections of the brain, spinal cord and peripheral nerves revealed no detectable differences between the two groups of chicks.

Alimentary canal. The esophagus showed the most consistent change of any structure in the alimentary canal. The

normal esophagus is made up of a basal cell layer arranged in a palisade with a sharp demarcation between this layer and the underlying fibrous stroma of supporting tissue. The basal layer normally shows some degree of mitotic activity. Toward the esophageal lumen several layers of stratified squamous cells are found with oval or round nuclei. As the lumen is approached the cells become flattened, the nuclei pyknotic, and keratinization or scaling occurs (fig. 15). In the zinc-deficient chicks the basal layer contained an increased number of cells but no apparent increase in mitotic figures (fig. 16). The number of cells overlying the basal layer was approximately doubled and the cells retained the morphology of the lower strata to a level near the lumen. There the cells appeared to be piled up in an irregular manner. The flattened cells near the lumen were embedded in a pink-staining granular material and the mass contained large numbers of gram positive bacteria. The submucosa, connective tissue and musculature appeared normal. The total picture in the esophagus of the birds on the zinc-low diet was similiar to the parakeratosis described in the skin of zinc-deficient swine. The changes extended the length of the esophagus but were not observed elsewhere in the digestive tract. The pancreas exhibited a hyperplasia and increase in number of islets of Langerhans but many of the islet cells had undergone hydropic degeneration.

DISCUSSION

Although quantitative data on the requirement of the rat for zinc are not available, the requirement for growth is only a few parts per million of diet (Stirn et al., '35). In view of the low requirement of the rat, it is somewhat surprising that swine and poultry require at least 30 to 40 p.p.m. of zinc in the diet. Most practical swine and poultry rations of the corn-soya type contain about 30 to 40 p.p.m. of zinc and thus under certain conditions are probably on the borderline of adequacy. The purified diets that are commonly used for experimental purposes contain somewhat less than the require-

ment as determined in this study and their adequacy depends on the zinc content of the protein, the level of calcium in the diet, and on the availability of non-dietary sources of zinc. When chicks are kept in galvanized batteries the requirement may be nearly fulfilled but there is a possibility that a mild deficiency will exist. The chances for a zinc deficiency under such conditions will be enhanced by a high-calcium level in the diet, the use of distilled water, the use of chicks from zinc-depleted hens and possibly by the use of newly galvanized cages. In view of these considerations, it seems possible that some of the work which led to the postulation of unidentified minerals required by the chick (Dannenburg et al., '55; Morrison et al., '55 and Menge et al., '56), was complicated by a zinc deficiency. The same possibility of complication exists in the case of the many postulates of organic growth factors.

Morrison et al. ('56) reported that an unidentified mineral found in the ash of crude feedstuffs was involved in bone formation. The malformation was characterized by enlargement and elongation of the intertarsal or hock joint. The tibiotarsae of the chick on the basal diet contained less ash, were slightly shorter and had a lower breaking strength than the bones from ash-supplemented chicks. Although the ash had no effect on the blood picture, the hematocrits were low (26%) in all groups. The active component of the ash behaved as a cation in acid solution. The properties of the active ion as well as the symptoms of the deficiency suggest that zinc may have been involved. Morrison et al. ('56) supplemented their diet with a reconstituted ash which supplied 3 p.p.m. of zinc and it failed to improve the rate of growth, but no mention was made of its effect on the bone development. It is not implied here that zinc is the only deficiency in their diet that is corrected by the ash of natural products, but the question may be raised whether or not sufficient zinc was supplied to insure maximum growth and normal bone formation.

The effect of high-calcium intake on the zinc requirement of the chick is analogous to that observed in swine but the effect appears to be much less marked. In the presence of

adequate zinc the chick thrives well even when the calcium level is as high as 2.1%. In the absence of supplemental zinc there was little or no difference between 2.1 and 1.6% of calcium as regards growth and gross appearance, but at a calcium level of 1.1% there was a noticeable improvement in all respects.

Since the basal diet used in this study contained about 15 p.p.m. of zinc it is doubtful that the chicks were as depleted of zinc as has been achieved in the rat. Although there was little or no mortality in the 4-week period, the severity of the pathology observed in the chick approached that observed in the rat (Follis et al., '41). The failure to find a decrease in the carbonic anhydrase activity of the blood is not surprising in view of the failure of Hove et al. ('40) to find a change in severely depleted rats. The pathology of the bone observed in this study is circumstantial evidence that zinc is involved in calcification and possibly with the phosphatase activity. The parakeratosis-like effect in the esophagus is entirely analogous to that observed in zinc-deficient swine whereas the skin lesion in the chick is a hyperkeratosis rather than parkeratosis. The degeneration of the feather follicles that results from the hyperkeratosis no doubt explains the poor feather development. The observations made here support the view that keratinization is an active process and that it requires zinc.

SUMMARY

Broiler strain chicks were maintained on a zinc-deficient diet (15 p.p.m.) for periods of 4 or 6 weeks. In part of the trials the chicks were kept in galvanized batteries and they developed mild symptoms of a zinc deficiency. In other trials the batteries were coated with lacquer and the chicks developed severe deficiency symptoms.

The symptoms of a mild deficiency included slow growth, shortening and thickening of the long bones, development of frizzled feathers, an abnormal respiration and an unsteady gait.

The more severely depleted chicks exhibited these same symptoms but to a greater degree and, in addition, developed a keratosis of the skin and an increased packed red cell volume. Microscopically there was evidence of parakeratosis in the esophagus and of poor calcification of the bone. There also appeared to be a failure of cartilage cell development in the epiphyseal plate region of the long bones and decreased osteoblastic activity in the thin bony collar.

The zinc requirement of chicks fed 1.6% of calicum, 0.7% of phosphorus and maintained in lacquered batteries was estimated to be 35 p.p.m. This requirement was decreased slightly by lowering the calcium to 1.1%, but did not appear to be increased by 2.1% of calcium. In the presence of adequate zinc the chicks grew equally well at all calcium levels.

The addition of the ash equivalent to 5% of dried distillers' solubles to a diet that contained adequate zinc did not improve the rate of gain significantly.

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LITERATURE CITED

ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS 1955 8th Ed., Washington, D. C.

CAMP, A. A., B. L. REID AND J. R. COUCH 1956 Growth promoting activity of ash when fed in practical diets to chicks. Poultry Sci., *35:* 621–627.

DANNENBURG, W. N., B. L. REID, E. E. ROZACKY AND J. R. COUCH 1955 An inorganic chick growth response. Ibid., *34:* 1023–1026.

FOLLIS, R. H., H. G. DAY AND E. V. MCCOLLUM 1941 Histological studies of the tissues of rats fed a diet extremely low in zinc. J. Nutrition, *22:* 223–237.

HOVE, E., C. A. ELVEHJEM AND E. B. HART 1940 The relation of zinc to carbonic anhydrase. J. Biol. Chem., *136:* 425–434.

LAERDAL, OSM. A., P. M. NEWBERNE, J. E. SAVAGE AND B. L. O'DELL 1957 A direct method for determination of digestibility in growing chickens. Poultry Sci., *36:* 815–820.

LEWIS, P. K. JR., W. G. HOEKSTRA AND R. H. GRUMMER 1957 Restricted calcium feeding versus zinc supplementation for the control of parakeratosis in swine. J. Animal Sci., *16:* 578–588.

LUECKE, R. W., J. A. HOEFER, W. S. BRAMMELL AND D. A. SCHMIDT 1957 Calcium and zinc in parakeratosis of swine. Ibid., *16:* 3–11.

MEHRING, A. L., J. H. BRUMBAUGH AND H. W. TITUS 1956 A comparison of the growth of chicks fed diets containing different quantities of zinc. Poultry Sci., *35:* 956–958.

MENGE, H., R. J. LILLIE, J. R. SIZEMORE AND C. A. DENTON 1956 An unidentified mineral required by the chick. Ibid., *35:* 244–245.

MORRISON, A. B., M. L. SCOTT AND L. C. NORRIS 1955 Evidence for an unidentified mineral required by the chick. Ibid., *34:* 738–740.

MORRISON, A. B., R. DAM, L. C. NORRIS AND M. L. SCOTT 1956 Furthur evidence on the requirement of the chick for unidentified minerals. J. Nutrition, *60:* 283–296.

O'DELL, B. L., AND J. E. SAVAGE 1957 Potassium, zinc and distillers' dried solubles as supplements to a purified diet. Poultry Sci., *36:* 459–460.

PHILPOT, F. J., AND J. S. L. PHILPOT 1936 A modified colorimetric estimation of carbonic anhydrase. Biochem. J., *30:* 2191–2193.

STIRN, F. E., C. A. ELVEHJEM AND E. B. HART 1935 The indispensability of zinc in the nutrition of the rat. J. Biol. Chem., *109:* 347–359.

TUCKER, H. F., AND W. D. SALMON 1955 Parakeratosis or zinc deficiency disease in the pig. Proc. Soc. Exp. Biol. Med., *88:* 613–616.

PLATES

PLATE 1

EXPLANATION OF FIGURES

1 Control chick, 6 weeks of age, on zinc-supplemented (63 p.p.m.) diet. White Rock male reared in galvanized battery.

2 Chick mildly deficient in zinc reared on basal diet in a galvanized battery. Note short, thick legs, frizzled feathers and panting reaction to handling.

3 Control chick, 4 weeks of age, on zinc-supplemented (63 p.p.m.) diet. White Cornish × White Rock cross, male.

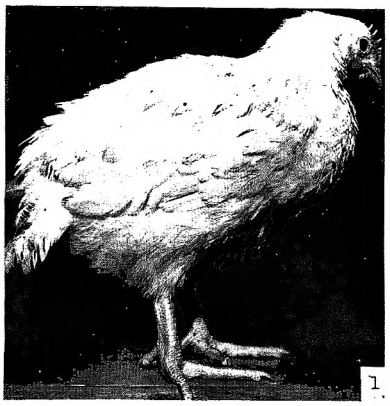
4 Chick, 4 weeks of age, showing acute deficiency of zinc. White Cornish × White Rock cross, reared in a lacquered cage. Note short legs, poor feathering, and scaly legs.

5 Same chick as in figure 3 showing wing feathers.

6 Chick from same group as the one in figure 4 showing poorly developed and broken wing primaries.

7 Same chick as in figure 3 showing close-up of feet. Note normal smooth texture.

8 Same chick as shown in figure 4 showing keratosis and dermatitis of the feet.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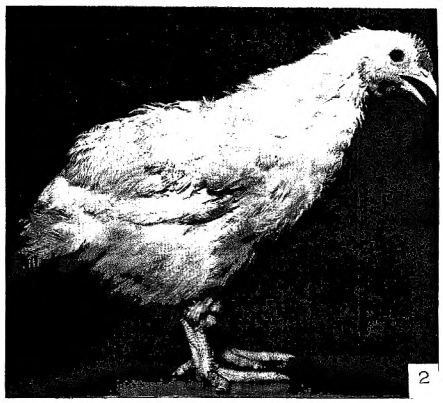
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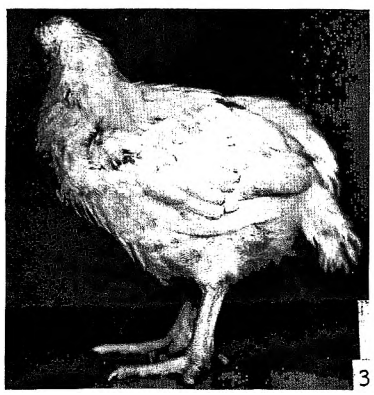
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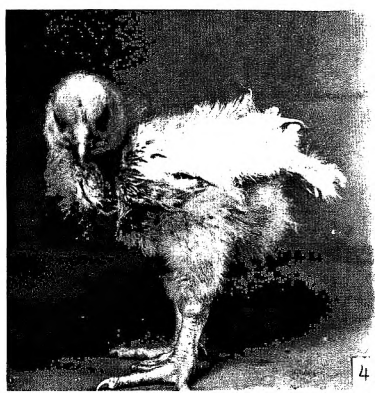
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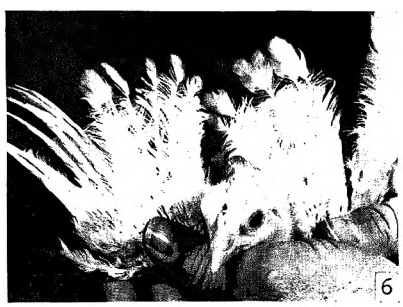
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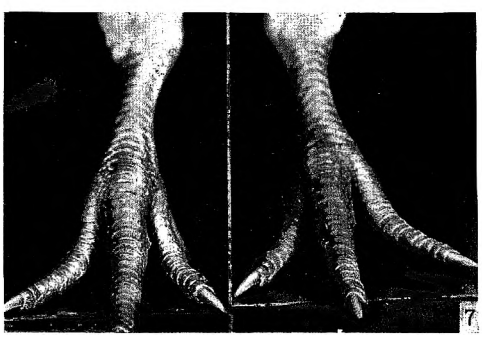
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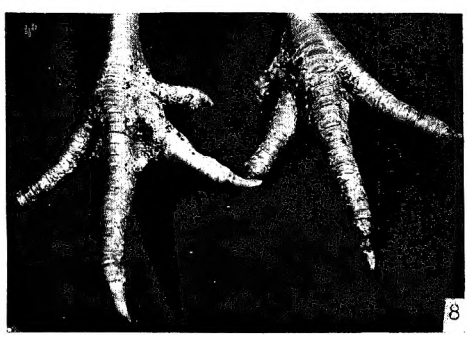
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PLATE 2

EXPLANATION OF FIGURES

9 Epiphyseal cartilage of a femur of a normal 4-week old chick. Hematoxylin and Eosin stain. × 90.

10 Epiphyseal cartilage of a femur from a 4-week old chick on a low zinc diet. Note narrow zone of proliferating cartilage cells which appear smaller and less active in maturing zone. Hematoxylin and Eosin stain. × 90.

11 Bony collar from shaft of femur of a normal 4-week old chick. Hematoxylin and Eosin stain. × 90.

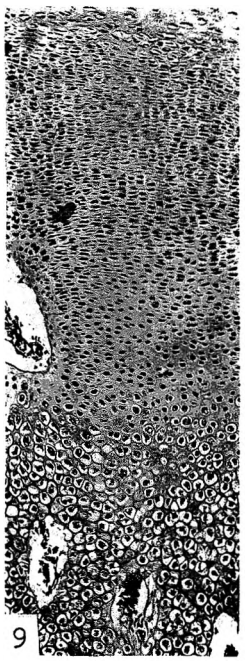
12 Bony collar from shaft of femur of a 4-week old chick on a low-zinc diet. Note large Haversian canals with active hemopoiesis and fewer osteoblasts than normal. Hematoxylin and Eosin stain. × 90.

13 Skin from shank (metatarsus) of 4-week old chick on low zinc diet to show hyperkeratinization of epithelium. Hematoxylin and Eosin stain. × 88.

14 Skin from shank (metatarsus) of normal 4-week old chick. Hematoxylin and Eosin stain. × 288.

15 Epithelium of esophagus from normal 4-week old chick. Hematoxylin and Eosin stain. × 288.

16 Epithelium of esophagus from 4-week old chick on low-zinc diet to show lack of keratinization and piling up of cells, debris and bacteria on surface. Hematoxylin and Eosin stain. × 288.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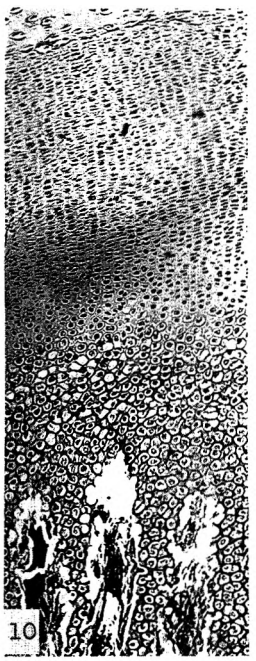
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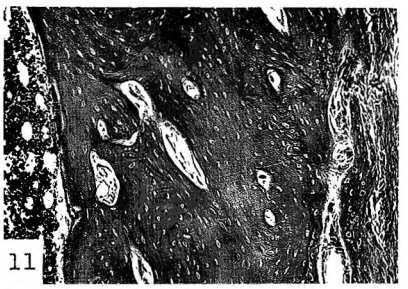
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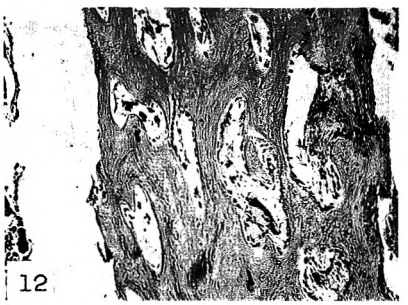
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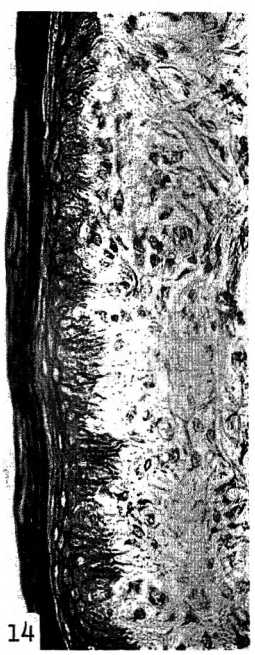
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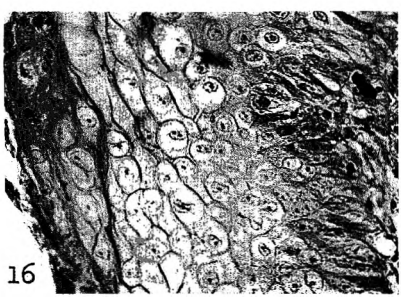
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EFFECT OF THYROPROTEIN AND PENICILLIN ON THE THIAMINE REQUIREMENT AND GROWTH OF NORMAL AND HYPERTHYROID RATS [1]

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The growth-promoting effects of antibiotics in experimental animals have been known for several years, although their exact mode of action is not yet clear. The production of experimental hyperthyroidism by the oral administration of thyroidally-active iodocasein is accompanied by a growth inhibition in rats. This growth inhibition can be overcome by the addition of certain antibiotics (Meites and Ogle, '51; Vogel, Hauge and Andrews, '58). Recent studies in our laboratories have demonstrated that certain antibiotics (penicillin, Aureomycin, and Ilotycin) depress the rate of oxygen consumption when administered by intramuscular injection or by stomach tube into either normal or hyperthyroid rats (Vogel, Hauge and Andrews, '58). Since changes in metabolic activity are usually associated with the thyroid gland, it was suggested that these effects may somehow involve the thyroid hormone.

Thyroid hormone activity is known to affect many vitamin requirements (Drill, '43). Experimental hyperthyroidism increases the quantitative requirement of rats for thiamine (Bumgardner, '52) and of guinea pigs for ascorbic acid (Pfandler, '52). In rats, antibiotics have a sparing action on many vitamins (Jones and Baumann, '55; Schendel and Johnson, '54). Since antibiotics appear to counteract the

[1] Journal paper no. 1178 of the Purdue Agricultural Experiment Station.
[2] Deceased November 26, 1957.

metabolic activity of induced hyperthyroidism, they may have a sparing action of the thiamine requirement of the hyperthyroid rat.

In this paper are reported the results of studies on the effect of procaine penicillin G on the thiamine requirement of the rat and the effects on the protein-bound iodine levels of the blood.

PROCEDURE

Since limiting growth effects of unknown factors had rendered any evaluation of the thiamine requirement by a growth response curve unfeasible in former studies (Bumgardner, '52), a liver concentrate was added to a thiamine-free basal ration in order to provide such unknown growth factors. Thiamine in the liver supplement was destroyed by treatment with sodium bisulfite at pH 1.5.

The basal ration, therefore, consisted of the following ingredients: casein, 18%; liver concentrate,[3] 1%; McCollum and Davis salt mixture 185, 4%; hydrogenated vegetable oil,[4] 5%; cod liver oil, 1%. Vitamins were added in milligrams/kilogram of diet) as follows: riboflavin, 20; pyridoxine, 20; calcium pantothenate, 100; inositol, 400; niacin, 60; *p*-aminobenzoic acid, 300; choline, 2000; biotin, 0.5; folic acid, 0.5; vitamin K, 10 and vitamin B_{12}, 0.15.

In the first series of experiments, weanling albino rats were fed the thiamine-free basal ration until growth plateaued and the symptoms of polyneuritis were evident. After the depletion period, 6 lots of rats, three males and three females per lot, were placed on each of 8 rations consisting of the basal ration and various levels of thyroprotein (0, 0.01, 0.03, and 0.05%) with and without procaine penicillin G (0.02%). Thiamine solution was administered daily in castor cups, the rats in the 6 lots receiving respectively 5, 10, 15, 20, 25, or 30 μg per rat. The test was continued for three weeks during which weekly measurements of weight gain and food con-

[3] Wilson's Liver Concentrate, N.F.

[4] Primex, Procter and Gamble Company, Cincinnati, Ohio.

sumption were made. The vitamin requirement of rats on each ration was obtained by plotting weight gain versus log dose. A straight line was drawn by the method of least squares, and its intersection with the horizontal line representing maximum weight gain was determined.

In a second series of experiments, albino rats were placed on a complete basal ration supplemented with 0.03% of thyroprotein for 14 days. After a 16-hour period of starvation, protein-bound iodine was determined (Barker, '48) in the blood serum of each rat two hours after receiving one of the following treatments: (a) intramuscular injection of 1.2 mg (0.2 ml)/100 gm body weight of procaine penicillin G in physiological saline solution, (b) stomach tube administration of 1.5 mg (0.25 ml)/100 gm body weight of an aqueous penicillin solution or (c) intramuscular injection of 0.2 ml/100 gm body weight of physiological saline solution. In order to enhance any possible antibiotic effect, a dose (1.5 mg/100 gm body weight) of penicillin was administered to hyperthyroid rats, the same dose repeated after three hours, and protein-bound iodine determined 6 hours after the initial treatment. Protein-bound iodine was also determined in rats fed basal, thyroprotein-supplemented, or antibiotic-thyroprotein-supplemented rations but without any treatment.

RESULTS

The results of the first series of experiments are summarized in tables 1 and 2. As previously reported (Bumgardner, '52), the requirement for thiamine was increased in the rats made hyperthyroid by the use of thyroprotein. The addition of antibiotic to the basal ration had no significant effect on the thiamine requirement but did increase growth at suboptimal levels of the vitamin. This agrees with the study of Mameesh et al. ('56). When hyperthyroidism was produced by feeding thyroprotein, however, penicillin exerted a marked effect in overcoming the increased thiamine requirement. Moreover, this effect was directly proportional to the level of thyroprotein in the ration. Similar results were noted

with food efficiency (table 1). The effect of thyroprotein and penicillin on maximum growth of rats is summarized in table 2. These data represent the average of all values after weight gain had plateaued. Under these conditions, the antibiotic was still able to promote a marked increase in growth. As

TABLE 1

Effect of varied degrees of hyperthyroidism and thiamine intake on growth and food efficiency of rats receiving a purified diet with and without procaine penicillin G

RATION	THIAMINE, μG PER DAY						
	2.5	5	10	15	20	25	30
	Average weekly gain, gm						
Basal		27.2	34.7	38.8	39.2	39.2	39.6
Basal + A [1]	26.5	31.7	36.9	39.1	38.6	41.2	39.1
Basal + 0.01% TP [2]		13.6	25.8	33.9	34.9	34.9	36.5
Basal + 0.01% TP + A		16.5	29.4	36.0	36.6	36.6	37.0
Basal + 0.03% TP		2.2	19.7	27.7	30.2	31.8	32.3
Basal + 0.03% TP + A		8.6	26.0	32.5	34.3	34.0	34.4
Basal + 0.05% TP		1.8	15.4	22.2	26.7	27.2	27.0
Basal + 0.05% TP + A		2.8	20.7	28.7	30.4	31.3	328.
	Food efficiency [3]						
Basal		3.2	2.7	2.7	2.8	2.8	2.7
Basal + A	3.5	3.0	2.7	2.6	2.6	2.8	2.7
Basal + 0.01% TP		5.0	3.8	3.1	3.2	3.4	2.9
Basal + 0.01% TP + A		4.8	3.5	3.0	2.9	3.2	3.1
Basal + 0.03%		29.0	4.7	3.8	3.8	3.7	3.5
Basal + 0.03% TP + A		19.2	4.0	3.5	3.4	3.6	3.5
Basal + 0.05% TP		36.1	6.2	4.6	3.9	4.5	4.3
Basal + 0.05% TP + A		24.6	4.5	3.8	3.4	3.8	3.5

[1] Procaine penicillin G (0.02%).

[2] Thyroprotein (iodinated casein).

[3] Grams food consumed/gram weight gain.

was the case with the requirements for thiamine, the effect of penicillin was greatest in the more hyperthyroid rats and actually was a direct, linear relationship to the growth inhibition induced by the thyroprotein (fig. 1).

In the second series of experiments, the effect of the various antibiotic treatments on protein-bound iodine in the serum of rats is summarized in table 3. Although a slight decrease

TABLE 2

Effect of thyroprotein and procaine penicillin G on the thiamine requirement and optimal growth in normal and hyperthyroid rats

RATION	EFFECT ON THIAMINE REQUIREMENT			EFFECT ON GROWTH		
	Calculated value of thiamine requirement	Increment due to thyroprotein	Counteraction by penicillin	Av. optimal weekly gain	Increment due to thyroprotein	Counteraction by penicillin
	μg/day			*gm*		
Basal	15.5	—	—	39.2	—	—
Basal + A [1]	15.6	—	+ 0.1	39.5	—	+ 0.3
Basal + 0.01 TP [2]	16.2	+ 0.7	—	35.0	— 4.2	—
Basal + 0.01 TP + A	15.4	—	— 0.8	36.5	—	+ 1.5
Basal + 0.03 TP	17.2	+ 1.7	—	31.4	— 7.8	—
Basal + 0.03 TP + A	15.5	—	— 1.7	34.2	—	+ 2.8
Basal + 0.05 TP	20.0	+ 4.5	—	27.0	— 12.2	—
Basal + 0.05 TP + A	16.6	—	— 3.4	31.5	—	+ 4.5

[1] Procaine penicillin G (0.02%).

[2] Thyroprotein (iodinated casein).

from the control group was observed in each case, the statistical analysis revealed that such effects were not significant ($P = 0.05$). The inclusion of procaine penicillin G as part of the ration also failed to alter the level of protein-bound iodine appreciably.

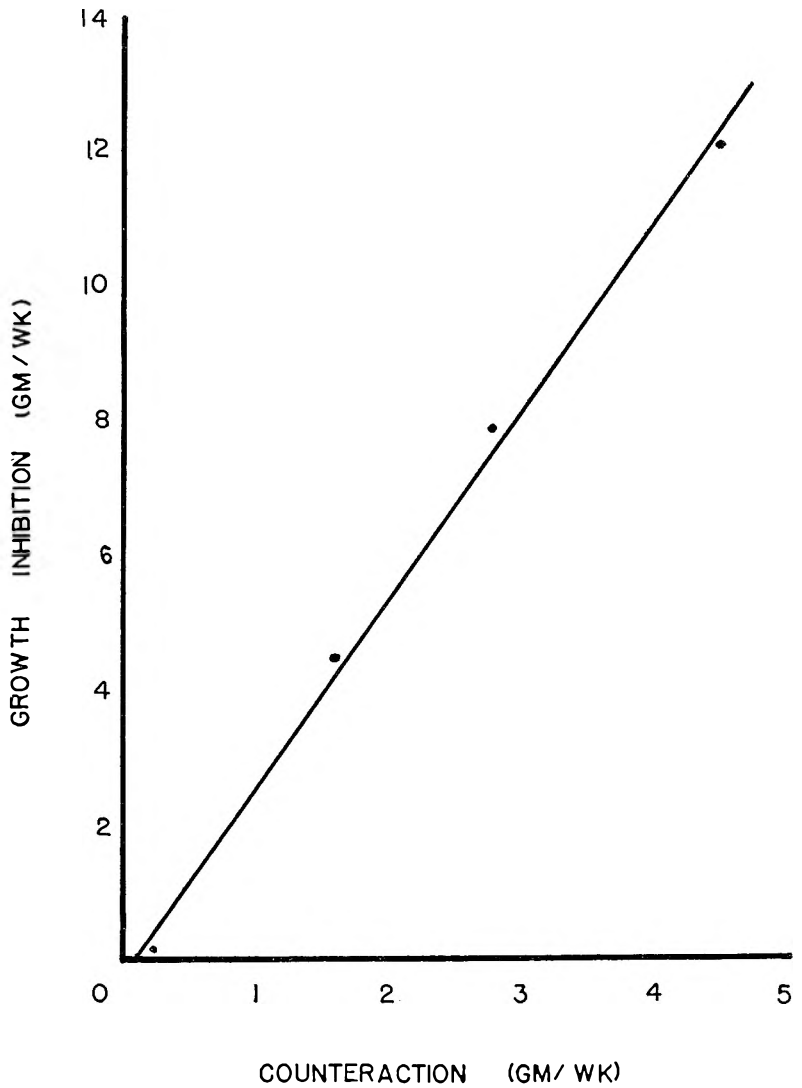

Fig. 1 Relationship of the growth inhibition produced by various levels of thyroprotein (0, 0.01, 0.03 and 0.05%) to the counteraction of this effect by penicillin.

TABLE 3

Effect of various antibiotic treatments on the protein-bound iodine (PBI) level in the serum of rats

TREATMENT	RATION	NO. OF DETERMINATIONS	PROTEIN-BOUND IODINE
			μg %
None	Basal	5	4.2 ± 0.3 [3]
None	Basal + 0.03% TP [1]	28	14.1 ± 2.1
None	Basal + 0.03% TP + 0.05% A [2]	5	13.6 ± 1.4
Intra-muscular injection of saline	Basal + 0.03% TP	16	13.6 ± 1.6
Intra-muscular injection of penicillin	Basal + 0.03% TP	25	13.3 ± 2.1
Stomach tube administration of penicillin, 1.5 mg	Basal + 0.03% TP	24	12.8 ± 1.5
Stomach tube administration of penicillin, 3.0 mg	Basal + 0.03% TP	5	13.7 ± 1.5

[1] Thyroprotein (iodinated casein).

[2] Procaine penicillin G.

[3] Mean error.

DISCUSSION

The first series of experiments indicates that penicillin was able to decrease the requirement for thiamine in the hyperthyroid rat by alleviating the effects induced by feeding thyroprotein. Since this effect was not constant at the various levels of thyroprotein administration and was minimum in rats on a normal ration, it appears that intestinal synthesis of the vitamin is probably not involved in the growth response due to antibiotic. Under the conditions of maximum growth, the counteraction by penicillin of the growth inhibition induced by feeding thyroprotein was found to be a direct, linear relationship (fig. 1). Since the various iodinated products in thyroprotein produce definite thyroidal activity (Reineke et al., '42), it may be suggested that some type of antagonism exists between the antibiotic and the activity of the thyroprotein.

Since the administration of penicillin decreased the requirement for thiamine in hyperthyroid rats and also reduced the oxygen consumption, it seemed plausible that the antibiotic may exert its effect by decreasing thyroidal activity

in the blood. Therefore, it was decided to measure the level of protein-bound iodine after certain antibiotic treatments. Despite the use of high doses, such studies demonstrated that the antibiotic did not have any appreciable effect either by intramuscular adminstration or when fed as a part of the ration. These findings indicate that penicillin apparently overcomes the effects of thyroprotein by altering the metabolism of the rat but in some manner independent of the level of serum organic iodine. Any further elucidation of the mode of action would require additional investigations.

SUMMARY

A series of experiments was conducted to determine the effect of dietary procaine penicillin G and thyroprotein on the thiamine requirements, growth at high levels of vitamins, and food efficiency of albino rats. The effects of thyroprotein on these factors were overcome in all cases by the addition of the antibiotic to the ration. Penicillin decreased the thiamine requirements and increased growth as well as food efficiency at high vitamin levels. These effects were directly proportional to the level of thyroprotein in the ration.

Subsequent experiments, in which protein-bound iodine was determined after various antibiotic treatments, revealed no significant changes in blood thyroidal activity (organic iodine).

LITERATURE CITED

BARKER, S. B. 1948 Determination of protein-bound iodine. J. Biol. Chem., *173:* 715.

BUMGARDNER, J. E. 1952 The Effect of Thyroid Activity on the Thiamin Requirement in the Rat. Ph.D. Thesis, Purdue University.

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

JONES, J. D., AND C. A. BAUMANN 1955 Relative effectiveness of antibiotics in rats given limiting B vitamins by mouth or by injection. J. Nutrition, *57:* 61.

MAMEESH, M. S., H. E. SCHENDEL, H. W. NORTON AND B. C. JOHNSON 1956 The effect of penicillin on the B_1 requirement of the rat. Brit. J. Nutrition, *10:* 23.

MEITES, J., AND R. C. OGLE 1951 Antithyrotoxic effects of antibiotics in rats. Proc. Soc. Exp. Biol. Med., *77:* 758.

PFANDLER, W. H. 1952 The effects of iodinated casein, thiouracil and cortisone on the ascorbic acid requirement of the male guinea pig. J. Nutrition, *47:* 487.

REINEKE, E. P., M. B. WILLIAMSON AND C. W. TURNER 1942 The effect of progressive iodination on the thyroidal activity of iodinated casein. J. Biol. Chem., *143:* 285.

SCHENDEL, H. E., AND B. C. JOHNSON 1954 Studies of antibiotics in weanling rats administered suboptimum levels of certain B vitamins orally and parenterally. J. Nutrition, *54:* 461.

VOGEL, G. R., S. M. HAUGE AND F. N. ANDREWS 1958 Effect of intramuscular administration of antibiotics on the oxygen consumption of normal and hyperthyroid rats. Am. J. Physiol., *192:* 73.

PRODUCTION AND STUDY OF VITAMIN E DEFICIENCY IN THE BABY PIG

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A review of attempts to produce unequivocal vitamin E deficiency in swine has not been encouraging, Adamstone et al. ('49) and Hill and Larson ('56) being among the more successful. Many other attempts (e.g., Hanson and Hathaway, '51) have not resulted in recognizable deficiency states, although in retrospect we can now recognize that the "cod-liver oil liver" and brownish body fat described in the literature 20 years ago (see Blaxter and Brown, '52) probably included a vitamin E deficiency among the causative factors (Hove and Siebold, '55; Gorham et al., '51).

The current studies were initiated with the aim of producing and characterizing vitamin E deficiency in baby pigs. In view of the results of previous investigators who had found the pig quite resistant to a deficiency of this vitamin, we accepted the necessity of using a stress factor. Cod-liver oil was chosen on the basis of its known role in precipitating vitamin E deficiency in several species of animals.

EXPERIMENTAL

Two experiments involving 37 baby pigs were conducted in the present investigation. These animals were obtained at three to 4 days of age from nearby farms and were all of mixed breeding. The composition of the basal diets used is shown in table 1. The synthetic milk diet containing 13.5% of dry matter, was prepared according to a modified Clark

procedure as described by Sheppard and Johnson ('57). In experiment 2 the "milk diet" was replaced after three weeks by the dry diet whose composition is shown in table 1. This dry diet was similar to the milk diet but was lower in protein and minerals. This change in the physical nature of the diet was instituted as a convenience. The lower protein content of the dry diet more closely approximated the requirement of the three-week-old pig. The difference in mineral content between the diets is more apparent than real, the major differ-

TABLE 1

Percentage composition of the dry matter of vitamin E-low basal diets

CONSTITUENT	MILK DIET EXP. 1 AND 2	DRY DIET EXP. 2
Casein [1]	27.3	20.0
DL-Methionine	0.5	0.3
Lard (Molecular distilled)	28.9 [2]	28.9 [2]
Cerelose	33.7	45.3
Minerals	9.6	3.5
Roughage [3]	—	2.0

[1] Labco, The Borden Co., New York.

[2] Replaced in part by cod-liver oil (feed grade) to give 2% in experiment 1, or 5 or 10% in experiment 2.

[3] Woodflock, The Brown Company, Chicago, Illinois.

ence being a result of using a large amount of hydroxides of calcium, potassium and sodium in order to solubilize the protein in the milk diets.

In the first experiment 17 baby pigs were used: 5 animals were placed on the vitamin E-low basal diet, 6 on the same diet supplemented with 2% of codliver oil (dry basis), and 6 on the basal diet supplemented with 10 mg of *dl*-alpha tocopherol acetate per 100 gm dry matter. The pigs were fed ad libitum for 8 weeks with feed intake measured daily and weights of the pigs taken twice weekly. Urine collections were made at two and at 8 weeks for the study of the ratio of total to preformed creatinine. At the termination of the experiment, cardiac and skeletal muscle samples were taken for

histological study, and blood samples were taken for analysis. Creatine and creatinine were determined in the urine by the method of Hare ('50); blood plasma alpha tocopherol was determined by the method of Quaife and coworkers as adapted to a macro scale by Farber et al. ('52). The only modification was an increase in the extraction time with xylene from 10 minutes to 20 minutes, which was necessary to obtain uniform results. Apparently extraction was not always complete in 10 minutes. Susceptibility of the red blood cells to hemolysis was tested using the dialuric acid method of Rose and György ('50). Samples for histological study were fixed in formalin-acetic acid and stained with hematoxylin and eosin. Maximum histologically observable muscle degeneration was given an arbitrary value of 4 in calculating the severity or "dystrophy score."

In the second experiment 20 baby pigs were used; 10 were given 5% cod-liver oil and 10 given 10% cod-liver oil in the dry matter of the diet. Half of each group received a supplement of vitamin E (10 mg per 100 gm dry matter of the diet). After three weeks the milk diet was replaced by a dry diet of essentially similar composition, but lower in protein, as cited above.

After an 8-week ad libitum feeding period the pigs were slaughtered and tissues taken for histological study. Urine collections were made during the final week, as were electrocardiograms. The latter were made by adapting the method of Beinfield and Lehr ('56). Light anesthesia was produced by intraperitoneal injection of 20 to 40 mg of sodium pentothal[1] per kilogram body weight. When under sedation, the pigs were suspended in a ventral position in a canvas sling and each leg was immersed in a container of 10% sodium chloride solution. The electrodes were then immersed in the saline and the electrocardiograms made with a Sanborn Cardiette.[2] Histological studies, red blood cell hemolysis tests and deter-

[1] Veterinary Nembutal solution.

[2] Mr. Lawrence D. Siler, Physical Environment Unit of the Graduate College, University of Illinois, made the electrocardiographic records of this experiment.

mination of urinary creatine and creatinine were made by the techniques described above.

RESULTS

In experiment 1 there were no statistical differences in weight gain or feed intake attributable to treatment. As shown in table 2, the ratio of total to preformed creatinine was not affected by time on experiment or by treatment.

TABLE 2

Vitamin E deficiency in baby pigs

TREATMENT	AV. DAILY GAIN	CREATININE RATIO		DYSTROPHY SCORE	PLASMA α-TOCOPHEROL
	gm	*2 weeks*	*8 weeks*		*mg/100 ml*
Experiment 1 [1]					
Group					
Basal diet	260	1.10	1.02	0	0
Basal + CLO	248	1.06	1.11	1.0	0
Basal + vitamin E	214	1.12	1.06	0	0.34
Experiment 2					
Group					
5% CLO basal	284		2.8	3.1	
5% CLO + vitamin E	284		1.1	0.3	
10% CLO basal	220		1.4	2.3	
10% CLO + vitamin E	280		1.1	0.6	

[1] Experiment 1, 17 pigs; experiment 2, 20 pigs.

Detectable amounts of alpha tocopherol were found only in the plasma of animals receiving a dietary supplement of this vitamin.

Gross pathology at autopsy, attributable to the diet, was present in only one pig, this one receiving cod-liver oil. A large muscle on the interior of the thigh showed an extensive Zenker's degeneration. The appearance was of white striated discoloration and the affected tissue was firmer than unaffected muscle. Histological sections of this lesion revealed almost complete degeneration of the muscle fibers and replacement with connective tissue. This animal had not shown observable signs of muscle weakness.

The skeletal muscle of two of the remaining 5 animals in this group showed only a slight degree of muscle degeneration upon histological examination, thus accounting for the dystrophy score of 1.0 shown in table 2.

The results of experiment 2, shown in part in table 2, were more definitive than those of experiment 1. The significantly smaller weight gain of the 10% cod-liver oil basal group originated only during the final two weeks, during which the pigs in this group decreased their feed intake markedly. The creatinine ratio was significantly raised in the 5% cod-liver oil basal lot only: the absence of such an increase in the 10% cod-liver oil basal group is not explainable. Increases in urinary creatine excretion, thus leading to an increased ratio of total to preformed creatinine, have been noted in vitamin E deficiency in other species by Mackenzie and McCollum ('40), van Wagtendonk et al. ('44), Draper et al. ('52), Verzar ('39), and Dinning ('55); these increases have been shown to accompany a decrease in muscle creatine content by Morgulis et al. ('36) and Dinning and Fitch ('58). Of the pigs receiving a vitamin E supplement, one in the 5% cod-liver oil basal lot showed a moderate degree of muscle degeneration histologically, while all of those receiving 10% cod-liver oil showed "doubtful or slight" degeneration. All pigs receiving no vitamin E supplement showed moderate to severe lesions upon histological examination of skeletal muscle. Cardiac muscle exhibited the same trends, but to a lesser degree.

During the 7th week of experiment 2, one pig in the 10% cod-liver oil basal group was found dead and was autopsied; two days later another pig in the same group showed very poor appetite, loss of coordination, and sensitiveness to touch. He was killed and autopsied immediately. Both pigs showed extensive degenerative changes in cardiac and skeletal muscles, a generalized brownish-yellow color in the body fat and degenerative changes in the parenchymal cells of the liver, ranging from albuminous degeneration to necrosis. The liver necrosis was grossly evidenced by a hob-nailed appearance of the surface. These findings were consistent with those at autopsy at

the end of the experiment. Only the animals receiving the 10% cod-liver oil basal diet showed the brown fat and necrotic liver, while those in both basal groups showed muscle lesions.

Examples of the histological findings are shown in figures 1 through 5. These appear to be typical of the muscular degeneration seen in other species as a result of vitamin E deficiency (Madsen et al., '35; Draper et al., '52; Olcott, '38).

Examples of the electrocardiograms are shown in figure 6. The PR, QRS, and QT intervals were not disturbed by the imposed treatments and averaged 0.07, 0.05, and 0.22 seconds, respectively. In the only available comparison with electrocardiograms of pigs, Rousseau et al. ('57) found intervals of 0.11, 0.06 and 0.27 seconds in the same order. The amplitude of the P, R and T waves averaged 0.15, 1.1 and 0.23 millivolts, respectively. The results were variable and no correlation could be detected attributable to treatment. The lack of electrocardiographic changes as a result of vitamin E deficiency is in agreement with results on the rat (Ensor, '46) and the monkey (Filer et al., '49), but not with the marked changes found in cattle (Gullickson and Calverly, '46; Blaxter et al., '51, sheep (Bacigalupo et al., '53; Draper et al., '52), rabbits (Gatz and Houchin, '51) and chickens (Sturkie et al., '54).

The urine of the pigs in experiment 2 was tested for pentose in view of a report by Minot et al. ('49) that human muscle dystrophy is accompanied by pentosuria. We did not find pentosuria in our dystrophic pigs.

In both experiments the ability of dialuric acid to hemolyze red blood cells was investigated. The procedure used was that of Rose and György ('50), who demonstrated that erythrocytes of vitamin E-deficient rats, in contrast to normal rats, hemolyzed in a dilute solution of dialuric acid. In only one case in the present investigation was there any evidence of hemolysis associated with tocopherol deficiency, and in this instance hemolysis was incomplete.[3]

[3] E. G. Hill (Personal communication, '58), using a modification of the hemolysis test of György, Cogan and Rose ('52), has observed an accelerated hemolysis of erythrocytes from vitamin E-deficient pigs in dilute hydrogen peroxide.

Fig. 1 Normal skeletal muscle.

Fig. 2 Moderate dystrophy. Degeneration of fibers and infiltration with reticular connective tissue. Area of calcification in lower left.

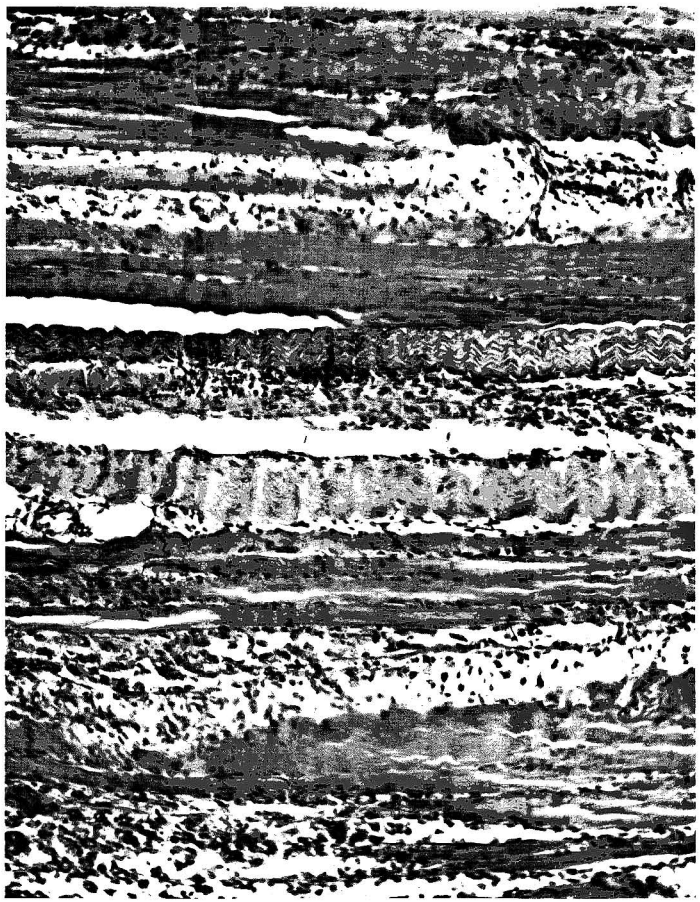

Fig. 3 Severe dystrophy. Many fibers still intact, but much infiltration with reticular connective tissue.

Fig. 4 Maximum dystrophy. Almost complete disorganization of the muscle fibers and many areas of calcification.

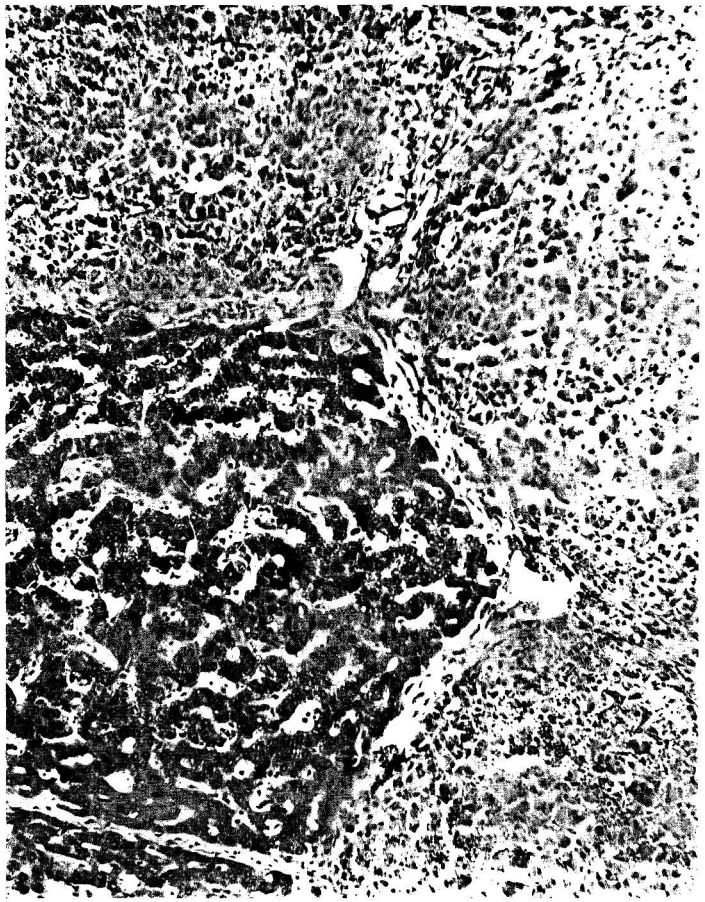

Fig. 5 Necrotic liver, showing a normal and several necrotic lobules. Albuminous degeneration and necrosis of parenchymal cells.

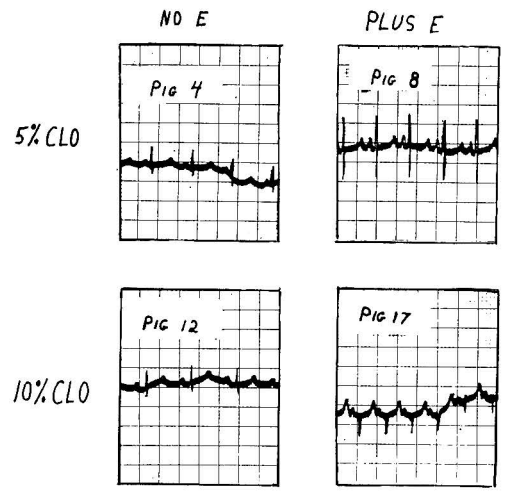

Fig. 6 Representative electrocardiograms from each group in experiment 2.

SUMMARY

Thirty-seven baby pigs were used in two experiments whose purpose was to produce and study a deficiency of vitamin E. Only under the conditions of a stress provided by at least 5% of cod-liver oil in the diet was an unmistakable deficiency produced. Symptoms of this deficiency included: death, creatinuria, degeneration of skeletal and cardiac muscle, degeneration of the liver and presence of a brownish-yellow substance in the adipose tissue. The deficiency was not accompained by changes in EKG or by ability of dialuric acid to hemolyze red blood cells. Vitamin E served to prevent appearance of these deficiency symptoms.

LITERATURE CITED

ADAMSTONE, F. B., J. L. KRIDER AND M. F. JAMES 1949 Response of swine to vitamin E-deficient rations. Ann. N. Y. Acad. Sci., *52:* 260.

BACIGALUPO, F. A., B. V. ALFREDSON, R. W. LUECKE AND F. THORP 1953 Electrocardiographic changes in vitamin E-deficient lambs. Am. J. Vet. Res., *14:* 214.

BEINFIELD, W. H., AND D. LEHR 1956 Advantages of ventral position in recording electrocardiograms of the rat. J. App. Physiol., *9:* 153.

BLAXTER, K. L., AND F. BROWN 1952 Vitamin E in the nutrition of farm animals. Nutr. Abs. Rev., *22:* 1.

BLAXTER, K. L., P. S. WATTS AND W. A. WOOD 1951 Experimental muscular dystrophy in young calves. Brit. J. Nutrition, *5:* ii.

DINNING, J. S. 1955 The nature of the creatinuria of nutritional muscular dystrophy in the rat. J. Nutrition, *55:* 209.

DINNING, J. S., AND C. D. FITCH 1958 Creatine metabolism in vitamin E deficiency. Proc. Soc. Exp. Biol. Med., *97:* 109.

DRAPER, H. H., M. F. JAMES AND B. C. JOHNSON 1952 Tri-o-cresyl phosphate as a vitamin E antagonist for the rat and lamb. J. Nutrition, *47:* 583.

ENSOR, C. R. 1946 The electrocardiogram of rats on vitamin E deficiency. Am. J. Physiol., *147:* 477.

FARBER, M., A. T. MILHORAT AND H. ROSENKRANTZ 1952 Rapid determination of plasma tocopherols. Proc. Soc. Exp. Biol. Med., *79:* 225.

FILER, L. J., R. E. RUMERY, P. N. G. YU AND K. E. MASON 1949 Studies on vitamin E deficiency in the monkey. Ann. N. Y. Acad. Sci., *52:* 284.

GATZ, A. J., AND O. B. HOUCHIN 1951 Studies on the heart of vitamin E deficient rabbits. Anat. Rec., *110:* 249.

GORHAM, J. R., N. BOE AND G. A. BAKER 1951 Experimental "yellow fat" disease in pigs. Cornell Vet., *41:* 332.

GULLICKSON, T. W., AND C. E. CALVERLY 1946 Cardiac failure in cattle on vitamin E-free rations as revealed by electrocardiograms. Science, *104:* 312.

GYÖRGY, P., G. COGAN AND C. S. ROSE 1952 Availability of vitamin E in the newborn infant. Proc. Soc. Exp. Biol. Med., *81:* 536.

HANSON, L. E., AND I. L. HATHAWAY 1951 Vitamin E requirements for growth and reproduction in swine. Sixty-third annual report of the Nebraska Agr. Exp. Sta.

HARE, R. S. 1950 Endogenous creatinine in serum and urine. Proc. Soc. Exp. Biol. Med., *74:* 148.

HILL, E. G., AND N. L. LARSON 1956 Nutritive requirements of swine. Ann. Rept. Hormel Inst. (1955–56): 83.

HOVE, E. L., AND H. R. SIEBOLD 1955 Liver necrosis and altered fat composition in vitamin E-deficient swine. J. Nutrition, *56:* 173.

MACKENZIE, C. G., AND E. V. MCCOLLUM 1940 The cure of nutritional muscular dystrophy in the rabbit by alpha-tocopherol and its effect on creatine metabolism. Ibid., *19:* 345.

MADSEN, L. L., C. M. MCCAY AND L. A. MAYNARD 1935 Synthetic diets for herbivora, with special reference to the toxicity of cod liver oil. Cornell Univ. Agr. Expt. Sta. Memoirs 178.

MINOT, A. S., H. FRANK AND D. DZIEWIATKOWSKI 1949 The occurrence of pentose and phosphorus-containing complexes in the urine of patients with progressive muscular dystrophy. Arch. Biochem., *20:* 394.

MORGULIS, S., AND H. C. SPENCER 1936 Metabolism studies in nutritional muscular dystrophy. J. Nutrition, *12:* 191.

OLCOTT, H. S. 1938 The paralysis in the young of vitamin E deficient female rats. Ibid., *15:* 221.

ROSE, C. S., AND P. GYÖRGY 1950 Tocopherol requirements of rats by means of the hemolysis test. Proc. Soc. Exp. Biol. Med., *74:* 411.

ROUSSEAU, J. E., JR., M. W. DICKS, R. TEICHMAN, C. F. HELMBOLDT, E. L. BACON, R. M. PROUTY, K. L. DOLGE, H. D. EATON, E. L. JUNGHERR AND G. BEALL 1957 Relationships between plasma, liver and dietary tocopherols in calves, lambs and pigs. J. Animal Sci., *16:* 612.

SHEPPARD, A. J., AND B. C. JOHNSON 1957 Pantothenic acid deficiency in the growing calf. J. Nutrition, *61:* 195.

STURKIE, P. D., E. P. SINGSEN, L. D. MATTERSON, A. KOZEFF AND E. L. JUNGHERR 1954 The effects of dietary deficiencies of vitamin E and the B complex vitamins on the electrocardiogram of chickens. Am. J. Vet. Res., *15:* 457.

VERZAR, F. 1939 Der Kreatin-Stoffwechsel bei der Muskeldystrophie durch E-vitaminmangel und sein Reeinflussung durch Tocopherol. Zeitschr. Vitaminforsch., *9:* 242.

VAN WAGTENDONK, W. J., V. SCHOCKEN AND R. WULZEN 1944 A dietary factor essential for guinea pigs. II. A comparative study of the creatine excretion of animals on a diet deficient in this factor and in vitamin E. Arch. Biochem., *3:* 305.

A STUDY OF THE EFFECT OF DEOXYPYRIDOXINE OR ISONIAZID UPON MINERAL RETENTION AND LIVER ENZYME ACTIVITIES OF PYRIDOXINE-DEFICIENT MALE RATS [1]

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INTRODUCTION

Convulsive seizures caused by vitamin B_6 deprivation in the chick, rat, dog and pig were reported many years ago (e.g., the work of Chick et al., '40). Snyderman, Holt and associates ('50, '53), reporting convulsions in infants upon vitamin B_6 deprivation, pointed out dramatically the essentiality of this vitamin in human nutrition. Coursin ('54) reported that an infant experiencing clinically and electroencephalographically demonstrable *status epilepticus* was improved after intramuscular vitamin B_6 injection, and pointed out that a better understanding of the role of vitamin B_6 in nerve cell metabolism would be of great value.

Hansen et al. ('54) showed that vitamin B_6 supplementation of a milk preparation suspected of being convulsigenic resulted in increased retention of N, P, Cl, Ca, Mg, Na, and K in a 4-month-old male child. However, the intake of milk during the period of supplementation was 37% greater than during the period of non-supplementation. Hsu et al. ('56) noted that a vitamin B_6 deficiency in rats caused a significant increase in concentration of serum Na but not of serum K.

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The concentration of serum K was lowered in rats receiving deoxypyridoxine with a pyridoxine-free diet. The same authors observed marked elevation of muscle K, definite reduction of muscle Na, and no alterations in Na or K concentrations in liver, heart and kidneys of vitamin B_6-depleted animals.

Pyridoxal, functioning as its coenzyme form pyridoxal phosphate, is required by at least 15 known animal enzyme systems. In vitamin B_6 deprivation, decreases in the blood level and in the hepatic and renal levels of various vitamin B_6-containing enzymes have been demonstrated (e.g., the work of Thompson and Guerrant ('53) with hepatic cysteine desulfhydrase in the rat). That the vitamin B_6 deficiency induced by the use of an antagonist is not equivalent biochemically to that induced by a vitamin B_6 deficiency was shown by Dietrich and Shapiro ('53). They observed no decrease in hepatic cysteine desulfhydrase activity concurrent with decreases in activities of hepatic transaminase and DOPA decarboxylase of mice receiving a deoxypyridoxine-containing vitamin B_6-adequate stock diet. Caldwell and McHenry ('53) found an increase of hepatic transaminase in the rat being fed deoxypyridoxine.

Because of the paucity of information in the literature concerning mineral metabolism in vitamin B_6 deprivation, fundamental investigation of the problem seems warranted—the logical experimental pilot animal being the laboratory rat. Since the study of mineral metabolism should be attempted under the condition of equalization of food intake between treatments, an excellent opportunity also exists for making a comparative evaluation of hepatic vitamin B_6-containing enzymes. Accordingly, this report presents data of body retentions of Ca, Mg, Na and K as well as hepatic contents of glutamic-aspartic transaminase and cysteine desulfhydrase observed under moderate and severe vitamin B_6 deprivation, both with and without vitamin B_6 antagonists and with equal food intake between treatments.

EXPERIMENTAL

Experiment 1. Thirty-two male Wistar strain albino rats ranging from 43 to 70 gm in weight were, on the basis of body weight and litter, assigned to 8 groups of 4 rats each. Each group of animals served as a replicate of 4 experimental treatments with the food intake within each group being limited to the amount consumed by the animal eating the least. Distilled water was given ad libitum. All animals were placed in screen-bottom cages and received for a 6-week period the vitamin B_6-deficient basal diet (diet A) having the following percentage composition: vitamin-test casein[3] 18, cerelose 30.5, sucrose 35.5, corn oil[4] 5, mineral mix[5] 4, vitamin mix[6] 5, and cellulose 2. At the end of the 6-week period, when many of the animals were exhibiting signs of a vitamin B_6 deficiency, rat 1 of each quadruplicate was continued on the basal diet, but rats 2, 3 and 4 were placed on diets identical to the basal to which had been carefully admixed, at the rate of 5 gm/kg, cerelose premixes of deoxypyridoxine (DOP), isoniazid (INH), and pyridoxine, so that the final diets contained per gram, respectively, the following additives: diet B 5 μg DOP, diet C 25 μg INH, and diet D 10 μg pyridoxine-HCl.

After the animals had been on the revised dietary regime for a one-week period they were placed in special metabolism cages for the quantitative collection of excreta. Seven-day collections of feces and urine were made and the urine composites for individual animals were preserved by making the weekly urine and washings approximately 0.2 N with added sulfuric acid. Carmine served as the feces marker.

[3] Nutritional Biochemicals Corporation, Cleveland, Ohio.

[4] Oleum Percomorphum (Mead Johnson & Co., Evansville, Ind.) and α-tocopheryl acetate added to the corn oil so that the final diet contained 2,000 I.U. vitamin A, 283 I.U. vitamin D, and 10 mg vitamin E per 100 gm.

[5] Jones and Foster ('42).

[6] The vitamin mix had the following composition: thiamine hydrochloride 250 mg, riboflavin 500 mg, Ca pantothenate 2 gm, nicotinic acid 2.5 gm, folic acid 50 mg, menadione 50 mg, vitamin B_{12} 2.5 mg, biotin 5 mg, choline chloride 100 gm, and cerelose to make 2,500 gm total.

The urine composites were filtered and made to a total volume of one liter. Urinary K assays were run directly on the urine composites by flame photometry at $\lambda = 769$ mμ using a Beckman Model B spectrophotometer; Na assays were run on 10-fold dilutions of the urine composites by flame photometry at $\lambda = 589$ mμ. For urinary Ca and Mg analyses 250 ml portions of the weekly composites were evaporated on a steam bath, then ashed at 550°C for approximately 15 hours. The residue was extracted by warming it for 10 minutes on a steam bath with each of three 20 ml portions of 1:1 HCl. The extract was filtered, the filter was washed with hot water, and the filtrate made to volume. The filtrate was analyzed for Ca by the permanganate titrimetric method (A.O.A.C., '55) and for Mg by the Titan yellow colorimetric method (Kunkel, et al., '47).

The feces for each animal here composited, ashed at 550°C for approximately 15 hours, and the residue dissolved by heating with several portions of 0.3 N HCl and made to volume. Determinatons of K, Na, Ca and Mg were made on the extracts by the methods previously cited for urine. Analyses were similarly made for the same elements in the experimental diets and the few diet refusals of individual animals.

Approximately 30 days after the completion of the metabolism period the animals were killed by a sharp blow on the head and exsanguination, the livers were quickly removed and blotted on filter paper, and 10% homogenates prepared with 0.1 M phosphate buffer of pH 7.0, using a Potter-Elvehjem homogenizer. These homogenates were assayed for cysteine desulfhydrase activity and 1% homogenates (10-fold dilutions of 10% homogenates) were assayed for glutamic-aspartic transaminase activity (Tonhazy et al., '50). The cysteine desulfhydrase assay is a modification of the procedure of Greenstein and Leuthardt ('44). One-half milliliter of L-cysteine solution (25 μM/ml) was placed in reaction vessels (15-ml centrifuge tubes) containing 0.5- or 1-ml aliquots of the previously described liver homogenates. The total volume was brought to 2 ml with buffer. The tubes were

incubated two hours with shaking in a 37°C water bath. The reaction was stopped by addition of 0.2 ml of 100% (w/v) trichloroacetic acid and the concentration of pyruvate measured as described in the glutamic-aspartic transaminase method of Tonhazy et al. ('50).

Experiment 2. Twenty-eight male albino rats ranging in body weight from 34 to 65 gm were, on the basis of weight and litter, assigned to 7 groups of 4 rats each. In a manner identical to that of experiment 1 each group of animals served as a replicate of 4 experimental treatments with food intakes within replicates being equalized. All animals were fed a ground rat diet[7] for a period of 31 days. Then the animals, ranging in body weight from 129 to 150 gm, were placed on the experimental diets. Rats 1 to 4 of each replicate were given diets A′, B′, C′, and D′, respectively, which differed from the corresponding diets of experiment 1, as follows: the protein (24-hour, 95% ethanol-extracted vitamin test casein) content of each diet was increased by 6% at the expense of 3% decreases of both cerelose and sucrose, and the INH content of diet C′ was raised to 50 μg/gm.

The animals were fed the diets for a period of 18 days after which quantitative 7-day collections of excreta were made. The assays for K, Na, Ca, and Mg of urine, feces, diets and food refusals were carried out in a manner identical to that of experiment 1, except that the individual urine composites were made approximately 0.2 N with hydrochloric instead of sulfuric acid. At the end of the collection period the animals were killed and the livers after being blotted with filter paper were frozen in dry ice-acetone and stored frozen for approximately 16 months, when they were assayed, in a manner identical to that of experiment 1, for enzyme activity.

RESULTS AND DISCUSSION

The typical symptoms of pyridoxine deficiency including acrodynia developed in the animals receiving diets A and A′. The animals in experiment 1 upon being given DOP rapidly

[7] Rockland rat diet, Arcady Farms Milling Company, Chicago, Ill.

developed stiffness of joints, severe acrodynia, and inanition. These rats died before collection periods were well under way. In the same experiment rats upon being given INH evidenced intensity of deficiency symptoms together with fits in several cases. Upon being given pyridoxine the animals rapidly returned to normal. In experiment 2 the inanition of animals receiving DOP was mild enough to allow collection periods to be made and no fits were observed in any experimental group.

The results of both experiments appear in table 1 where mean values for the experimental criteria are given together with their respective standard errors. Such pooled standard errors are the square roots of the respective mean variances. The criteria evaluated appear in column 1 and are largely self-explanatory. The activities of liver enzymes are shown on lines 2 and 3 of the table and are expressed both in terms of concentration and total hepatic content. The aspartic-glutamic transaminase values found in experiment 2 were much lower than corresponding ones observed in experiment 1. Since one would expect the reverse to be true because of the lesser severity of pyridoxine deficiency in experiment 2 and since the activity found in experiment 1 is in line with rat and mouse hepatic activities reported by Caldwell and McHenry ('53) and Dietrich and Shapiro ('53), respectively, it appears that the prolonged period of frozen storage may have depressed this transaminase activity. During a 26-day period of frozen storage of blood Marsh et al. ('55) reported great stability of transaminase; in the second experiment no basis exists for estimating stability during frozen storage because no enzyme assays were conducted at the time the animals were killed. The hepatic cysteine desulfhydrase activities of the animals receiving diets D and D′ are in line with the activities of normal mouse and rat livers reported by Dietrich and Shapiro ('53) and Thompson and Guerrant ('53), respectively. The reason for the hepatic cysteine desulfhydrase activity of the rats receiving diets A and C (experiment 1) being so much lower than that of animals re-

TABLE 1

Mean responses of severely and moderately vitamin B_6-depleted male albino rats to semisynthetic diets containing pyridoxine and to pyridoxine-deficient diets with and without added pyridoxine antagonists

ITEM	GROUP MEANS									
	Experiment 1 [1] (severe depletion)					Experiment 2 [2] (moderate depletion)				
	Diet A basal	Diet B basal + DOP [3]	Diet C basal + INH [4]	Diet D basal + vitamin B_6	S. E. of the means	Diet A′ basal	Diet B′ basal + DOP	Diet C′ basal + INH	Diet D′ basal + vitamin B_6	S. E. of the means
1. Body weight gain, gm [5]	21.5	All animals died during collection period	14.8	34.2	± 3.2	32.6	27.9	31.6	32.6	± 3.1
2. Liver aspartic-glutamic transaminase activity: μl CO_2/mg liver D.M./hr.	411.9		380.0	626.4	± 21.9	43.9	25.6	43.3	86.9	± 22.3
(μl CO_2/total liver/hr.) $\times 10^{-2}$	630.4		575.4	862.9	± 34.6	72.0	36.9	64.1	141.0	± 36.8
3. Liver cysteine desulfhydrase activity: μM NH_3/gm liver D.M./hr.	0.31		0.10	9.28	± 0.47	10.2	8.7	9.9	16.1	± 6.6
μM NH_3/total liver/hr.	0.46		0.14	12.80	± 0.83	17.1	14.1	15.0	26.2	± 11.3
4. Ca retention, mg/100 gm body weight/week	77.81 [2]		104.62 [6]	95.69 [2]	± 7.81	65.12	57.92	60.91	67.44	± 5.90
5. Mg retention, mg/100 gm body weight/week	6.88 [6]		7.59 [7]	6.06 [7]	± 0.87	0.07	— 2.47	— 0.78	— 0.22	± 0.82
6. Na retention, mg/100 gm body weight/week	— 4.25 [2]		8.04 [6]	8.70 [2]	± 1.26	5.55	2.12	5.01	2.72	± 1.44
7. K retention, mg/100 gm body weight/week	— 29.57 [2]		— 18.08 [6]	— 15.02 [2]	± 3.76	— 22.24	— 35.54	— 25.41	— 26.26	± 2.60

[1] Eight observations/group except where noted.
[2] Seven observations/group.
[3] Deoxypyridoxine.
[4] Isoniazid.
[5] Experiment 1: 37 days; experiment 2: 25 days.
[6] Six observations/group.
[7] Five observations/group.

ceiving the corresponding diets A′ and C′ (experiment 2) appears to be the greater severity of the pyridoxine depletion in the former experiment. The enzyme activities of experiment 2 have larger coefficients of variation than those of experiment 1 and the reason is not apparent. The total one week retention of minerals is divided, in each case, by the body weight expressed in 100 gm; the resulting values appear in lines 4 to 7 of table 1. With the exceptions of Mg in experiment 2 and K in both experiments, generally the mineral balances were positive. In attempting to explain the strongly negative K balances a constant error in fecal or urinary K assays was sought. The total amounts of K contained in feces were too small to be critical in this respect and recoveries of K added to urine were satisfactory. Further, ashing of the urine before assay had no effect on assay values.

Statistical evaluation of the data appears in table 2 (experiment 1) and table 3 (experiment 2). In these tables the items in column 1 are identical to the ones appearing in column 1 of table 1. The numerical values appearing in columns 2–7 of each table represent the value obtained by subtracting from the group mean for treatment A the corresponding value for treatment B, and so on. The minus sign merely indicates that the treatment mean listed second exceeds that listed first. The statistical significance of the differences, indicated by asterisks, was determined by Tukey's test as modified by Hartley (Snedecor, '56). In further discussion the terms significant or significantly are understood to denote statistical significance.

The data of table 2, describing the responses of rats having severe pyridoxine depletion alone and with added INH, indicate the following: weight gains of animals receiving the complete diet significantly exceeded those receiving the deficient diet either alone or with INH added. Although the antagonist further reduced the growth inhibition of deprivation, the amount of inhibition was not significant. The hepatic activity of each enzyme, by either method of expression, was significantly decreased in pyridoxine deficiency. When INH

TABLE 2

Comparison of responses of severely vitamin B_6-depleted male albino rats to semi-synthetic diets containing pyridoxine and to pyridoxine-deficient diets with and without added pyridoxine antagonists

Experiment 1

ITEM	COMPARISONS OF TREATMENT MEANS					
	A — B [1]	A — C	A — D	B — C	B — D	C — D
1. Body weight gain, gm (37 days)		6.7 [2]	— 12.7 [3]			— 19.4 [3]
2. Liver aspartic-glutamic transaminase activity:						
μl CO_2/mg liver D.M./hr.		31.9	— 214.5 [3]			— 246.4 [3]
(μl CO_2/total liver/hr.) $\times$ 10^{-3}		55.0 [3]	— 232.5 [3]			— 287.5 [3]
3. Liver cysteine desulfhydrase activity:						
μM NH_3/gm liver D.M./hr.		0.21	— 8.97 [3]			— 9.18 [3]
μM NH_3/total liver/hr.		0.32	— 12.34 [3]			— 12.66 [3]
4. Ca retention, mg/100 gm body weight/week		— 26.81	— 17.88			8.93
5. Mg retention, mg/100 gm body weight/week		— 0.71	0.82			1.53
6. Na retention, mg/100 gm body weight/week		— 12.29 [3]	— 12.95 [3]			— 0.66
7. K retention, mg/100 gm body weight/week		— 11.49 [3]	— 14.55 [3]			— 3.06

[1] Treatment A = basal (— pyridoxine); B = basal + 5 μg deoxypyridoxine (DOP)/gm of diet; C = basal + 25 μg isoniazid (INH)/gm of diet; and D = basal + 10 μg pyridoxine/gm of diet.

[2] Values equal mean differences between treatments.

[3] Significant at $P = 0.05$.

TABLE 3

Comparison of responses of moderately vitamin B_6-depleted male albino rats to semi-synthetic diets containing pyridoxine and to pyridoxine-deficient diets with and without added pyridoxine antagonists

Experiment 2

ITEM	COMPARISONS OF TREATMENT MEANS					
	A′— B′ [1]	A′— C′	A′— D′	B′— C′	B′— D′	C′— D′
1. Body weight gain, gm (25 days)	4.7 [2]	1.0	0	— 3.7	— 4.7	— 1.0
2. Liver aspartic-glutamic transaminase activity:						
μl CO_2/mg liver D.M./hr.	18.3	0.6	— 43.0	— 17.7	— 61.3	— 43.6
(μl CO_2/total liver/hr.) $\times 10^{-3}$	35.1	7.9	— 69.0	— 27.2	— 104.1	— 76.9
3. Liver cysteine desulfhydrase activity:						
μM NH_3/gm liver D.M./hr.	1.5	0.3	— 5.9	— 1.2	— 7.4	— 6.2
μM NH_3/total liver/hr.	3.0	2.1	— 9.1	— 0.9	— 12.1	— 11.2
4. Ca retention, mg/100 gm body weight/week	7.20	4.21	— 2.32	— 2.99	— 9.52	— 6.53
5. Mg retention, mg/100 gm body weight/week	2.54	0.85	0.29	— 1.69	— 2.25	— 0.56
6. Na retention, mg/100 gm body weight/week	3.43	0.54	2.83	— 2.89	— 0.60	2.29
7. K retention, mg/100 gm body weight/week	13.30 [3]	3.17	4.02	— 10.13 [3]	— 9.28 [3]	0.85

[1] Treatment A′ = basal (— pyridoxine); B′ = basal + 5 μg deoxypyridoxine (DOP)/gm of diet; C′ = basal + 50 μg isoniazid (INH)/gm of diet; and D′ = basal + 10 μg pyridoxine/gm of diet.

[2] Values equal mean differences between treatments.

[3] Significant at P = 0.05.

treatment was imposed with the vitamin deficiency the activities were further reduced, but not significantly so. Calcium, Na and K retention by animals receiving the complete diet exceeded that by animals made pyridoxine deficient—with significance being attained in the case of Na and K. A striking finding is that the addition of INH to the pyridoxine-deficient diet increased the retention of all 4 minerals, with significance being attained in the case of Na and K. Further, no significant differences existed between the Na and K retentions of rats receiving diets C and D. In connection with K retention it is interesting to note that Hsu et al. ('56) reported marked elevation in muscle K in pyridoxine- depleted rats. It seems likely that the pyridoxine depletion in the present work was more severe than that in the work cited.

The data of table 3 compare the responses of rats having moderate pyridoxine deficiency alone and with either DOP or INH therapy, and indicate the following: no significant differences were evident in weight gains, due, no doubt, to the shorter experimental period, the larger size of animal and the absence of a long depletion period. Although significant differences in hepatic enzyme activities were not found, a trend toward further depression of aspartic-glutamic transaminase activity of pyridoxine-deficient rats by DOP was indicated. The same did not appear to be true of INH. Cysteine desulfhydrase activity did not appear to be depressed by the addition of either DOP (in agreement with the findings of Dietrich and Shapiro ('53) or INH to a pyridoxine-deficient diet. Mineral retention of animals receiving the complete diet in no case exceeded significantly that of animals receiving the pyridoxine-deficient (A′) diet. The animals receiving the pyridoxine-deficient diet containing DOP retained significantly less K than those receiving any of the three remaining diets. INH reduced the K retention in a pyridoxine deficiency, but not significantly so. No significant differences between treatments existed in the retention of the other minerals. Of importance is the fact that as the pyridoxine depletion decreased in severity the significant differences in Na retention between treatments disappeared.

SUMMARY AND CONCLUSIONS

Based upon the responses (growth rate, hepatic glutamic-aspartic transaminase and cysteine desulfhydrase activities, and retention of dietary Ca, Mg, Na and K) of 15 groups of 4 rats fed a pyridoxine-free basal diet or the same plus either deoxypyridoxine (DOP), isoniazid (INH), or pyridoxine, the following conclusions seem warranted:

1. Under moderate pyridoxine depletion hepatic glutamic-aspartic transaminase activity appears to be reduced. Treatment with DOP, but not INH, appears to reduce such activity further. Under the same conditions hepatic cysteine desulfhydrase activity appears to be reduced below that of rats receiving complete diets and also appears not be reduced further by treatment with DOP or INH.

2. Under severe pyridoxine depletion the hepatic glutamic-aspartic transaminase and cysteine desulfhydrase activities are reduced significantly. The addition of INH to pyridoxine-deficient diets does not reduce the activities of these enzymes to any significant degree.

3. Calcium and Mg retention are not altered significantly in moderate or severe pyridoxine depletion. Under moderate pyridoxine depletion neither Na or K retention is reduced significantly. However, when DOP is given the retention of K, but not of Na, is reduced significantly. Under severe pyridoxine depletion the retention of both Na and K are reduced significantly. The addition of INH to pyridoxine-deficient diets does not reduce the retention of these minerals to any significant extent.

ACKNOWLEDGMENT

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LITERATURE CITED

ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS 1955 Methods of Analysis Washington 4, D. C., 8th edition.

CALDWELL, E. F., AND E. W. MCHENRY 1953 Deoxypyridoxine and liver transamination in the rat. Arch. Biochem. Biophys., *45:* 466.

CHICK, H., M. M. EL SADR AND A. N. WORDEN 1940 Occurrence of fits of epileptiform nature in rats maintained for long periods on diet deprived of vitamin B_6. Biochem. J., *34:* 595.

COURSIN, D. B. 1954 Convulsive seizures in infants with pyridoxine-deficient diet. J. Am. Med. Assoc., *154:* 406.

DIETRICH, L. S. AND D. M. SHAPIRO 1953 Antagonist studies: Comparison of desoxypyridoxine and pyridoxine-deficiency therapy on various vitamin B_6 enzymes. Proc. Soc. Exp. Biol. Med., *84:* 555.

GREENSTEIN, J. P., AND F. M. LEUTHARDT 1944 Degradation of cystine peptides by tissues. I. Exocystine desulfurase and dehydropeptidase in rat liver extracts. J. U. S. Natl. Cancer Inst., *5:* 209.

HANSEN, A. E., H. F. WIESE, D. J. D. ADAM, D. R. BUSSEY AND A. G. WORSHAM 1954 Influence of pyridoxine on mineral balance in infant maintained on a convulsigenic milk preparation. Federation Proc., *13:* 460.

HSU, J. M., E. B. MCCOLLUM AND B. F. CHOW 1956 Sodium and potassium imbalance in pyridoxine-deficient rats. Abst. A. C. S. Meeting, Sept. 16–21, 1956, Atlantic City, N. J., p. 49C.

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.

KUNKEL, H. O., P. B. PEARSON AND B. S. SCHWEIGERT 1947 The photoelectric determination of magnesium in body fluids. J. Lab. Clin. Med., *32:* 1027.

MARSH, M. E., L. D. GREENBERG AND J. F. RINEHART 1955 The relationship between pyridoxine ingestion and transaminase activity. J. Nutrition, *56:* 115.

SNEDECOR, G. W. 1956 Statistical Methods Applied to Experiments in Agriculture and Biology. Fifth edition. The Iowa State College Press, Ames. pp. 251–253.

SNYDERMAN, S. E., R. CARRETERO AND L. E. HOLT, JR. 1950 Pyridoxine deficiency in the human being. Federation Proc., *9:* 371.

SNYDERMAN, S. E., L. E. HOLT, JR., R. CARRETERO AND K. JACOBS 1953 Pyridoxine deficiency in the human infant. J. Clin. Nutrition, *1:* 200.

THOMPSON, R. Q., AND N. B. GUERRANT 1953 Effect of dietary protein and vitamin B_6 on hepatic thionase activity. J. Nutrition, *50:* 161.

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.

STUDIES OF THE METABOLIZABLE AND PRODUCTIVE ENERGY OF GLUCOSE FOR THE GROWING CHICK

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A first requisite in the study of the energy nutrition of avian species is an accurate knowledge of the energy values of purified nutrients. An earlier paper from this laboratory (Hill and Anderson, '58) has described studies comparing metabolizable energy and productive energy determinations with growing chicks, showing that metabolizable energy is the preferred measure both for accuracy and reproducibility.

The purpose of the studies to be described was to establish the energy value of glucose for the chick as a primary standard for the determination of energy values of other materials.

EXPERIMENTAL

The composition of the diet used in all of the experiments to be described is shown in table 1. The experimental subjects were male cross-bred chicks (RIR $\times$ BPR), reared to 9 or 10 days of age on the standard reference diet and then divided into uniform groups by a method similar to that of McKittrick ('47) to equalize both weight and rate of gain. The experimental groups contained 10 chicks each, and the experiments were conducted for 14 days following the preliminary period. The chicks were maintained in electrically heated, wire-floored battery cages. The methods used for the

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determination of productive energy by a carcass analysis method similar to that of Fraps ('46), and for the determinations of metabolizable energy corrected for nitrogen retention, have been described in detail in the previous paper (Hill and Anderson, '58). Metabolizable energy measurements were based on pooled samples of excreta collected during the last 4 days of the two-week experimental period. All data and computations presented in this paper are expressed on a dry matter basis.

TABLE 1

Composition of diet E 9

COMPONENT	AMOUNT	COMPONENT	AMOUNT
	%		%
Glucose (Cerelose)	44.1	Fish solubles (dry basis)	1.0
Ground wheat	9.0	Hydrogenated vegetable fat [1]	2.5
Soybean oil meal (44% protein)	17.5	Ground limestone	2.0
Crude casein	10.5	Dicalcium phosphate	1.0
Gelatin	2.5	Salt (iodized)	0.5
Fish meal (menhaden)	4.0	Mineral mixture [2]	0.4
Dried brewers' yeast	2.5	Vitamin mixture [2]	0.5
Dried whey	2.0		100.0

[1] Hydora, Lever Bros.

[2] The mineral and vitamin mixtures supply, in milligrams per 100 gm of diet: 220 K_2HPO_4, 120 $MgSO_4$, 30 $MnSO_4$, 30 $FeSO_4 \cdot 7H_2O$, 0.8 $CuSO_4 \cdot 5H_2O$, 0.3 thiamine, 0.4 riboflavin, 1.0 calcium pantothenate, 0.5 pyridoxine, 2.6 niacin, 0.07 folacin, 0.09 menadione, 0.01 biotin, 0.001 vitamin B_{12}, 130 choline chloride, 1000 U.S.P. units vitamin A, 100 I.C. units vitamin D_3, 2.2 mg alpha tocopheryl acetate.

Two kinds of experiments were conducted to determine the energy value of glucose. In the first, diet E9 was altered by omitting 25 parts of glucose[2] per 100 parts of diet; the altered diet was fed to one group of chicks at a level equal to 75% of the ad libitum consumption of the complete diet by another group. This procedure equalized the intake of non-glucose components of the diet. The basis of the experiment was the assumption that the difference between the metabolizable energy per gram of diet E9 and per 0.75 gram of the

[2] Cerelose, Corn Products Refining Company.

altered diet, each corrected for nitrogen retention, would be equal to the metabolizable energy of 0.25 gram of Cerelose. At the same time, each of these respective diets was fed at a lower plane of intake in order to make possible simultaneous determination of productive energy. In a further experiment, the metabolizable energy values of the reference diet intact and with 20% of Cerelose omitted were determined under ad libitum feeding, and the energy of Cerelose estimated from the difference between them.

In the second approach, graded levels of cellulose[3] (0, 5, 10 and 20%) were substituted for glucose. Each of the experimental diets was fed at a level of daily intake equal to that of the ad libitum consumption of diet E9. At the same time, each of the respective diets was also fed at 60% of this level in order to permit the simultaneous determinations of productive energy. Two replicate experiments of this kind were conducted, at different times but using the same diet ingredients. The basis of this experiment was the assumption that if the digestibility of cellulose is zero, the difference in metabolizable energy content of the respective diets would be equal to the energy content of the glucose replaced. Evidence to support the assumption that the digestibility of cellulose is indeed negligible was obtained in the course of the experiment. A further experiment was also conducted in which the metabolizable energy of the reference diet with and without 20% cellulose was determined under ad libitum feeding, and the value of Cerelose estimated from the difference.

Metabolizable energy determinations

Metabolizable energy data for the diets used in the first experiment, in which glucose was deleted and the diets were pair-fed, are shown in table 2, experiment 1. Two comparisons were possible, one between the intact and altered diet based on ad libitum consumption of diet E9, and the other in which both diets were fed at a lower plane of intake. In

[3] Solka Floc, obtained from The Brown Company, New York, New York.

TABLE 2

Estimation of metabolizable energy of glucose by deletion and by substitution with cellulose

DIET AND QUANTITY	METABOLIZABLE ENERGY OF DIETS		DIFFERENCE, EQUIVALENT TO GLUCOSE REMOVED	APPARENT METABOLIZABLE ENERGY OF GLUCOSE
	Replicates	Average		
	Cal.	*Cal.*	*Cal.*	*Cal./gm*
Exp. 1, glucose deletion, pair-fed				
Diet E9, fed ad lib., per gram		3.337		
Diet E9 minus 25% glucose, per 0.75 gm		2.429	0.908	3.63
Diet E9, fed 60% ad lib., per gram		3.347		
Diet E9 minus 25% glucose, per 0.75 gm		2.452	0.895	3.58
				Average = 3.60
Exp. 2, glucose deletion ad libitum feeding				
Diet E9, per gram	3.385			
	3.362	3.374		
Diet E9 with 20% glucose	2.633		0.738	3.69
deleted, per 0.80 gm	2.638	2.636		
Exp. 4, cellulose substitution, ad libitum feeding				
Diet E9, per gram	3.385			
	3.362	3.374		
Diet E9, with 20% cellulose	2.680		0.700	3.50
replacing glucose, per gram	2.668	2.674		

this table are shown the computations of the difference in metabolizable energy from 1 gm of diet E9 and 0.75 gm of the reference diet with 25% glucose omitted, all data corrected to a condition of nitrogen equilibrium. The difference was considered to be equal to the energy of 0.25 gm of glucose, and yielded estimated metabolizable energy values of 3.63 and 3.58 Cal. per gram of glucose dry matter, averaging 3.60.

TABLE 3

Metabolizable energy of diet E9 with graded levels of cellulose substituted for glucose

CELLULOSE IN DIET E9	METABOLIZABLE ENERGY OF DIET	
	Fed ad libitum	Fed at 60% ad lib.
%	*Cal./gm*	*Cal./gm*
0	3.36	3.34
	3.28	3.37
	3.32 [1]	*3.35* [1]
5	3.15	3.16
	3.06	3.06
	3.11	*3.11*
10	2.92	2.98
	2.86	2.89
	2.89	*2.93*
20	2.58	2.58
	2.61	2.54
	2.59	*2.56*

[1] Values in italic figures are averages of replicate determinations.

Data from a similar experiment in which 20% glucose was deleted and the diets were fed ad libitum are also presented in table 2 as experiment 2. In this experiment a metabolizable energy of 3.69 Cal. per gram of glucose dry matter was obtained.

Data from the two experiments in which graded levels of cellulose were substituted for glucose are shown in table 3. As expected, there was a stepwise reduction in metabolizable energy of the diets as the proportion of cellulose was increased. In this table, the first figure for each diet represents the value obtained in the first replicate experiment, and the

second value was derived from the second experiment. Analysis of variance showed no significant difference between replicate experiments or between the two levels of feed intake. Accordingly, the data were combined for the calcula-

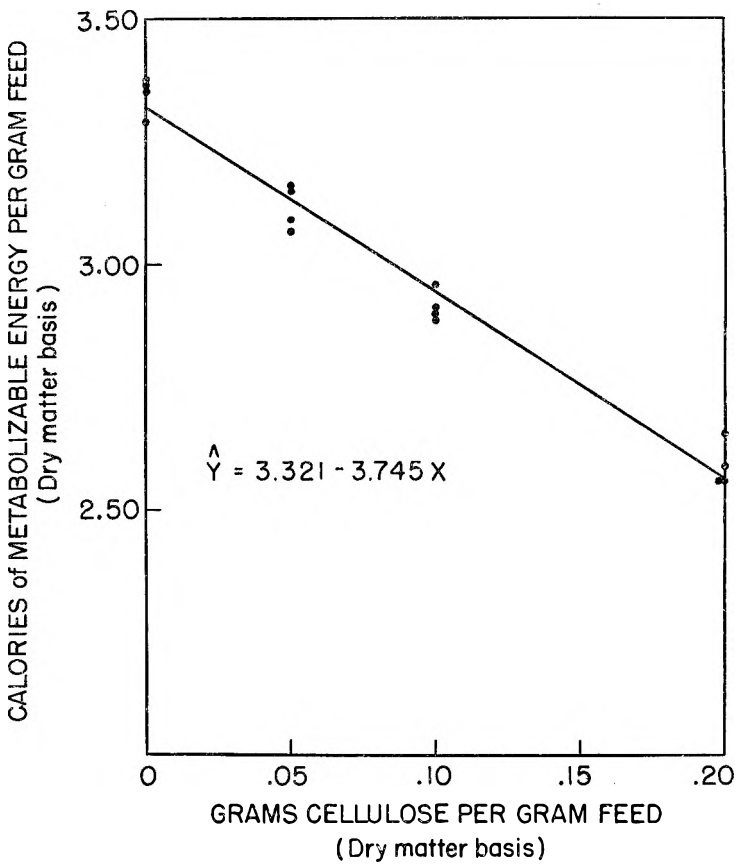

Fig. 1 Linear regression of metabolizable energy of diet on level of cellulose.

tion of a single linear regression equation relating the effect of level of cellulose to metabolizable energy of the diet, yielding the equation:

$$\hat{Y} = 3.321 - 3.745X$$

in which $\hat{Y}$ is metabolizable energy in Calories per gram and X is the proportion of cellulose in the diet (fig. 1).

On the assumption that cellulose had zero metabolizable energy value, these data indicated a metabolizable energy of 3.745 Cal. per gram of glucose dry matter. This value was higher than that estimated from deleting glucose in the earlier experiment, and suggested the possibility that cellulose exerted a slightly negative effect on the digestibility of other diet components, or caused a greater loss of intestinal secretions which were carried into the excreta by the greater indigestible residue of the cellulose-containing diets. Data pre-

TABLE 4

Constancy of excreted energy derived from non-cellulose portion of diets containing graded levels of cellulose [1]

CELLULOSE IN DIET E9	(a) ENERGY OF EXCRETA PER GRAM DIET AT N EQUILIBRIUM	(b) ENERGY OF CELLULOSE PER GRAM DIET	(a − b) ENERGY OF NON-CELLULOSE EXCRETA
%	*Cal.*	*Cal.*	*Cal.*
Ad libitum series			
0	0.94	—	0.94
5	1.18	0.21	0.97
10	1.41	0.42	0.99
20	1.75	0.84	0.91
Restricted series			
0	0.90	—	0.90
5	1.17	0.21	0.96
10	1.37	0.42	0.95
20	1.79	0.84	0.95

[1] All data on dry matter basis.

sented in table 4 indicate that cellulose did not exert such a negative effect. These data show the excreted energy derived per gram of diet (a), corrected to nitrogen equilibrium, for each of the respective diets at the two planes of intake. Assuming that the cellulose was not digested, its energy content (b) could be subtracted from the known excreta energy for each diet, yielding an estimate of the excreta energy derived from the non-cellulose components of each respective diet. These data show no evidence of any relationship between the amount of energy from non-cellulose excreta and the proportion of cellulose fed, indicating that cellulose had

no apparent effect on the utilization of other diet components. If the use of cellulose had caused either a negative effect on digestibility or a greater loss of intestinal secretions, it would be expected that the apparent excreted energy derived from the non-cellulose components would increase as the proportion of cellulose was increased. These data show that cellulose was essentially inert under these conditions.

The second experiment comparing cellulose and glucose is summarized in table 2, experiment 4, which shows the metabolizable energy for the reference diet intact and with 20% cellulose substituted for glucose under ad libitum feeding conditions. The metabolizable energy of glucose, estimated from the average of replicate determinations, was 3.50 Cal. per gram. The apparent disagreement between the two experiments using cellulose is discussed in a later section.

The mean metabolizable energy value of glucose from the 4 different experiments was 3.64 Cal. per gram dry matter.

Productive energy estimations

Summarized in table 5 are data on growth rates, tissue composition, energy gains, and feed consumption from the three experiments conducted. From these data, the productive energy values of the respective diets were calculated by the procedure described in the previous paper (Hill and Anderson, '58). The derivation of the productive energy values was based on the use of average weight by periods as developed by Fraps ('46), and the values 5.66 Cal. per gram of tissue protein and 9.35 Cal. per gram of tissue fat for estimating energy gains during growth. As found in our previous experiments and discussed earlier, the productive energy estimations showed a higher degree of variability than the measurements of metabolizable energy.

The data obtained were used as indicated in table 6 to estimate the productive energy of glucose. The difference between the productive energy of diet E9 and the productive energy of 0.75 gm of diet E9 with 25% of glucose omitted

TABLE 5

Productive energy determinations for diets used to establish the energy of glucose

SUPPLEMENT TO DIET E9	FEED INTAKE [1] PER CHICK	AVERAGE WEIGHT		FINAL COMPOSITION		ENERGY GAIN	PRODUCTIVE ENERGY OF DIET
		Initial	Final	Fat	Protein		
	gm	*gm*	*gm*	*gm*	*gm*	*Cal.*	*Cal./gm*
Deletion of glucose							
1. None	338	123	346	31.1	61.4	443.5	2.34
2. None	203	125	247	16.1	44.6	204.4	
3. Minus 25% glucose	253	121	287	16.9	52.0	261.1	2.01
4. Minus 25% glucose	190	120	245	11.0	43.8	160.7	
Substitution of cellulose							
Replicate 1							
5. None	331	104	323	30.2	57.7	430.0	2.41
6. None	200	110	228	14.2	42.4	184.9	
7. 5% cellulose	328	107	334	28.0	61.0	423.8	2.63
8. 5% cellulose	200	109	230	12.6	44.5	182.8	
9. 10% cellulose	322	107	312	24.9	56.5	369.3	2.68
10. 10% cellulose	200	105	221	10.0	40.9	145.3	
11. 20% cellulose	335	107	288	17.1	52.2	272.4	1.91
12. 20% cellulose	201	107	216	6.3	37.2	86.7	
Replicate 2							
13. None	378	115	360	35.5	63.2	489.6	2.47
14. None	220	114	227	12.9	42.7	164.8	
15. 5% cellulose	365	116	326	29.5	57.9	402.5	2.14
16. 5% cellulose	218	116	229	13.4	42.4	164.5	
17. 10% cellulose	364	113	331	26.2	58.8	382.2	2.44
18. 10% cellulose	219	115	230	10.9	42.4	143.1	
19. 20% cellulose	364	114	297	17.8	54.6	278.1	1.98
20. 20% cellulose	218	114	211	6.7	39.1	85.6	

[1] All even-numbered lots were restricted in feed consumption as described in text.

was considered to be the productive energy of 0.25 gm of glucose; this yielded an estimate of 3.32 Cal. per gram of glucose, approximating 90% of its metabolizable energy. This value seems quite high in comparison to the relationship previously found between productive energy and metabolizable energy values wherein productive energy approximated 65 to 75% of metabolizable energy.

TABLE 6

Estimation of the productive energy of glucose

I. By deletion:

	Calories
(a) Productive energy of diet E9, per gram	2.34
(b) Productive energy of diet E9 minus 25% glucose, per 0.75 gm	1.51
(a — b) = Productive energy of 0.25 gm glucose	0.83
(a — b)/0.25 = Productive energy per gram glucose	3.32

II. By substitution of cellulose:

DIET	PRODUCTIVE ENERGY		
	Diet	E9 minus cellulose diet	Apparent value of glucose
	Cal./gm	*Cal./gm*	*Cal./gm*
E9	2.438		
E9 + 5% cellulose	2.385	0.053	1.06
E9 + 10% cellulose	2.562	— 0.12	—
E9 + 20% cellulose	1.942	0.496	2.48

The data obtained with diets containing graded levels of cellulose were also employed to compute the apparent productive energy of glucose as shown in table 6. The difference in energy value between diet E9 and the diets containing 5 to 10% of cellulose were probably too small to be measured with any degree of reliability by the productive energy method. Accordingly, it seems probable that the only estimate worth considering is that based on the difference between E9 and the diet containing 20% of cellulose; the apparent value of glucose estimated from this difference was 2.48 Cal. per gram.

The average of the two methods for determining the productive energy of glucose was 2.90 Cal. per gram dry matter, which approximates 80% of its metabolizable energy.

DISCUSSION

The data obtained in these experiments show that the combustible energy of glucose (Cerelose) is essentially quantitatively utilized by the growing chick, approximately 97% appearing as metabolizable energy.

In order to use glucose as a primary reference standard for the determination of metabolizable energy values of other substances, its energy value must be known accurately. Any error in the assumed reference value will appear as a constant error in all energy estimates derived from it; i.e., if the value for glucose is in error by +0.10 Cal. per gram, all estimates for other materials derived by substituting them for glucose in the reference diet will likewise err by +0.10 Cal. per gram. It was the recognition of the need for a well established value for glucose which led us to use two independent methods for its estimation.

The two methods, deletion of glucose and its replacement by cellulose, gave values ranging from 3.50 to 3.74 Cal. per gram. The true value of glucose for the chick certainly lies within this range. Because our data gave no evidence of any negative effect of cellulose on diet utilization, it is considered appropriate to average all of the values obtained by the two methods. Accordingly, on the basis of these data, the value of 3.64 Cal. per gram (dry matter basis) has been taken to represent the metabolizable energy of glucose (Cerelose) as a reference standard to be used in further studies on the metabolizable energy of other materials. This value agrees well with data obtained by Forbes and Swift ('44) for the rat, which showed utilization of 97.6% of the gross energy of Cerelose as metabolizable energy, or approximately 3.66 Cal. per gram of dry matter.

The replicability of the estimates of glucose value appear to be less than our previous studies had shown to be possible for successive determinations of metabolizable energy of a single diet. The range of glucose values from the separate experiments approximated ±4% of the average. This was due largely to the effect of assigning all of the difference

between comparative diets to the substituted or deleted glucose, which magnified differences and errors by 4- or 5-fold. In our previous work, the precision of metabolizable energy measurements was approximately 1% (standard deviation). The variations between separate experiments on the value of glucose are consistent with this precision of measurement of the complete diets.

The largest discrepancy (3.74 vs. 3.50) occurred between the two experiments using cellulose. The standard error of the regression slope obtained in the analysis of the first of these experiments was ± 0.16, which includes within its range the average value for all experiments (3.64) and is sufficiently large to indicate that the difference between the two experiments was not significant by usual standards. It is unlikely that the discrepancy between these experiments was due to differences in level of food intake, since in our previous work as well as the present study (table 3) rate of feed consumption was found to have no effect on metabolizable energy value.

Combining the data to obtain a mean value for glucose by taking a simple average of the 4 values from the separate experiments would appear to disregard the greater number of observations made in experiments using cellulose. This procedure was used mainly because it was considered appropriate to give equal weight to the two independent approaches to the problem, and also because the experiments with cellulose appeared to be more variable. The slight increase in average value which would result from giving added weight to the more extensive experiment with cellulose seems of little significance in view of the error associated with these estimates.

The data obtained in these experiments for the productive energy of the experimental diets and the computation of a value for glucose from them illustrate again the extreme variability of this measure of energy value. The average productive energy of glucose, 2.90 Cal. per gram, is approximately 80% of its metabolizable energy, which is not greatly

different from the relation between productive and metabolizable energy values observed in our previous work. It is based on two widely discrepant estimates, 3.32 and 2.48 Cal. per gram, derived from the two different experimental procedures used. These estimates approximate 90 and 68% of the metabolizable energy of glucose, respectively, a difference which invites speculation concerning the possible effect of extra work incurred by the feeding of cellulose. However, the variability associated with productive energy measurements on the complete diets, and the consequent errors of computations based on differences between diets, are such that the present data can only be considered suggestive of a possible relationship. Since net energy is known to change with the nutrient balance of the diet, it is reasonable to conclude that the change in nutrient balance produced by deleting or replacing glucose may have (1) so changed the net energy of the diet that a valid estimate of glucose value could not be obtained, or (2) increased the already considerable variability characteristic of the productive energy method. In either case, the establishment of a reliable reference value for glucose productive energy by this procedure appears most difficult.

SUMMARY

The metabolizable and productive energy of glucose for the growing chick have been established by two methods. In the first, a known proportion of glucose was omitted from the standard reference diet, and the altered diet was pair-fed to equalize the intake of non-glucose components. In the second, graded levels of cellulose were used to replace equal amounts of glucose. Simultaneous measurements of metabolizable energy (by combustion of feed and excreta) and productive energy (by carcass analysis) were made.

The average metabolizable energy of glucose was found to be 3.64 Cal. per gram of dry matter by the two methods. The experiments with cellulose showed no evidence of any negative effect of cellulose on utilization of other components of the diet.

The productive energy of glucose was estimated to be 3.32 and 2.48 Cal. per gram by the two respective methods. The average, 2.90 Cal. per gram, is approximately 80% of its metabolizable energy, which is in general agreement with the relation of productive energy and metabolizable energy values observed in previous work.

LITERATURE CITED

FORBES, E. B., AND R. W. SWIFT 1944 Associative dynamic effects of protein, carbohydrate and fat. J. Nutrition, *27:* 453–468.

FRAPS, G. S. 1946 Composition and productive energy of poultry feeds and rations. Texas Agr. Exp. Sta. Bull. 678.

HILL, F. W., AND D. L. ANDERSON 1958 Comparison of metabolizable energy and productive energy determinations with growing chicks. J. Nutrition, *64:* 587–603.

MCKITTRICK, D. S. 1947 The selection of chicks for growth experiments and the evaluation of growth. Growth, *11:* 89–100.

AN UNIDENTIFIED FACTOR IN ALFALFA WHICH COUNTERACTS MINERAL OIL TOXICITY IN THE RAT AND MOUSE [1]

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Data are available indicating that rats and mice fed low-fat diets are particularly sensitive to a number of stressor agents including x-irradiation (Decker et al., '50; Cheng et al., '52), administration of desiccated thyroid (Greenberg and Deuel, '50; Greenberg, '52) and toxic doses of succinylsulfathiozole (SST), atabrine, iodinated casein, triacetin and a combination of SST and dihydrostreptomycin undecylenate (Bosshardt et al., '50; Bosshardt and Huff, '53). A significant protective effect was obtained against each of the above stressor agents by increasing the fat content of the diet. Rats and mice fed low-fat diets are also markedly sensitive to mineral oil administration as evidenced by growth retardation, alopecia, priapism, humped posture, spastic gait and death on rations containing 7.5 or 10% of mineral oil (Bacon et al., '52; Ershoff and Greenberg, '54; Greenberg and Ershoff, '55). As in the other experiments indicated above, the deleterious effects of this stressor agent could be largely counteracted by increasing the fat content of the diet. In the present communication data are presented indicating that alfalfa meal is also effective in counteracting the toxic effects of mineral oil administration in rats and mice on a low-fat ration. The pro-

[1] Communication no. 446 from the Department of Biochemistry and Nutrition, University of Southern California.

tective factor is retained in the defatted fraction of alfalfa and is distinct from any of the known nutrients.

PROCEDURE AND RESULTS

Experiments with immature rats

A series of experiments was designed to study the effects of alfalfa meal and fractions thereof on the growth and appearance of immature rats fed a purified low-fat ration supplemented with 7.5 or 10% of mineral oil. The basal low-fat ration employed in these experiments consisted of sucrose, 71; casein,[2] 24; and salt mixture,[3] 5%. To each kilogram of the above diet were added the following synthetic vitamins: thiamine hydrochloride, 20 mg; riboflavin, 20 mg; pyridoxine hydrochloride, 20 mg; calcium pantothenate, 60 mg; nicotinic acid, 100 mg; ascorbic acid, 200 mg; biotin, 4 mg; folic acid, 10 mg; para-aminobenzoic acid, 400 mg; inositol, 800 mg; vitamin B_{12}, 150 μg; 2-methyl-naphthoquinone, 5 mg; choline chloride, 2 gm; vitamin A, 5000 U.S.P. units; vitamin D_2, 500 U.S.P. units; and alpha-tocopherol acetate, 100 mg. The vitamins were added in place of an equal amount of sucrose. Mineral oil and the various test supplements were incorporated in the above diet in the amounts listed in tables 1 and 2, replacing equal amounts of sucrose. Male rats of the Holtzman and Long-Evans strains were selected at 21 to 24 days of age and a body weight between 40 and 50 gm for the present series of experiments. The rats were housed in metal cages with raised screen bottoms (two or three animals per cage) and were provided with food and water ad libitum. Diets were made up bi-weekly and stored under refrigeration when not in use. The animals were fed daily and all food not consumed 24 hours after feeding was discarded. In each experiment one group was fed the basal low-fat ration; the remaining groups were fed the basal

[2] Vitamin-free Test Casein, General Biochemicals, Inc., Chargrin Falls, Ohio.

[3] Hubbell, Mendel and Wakeman Salt Mixture, General Biochemicals, Inc., Chagrin Falls, Ohio.

TABLE 1

Comparative effects of alfalfa meal, alfalfa fractions, and corn oil on symptoms of mineral oil toxicity in the rat (10 animals per group)

SUPPLEMENTS FED WITH BASAL RATION [1]	BODY WEIGHT		SYMPTOMS [3]	BODY WEIGHT		SYMPTOMS [3]
	Initial	Average gain after 6 weeks [2]		Initial	Average gain after 6 weeks [2]	
	gm	*gm*		*gm*	*gm*	
	Holtzman Strain			*Long-Evans Strain*		
None	44.9	163 ± 4.3	0	47.2	152 ± 9.9	0
10% Corn oil	45.1	233 ± 4.8	0	47.0	244 ± 5.2	0
7.5% Mineral oil	44.8	104 ± 6.2	++	47.2	96 ± 7.2	++
7.5% Mineral oil and 20% alfalfa meal	45.0	219 ± 3.8	0	47.0	212 ± 9.6	0
7.5% Mineral oil and 20% defatted alfalfa meal [4]	45.0	215 ± 3.2	0	46.7	208 ± 9.0	0
7.5% Mineral oil and 10% defatted alfalfa meal	45.1	203 ± 4.7	0	46.6	199 ± 8.4	0
7.5% Mineral oil and 5% defatted alfalfa meal	45.0	168 ± 6.7	0	46.6	172 ± 6.8	0
7.5% Mineral oil and 15% alfalfa residue	45.0	215 ± 3.2	0	46.5	204 ± 7.1	0
7.5% Mineral oil and 5% dried alfalfa juice	45.0	152 ± 8.8	+	46.6	146 ± 9.8	+
7.5% Mineral oil and 5% water soluble extract of alfalfa				46.6	138 ± 8.8	+
7.5% Mineral oil and 1% alfalfa fat	45.0	176 ± 5.8	+	46.7	147 ± 14.6	+
7.5% Mineral oil and 1% corn oil	45.0	198 ± 5.9	0	46.6	184 ± 6.0	0
7.5% Mineral oil and 5% corn oil	45.1	225 ± 5.3	0	46.4	242 ± 5.5	0
7.5% Mineral oil and 10% corn oil	45.1	232 ± 5.6	0	46.5	238 ± 8.6	0

[1] The alfalfa samples were kindly provided by the Research and Development Division of Nutrilite Products, Inc., Buena Park, California.

[2] Including standard error of the mean calculated as follows:

$$\pm \frac{\sqrt{\epsilon \cdot \frac{d^2}{n}}}{\sqrt{n}}$$

where "d" is the deviation from the mean and "n" is the number of observations.

[3] Degree of alopecia was classified as follows: 0, none; + mild; ++, intermediate; +++, severe.

[4] Alfalfa meal was hammer-milled through an 80-mesh screen, then exhaustively extracted with 30% acetone and 70% hexane. Five separate cycles each consisting of thorough extraction followed by drying at room temperature were employed.

TABLE 2

Comparative effects of alfalfa meal and supplements of the known nutrients on symptoms of mineral oil toxicity in the rat [1] *(10 animals per group)*

SUPPLEMENTS FED WITH BASAL RATION [2]	BODY WEIGHT		SYMPTOMS [4]
	Initial	Average gain after 6 weeks [3]	
	gm	*gm*	
None	43.3	168 ± 7.6	0
7.5% Mineral oil	43.3	103 ± 11.9	++
7.5% Mineral oil and 20% defatted alfalfa meal [5]	43.2	222 ± 8.4	0
7.5% Mineral oil and 10% cottonseed oil	43.0	240 ± 9.6	0
7.5% Mineral oil and B vitamins, C and K [6]	44.3	98 ± 9.2	++
7.5% Mineral oil and vitamins A, D and E [7]	43.4	88 ± 11.7	++
7.5% Mineral oil and 10% casein	42.8	99 ± 12.0	++
7.5% Mineral oil and 5% salt mixture	42.6	107 ± 10.6	++
7.5% Mineral oil and 5% cellulose	42.6	139 ± 4.0	+
7.5% Mineral oil and combined supplements [8]	42.8	150 ± 7.8	+
10% Mineral oil and 5% cellulose	43.2	93 ± 6.8	+
7.5% Mineral oil and 2.5% alfalfa ash	43.2	72 ± 8.7	++
7.5% Mineral oil and 2% flavonoids [9]	43.0	124 ± 7.8	++
7.5% Mineral oil and aureomycin HCl [10]	42.8	98 ± 8.8	++
7.5% Mineral oil and 10% yeast	43.0	121 ± 8.6	++
10% Mineral oil	44.0	66 ± 10.8	+++
10% Mineral oil and 10% defatted alfalfa meal	43.3	165 ± 10.2	0
10% Mineral oil and 20% defatted alfalfa meal	43.0	182 ± 9.6	0
10% Mineral oil and 10% cottonseed oil	43.2	198 ± 8.8	0
10% Mineral oil and 5% dried alfalfa juice	43.4	87 ± 8.0	+
10% Mineral oil and 15% alfalfa residue	43.0	166 ± 7.8	0
10% Mineral oil and 10% cellulose	43.0	105 ± 7.2	+

[1] Long-Evans strain.

[2] The alfalfa samples were provided by the Research and Development Division of Nutrilite Products, Inc., Buena Park, California. The casein (Vitamin-free Test Casein) and salt mixture (Hubbell, Mendel and Wakeman Salt Mixture) were obtained from General Biochemicals, Inc., Chagrin Falls, Ohio; the cellulose (Solka-Floc) from the Brown Co., Boston, Massachusetts; and yeast (Primary Dried Yeast, Strain 200) from Anheuser, Busch, Inc., St. Louis, Missouri.

[3] Including standard error of the mean. See footnote 2, table 1.

[4] Degree of alopecia was classified as follows: 0, none; +, mild; ++, intermediate; +++, severe.

[5] See footnote 4, table 1.

[6] The following vitamins were added per kilogram of diet: thiamine hydrochloride, 20 mg; riboflavin, 20 mg; pyridoxine hydrochloride, 20 mg; calcium pantothenate, 60 mg; nicotinic acid, 100 mg; ascorbic acid, 200 mg; biotin, 4 mg; folic acid, 10 mg; para-aminobenzoic acid, 400 mg; inositol, 800 mg; vitamin B_{12}, 150 μg; choline chloride, 2 gm; and 2-methyl-naphthoquinone, 5 mg.

[7] Vitamin A, 5000 U.S.P. units; vitamin D_2, 500 U.S.P. units; and alpha-tocopherol acetate, 100 mg per kilogram of diet.

[8] Casein, 10%; cellulose, 5%; salt mixture, 2.5%; and the vitamin supplements indicated in footnotes 6 and 7.

[9] The following flavonoids were each fed at a 0.4% level in the diet: calcium flavonate glycoside, naringin (naringenin-5-rhamnosidoglucoside), hesperidin complex, hesperidin methyl chalcone and lemon bioflavonoid complex.

[10] Aureomycin·HCl, 100 mg per kilogram of diet.

ration with the supplements listed in tables 1 and 2 (10 animals per group). Feeding was continued for 6 weeks.

Experiment 1. Beneficial effects of alfalfa meal on symptoms of mineral oil toxicity in the rat. In agreement with previous findings (Bacon et al., '52; Greenberg and Ershoff, '55), the addition of mineral oil at a 7.5% level to a purified low-fat ration resulted in a significant retardation in growth and other symptoms of mineral oil toxicity including humped posture, spastic gait, priapism and a "combed, greasy" appearance. These effects were counteracted by the concurrent feeding of corn oil at levels of 10, 5 and 1% of the diet, the growth increment being proportional to the level of fat administered. Alfalfa meal, when incorporated at a 20% level in the diet, was also effective in counteracting all symptoms of mineral oil toxicity. The protective factor (or factors) was retained in the alfalfa residue fraction (the water-washed pulp remaining after the extraction of the juice). Dried alfalfa juice and the water-soluble fraction of alfalfa when fed in amounts corresponding to that present in 20% alfalfa meal had little if any activity. Defatted alfalfa meal, when fed at a 20% level in the diet, was as effective as an equal amount of whole alfalfa meal in counteracting symptoms of mineral oil toxicity (fig. 1). Significant activity was also exhibited by defatted alfalfa meal at 5% and 10% levels of supplementation, but the effects obtained were less marked than that obtained at the 20% level. Alfalfa fat when fed at a 1% level in the diet (corresponding to the amount provided by 20% whole alfalfa meal in the ration) also exhibited some activity although less than that obtained with 20% alfalfa meal (either whole or defatted). Similar results were obtained with both the Holtzman and the Long-Evans strains (table 1).

Experiment 2. Comparative effects of defatted alfalfa meal and supplements of the known nutrients on symptoms of mineral oil toxicity in the rat. In agreement with findings in experiment 1, defatted alfalfa meal when fed at a 20% level in the diet completely counteracted all symptoms of mineral oil toxicity in the rat. The protective factor (or factors)

appears to be distinct from any of the known nutrients. This is indicated by the fact that supplements of all the known vitamins, protein in the form of casein, cellulose or salt mixture, either when fed alone or with one another in amounts equal to or exceeding the amount of such nutrients in 20% defatted alfalfa meal, had little if any protective effect. Alfalfa ash at a level corresponding to the amount provided by 20% alfalfa meal in the diet, aureomycin HCl at a level of 100 mg per kilogram of diet, mixed flavonoids at a 2% level in the diet and yeast at a level of 10% of the diet had little if any significant effect. Findings are summarized in table 2.

Experiments with immature mice

A series of experiments was conducted similar to the above to determine the effects of alfalfa and fractions thereof on the growth, appearance, and incidence of survival of immature mice fed a low-fat diet supplemented with massive doses of mineral oil. The basal low-fat ration employed in these studies consisted of cerelose, 69%; casein,[2] 24%; salt mixture,[3] 5%; and cellulose,[4] 2%. To each kilogram of the above diet were

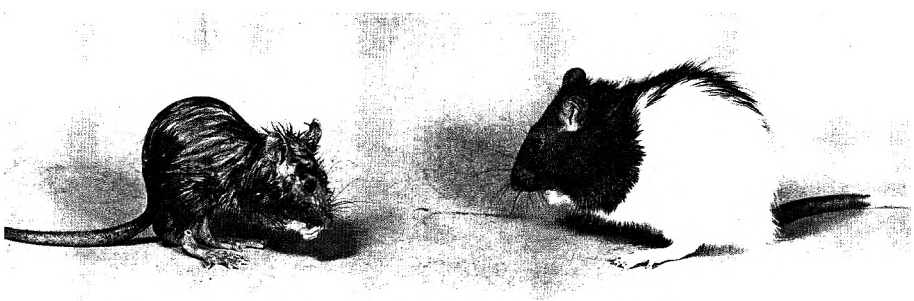

Fig. 1 The rat on the left received a purified low-fat diet containing 10% of mineral oil. The rat on the right received a similar diet supplemented with 20% of defatted alfalfa meal. Photograph was taken after 30 days of feeding.

[2] Footnote, see p. 576.
[3] Footnote, see p. 576.
[4] Solka Floc, Brown Co., Boston, Mass.

added the following synthetic vitamins: thiamine hydrochloride, 10 mg; riboflavin, 10 mg; pyridoxine hydrochloride, 10 mg; calcium pantothenate, 60 mg; nicotinic acid, 100 mg; ascorbic acid, 200 mg; biotin, 4 mg; folic acid, 10 mg; para-aminobenzoic acid, 400 mg; inositol, 800 mg; vitamin B_{12}, 150 μg, 2-methyl-naphthoquinone, 5 mg; choline chloride, 2 gm; vitamin A, 5000 U.S.P. units; vitamin D_2, 500 U.S.P. units; and alpha-tocopherol acetate, 100 mg. The vitamins were added in place of an equal amount of cerelose. Mineral oil and the various test supplements were incorporated in the above diet in the amounts listed in tables 3 and 4, replacing equal amounts of cerelose. Male mice of the Webster strain were selected between 11 and 14 gm in body weight and were fed the various diets indicated in tables 3 and 4. The mice were placed in metal cages with raised screen bottoms (6 animals per cage) and were provided with food and water ad libitum. The animals were fed daily and all food not consumed 24 hours after feeding was discarded. Twelve mice were employed in each group. Data were computed however, on the basis of the top 10 animals in each group to minimize variations in averages due to early deaths and atypical responses on the part of individual mice. Feeding was continued for two to 4 weeks. Results are summarized in tables 3 and 4.

In agreement with previous findings (Ershoff and Greenberg, '54), the addition of mineral oil at a 7.5 or 10% level to a purified low-fat ration resulted in a significant retardation in growth, alopecia and a high incidence of mortality within a 4-week period in the immature mouse. These effects were largely counteracted by the concurrent feeding of cottonseed oil at a 5 or 10% level in the diet. Present findings indicate that defatted alfalfa meal when fed at levels of 5, 10 or 20% of the diet was also effective in counteracting the above symptoms, an optimal effect being observed at the 10% level of supplementation (fig. 2). Dried alfalfa juice or the water-soluble extract of alfalfa when fed in an amount corresponding to that present in 20% alfalfa meal had little if any protective effect. Increasing the casein or salt content, doubling the B

TABLE 3

Effects of alfalfa meal, alfalfa fractions and cottonseed oil on symptoms of mineral oil toxicity in the mouse [1] (10 animals per group)

SUPPLEMENTS FED WITH BASAL RATION [2]	INITIAL BODY WT.	AVERAGE GAIN IN BODY WT. AFTER FOLLOWING DAYS OF FEEDING			PERCENT SURVIVAL [3]	ALOPECIA [4]
		14th	21st	28th		
	gm	*gm*	*gm*	*gm*		
Experiment 3 (a)						
None	12.2	7.0 (10)[5]	10.1 (10)	11.9 (10)	100	0
7.5% Mineral oil	12.3	3.4 (10)	4.2 (8)	2.8 (4)	40	+++
7.5% Mineral oil and 5% defatted alfalfa meal	12.2	8.4 (10)	10.3 (10)	11.8 (10)	100	+
7.5% Mineral oil and 10% defatted alfalfa meal	12.0	8.1 (10)	11.0 (10)	13.1 (10)	100	0
7.5% Mineral oil and 20% defatted alfalfa meal	11.8	9.1 (10)	11.4 (10)	13.6 (10)	100	0
7.5% Mineral oil and 5% dried alfalfa juice	12.3	6.0 (10)	8.2 (5)	4.1 (1)	10	+++
7.5% Mineral oil and 15% alfalfa residue	12.0	7.8 (10)	11.3 (10)	12.6 (10)	100	0
7.5% Mineral oil and 15% defatted alfalfa residue	12.1	8.6 (10)	11.4 (10)	12.8 (10)	100	0
7.5% Mineral oil and 10% cottonseed oil	12.0	9.2 (10)	12.8 (10)	14.6 (10)	100	+
10% Cottonseed oil	12.0	9.7 (10)	14.2 (10)	14.9 (10)	100	0
Experiment 3 (b)						
None	13.0	12.8 (10)	15.8 (10)		100	0
10% Mineral oil	13.3	5.0 (10)	2.2 (3)		30	+++
10% Mineral oil and 5% defatted alfalfa meal	12.9	9.1 (10)	10.8 (10)		100	+
10% Mineral oil and 10% defatted alfalfa meal	12.8	12.2 (10)	13.4 (10)		100	0
10% Mineral oil and 20% defatted alfalfa meal	12.9	13.3 (10)	14.0 (10)		100	0
10% Mineral oil and 5% water-soluble extract of alfalfa	13.2	2.4 (6)	2.2 (4)		40	+++
10% Mineral oil and 15% alfalfa residue	12.8	11.5 (10)	12.3 (10)		100	0
10% Mineral oil and 15% defatted alfalfa residue	12.9	14.6 (10)	15.7 (10)		100	0
10% Mineral oil and 1% cottonseed oil	13.0	8.2 (10)	9.3 (7)		70	+++
10% Mineral oil and 5% cottonseed oil	13.0	12.8 (10)	14.0 (10)		100	++
10% Mineral oil and double vitamins [6]	12.8	3.4 (10)	—		0	+++
10% Mineral oil and 5% salt mixture	13.0	4.9 (1)	3.8 (1)		10	+++
10% Mineral oil and 10% casein	13.0	5.9 (10)	1.3 (6)		60	+++
10% Cottonseed oil	12.9	11.7 (10)	14.6 (10)		100	0

[1] Mice employed in experiment 3(a) were obtained from Curd's Caviary and Animal Supply, Downey, California, those employed in experiment 3(b) from the Taconic Farms, Inc., Germantown, New York. Although mice from both sources were described by the suppliers as "Swiss mice, Webster strain," considerable differences in growth between the two groups occurred on identical diets.

[2] See footnote 1, table 1.

[3] The duration of the experiment was 28 and 21 days respectively for experiments 3(a) and 3 (b).

[4] Degree of alopecia was classified as follows: 0, none; +, slight; ++, moderate; +++, virtually complete.

[5] The values within parentheses indicate the numbers of animals which survived and on which averages are based.

[6] Water-soluble and fat-soluble vitamins were added in amounts equal to those present in the basal ration.

TABLE 4

Effects of alfalfa fractions, the known nutrients and other materials of plant and animal origin on symptoms of mineral oil toxicity in the mouse [1] (10 animals per group)

SUPPLEMENTS FED WITH BASAL RATION [2]	INITIAL BODY WT.	AVERAGE GAIN IN BODY WT. AFTER 14 DAYS OF FEEDING	PERCENT SURVIVAL [3]	ALOPECIA [4]
	gm	*gm*		
Experiment 3 (c)				
None	11.8	6.4 (10)[5]	100	0
10% Mineral oil	12.0	1.3 (9)	90	++
10% Mineral oil and 20% defatted alfalfa meal	11.8	7.1 (10)	100	0
10% Mineral oil and 5% dried alfalfa juice	12.0	—0.7 (5)	50	+++
10% Mineral oil and 5% water-soluble extract of alfalfa	12.2	—0.2 (6)	60	+++
10% Mineral oil and 15% alfalfa residue	11.9	7.8 (10)	100	0
10% Mineral oil and 15% defatted alfalfa residue	11.8	8.2 (10)	100	0
10% Mineral oil and 2.5% alfalfa ash	12.1	—0.1 (7)	70	+++
10% Mineral oil and 2.5% alfalfa lipids	12.0	—	0	+++
10% Mineral oil and B vitamins, C and K [6]	12.1	1.1 (9)	90	++
10% Mineral oil and vitamins A, D and E [7]	12.0	3.0 (10)	100	++
10% Mineral oil and 2.5% salt mixture	12.1	2.2 (10)	100	++
10% Mineral oil and 10% casein	11.9	2.3 (8)	80	++
10% Mineral oil and 2% flavonoids [8]	12.1	3.2 (9)	90	++
10% Mineral oil and 5% cellulose	12.0	4.5 (10)	100	+
10% Mineral oil and 20% rye grass	11.8	6.8 (10)	100	0
10% Mineral oil and 20% orchard grass	11.7	6.4 (10)	100	0
10% Mineral oil and 20% wheat grass	11.9	6.5 (10)	100	0
10% Mineral oil and 20% fescue grass	12.0	6.2 (10)	100	0
10% Mineral oil and 20% oat grass	12.0	6.1 (10)	100	0
10% Mineral oil and 10% desiccated whole liver	11.8	0.5 (1)	10	+++
10% Mineral oil and 10% yeast	12.1	—1.2 (1)	10	+++
10% Mineral oil and 10% defatted fish meal	11.9	1.7 (5)	50	++
10% Mineral oil and 10% cottonseed oil	12.0	8.6 (10)	100	+
10% Cottonseed oil	11.8	6.2 (10)	100	0

[1] Mice were obtained from Curd's Caviary and Animal Supply, Downey, California.

[2] The alfalfa samples, casein, salt mixture, cellulose and yeast were obtained from the sources indicated in footnote 1, table 2. The dehydrated rye grass, orchard grass, wheat grass, fescue grass and oat grass were obtained from the National Chlorophyll & Chemical Co., Lamar, Colorado; liver (Desiccated Liver N. F.) from Wilson Laboratories, Chicago, Ill.; and defatted fish meal from VioBin Corp., Monticello, Illinois.

[3] The duration of the experiment was 14 days.

[4] Degree of alopecia was classified as follows: 0, none; +, slight; ++, moderate; +++, virtually complete.

[5] The values within parentheses indicate the numbers of animals which survived and on which averages are based.

[6] The following vitamins were added per kilogram of diet: thiamine hydrochloride, 10 mg; riboflavin, 10 mg; pyridoxine hydrochloride, 10 mg; calcium pantothenate, 60 mg; nicotinic acid, 100 mg; ascorbic acid, 200 mg; biotin, 4 mg; folic acid, 10 mg; para-aminobenzoic acid, 400 mg; inositol, 800 mg; vitamin B_{12}, 150 μg; 2-methyl-naphthoquinone, 5 mg; and choline chloride, 2 gm.

[7] The following were added per kilogram of diet: 5000 U.S.P. units of vitamin A, 500 U.S.P. units of vitamin D and 100 mg of alpha-tocopherol acetate.

[8] The following flavonoids were each fed at a 0.4% level in the diet: calcium flavonate glycoside, naringen (naringenin-5-rhamnosidoglucoside), hesperidin complex, hesperidin methyl chalcone and lemon bioflavonoid complex.

vitamin, ascorbic acid and 2-methyl-naphthoquinone content or doubling the vitamin A, D, and tocopherol content of the basal ration were also without protective effects. Alfalfa ash at a level corresponding to the amount provided by 20% defatted alfalfa meal in the diet and alfalfa lipids at a 2.5% level in the diet were also without protective effect. Alfalfa residue (the water-washed pulp remaining after extraction of the juice) and defatted alfalfa residue were as active as defatted alfalfa meal in counteracting symptoms of mineral oil toxicity. A supplement of 5% of purified cellulose (corresponding to approximately twice the total fiber provided by 10% of defatted alfalfa meal in the diet) also exhibited some activity although less than that obtained with defatted alfalfa meal. Dehydrated rye grass, orchard grass, wheat grass, fescue grass, and oat grass also had significant activity. Desiccated liver and yeast when fed at a 10% level in the diet and desiccated and defatted fish meal at a similar level of supplementation were without protective effect. The findings indicate that mice differ significantly from rats in their comparative response to cottonseed oil and defatted alfalfa meal supplements when fed with a low-fat ration containing toxic amounts of mineral oil. Whereas both supplements were effective in both species in counteracting symptoms of mineral oil toxicity, in the rat, cottonseed oil at levels of 5 or 10% of the diet was at least as active as 20% of defatted alfalfa meal in counteracting the

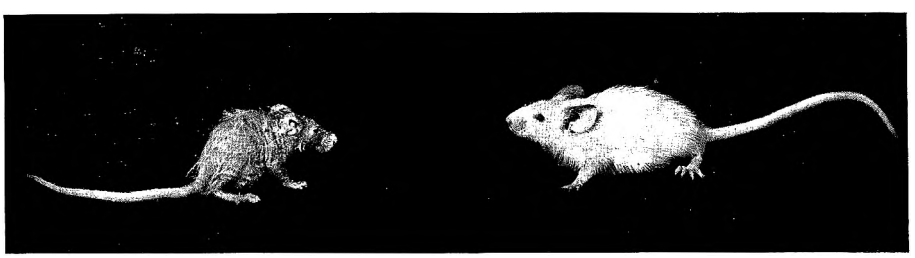

Fig. 2 The mouse on the left received a purified low-fat diet containing 7.5% of mineral oil. The mouse on the right receivd a similar diet supplemented with 10% of defatted alfalfa meal. Photograph was taken after 24 days of feeding.

deleterious effects of mineral oil administration. In the mouse, however, although cottonseed oil at levels of 5 or 10% of the diet counteracted the growth retardation and prevented the death of mice fed the mineral oil-containing diets, these supplements were only moderately effective in preventing alopecia. Even at the 10% level of cottonseed oil supplementation, approximately one third to one half of the mice administered the mineral oil-containing rations showed extensive alopecia; in contrast, no alopecia occurred in any of the mice fed comparable amounts of mineral oil in conjunction with the 10 or 20% defatted alfalfa meal supplements.

DISCUSSION

Present findings indicate that defatted alfalfa meal contains a factor or factors distinct from any of the known nutrients that counteracted symptoms of toxicity in immature rats and mice fed a purified low-fat ration supplemented with massive doses of mineral oil. Supplements of dried alfalfa juice, the water-soluble extract of alfalfa, alfalfa ash or alfalfa lipids had little if any protective effect. Alfalfa residue (the water-washed pulp remaining after extraction of the juice) was a potent source of the active factor. This is the same alfalfa fraction that (1) counteracted the inhibitory effects of massive doses of estradiol on ovarian development in the immature rat (Ershoff et al., '56), (2) prolonged the survival of immature hamsters fed highly purified diets (Ershoff, '56), (3) promoted growth of immature guinea pigs fed a mineralized dried milk ration (Ershoff, '57a), and (4) counteracted the toxic effects of massive doses of glucoascorbic acid in the immature rat (Ershoff, '57b), effects that could not be obtained with supplements of the known nutrients, either alone or in combination. The term "PR (Plant Residue) Factor" has been suggested as a generic term for the substance (or substances) in alfalfa residue (and other succulent plants) responsible for the effects indicated above (Ershoff, '58). A rapid bioassay procedure for measuring PR Factor activity has been de-

scribed employing toxic doses of glucoascorbic acid as a stressor agent (Ershoff, '57b). A high correlation has been observed in the PR Factor activity of alfalfa fractions as determined by the above assay procedure and the activity of the same alfalfa fractions under the experimental conditions indicated above.

No data are available to indicate the *modus operandi* whereby defatted alfalfa meal exerted its protective effect under conditions of the present experiment. It has been established that low-fat diets containing mineral oil at levels of 7.5 or 10% of the ration when fed to immature rats and mice result in retardation of growth and other symptoms which can be corrected by the administration of methyl linoleate, cottonseed oil and other fats (Bacon et al., '52; Ershoff and Greenberg, '54; Greenberg and Ershoff, '55). Bosshardt and Huff ('53) have suggested that fat *per se* is an essential nutrient which, if not supplied in adequate amounts by the diet, must be synthesized by the animal. The capacity to synthesize certain long-chain fatty acids is markedly impaired by various stressor agents. When animals on a low-fat ration are subjected to these stressor agents, nutritive failure occurs which can be counteracted by providing the long-chain fatty acids indicated above. Bosshardt and Huff ('53) have pointed out, however, that certain natural foodstuffs contain a factor or factors apparently distinct from any of the known nutrients, which is also effective in correcting the nutritive failure induced by exposure to various stressor agents, presumably by facilitating the synthesis of fat. Present findings suggest that alfalfa meal and, more specifically, its defatted, water-insoluble fraction, is a potent source of such a factor. It is possible, however, that the protective effects of cottonseed oil or corn oil in the present experiment were due not to these supplements *per se* but to their favorable influence on the synthesis (either by the intestinal flora or the animals' own tissues) of some factor or factors similar to that present in the defatted water-insoluble alfalfa residue. The greater efficacy of defatted alfalfa as contrasted to cottonseed oil in counter-

acting symptoms of mineral oil toxicity in the immature mouse would be in accord with the latter hypothesis.

Although mineral oil was at one time believed to pass through the intestinal tract unabsorbed (see Becker, '52, for a historical review of the subject), it is now established that mineral oil is absorbed in sufficient quantities to be demonstrable in the lymph, liver, and other tissues (Stryker, '41; Frazer et al., '42). Frazer et al. ('42) demonstrated histologically, biochemically, and by actual count of the oil droplets through special staining techniques, that 35 to 60% of *emulsified* mineral oil is absorbed through the bowel wall. It is well established that mineral oil impairs the absorption of carotene and several of the fat-soluble vitamins, presumably by dissolving these nutrients from food in the intestine with the result that they are excreted with the mineral oil in the feces. It has been suggested that mineral oil may impair the *utilization* of nutrients as well (Greenberg and Ershoff, '55). Further studies are indicated to determine to what extent the protective effects of cottonseed or corn oil and defatted alfalfa meal in the present experiment may have been due to the possible effects of these supplements in promoting the transport, breakdown, detoxification or excretion of absorbed mineral oil.

SUMMARY

Immature rats and mice were fed purified low-fat rations to which were added mineral oil at levels of 7.5 and 10% of the diet. The growth retardation and other toxic manifestations of mineral oil administration under these conditions were largely counteracted by the concurrent administration of either cottonseed or corn oil or defatted alfalfa meal. The protective factor (or factors) in alfalfa was retained in the residue fraction (the water-washed pulp remaining after extraction of the juice). Dried alfalfa juice, the water-soluble extract of alfalfa, alfalfa ash or alfalfa lipids had little if any activity. The protective factor in defatted alfalfa was distinct from any of the known nutrients.

LITERATURE CITED

BACON, E. K., S. LASSEN, S. M. GREENBERG, J. W. MEHL AND H. J. DEUEL, JR. 1952 The influence of ingested mineral oil upon the development of an essential fatty acid deficiency in the rat. J. Nutrition, *47:* 383.

BECKER, G. L. 1952 The case against mineral oil. Am. J. Dig. Dis., *19:* 344.

BOSSHARDT, D. K., AND J. W. HUFF 1953 Nutritional deficiencies in mice caused by stress agents. J. Nutrition, *50:* 117.

BOSSHARDT, D. K., W. J. PAUL, R. H. BARNES AND J. W. HUFF 1950 Production of a nutritional deficiency in mice fed a low fat diet. Proc. Soc. Exp. Biol. Med., *75:* 722.

CHENG, A. L. S., G. D. KRYDER, L. BERGQUIST AND H. J. DEUEL, JR. 1952 The effect of fat level of the diet on general nutrition. IX. The relationship of radiation injury in the rat to the fat content of the diet. J. Nutrition, *48:* 161.

DECKER, A. B., D. L. FILLERUP AND J. F. MEAD 1950 Chronic essential fatty acid deficiency in mice. Ibid., *41:* 507.

ERSHOFF, B. H. 1956 Beneficial effects of alfalfa, aureomycin and cornstarch on the growth and survival of hamsters fed highly purified rations. Ibid., *59:* 579.

———— 1957a Beneficial effects of alfalfa and other succulent plants on the growth of immature guinea pigs fed a mineralized dried milk ration. Ibid., *62:* 295.

———— 1957b Beneficial effects of alfalfa and other succulent plants on glucoascorbic acid toxicity in the rat. Proc. Soc. Exp. Biol Med., *95:* 656.

———— 1958 Unidentified nutritional factors and resistance to stress. Proc. Am. Pharm. Manufacturers' Assn., Western Section, 12th Annual Conference.

ERSHOFF, B. H., AND S. M. GREENBERG 1954 Comparative effects of dietary fat and dextrin on mineral oil toxicity in the mouse. Amer. J. Dig. Dis., *21:* 363.

ERSHOFF, B. H., H. J. HERNANDEZ AND J. H. MATTHEWS 1956 Beneficial effects of alfalfa on the ovarian development of immature rats fed massive doses of alpha-estradiol. J. Nutrition, *59:* 147.

FRAZER, A. C., H. C. STEWART AND J. H. SCHULMAN 1942 Emulsification and absorption of fats and paraffins in the intestines. Nature, *149:* 167.

GREENBERG, S. M. 1952 The protective effect of dietary fat on immature rats fed thyroid. J. Nutrition, *47:* 31.

GREENBERG, S. M., AND H. J. DEUEL, JR. 1950 The protective effect of high-fat diets on immature rats fed thyroid. Ibid., *42:* 279.

GREENBERG, S. M., AND B. H. ERSHOFF 1955 Comparative effects of liver residue and dietary fat on mineral oil toxicity in rats fed a low fat ration. Exp. Med. Surg., *13:* 143.

STRYKER, W. A. 1941 Absorption of liquid petrolatum ("mineral oil") from the intestine. A histologic and chemical study. Arch. Path., *31:* 670.

NUTRITION OF SALMONOID FISHES

VI. PROTEIN REQUIREMENTS OF CHINOOK SALMON AT TWO WATER TEMPERATURES [1,2]

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INTRODUCTION

Previous studies with fish, most recently by Gerking ('55a) with goldfish, indicate that the higher the protein intake, the higher the nitrogen retention and gain in weight. Prior to investigating the quantitative amino acid requirements of salmon, it seemed desirable to confirm this relationship, and to establish a minimum protein level that would produce maximum growth. Recent studies by Bressani and Mertz ('56) have shown the importance of maintaining the protein level above the minimum protein requirement when determining the quantitative requirement for an essential amino acid.

Since fish are poikilothermous animals, there is an additional variable not encountered in most animal studies. Pentalow ('39) showed that consumption of natural foods and growth rates of young brook trout, another salmonid, increased with rising temperature up to 60°F then decreased with rising temperature. Baldwin ('57) confirmed these results but found the maximum to be at 56°F.

[1] Journal Paper no. *1253*, Purdue Agricultural Experiment Station. The experimental data in this paper are taken from a thesis submitted by Donald C. DeLong in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry, Purdue University.

[2] Presented at the meetings of the American Institute of Nutrition, Chicago, Ill., April, 1957.

In the present studies, water supplies at 47° and 58° F were used to determine the effect of temperature on the minimum protein level for maximum growth.

Studies on production diets for salmonoid fishes showed that the protein levels of these production diets vary from 60.7% to 64.6% (Wood et al., '57a). Since the protein component of the diet is usually the most expensive major ingredient, information on minimum requirements should also prove useful to the production hatchery.

EXPERIMENTAL

The experiments were designed to determine the minimum amount of protein needed to give optimum growth for this species. All lots were fed diets containing an amino acid mix, casein, and gelatin with a balance of essential amino acids similar to that in whole egg protein (table 1). Other components of the diet (table 2) were the same as those previously reported (Halver, '57). Variations in the total crude protein content were obtained by reducing the amount of each of the three nitrogenous components proportionately. As the protein components were reduced in the diet, they were replaced by an equivalent weight of dextrin. The levels in the first feeding trial were 13, 39, 52 and 65% protein. The second feeding trial was made with 9 protein levels at 5% increments from 25 to 65%.

The methods of diet preparation and general experimental feeding techniques were the same as those previously reported (Halver, '57). The diet was bound with carboxymethylcellulose to give the consistency of bread dough. Water added during mixing of the diet was heated to 40°C to avoid utilization of the binding property of gelatin, since the amount of gelatin varied. After preparation, the diets were either frozen or stored under refrigeration until used.

Chinook salmon fingerlings obtained from the Willard Fish Cultural Station at Cook, Washington, were used in these experiments. Five lots of 200 fish each were used at both

TABLE 1

Source and amino acid composition of 65% protein diet

AMINO ACID	AMINO ACIDS IN 70 GM WHOLE EGG PROTEIN	AMINO ACIDS SUPPLIED BY 40 GM CASEIN	AMINO ACIDS SUPPLIED BY 10 GM GELATIN	AMINO ACIDS SUPPLIED BY CRYST. AMINO ACIDS	PROTEIN SUPPLIED BY CRYST. AMINO ACIDS
	gm	*gm*	*gm*	*%*	*%*
Arginine	4.48	1.68	0.82	1.98	3.98
Histidine	1.47	1.28	0.09	0.10	0.17
Lysine	5.04	3.40	0.50	1.14	1.37
Phenylalanine	4.41	2.52	0.23	1.66	0.88
Tyrosine	3.15	2.56	0.05	0.54	0.26
Tryptophan	1.05	0.52	0.00	0.53	0.45
Cystine	1.68	0.16	0.01	1.51	1.10
Methionine	2.87	1.40	0.08	1.39	0.82
Threonine	3.01	1.80	0.19	1.02	0.74
Leucine	6.44	4.00	0.35	2.09	1.40
Isoleucine	5.60	3.00	0.17	2.43	1.62
Valine	5.11	3.08	0.28	1.75	1.31
Glycine				5.06	5.90
Total	44.31	25.40	2.77	21.20	20.00

temperatures in the first experiment, and 9 lots of 150 fish each at both temperatures in the second experiment. Fish were housed in screened wood-plank troughs which had been sealed with an inert plastic film. Water was maintained at a constant level and drained off at about three gallons per minute. In the first experiment, the water at the lower water temperature was obtained from the Little White Salmon River where the water temperature varied from 45 to 49°F. In the second experiment, the water came from a spring with a constant

TABLE 2

Components of diet

COMPONENT	RANGE
	gm
Casein	8–40
Gelatin	2–10
Amino acid mix	4.5–22
Dextrin	61.5–4
Corn oil	5
Cod liver oil	2
Minerals [1]	4
Alpha cellulose + vitamins [1]	3
Carboxymethylcellulose	10
	100
Water	100
Total	200

[1] See Halver ('57).

temperature of 47°F. Both of these water supplies were at the Western Fish Nutrition Laboratory, Cook, Washington. The warmer water came from a spring at the Fish Nutrition Laboratory, Hagerman, Idaho, and had a constant temperature of 58°F. The river water supply passes through filters, and varies only in mineral content and temperature. The spring water supplies were from covered reservoirs.

The fish were fed a slowly sinking diet expelled through a garlic press into the upper portion of the water. Diets were fed as long as the fish accepted them, and feeding was stopped as soon as any portion reached the bottom of the trough. The

fish would eat off the bottom, but this was avoided as much as possible since leaching of nutrients from the diets occurs. The fish were gradually adapted to the 65% protein diet prior to the start of the experiment (see Halver et al., '57). Fish were fed three times daily, 6 days weekly, on a rigid schedule. The entire population of each trough was weighed bi-weekly. Troughs were cleaned partially daily without removing the fish, and were drained, cleaned, and disinfected during the bi-weekly weighing period.

After completion of each experiment, 10 representative samples were taken from each lot and were examined with the aid of a trained histologist for gross pathological changes of the gills, liver, kidney, spleen, gastrointestinal tract, visceral fat, tail and fins. Ten per cent of the remaining fish were taken for proximate analysis and ash, total lipid and total nitrogen determined as described by Wood et al. ('57b).

RESULTS

Table 3 summarizes the average individual weight gains obtained in 10 weeks on different protein levels with water temperatures of 47°F at Cook, Washington. In the first feeding test with protein levels of 13 to 65%, the fish were small at the start, averaging about 1.5 gm. The final weights averaged from 1.95 to 2.34 gm, and the highest average gain of 0.85 gm was observed with the 39% protein level. A growth depression was observed at higher levels of crude protein. In the second feeding tests, 9 levels of crude protein were used, and the hatchery fish available were about three times heavier than those used earlier in the summer, averaging about 5.6 gm. The final individual average weights ranged from 6.0 to 7.6 gm, and the fish at the 40% protein level made an individual average gain of 1.64 gm. Higher protein levels showed no significant improvement over the 40% level and no growth depression was observed.

In figure 1, per cent protein in the diet has been plotted against per cent gain in body weight for the 10-week period. The upper curve represents the smaller fish in the first feed-

ing test. A hypothetical growth plateau representing maximum growth has been projected. In the lower curve are plotted the per cent gains of the larger fish in the second feeding trial. The curves suggest a minimum requirement of 40% crude protein for fingerling chinook salmon at a water temperature of 47°F.

TABLE 3

Protein requirement of chinook salmon

CRUDE PROTEIN	REQUIREMENT AT 47°F			REQUIREMENT AT 58°F		
	Av. initial weight	Av. final weight	Av. gain	Av. initial weight	Av. final weight	Av. gain
%	gm	gm	gm	gm	gm	gm
13	1.49	1.97	0.48 (12) [1]	2.77	3.80	1.03 (57) [1]
26	1.49	2.13	0.64 (12)	2.50	4.95	2.45 (28)
39	1.49	2.34	0.85 (7)	2.60	6.65	4.05 (20)
52	1.47	1.99	0.52 (19)	2.57	7.68	5.11 (22)
65	1.48	1.95	0.47 (19)	2.46	8.04	5.58 (32)
25	5.50	6.19	0.69 (4)	5.82	8.36	2.54 (33)
30	5.48	6.03	0.55 (3)	5.80	8.71	2.75 (4)
35	5.50	6.74	1.24 (0)	5.83	8.84	3.01 (3)
40	5.56	7.20	1.64 (0)	5.56	9.22	3.66 (2)
45	5.66	7.33	1.67 (3)	5.56	8.84	3.28 (1)
50	5.47	6.96	1.49 (2)	6.00	10.30	4.30 (1)
55	5.80	7.46	1.66 (1)	5.55	10.18	4.75 (0)
60	5.34	6.87	1.53 (3)	5.44	9.56	4.18 (5)
65	5.80	7.60	1.80 (0)	5.54	10.05	4.51 (19)

[1] Fish mortality is given within parentheses. Two hundred fish in each of first 5 lots and 150 in each of last 9 lots, at start of experiment.

Table 3 also summarizes the average individual weight gains obtained in 10 weeks on different protein levels with a water temperature of 58°F at Hagerman, Idaho. In the first feeding test with protein levels of 13 to 65%, the initial individual average weights were about 2.6 gm. The final weights averaged from 3.8 to 8.0 gm, and the highest average gain of 5.58 gm was observed at the 65% protein level. The mortality was consistently high in this test. In spite of this, the fish on the 52 and 65% protein levels tripled their weights in 10 weeks.

In the second feeding test, 9 levels of crude protein were used and the fish were heavier, with initial individual average weights per lot of 5.4 to 6.0 gm. The final individual average weights ranged from 8.36 to 10.30 gm, and the fish at the 55% and the 65% protein levels made the highest individual average gains, 4.75 and 4.51 gm, respectively.

In figure 2, the per cent protein in the diet has been plotted against per cent gain in body weight for the 10-week period. Projecting horizontal lines through the highest points and determining the intercept with lines representing the slopes

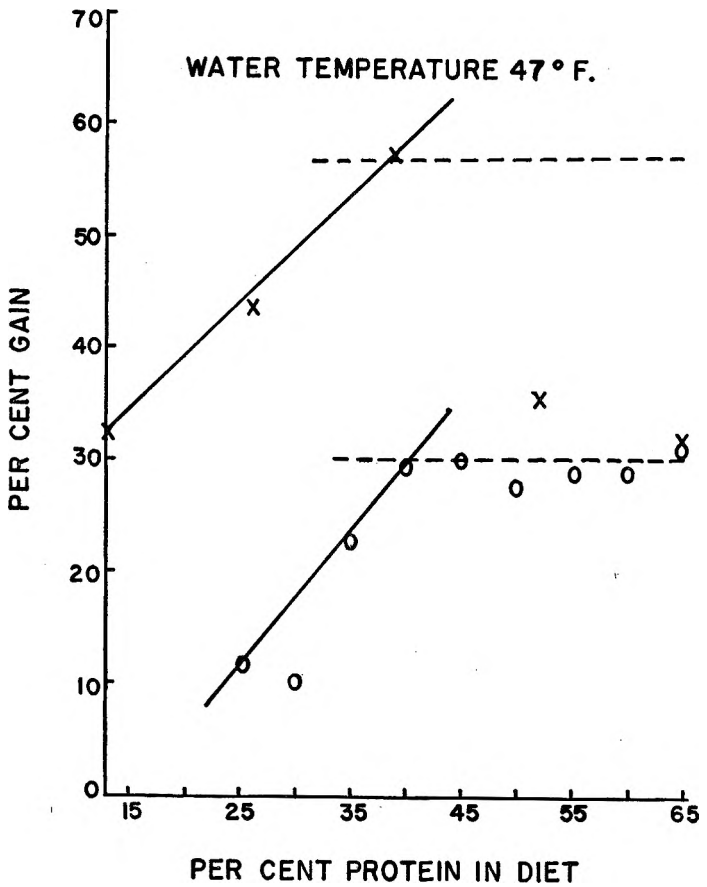

Fig. 1 Protein requirement of chinook salmon at 47°F.

of the rising part of the curves gives a minimum requirement value of about 55% protein.

Gross post mortem examination showed bright pink gills, and smooth glistening dark red kidney and spleen, and livers of normal size, in all groups. The visceral fat was varied but showed no relation to the diet fed, and if present was considered to be the result of rapid growth on the synthetic diet. Frayed tails and fins were noticed in about 60% of each lot at the higher temperature only. Also at the higher water temperatures, there were a few cases of distended stomachs with

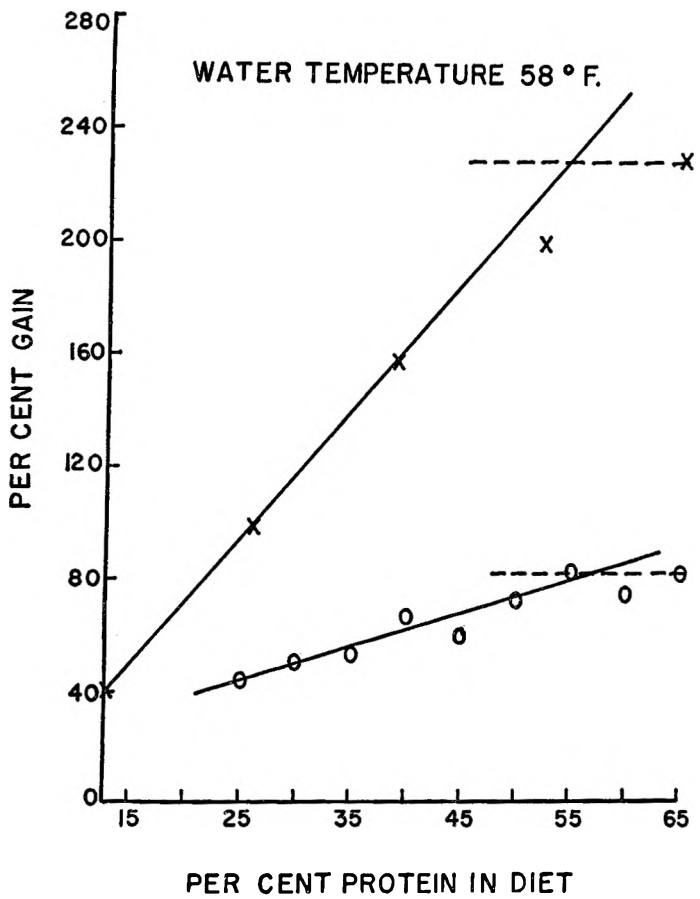

Fig. 2 Protein requirement of chinook salmon at 58°F.

a deposit of carboxymethylcellulose at the pyloric sphincter, indicating that when the intake was greatly increased the fish were unable to handle this carbohydrate derivative. There were no gross pathological findings that could be attributed to protein deficiency or protein excess.

Proximate analysis of representative fish did not show any differences in ash, total lipid or total nitrogen between diets. The total lipid content of the fish at the higher water temperature was about 30% higher than at the lower water temperature, which was attributed to a larger intake of food and more rapid growth.

DISCUSSION

In the absence of knowledge of the quantitative amino acid requirements of this species, the amino acid pattern for the 10 essential amino acids was adjusted to that of whole egg protein. Nasset ('57) has shown, with rats, that mixtures of amino acids simulating egg protein are superior to mixtures of essential amino acids based on the mean minimum requirements. Fish differ qualitatively from the rat with respect to arginine (Halver et al., '57); but, as can be seen from table 1, arginine would not be expected to be a limiting factor in these rations.

Phillips et al. ('48) reported that the safe level of digestible carbohydrate in fish diets was about 9%. Levels over this could cause "high glycogen" livers which were swollen, glossy in appearance and pale in color. This value was calculated with a meat diet and actually corresponds to 33% of the diet as carbohydrate on a dry weight basis. McLaren et al. ('46) have shown that the liver damage due to excess carbohydrate could be reduced if 5% liver or a vitamin supplement was included in the diet. In the present experiment, levels of carbohydrate as high as 61% did not produce liver damage as described by Phillips et al. and by McLaren and coworkers. There was some indication of increased mortality at the higher water temeperature, but these protein levels were low

with respect to the requirement. It should be noted that the diet outlined in tables 1 and 2 contained an adequate supply of vitamins (Halver, '57).

These experiments suggest that at the lower water temperature of 47°F, high levels of protein may even be toxic to salmon weighing less than 2 gm. Thus, in our feeding tests (table 3) the fish gained less weight at the 52 and 65% protein levels than at the 13 and 26% protein levels.

A similar growth depression was not observed with older fish weighing about 6 gm. Here the gains reached a plateau at 40% of crude protein. It is possible that the metabolic activity at low water temperature is at such a reduced level that it does not permit rapid elimination of excess amino acids in the amino acid pool of very young fish receiving high levels of peptide-bound and free amino acids. At higher water temperatures, however, metabolism and enzymatic activity in the fish would be at a sufficiently high level to permit adequate utilization of protein.

The observations of possible toxicity at high levels of protein at 47°F in small fish, and of a minimum requirement of 40% protein at 47°F, should prove useful for the development of standardized production diets.

Gerking ('55b) has found, in controlled intake studies on goldfish, that in order to obtain maximum utilization of protein it is necessary to feed the fish at maximum rates. All diets in the present experiment were fed as long as the fish accepted food. The diets were isocaloric on a total energy basis. If the fish eats to meet its caloric requirement, the values found for the optimum protein level should be true percentages of the diet.

The higher value for the protein requirement at the higher temperature might be attributed to an increased endogenous nitrogen excretion due to increased nitrogen catabolism. This would indicate not an increased requirement for growth but an increased maintenance requirement. No direct evidence can be given at this time to prove this hypothesis, however.

SUMMARY

The data presented suggest that the optimum protein level for chinook salmon is dependent upon water temperature. The requirement at 47°F was found to be 40% of the diet and the requirement at 58°F, 55% of the diet. The feeding tests also show that the protein requirements of fish are two to 4 times higher than those of birds and mammals, when expressed as a percentage of the dry diet.

LITERATURE CITED

BALDWIN, N. S. 1957 Food consumption and growth of brook trout at different temperatures. Trans. Amer. Fish Soc., *87:* 323.

BRESSANI, R., AND E. T. MERTZ 1956 Relationship of protein level to the minimum lysine requirement of the rat. Federation Proc., *15:* 544.

GERKING, S. D. 1955a Influence of rate of feeding on body composition and protein metabolism of bluegill sunfish. Physiol. Zool., *28:* 267.

———— 1955b Exogenous excretion of bluegill sunfish. Ibid., *28:* 283.

HALVER, J. E. 1957 Nutrition of salmonoid fishes. III. Water-soluble vitamin requirements of chinook salmon. J. Nutrition, *62:* 225.

HALVER, J. E., D. C. DELONG AND E. T. MERTZ 1957 Nutrition of salmonoid fishes. V. Classification of essential amino acids for chinook salmon. Ibid., *63:* 95.

MCLAREN, B. A., E. F. HERMAN AND C. A. ELVEHJEM 1946 Nutrition of rainbow trout. Studies with purified rations. Arch. Biochem., *10:* 433.

NASSET, E. S. 1957 Comparison of amino acid mixtures and egg protein as sources of dietary nitrogen. J. Nutrition, *61:* 555.

PENTALOW, F. T. K. 1939 The relation between growth and food consumption in the brown trout (*S. trutta*). J. Exp. Biol., *16:* 446.

PHILLIPS, A. M., A. V. TUNISON AND D. R. BROCKWAY 1948 The utilization of carbohydrates by trout. N.Y. Cons. Dept. Fish. Res. Bull. No. 11, 44 pp.

WOOD, E. M., W. T. YASUTAKE, A. N. WOODALL AND J. E. HALVER 1957a The nutrition of salmonoid fishes II. Studies on production diets. J. Nutrition, *61:* 479.

———— 1957b The nutrition of salmonoid fishes. I. Chemical and histological studies of wild and domestic fish. Ibid., *61:* 465.

STUDIES ON THE NUTRITIVE EFFECTS OF SELENIUM FOR CHICKS [1]

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Selenium, in minute amounts, has recently been shown (Scott et al., '57; Stokstad et al., '57; Schwarz et al., '57, and Patterson et al., '57) to prevent exudative diathesis in vitamin E-deficient chicks. Thus selenium, or an organic factor containing selenium, appears to be the active substance in dried brewers' yeast previously reported by Carlson ('49), Miller et al. ('55) and Scott et al. ('55a) as able to replace vitamin E for prevention of this disorder in chicks. Earlier last year Schwarz and Foltz ('57) demonstrated that selenium is the active component of the unknown "factor 3" in brewers' yeast, hog kidney and casein which is required for prevention of dietary liver necrosis in rats fed diets containing torula yeast and no added vitamin E. It was found in these studies that selenium levels as low as 0.1 p.p.m. were adequate for prevention of exudative diathesis in chicks, and 0.04 p.p.m. prevented dietary liver necrosis in rats.

The objectives of this report are (1) to show that selenium deficiency in chicks can be produced with purified diets containing no torula yeast; (2) to indicate that selenium is a required nutrient *per se,* necessary for growth as well as for prevention of exudative diathesis; (3) to show that selenium is effective in increasing the serum albumin levels in vitamin

[1] This work was aided by grants from the Muscular Dystrophy Associations of America, Inc.; the Nutrition Foundation, Inc.; and Distillation Products Industries, who also supplied the stripped lard and *d*-α-tocopheryl acetate used in the studies.

E-deficient chicks; (4) to show that selenium is at least partially effective in the prevention of muscular dystrophy in chicks fed diets low in methionine and vitamin E; (5) to present more precise evidence concerning the amounts of selenium required by chicks, both in the presence and absence of vitamin E in the diet; and (6) to show that the petroleum ether-insoluble residues of some natural feedstuffs are relatively rich sources of a factor, presumably a selenium compound, which prevents exudative diathesis in chicks.

EXPERIMENTAL

The White Plymouth Rock chicks used in all experiments were housed in thermostatically-controlled, electrically-heated pens with wire mesh floors. All experimental lots contained 17 to 20 chicks at the start. Except where indicated, male chicks were used. Feed and water were supplied ad libitum from two days of age until the end of each experiment. The formulas of the basal diets used are presented in table 1. All selenium additions were made as sodium selenite except where otherwise specified. Vitamin E was supplied as *d*-α-tocopheryl acetate.

Selenium effects in a purified diet based on soybean protein. All original work on the effectiveness of selenium against exudative diathesis and liver necrosis was conducted with basal diets containing torula yeast as the source of protein. In order to determine if similar results could be obtained with another diet, an experiment was conducted in which isolated soybean protein replaced torula yeast as the source of protein (diet A, table 1). Groups of chicks received this basal diet alone, and supplemented with 0.1 p.p.m. of selenium as sodium selenite, 0.1 p.p.m. of selenium as seleno-cystathionine,[2] and 2.0 p.p.m. of metallic selenium. Other lots of chicks received 20 I.U. of vitamin E per pound of diet or 10% of dried brewers' yeast, since both of these supplements had been shown in previous studies to prevent exudative diathesis in chicks receiving this basal diet.

[2] The authors are indebted to Dr. K. Schwarz, National Institutes of Health, Bethesda, Md., for the sample of seleno-cystathionine.

The results of the first experiment are shown in table 2. Although the incidence of exudative diathesis was not as great on this diet as had been observed in previous experiments using the torula yeast-containing diet, the disorder was sufficiently severe to allow good evaluation of the various treatments. Selenium, as selenite or in seleno-cystathionine, was as effective as dried brewers' yeast or vitamin E in preventing exudative diathesis. Metallic selenium was not measurably effective in this experiment.

TABLE 1

Experimental diets

INGREDIENTS	DIET A	DIET B	DIET C
	%	%	%
Isolated soybean protein	27.76		
Torula yeast		58.50 [1]	
Casein			15.00
Gelatin			10.00
Cellulose	3.00		3.00
Glucose	56.89	30.18	61.77
Vitamin E-free lard	5.00	5.00	4.00
DL-Methionine	0.70	0.30	
Glycine	0.30	0.40	
DL-Phenylalanine		0.10	
L-Arginine·HCl		0.20	
Mineral mixture	5.43 [2]	4.45 [3]	5.33 [2]
Vitamin mixture	0.92 [2]	0.87 [3]	0.92 [2]
Antioxidant			+ [4]

[1] Torafeed, obtained from Red Star Yeast Corp., Milwaukee, Wis.

[2] For vitamin and mineral mixtures see Morrison et al. ('55), except that in diet C, Mg was supplied as $MgCO_3$ and Fe was supplied as $FePO_4 \cdot 4H_2O$.

[3] For vitamin and mineral mixtures see Scott et al. ('55a).

[4] Diphenyl-*p*-phenylenediamine added at a level of 100 mg per pound of diet.

Studies on the selenium requirement of chicks for growth and prevention of exudative diathesis. Although levels as low as 0.1 p.p.m. of selenium were found to be effective in preventing exudative diathesis, no evidence has been reported concerning the minimum selenium requirement of chicks. An experiment was designed, therefore, to test the effectiveness of additions to the torula yeast-containing diet (diet B, table

1) of 0.025, 0.05, 0.1, 0.2 and 0.5 p.p.m. of selenium as sodium selenite. Groups of chicks also received 50 I.U. of vitamin E per pound of diet fed alone and together with 0.1 p.p.m. of selenium. The results of this experiment are shown in table 3 (experiment 1). Selenium at levels of 0.025 and 0.05 p.p.m. markedly reduced the incidence of exudative diathesis as compared to that observed with chicks receiving the basal diet, but in this experiment, 0.1 p.p.m. of selenium was required to completely prevent these symptoms. Vitamin E at 50 I.U. per pound of diet, did not completely prevent exu-

TABLE 2

Comparison of selenium compounds, vitamin E, and torula yeast in prevention of exudative diathesis in chicks receiving the vitamin E-deficient, isolated soybean-protein diet

TREATMENT	EXUDATIVE DIATHESIS 4 WEEKS
	Number
Basal (diet A, table 1)	10/26 [1]
Vitamin E, 20 I.U./lb	0/16
Dried brewers' yeast, 10%	0/16
Selenium as Na_2SeO_3, 0.1 p.p.m.	0/16
Selenium (metallic), 2 p.p.m.	5/12
Selenium, as seleno-cystathionine, 0.1 p.p.m.	0/16

[1] Expressed as number of chicks showing symptoms (numerator) over number of chicks surviving (denominator) until symptoms appeared.

dative diathesis and the chicks receiving this level of vitamin E grew at a considerably slower rate than those receiving 0.1 p.p.m. of selenium alone or in combination with 50 I.U. of vitamin E per pound of diet. The combination of vitamin E and selenium produced no better results than were obtained with selenium alone. Two cases of encephalomalacia, as determined by symptoms and histological examination of the cerebellum, occurred in the lots receiving 0.1 p.p.m. of selenium.

In view of the fact that the above experiment failed to pinpoint the selenium requirement of chicks receiving the torula yeast diet, a further experiment was conducted in which selen-

ium was fed at levels of 0.02, 0.04, 0.06, 0.08 and 0.1 p.p.m. The results of this experiment, also represented in table 3 (experiment 2), show that, in the absence of added vitamin E, the minimum level of selenium required for prevention of

TABLE 3

Levels of selenium [1] *effective against exudative diathesis*
Torula yeast basal diet

TREATMENT	AVERAGE WEIGHT 26 DAYS	EXUDATIVE DIATHESIS [2]
	gm	
Experiment 1		
Basal (diet B, table 1)	— (0) [3]	16/16
0.025 p.p.m. Se	252 (8)	9/14
0.05 p.p.m. Se	263 (14)	5/15
0.10 p.p.m. Se	317 (31) [4]	0/31
0.20 p.p.m. Se	310 (16)	0/16
0.50 p.p.m. Se	324 (15)	0/15
50 I.U. vitamin E/lb.	257 (25) [4]	3/25
50 I.U. vitamin E/lb. + 0.1 p.p.m. Se	305 (33) [4]	0/33
TREATMENT	AVERAGE WEIGHT 21 DAYS	EXUDATIVE DIATHESIS [2]
Experiment 2		
Basal (diet B, table 1)	151 (10)	16/16
0.02 p.p.m. Se	180 (12)	14/16
0.04 p.p.m. Se	192 (15)	5/15
0.06 p.p.m. Se	222 (17)	1/17
0.08 p.p.m. Se	218 (16)	0/16
0.10 p.p.m. Se	197 (16)	0/16

[1] As Na_2SeO_3.

[2] See footnote to table 2.

[3] Figures within parentheses show chicks surviving at 26 days of age. Seventeen chicks per lot started.

[4] Averages of duplicate lots of chicks.

exudative diathesis in chicks receiving the torula yeast diet was approximately 0.08 p.p.m.

The third series of experiments was conducted to observe the effectiveness of selenium and vitamin E in preventing exudative diathesis and supporting chick growth over a longer period of time. Groups of chicks were fed the torula yeast

basal diet supplemented with 0.1 p.p.m. of selenium, 50 I.U. of vitamin E per pound of diet and a combination of 50 I.U. of vitamin E per pound of diet and 0.1 p.p.m. of selenium. The chicks received these diets from two days of age to 59 days of age. The results of this experiment are given in table 4. The effect of selenium in increasing the growth of chicks as compared to those receiving the torula yeast diet supplemented with vitamin E alone, was confirmed. Survival was excellent in the chicks receiving selenium alone or selenium plus vitamin E, whereas very severe mortality occurred

TABLE 4

Growth and survival of chicks fed a selenium-deficient diet to 59 days of age

TREATMENT	WEIGHT		
	28 days	42 days	59 days
	gm	*gm*	*gm*
50 I. U. vitamin E/lb. of diet (diet B, table 1)	294 (26) [1]	426 (12)	— (3)
0.1 p.p.m. selenium [2]	422 (39)	699 (37)	1093 (36)
0.1 p.p.m. selenium [2] + 50 I.U. vitamin E/lb. of diet	420 (40)	684 (39)	1098 (39)

[1] Figures within parentheses indicate number of surviving chicks at the given age. Duplicate lots of 20 chicks each per treatment at start.

[2] As Na_2SeO_3.

in the chicks receiving vitamin E alone. Seven of these chicks developed exudative diathesis before they died. Other chicks showed no exudates but became very inactive and appeared to suffer from anorexia before death. No cases of exudative diathesis were observed in any of the chicks receiving selenium during the experimental period. One case of encephalomalacia was observed in one of the groups receiving selenium.

The previous experiments have shown that 0.1 p.p.m. of selenium added to the torula yeast basal diet is sufficient to prevent exudative diathesis and support growth maximum for this diet. A further experiment was designed to test the effect of lower levels of selenium in the presence of vitamin

E, to determine whether the presence of vitamin E reduces the amount of selenium required for maximum growth.

Duplicate lots of chicks were fed a high level of vitamin E alone and in combination with 0.02, 0.04, and 0.1 p.p.m. of selenium added as sodium selenite. Levels of 0.04, and 0.1 p.p.m. of selenium also were fed in the absence of supplementary vitamin E. The chicks used in this experiment were from a flock of hens maintained at Cornell University on a diet low in vitamin E in an effort to reduce the stores of vita-

TABLE 5

Effect of vitamin E on the selenium requirement for growth

TREATMENT	AVERAGE [1] WT. AT 7 WEEKS	NO. OF SURVIVING CHICKS	NO. WITH EXUDATIVE DIATHESIS
	gm		
100 I.U. vitamin E/lb. of diet (diet B, table 1)	641	32	0
100 I.U. vitamin E/lb. of diet + 0.02 p.p.m. selenium [2]	641	32	0
100 I.U. vitamin E/lb. of diet + 0.04 p.p.m. selenium	740	35	0
100 I.U. vitamin E/lb. of diet + 0.1 p.p.m. selenium	748	35	0
No vitamin E + 0.04 p.p.m. selenium	660	26	6
No vitamin E + 0.1 p.p.m. selenium	702	32	0

[1] Average of duplicate lots; 18 chicks per lot at start.

[2] As Na_2SeO_3.

min E received by the chicks from the yolk sac. Equal numbers of male and female chicks were used in this experiment. The results of the experiment are recorded in table 5.

The chicks receiving no supplementary selenium grew at a significantly lower rate than chicks receiving 0.04 or 0.1 p.p.m. of selenium in the presence of vitamin E or 0.1 p.p.m. of selenium in the absence of vitamin E. In the presence of vitamin E, therefore, 0.04 p.p.m. of added selenium appears to approximate the minimum requirement for growth on this diet. Selenium, at a level of 0.04 p.p.m. in the absence of supple-

mentary vitamin E, or 0.02 p.p.m. of selenium in the presence of vitamin E was not adequate to support maximum growth. Six cases of exudative diathesis were observed in the lots receiving 0.04 p.p.m. of selenium without vitamin E. The effects of no selenium supplementation to the diet adequate in vitamin E were not as pronounced as in the previous experiment.

Blood serum proteins. Chicks suffering from exudative diathesis were shown to have a very low level of blood serum albumin (Goldstein and Scott, '56). This condition was corrected by the addition of vitamin E. An experiment was conducted to determine the effects of selenium on the blood albumin level of chicks deficient in vitamin E. Hanging-strip paper electrophoresis patterns of blood proteins were run on pooled samples of blood from chicks in the second experiment. Samples of blood were taken by heart puncture from 4 chicks showing exudative diathesis in the lot receiving 0.025 p.p.m. of selenium, and from 4 chicks in each of the two lots receiving 0.1 p.p.m. of selenium, 50 I.U. of vitamin E per pound of diet, and 50 I.U. of vitamin E per pound of diet plus 0.1 p.p.m. of selenium. The samples within each lot were pooled and electrophoretic blood protein patterns and total blood protein values were obtained by procedures described by Goldstein and Scott ('56). The blood samples were taken when the chicks were 28 days of age. Since no chicks were alive in the lot receiving no vitamin E or selenium at this age, the lot receiving 0.025 p.p.m. of selenium was chosen as a negative control. The data from these chicks are given in experiment A, table 6. In another experiment, blood samples were taken from 4 chicks each in lots receiving the torula yeast basal diet plus no added selenium or vitamin E, 0.1 p.p.m. of selenium, and 50 I.U. of vitamin E per pound of diet. These chicks were 19 days of age when blood samples were taken for electrophoresis and total protein measurements. The data from these chicks are included in table 6, experiment B. In the chicks from one series, the combination of selenium plus vitamin E in the diet appeared to produce a higher A/G

ratio than was obtained with either of these factors when fed alone. However, in view of variation encountered among serum proteins of chicks, this difference may not be meaningful. In the second series of electrophoresis determinations, vitamin E and selenium appeared to be equally effective in maintaining the A/G ratios at levels apparently maximum for this diet.

TABLE 6

Serum albumin-globulin ratios and total protein in chicks receiving selenium and vitamin E

TREATMENT	EXPERIMENT A		EXPERIMENT B	
	A/G	Total protein	A/G	Total protein
		%		%
Basal (diet B, table 1) (exudative diathesis)	—	—	0 [1]	2.28
0.025 p.p.m. selenium [2] (exudative diathesis)	0.16	2.58	—	—
0.1 p.p.m. selenium	0.36	2.89	0.37	2.96
50 I.U. vitamin E/lb. diet	0.30	2.84	0.44	3.42
50 I.U. vitamin E/lb. diet + 0.1 p.p.m. selenium	0.44	3.03	—	—

[1] The albumin level in chicks from this lot was too low to be measured accurately.
[2] As Na_2SeO_3.

Effectiveness of injected selenium. The results of injecting selenium subcutaneously upon recovery of chicks from exudative diathesis are presented in table 7. Subcutaneous injections on alternate days of 6 μg of selenium per chick, in water solution, brought about complete recovery from the exudative diathesis.

Selenium response not affected by arsanilic acid. Since inorganic and organic arsenic compounds have been reported to alleviate symptoms of selenium toxicity in animals fed diets high in selenium (Moxon, '38; Hendrick et al., '53), one lot of chicks was fed 0.01% of arsanilic acid. This treatment had no effect on the prevention of exudative diathesis in

chicks receiving 0.1 p.p.m. of selenium. These results also are shown in table 7.

Prevention of exudative diathesis by petroleum ether-extracted residues of certain natural feedstuffs. In the first of two experiments several natural feed ingredients were tested to determine the effect of the petroleum ether-insoluble residues and the petroleum ether-soluble extracts of natural feedstuffs on the incidence of exudative diathesis in chicks fed the torula yeast basal diet. Presumably any activity of the petroleum ether-soluble portions would be due to vitamin E or

TABLE 7

Effect of injected selenium and dietary arsanilic acid on exudative diathesis

DIET FED	INCIDENCE OF EXUDATIVE DIATHESIS [1]	
	2 weeks	3 weeks
Basal (diet B, table 1)	9/15	13/15
Same as basal — before injecting selenium	9/16	—
Same as basal — after injecting selenium [2] for one week	—	0/15
Basal + 0.1 p.p.m. selenium [3] + 0.1% arsanilic acid	0/15	0/13

[1] See footnote to table 2.

[2] As Na_2SeO_3, 6 μg Se/chick, on alternate days.

[3] As Na_2SeO_3.

any selenium extracted from the feed material, while any activities from the petroleum ether-insoluble residues may represent a factor other than vitamin E which could be selenium.

The natural feed ingredients were extracted by continuous extraction with petroleum ether for a 24-hour period. The residues were dried and the petroleum ether was evaporated from the extracts.

The petroleum ether-insoluble residues and the extracts were added to the torula yeast diet at the expense of glucose. In the first experiment, the feeds studied were: corn, wheat, oats, dried distillers' solubles and standard middlings. The results are listed in table 8. Except for corn, the petroleum ether-extracted residues all were more effective in preventing exudative diathesis than were the corresponding extracts.

In corn, the incidence of exudative diathesis was approximately the same in groups of chicks fed either the petroleum ether-extract or the extracted residue. Neither fraction caused any marked reduction in the incidence of exudative

TABLE 8

Effect of natural feed materials on the exudative diathesis

INGREDIENTS	INCIDENCE OF EXUDATIVE DIATHESIS AT 3 WEEKS OF AGE [1]
Experiment 1	
Torula yeast basal diet (diet B, table 1)	12/16
10 I.U. vitamin E/lb. of diet	4/16
Extracted residue [2] ≏ 30% corn	10/18
Extract ≏ 30% corn	9/18
Extracted residue ≏ 30% oats	2/16
Extract ≏ 30% oats	10/18
Extracted residue ≏ 30% wheat	3/18
Extract ≏ 30% wheat	10/18
Extracted residue ≏ 30% standard middlings	0/15
Extract ≏ 30% standard middlings	4/17
Extracted residue ≏ 5% dried distillers' solubles	6/17
Extract ≏ 5% dried distillers' solubles	11/17
Experiment 2	
Torula yeast basal diet (diet B, table 1)	16/16
0.1 p.p.m. selenium	0/16
Extracted residue [2] ≏ 30% corn	15/16
Extracted residue ≏ 20% wheat	13/15
Extracted residue ≏ 20% oats	10/13
Extracted residue ≏ 15% corn gluten meal	14/16
Extracted residue ≏ 15% soybean oil meal	0/15
Extracted residue ≏ 15% standard middlings	0/15

[1] See footnote to table 2.

[2] The natural feed materials were extracted with petroleum ether, b.p. 30 to 60°C.

diathesis. The petroleum ether-extracted residue equivalent to 30% of standard middlings was particularly active in preventing exudative diathesis.

In a second experiment, the petroleum ether-extracted residues of corn, wheat, oats, and standard middlings were fed again along with those of corn gluten meal and soybean oil

meal. The residues of wheat, oats and standard middlings were fed at reduced levels corresponding to 20, 20 and 15% respectively of the original materials. The results of this experiment are also given in table 8. The petroleum ether-extracted residues from soybean oil meal and standard middlings completely prevented exudative diathesis. The residues from corn, wheat, oats and corn gluten meal did not greatly reduce the incidence of exudative diathesis at the levels fed in this experiment.

From these experiments, soybean oil meal and standard middlings appeared to be especially high in a factor that prevented exudative diathesis. Since selenium is known to be widely distributed in plants and is especially associated with their proteins, chemical analyses for selenium were conducted on these materials by the official method of the Association of Official Agricultural Chemists for the determination of selenium in feedstuffs. The soybean oil meal contained approximately 0.6 p.p.m. and standard middlings contained 0.8 p.p.m. of selenium. Therefore, these materials supplied enough selenium to the diet at the levels fed to account for their activity in preventing exudative diathesis.

Effect of selenium on muscular dystrophy in chicks. Since Machlin and Shalkop ('56) showed that vitamin E is effective in preventing muscular dystrophy in chicks fed a purified diet low in methionine, experiments were conducted to determine the effects of selenium on the prevention of muscular dystrophy in chicks receiving a similar vitamin E-deficient, methionine-low diet. The composition of the diet is presented in table 1 (diet C).

One group of 21 chicks, 11 males and 10 females, received the basal diet alone. Comparable lots of chicks were fed this diet supplemented with 1.0 p.p.m. of selenium or 50 I.U. of vitamin E per pound of diet. The results of the experiment are presented in table 9. This experiment was repeated using selenium at a level of 5.0 p.p.m.

The extremely high incidence of muscular dystrophy which developed in chicks receiving the basal diet was completely

prevented by supplementing the diet with vitamin E. Although selenium alone did not completely prevent muscular dystrophy, it produced a definite decrease in the incidence of this disorder under these experimental conditions. Other experiments, using the torula yeast containing diet, without added methionine or vitamin E, gave similar results. However, the occurrence of exudative diathesis in chicks receiving the basal torula yeast diet complicated evaluation and interpretation of these results. In all instances, however, mus-

TABLE 9

Effect of selenium on the incidence of muscular dystrophy

TREATMENT	AVERAGE WEIGHT 5WEEKS OF AGE	INCIDENCE OF MUSCULAR DYSTROPHY [1]
	gm	
Basal diet (diet C, table 1)	336	19/20
Basal plus 1.0 p.p.m. of selenium [2]	346	7/21
Basal plus 50 I.U. vitamin E per pound of diet	336	0/21

[1] See footnote to table 2.

[2] In a subsequent experiment, upon the addition of 5.0 p.p.m. of selenium, as Na_2SeO_3, the incidence of muscular dystrophy was 4/13, whereas it was 13/17 in the chicks receiving the basal diet.

cular dystrophy was readily recognized by the appearance of grossly-visible white striations of the breast muscle. Positive identification of this as muscular dystrophy was confirmed by histological examination of the affected muscle tissues.

DISCUSSION

The results of these experiments show that selenium prevents exudative diathesis when added to vitamin E-deficient diets containing either torula yeast or isolated soybean protein as the source of protein. Selenium also improves growth when added to a torula yeast-containing diet adequate in all recognized nutrients, including vitamin E.

The torula yeast basal diet used in the experiment presented in table 5, was analyzed by the Oak Ridge National Laboratory by the neutron activiation analysis method (Co-

mar, '55) and was found to contain 0.056 p.p.m. of selenium. This was supplied to the diet almost entirely by the torula yeast which contained approximately 0.09 p.p.m. of selenium. Calculations show that the torula yeast supplied 0.052 p.p.m. of selenium.

In the absence of vitamin E, 0.08 p.p.m. of added selenium was necessary to prevent exudative diathesis. Thus, the total amount of selenium required for prevention of this disorder was approximately 0.14 p.p.m. Addition of 0.04 p.p.m. of selenium to the basal diet in the presence of vitamin E was sufficient to produce maximum growth. In the absence of vitamin E, this level of selenium was insufficient for maximum growth. Therefore, the selenium requirement for growth in the presence of vitamin E is approximately two-thirds the requirement for preventing exudative diathesis, when fed alone.

In the experiments referred to in tables 3 and 4, the deficiency of selenium in the absence of vitamin E was more severe than in subsequent experiments conducted in this laboratory. In these experiments, 50 I.U. of vitamin E per pound of diet did not prevent exudative diathesis completely. This level of vitamin E is approximately 4 to 5 times the requirement for the chick found by Singsen et al. ('55) for preventing encephalomalacia and by Scott et al. ('55b) for preventing exudative diathesis to three weeks of age. This level of selenium also promoted a marked increase in growth as compared to that obtained on the basal diet. Although vitamin E alone usually prevented exudative diathesis, it did not produce growth equivalent to that obtained with selenium alone.

A different supply of torula yeast was used in the later experiments, on which the selenium analyses were conducted. A possible explanation of the different results may lie in differences in selenium content of the torula yeast samples. The Activation Analyses Group at the Oak Ridge National Laboratory stated that they have found wide variations in selenium concentrations in the analysis of torula yeast. Schwarz and Foltz ('58) also reported widely varying amounts of selenium in torula yeast samples analyzed by activation

analysis. Thus, the amount of selenium required to adequately supplement the basal diet can be expected to vary depending on the sample of torula yeast used. Other factors that affect vitamin E requirements, such as the amount and type of fat in the diet, may also affect the selenium requirement.

The improvement in growth of chicks fed the torula yeast basal diet in response to selenium, over that obtained when vitamin E alone was fed, was not observed when the isolated soybean protein basal diet was used (Norris et al., '57). A sample of isolated soybean protein analyzed by neutron activation analysis was found to contain 0.36 p.p.m. of selenium. The basal diet used by the above workers contained approximately 25% of the isolated soybean protein. This supplied approximately 0.09 p.p.m. of selenium to the basal diet. This level of selenium is approximately equal to that found to be required for maximum growth on the torula diet when it was supplemented with vitamin E. However, this level of selenium is not sufficient for prevention of exudative diathesis in the absence of vitamin E, thereby accounting for the finding that selenium supplementation was effective in the isolated soybean protein diet for prevention of this disorder.

It is possible that both vitamin E and selenium are needed to prevent exudative diathesis. The chicks used in these experiments undoubtedly had some stores of vitamin E which may have persisted for the duration of the expermients. Nason ('57) found that vitamin E is removed with difficulty from isolated animal tissues.

Since vitamin E and selenium both affect exudative diathesis in chicks, these nutrients appear to be interrelated in their metabolic functions. Moreover, it appears from these experiments, that vitamin E cannot completely replace the need for selenium. More evidence is necessary to ascertain whether selenium can replace vitamin E entirely for the prevention of exudative diathesis.

Other manifestations of vitamin E deficiency in chicks are encephalomalacia and muscular dystrophy. In two experiments, a few chicks in lots receiving 0.1 p.p.m. of selenium

showed symptoms of encephalomalacia. No symptoms were seen in the lots receiving vitamin E. Recently, Dam et al. ('57) reported that 5 p.p.m. of selenium had no effect on the occurrence of encephalomalacia.

Results of several experiments indicated that selenium is at least partially effective against muscular dystrophy induced by feeding diets low in methionine and vitamin E. Dam and Sondergaard ('57) reported that selenium, at a level of 5 p.p.m., was markedly effective in reducing, but not completely preventing muscular dystrophy in chicks receiving a diet similar to that used in the present studies with the exception that it was low in protein as well as in methionine and vitamin E.

The diet used in the muscular dystrophy studies presented in table 9, contained, by neutron activation analysis, approximately 0.14 p.p.m. of selenium. This amount of selenium is sufficient for prevention of exudative diathesis and for maximum growth. No exudative diathesis was observed in the chicks receiving this diet. Thus, it appears that the amount of selenium needed for maximum effect upon muscular dystrophy is greater than that needed for growth or prevention of exudative diathesis.

SUMMARY

Results of experiments on the nutritive effects of selenium for chicks indicated that:

1. Selenium was effective in preventing exudative diathesis when chicks were fed a diet containing isolated soybean protein as a source of protein as well as on a torula yeast-containing diet.
2. Selenium is a required nutrient for maximum growth. Injected selenium was also effective in bringing about recovery of chicks already suffering from exudative diathesis.
3. Selenium was effective in increasing serum albumin levels in vitamin E-deficient chicks.
4. Selenium reduced the incidence of muscular dystrophy in chicks fed diets low in methionine and vitamin E but did not completely prevent it.

5. Approximately 0.08 p.p.m. of added selenium were necessary to prevent exudative diathesis in chicks fed the torula yeast diet in the absence of supplementary vitamin E. In the presence of vitamin E, 0.04 p.p.m. of added selenium resulted in maximum growth for the torula yeast diet. Under the conditions of these experiments, therefore, the total selenium requirement for prevention of exudative diathesis in the absence of supplemental vitamin E appeared to be approximately 0.14 p.p.m.; and the total requirement for maximum growth in the presence of vitamin E was approximately 0.10 p.p.m. of selenium as sodium selenite.

6. Petroleum ether-insoluble residues of certain natural feedstuffs were relatively rich sources of a factor, presumably a selenium compound, which prevented exudative diathesis in chicks.

LITERATURE CITED

ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS 1955 Official and Tentative Methods of Analysis, 8th Ed., Washington, D. C.

CARLSON, C. W. 1949 Further studies on unidentified factors required in chick nutrition. Thesis for Degree of Doctor of Philosophy, Cornell University, Ithaca, New York.

COMAR, C. L. 1955 Radioisotopes in Biology and Agriculture. McGraw-Hill Book Company Inc. New York, Toronto, London.

DAM, H., G. K. NIELSEN, I. PRANGE AND E. SONDERGAARD 1957 The exudative diathesis produced by torula yeast. Experientia, *13:* 493.

DAM, H., AND E. SONDERGAARD 1957 Prophylactic effect of selenium dioxide against degeneration (white striation) of muscles in chicks. Ibid., *13:* 494.

GOLDSTEIN, J., AND M. L. SCOTT 1956 An electrophoretic study of exudative diathesis in chicks. J. Nutrition, *60:* 349.

HENDRICK, C., H. L. KLUG AND O. E. OLSON 1953 Effect of 3-nitro-4 hydroxyphenylarsonic acid on selenium poisoning in the rat. Ibid., *51:* 131.

MACHLIN, L. J., AND W. T. SHALKOP 1956 Muscular degeneration in chickens fed diets low in vitamin E and sulfur. Ibid., *60:* 87.

MILLER, R. F., G. SMALL AND L. C. NORRIS 1955 Studies on the effect of sodium bisulfite on the stability of vitamin E. Ibid., *55:* 81.

MORRISON, A. B., M. L. SCOTT AND L. C. NORRIS 1955 Evidence for an unidentified mineral required by the chick. Poultry Sci., *34:* 738.

MOXON, A. L. 1938 The effect of arsenic on the toxicity of seleniferous grains. Science, *88:* 81.

NASON, A., K. O. DONALDSON AND I. R. LEHMAN 1957 The role of vitamin E at the enzymatic level. Trans. N. Y. Acad. Sci., *20:* 27.

NORRIS, L. C., R. M. LEACH, JR. AND T. R. ZEIGLER 1957 Proceedings of the 1957 Cornell Nutrition Conference for Feed Manufacturers, p. 33.

PATTERSON, E. L., R. MILSTREY AND E. L. R. STOKSTAD 1957 Effect of selenium in preventing exudative diathesis in chicks. Proc. Soc. Exp. Biol. Med., *95:* 617.

SCHWARZ, K., J. G. BIERI, G. M. BRIGGS AND M. L. SCOTT 1957 Prevention of exudative diathesis in chicks by factor 3 and selenium. Ibid., *95:* 621.

SCHWARZ, K., AND C. M. FOLTZ 1957 Selenium as an integral part of factor 3 against dietary necrotic liver degeneration. J. Am. Chem. Soc., *79:* 3292.

———— 1958 Factor 3 potencies of selenium compounds. Federation Proc., *17:* 492.

SCOTT, M. L., J. G. BIERI, G. M. BRIGGS AND K. SCHWARZ 1957 Prevention of exudative diathesis by factor 3 in chicks on vitamin E-deficient torula yeast diets. Poultry Sci., *36:* 1155.

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

SCOTT, M. L., L. C. NORRIS, G. F. HEUSER AND T. S. NELSON 1955b Further studies on vitamin E and a vitamin E-like factor in dried brewers' yeast. Poultry Sci., *34:* 1220.

SINGSEN, E. P., R. H. BUNNELL, L. D. MATTERSON, A. KOZEFF AND E. L. JUNGHERR 1955 Studies on encephalomalacia in the chick. 2. The protective action of diphenyl-p-phenylene-diamine against encephalomalacia. Ibid., *34:* 1234.

STOKSTAD, E. L. R., E. L. PATTERSON AND R. MILSTREY 1957 Factors which prevent exudative diathesis in chicks on torula yeast diets. Ibid., *36:* 1160.

WHEAT CEREAL DIETS, RAT CARIES, LYSINE AND MINERALS

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The studies reported in this paper are a continuation of those previously described concerning the development of rat caries by a heat-processed cereal diet (McClure, '52). The new data pertain to the cariogenic effect of a single cereal, i.e., wheat, present in the diet either as flour, dry bread or a commercial breakfast food; the heat-processing of wheat flours and toasting of bread as related to experimental caries; and the effect of mineral and also lysine supplementation on the dental caries produced by some of the diets.

Other investigators have used wheat flours in diets producing experimental caries with varying degrees of success (Cox, '52). The effect of metallic and other compounds on experimental caries has been recently reviewed (Hein, '55). Previous experiments of particular interest are those in which mineral supplements affected experimental caries (Stralfors, '56; Mitchell, Helman and Chernausek, '52; Sognnaes and Shaw, '54; Constant, Sievert, Phillips and Elvehjem, '54; Nizel and Harris, '55; McClure, Folk and Rust, '56) and variations in minerals were studied in relation to the mineral composition of the teeth (Shaw, '55; Sobel, '55; Wynn, Haldi, Bentley and Law, '56, '57; McClure, Folk and Rust, '56).

EXPERIMENTAL

The general plan of these experiments follows previous studies (McClure and Folk, '53). Started at weaning age,

30 to 35 gm Sprague-Dawley or Holtzman rats from the National Institutes of Health colony were housed two per cage, fed *ad libitum* and continued on experiment for 60 days. The components and the analyses of typical diets are presented in table 1. Data relative to caries are shown in tables 2 and 3, and ash, calcium and phosphorus analyses appear in table 4.

These cereal diets without mineral supplementation are very deficient in calcium and low in phosphorus, i.e., as little as 0.03% calcium and 0.21% phosphorus (table 1). Some of the diets are inadequate in total protein, i.e., less than 12%. That lysine is the amino acid limiting growth on wheat protein has been amply confirmed (Howard, Monson, Bauer and Block, '58). A lysine deficiency was observed in two of the diets, one containing toasted bread, the other whole wheat flour autoclaved with lactose. The rats gained very little, particularly on the unsupplemented diets, but they were maintained with only minor mortality during the 60-day experimental period. The studies thus served the primary purpose of providing experimental information on the etiology of dental caries.

The wheat flour was heat processed, alone (diet 257) or mixed with the 18% cerelose of the diet (diet 258) or 8% lactose of the diet (diet 188), by placing the material in an open pan about an inch and a half deep and autoclaving 15 minutes at 15 pounds pressure. Calcium and phosphorus were determined by standard procedures (Kirk, '50; Gee and Dietz, '53). Upper and lower sound and carious molar teeth, incisor teeth and mandibles were pooled for analysis according to the diet (table 4).

After sacrifice the rats' heads were autoclaved, to facilitate removal of soft tissue, and placed under water in the cold prior to the caries diagnosis. Immediately on removing from the water the teeth were examined under low power magnification (7 ×). Slow drying in the heat of the microscope lamp facilitates detection of the initial white opaque lesions. As in previous studies these lesions are found most frequently on lower buccal surfaces. Severe occlusal caries, identified

TABLE 1

Composition and analysis of diets used in the caries studies reported in table 2

DIET NO.	256	186	249	187	228	229	208	230
	%	%	%	%	%	%	%	%
Maryland whole wheat [1]	80.0	—	—	—	78.3	—	—	—
Commercial whole wheat flour [2]	—	80.0	—	—	—	77.9	77.1	—
White bread	—	—	80.0	—	—	—	—	—
Shredded wheat biscuit [2]	—	—	—	80.0	—	—	—	78.3
Cerelose	18.0	10.0	18.0	10.0	18.0	18.0	10.0	18.0
Lactose	—	8.0	—	8.0	—	—	8.0	—
Liver powder	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Skim milk powder ash	—	—	—	—	—	—	2.9	—
$CaCO_3$ [3]	—	—	—	—	1.1	1.3	—	1.2
Na_2HPO_4 [3]	—	—	—	—	0.6	0.8	—	0.6
Vitamins [4]								
	Analysis of diets							
Protein (N × 6.25)	10.81	14.02	12.80	10.31	9.88	13.62	14.90	9.19
Ash	1.50	1.33	2.53	1.22	2.62	2.23	4.20	2.55
Ca	0.03	0.03	0.12	0.05	0.51	0.45	0.47	0.50
P	0.34	0.31	0.20	0.29	0.37	0.37	0.82	0.41
Ca: P	0.08	0.09	0.60	0.17	1.38	1.28	0.57	1.22

[1] Whole wheat grown in Montgomery County, Md., was ground to a flour.

[2] Purchased on open market.

[3] The fluorine content of several batches of Na_2HPO_4 was 8.3, 7.1, 4.6 and 3.6 p.p.m. No fluoride was detected in one batch of $CaCO_3$ and 1.4 p.p.m. in another.

[4] A concentrate given orally once a week provided approximately 1500 units of A, 105 units of D and 5 mg of E.

extensively with coarse-particle and high-sugar diets, occurred to only a limited extent. All the lesions observed on all the lower molars were recorded.

Severity of caries was evaluated as follows. Lingual, buccal and occlusal tooth surfaces were divided into three areas on the first molar, and two areas on the second and third molars. The posterior surface of the third molar and the anterior surface of the first molar were included as a part of the lingual and buccal areas of these teeth. Each carious lesion was evaluated as to whether it involved one, two or three areas of each surface and the lesion given a relative severity score of one, two or three. A maximum evaluation of caries in the first molar, for example, would be 9 areas involved and a severity score of 27.

RESULTS

Rats fed diets containing wheat flours and wheat biscuit unsupplemented, averaged approximately 0.5 to 0.9 gm daily weight gain. The white bread and toasted bread diets resulted in an average of 1.1 and 0.7 gm daily gain respectively. Rats fed lysine-supplemented diets 212 and 251 (table 2) showed an average daily gain of 1.6 and 0.9 gm respectively. The 5 mineral-supplemented diets (table 2) resulted in an average daily gain, varying from 1.0 to 1.2 gm. Rats given diet 232 averaged 1.1 gm daily gain.

Referring to the data of table 2 it is apparent that the unsupplemented basal diets containing wheat flour, bread or shredded wheat biscuit produced a high incidence of severe smooth-surface caries. In experiments 1 and 2, severity of caries was increased by autoclaving the Maryland wheat flour (alone and mixed with cerelose, 83.7 and 70.2% respectively, diets 257 and 258 vs. 256). By autoclaving whole wheat flour mixed with lactose, severity of caries was increased about 77% (diet 188 vs. 186). Shredded wheat biscuit and whole wheat flour, in diets 187 and 186 respectively, gave similar caries results. According to experiment 3, the toasting of bread did not increase its cariogenicity. A supplement of

TABLE 2

Incidence and severity of smooth-surface caries developed by diets containing wheat flours, bread and shredded wheat biscuit

EXP.[1] NO.	DIET NO.	CEREAL FOOD AND SUPPLEMENTS IN DIET	NO. OF RATS	RATS WITH CARIES	CARIES SEVERITY
				%	SCORE
		Effect of mineral and lysine supplements			
	256	Maryland wheat flour (MWF)	35	94.2	7.4 ± 1.0[4]
1	257	MWF, autoclaved	38	100.0	13.6 ± 1.6
	258	MWF + cerelose, autoclaved	34	97.0	12.6 ± 1.6
	186	Whole wheat flour (WWF)	31	82.1	8.7 ± 1.4
2	187	Shredded wheat biscuit (SWB)	33	86.2	9.4 ± 1.7
	188	WWF + lactose, autoclaved	28	93.8	13.4 ± 1.8
	249	White bread	39	82.1	6.2 ± 1.0
3	250	White bread, toasted	40	70.0	6.1 ± 1.0
	251	Diet 250 + 2.0% L-lysine	38	36.8	1.6 ± 0.8
4	188	WWF + lactose, autoclaved	38	100.0	16.7 ± 2.0
	212	Diet 188 + 2.0% L-lysine	39	100.0	8.1 ± 0.8
		Diets containing mineral supplements			
5	208	WWF + milk ash	36	55.6	1.7 ± 0.4
	209	WWF[2] + milk ash	34	58.8	3.1 ± 0.6
	228	MWF + $CaCO_3$ + Na_2HPO_4	38	47.4	2.9 ± 0.8
6	229	WWF + $CaCO_3$ + Na_2HPO_4	38	68.4	3.6 ± 0.7
	230	SWB + $CaCO_3$ + Na_2HPO_4	37	54.1	3.1 ± 0.7
7	187	SWB[3]	32	75.6	7.4 ± 1.6
	232	SWB + $CaCO_3$ + Na_2HPO_4 + lysine	40	15.8	0.7 ± 0.3

[1] In each experimental group litter-mate rats were compared; this was not true, of course, of comparisons of one group with another.

[2] Whole wheat flour was autoclaved.

[3] No mineral supplement added.

[4] Standard error.

2.0% of lysine in the toasted bread diet, however, caused a notable reduction in both incidence and severity of caries (diet 251 vs. 250). Similarly 2.0% of lysine reduced caries severity about 50% when present in a diet containing whole wheat flour plus lactose autoclaved (diet 212 vs. 188, exp. 4).

The objective of experiments 5 and 6 was to determine the caries potential of wheat cereal diets when the calcium and phosphorus content of the diet was adequate and at a level comparable to the calcium and phosphorus in our cariogenic skim milk powder diets (McClure and Folk, '53). With 5 of these mineral-supplemented diets (experiments 5 and 6) 47.4 to 68.4% of the rats were carious and the severity scores varied from a mean of 1.7 to 3.6. This result compares with a range of 70.0 to 100.0% caries incidence and from 6.1 to 16.7 mean caries severity scores in rats fed the unsupplemented cereal diets of experiments 1 to 4. Although not based on littermates the experiments studying mineral vs. non-mineral supplemented diets are nonetheless consistent and apply to 350 rats taken from 175 litters fed diets without mineral supplementation vs. 200 rats representing 70 litters fed mineral-supplemented diets. The results undoubtedly are very indicative of an important anti-caries role of these combined mineral supplements. In experiment 7, mineral plus lysine supplementation of the shredded wheat biscuit diet resulted in very little caries: 15.8% incidence and 0.7 average severity score.

To help resolve the indicated cariostatic role of $CaCO_3$ plus Na_2HPO_4 in these diets, the data shown in table 3 were obtained. Littermates were fed diets 277, 278, 279 and 280, the latter diet containing 2.0% of $CaHPO_4$. A cariostatic property of $CaHPO_4$ is indicated in previous studies by Stralfors ('56). The only caries reduction observed was in animals given the Na_2HPO_4 supplement (diet 279). The other three diets gave remarkably consistent results and no reduction in caries was due to $CaCO_3$ or $CaHPO_4$ supplements alone.

Prior to obtaining the analytical data shown in table 4 we had determined the ash, calcium and phosphorus in molar and

incisor dentin and enamel of three groups of rats fed three of the basal cereal diets with minerals added (diets 228, 229, 230, table 2) and three groups fed three unsupplemented diets (diets 256, 186, 188, table 2). All these analyses were indicative of the fact that the very low calcium content of these basal diets had not affected the over-all calcification in either the molar or incisor teeth. There were no differences in these data for ash, calcium and phosphorus which could be attributed to the mineral content of the diet.

TABLE 3

Components, analysis, and cariogenic property of wheat flour diet. Effect of $CaCO_3$, Na_2HPO_4 and $CaHPO_4$ as diet supplements, on smooth-surface dental caries

DIET NO.	277	278	279	280
	%	%	%	%
Whole wheat flour	80.0	79.0	78.4	78.0
Cerelose	18.0	18.0	18.0	18.0
Liver powder	2.0	2.0	2.0	2.0
$CaCO_3$	0.0	1.0	0.0	0.0
Na_2HPO_4	0.0	0.0	1.6	0.0
$CaHPO_4$[1]	0.0	0.0	0.0	2.0
	Analysis of diets[2]			
Protein (N × 6.25)	14.25	14.00	13.88	13.88
Ash	1.25	2.25	2.77	3.17
Ca	0.03	0.44	0.03	0.63
P	0.28	0.28	0.62	0.74
Ca: P	0.11	1.57	0.05	0.85
	Incidence and severity of caries			
No. of rats	38	38	37	40
Rats with caries, %	97.4	94.6	59.5	90.0
Carious lower teeth	3.3 ± 0.5[3]	3.4 ± 0.2	1.2 ± 0.2	3.1 ± 0.3
Carious areas	5.7 ± 0.6	6.2 ± 0.6	1.6 ± 0.3	5.6 ± 0.7
Caries score	6.4 ± 0.8	6.6 ± 0.6	1.6 ± 0.3	6.2 ± 0.9
Av. final weight, gm	90.8	88.2	85.6	86.9
Av. daily gain, gm	0.9	0.9	0.8	0.9

[1] The fluorine content of this mineral was 6.4 p.p.m. See table 1 for fluorine in $CaCO_3$ and Na_2HPO_4.

[2] Fluorine analyses of these diets showed an average content of 0.70 p.p.m. fluorine.

[3] Standard errors.

The data shown in table 4 were obtained, therefore, to explore further the effects of these mineral supplements on the calcification of the teeth as well as the mandibles. Again it became apparent (table 4) that the ash, calcium and phosphorus of the molar and incisor dentin and enamel remained essentially the same regardless of the calcium or phosphorus content of the diet (diets 277, 278, 279 and 280). However, without a calcium supplement the mandibles were very poorly calcified as shown by their unusually low content of ash, calcium and phosphorus (table 4).

DISCUSSION

Our previous caries results with a diet containing three heat-processed cereal foods were, in general, duplicated in this study using a diet of similar type but containing only one cereal food, namely, wheat. In addition, new data were obtained indicative of an association of cariogenicity with a non-heat-processed cereal food, wheat flour. As in previous studies with skim milk powders (McClure and Folk, '53), autoclaving this wheat flour as a procedure for heat-processing increased its cariogenic effect in the diet. As previously shown also (McClure and Folk, '55), a lysine supplement reduced this increased caries development. The type of smooth-surface caries was similar to that previously observed (McClure, '52; McClure and Folk, '53; McClure, '57; Losee and Nemes, '54). It seems likely that a lysine deficiency was responsible in part at least for the caries which developed from diets containing the non-heat-processed wheat flours. This question, however, remains unresolved by these studies.

Toasting of bread, as has been shown previously (Morgan and King, '26) rendered it less adequate for growth. However, it did not alter the caries potential of the bread diet (diet 249 vs. diet 250). The result recalls our previous evidence that the growth requirement for lysine perhaps is not related proportionally to the lysine-caries relationship McClure and Folk, '55). Previous results on growth vs. cario-

TABLE 4

Ash, calcium and phosphorus in teeth and mandibles of rats fed diets 277, 278, 279 and 280 (table 3)

(Results based on weight of dry-fat free mandibles and on dry weight of teeth)

DIET NO.	MANDIBLES			MOLAR TEETH						INCISOR TEETH					
				Enamel			Dentin			Enamel			Dentin		
	Ash	Ca	P	Ash	Ca	P	Ash	Ca	P	Ash	Ca	P	Ash	Ca	P
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
277	57.34	16.12	10.78	95.00	35.11	17.55	77.24	28.25	14.61	95.06	33.85	17.90	76.83	24.88	14.74
278	72.70	20.73	13.27	95.17	34.81	17.48	77.26	28.08	14.37	94.79	34.56	17.63	76.80	25.65	15.50
279	52.97	16.30	10.83	94.95	34.81	17.72	76.46	26.86	14.12	94.35	33.96	17.58	73.35	23.76	14.53
280	72.42	19.95	13.27	94.92	34.23	17.57	78.05	28.36	14.46	94.87	34.81	17.69	77.19	26.61	15.35

genicity associated with heat processing of skim milk powders have suggested also that "the cariogenic factor is possibly not the same as the growth inhibiting factor" (McClure and Folk, '53). Nonetheless, supplementing diet 250 with 2.0% of lysine resulted in an increase in average daily gain from 0.7 gm (diet 250) to 1.6 gm (diet 251). Similarly diet 188 (table 2) when supplemented with lysine gave a daily gain of 0.9 gm vs. 0.6 gm without lysine. Thus growth was improved as well as caries reduced by a lysine supplement in these diets. The association of growth and caries reduction accompanying lysine supplementation of lysine-deficient diets has been observed and discussed previously (McClure and Folk, '55; Bavetta and McClure, '57). Recent evidence has suggested that the anticaries effect of lysine may be due to an extraoral systemic effect (McClure, '57).

Special attention in this study was focused on the relation of dietary minerals to cariogenicity, obviously because of the extreme inadequacy of calcium and the low phosphorus in these cereal diets. Mineral supplementation, using $CaCO_3$ plus Na_2HPO_4 or milk ash, provided quantities of calcium and phosphorus comparable to that present in our mineral-unsupplemented but highly cariogenic skim milk powder diets. These mineral additives produced a relatively low caries incidence and low severity scores: a result which recalls our previous evidence that a salt mixture reduced the caries produced by a skim milk powder diet (McClure, Folk and Rust, '56). This later basal diet was, however, presumably adequate in calcium and phosphorus, present as an intrinsic part of the skim milk powder.

Calcification of the teeth appears to have been unrelated to the dental caries which developed on any of these diets. It should be mentioned that, with the possible exception of the third molars, rats at the age of starting these diets have their molar teeth already practically completely erupted and calcified. However, the continuously growing incisors are nearly entirely replaced by new growth during the course of a 60-day experiment. Without $CaCO_3$ or $CaHPO_4$ supple-

mentation, the mandibles were very deficient in ash, calcium and phosphorus. These data agree with other reports (Wynn, Haldi, Bentley and Law, '56, '57) of no significant change in the ash, calcium and phosphorus content of enamel and dentin, even though the calcium and phosphorus of the diets varied widely and were below minimum requirements for the rat's growth. As shown in the footnotes of tables 1 and 3, the fluoride content of the mineral-supplemented diets was not sufficient to account for any reduction in caries.

A suggestion previously made by other investigators (Nizel and Harris, '55) is that a caries-inhibitory effect of a mineral supplement may be exerted within the oral cavity. While our data thus far indicate that Na_2HPO_4 functions as an anticaries agent, its mode of action is a matter of speculation, and is the subject of continuing investigations.

SUMMARY

1. Diets containing wheat flours, dry or toasted bread, or shredded wheat biscuit, cerelose or lactose, and liver powder resulted in a high incidence of severe smooth-surface caries in white rats.

2. Autoclaving whole wheat flours with cerelose or lactose increased their caries potential whereas toasting of bread had no such influence. A lysine supplement reduced the dental caries produced by diets containing autoclaved wheat flour and toasted bread.

3. Mineral supplementation with calcium carbonate plus sodium acid phosphate or with milk ash, significantly reduced the caries produced by wheat cereal diets.

4. Neither calcium carbonate nor calcium acid phosphate alone was cariostatic, whereas sodium acid phosphate alone significantly reduced the caries produced by a wheat flour diet.

5. The percentage of ash, calcium and phosphorus of the dentin and enamel of both incisor and molar teeth was unaffected by extreme deficiency in dietary calcium and a low phosphorus content. These analytical data were unrelated to the smooth-surface caries as diagnosed in the molar teeth.

LITERATURE CITED

BAVETTA, L. A., AND F. J. McCLURE 1957 Protein factors and experimental rat caries. J. Nutrition, *63:* 107.

CONSTANT, M. A., H. W. SIEVERT, P. H. PHILLIPS AND C. A. ELVEHJEM 1954 Dental caries in the cotton rat. XIV. Further studies of caries production by natural diets with especial reference to the role of minerals, fat, and the stage of refinement of cereals. Ibid., *53:* 17.

COX, G. J. 1952 Experimental dental caries in animals. Chapter in A Survey of the Literature of Dental Caries. Publication 225. National Academy of Sciences, National Research Council.

GEE, A., AND V. R. DIETZ 1953 Determination of phosphate by differential spectrophotometry. Anal. Chem., *25:* 1320.

HEIN, J. W. 1955 Chapter in Advances in Experimental Caries Research. R. F. Sognnaes, Editor. Publication of the A.A.A.S., Washington, D. C.

HOWARD, H. W., W. J. MONSON, C. D. BAUER AND R. J. BLOCK 1958 The nutritive value of bread flour proteins as affected by practical supplementation with lactalbumin, nonfat dry milk solids, soybean proteins, wheat gluten and lysine. J. Nutrition, *64:* 151.

KIRK, P. L. 1950 Quantitative Ultramicroanalysis. John Wiley & Sons.

LOSEE, F. L., AND J. L. NEMES 1954 Human type caries distribution in Osborne-Mendel rats kept on heated skim milk powder diet. Proc. Soc. Exp. Biol. Med., *87:* 429.

McCLURE, F. J. 1952 Dental caries in rats fed a diet containing processed cereal foods and a low content of refined sugar. Science, *116:* 229.

———— 1957 Effect of lysine provided by different routes on cariogenicity of a lysine deficient diet. Proc. Soc. Exp. Biol. Med., *96:* 631.

McCLURE, F. J., AND J. E. FOLK 1953 Skim milk powders and experimental rat caries. Ibid., *83:* 21.

———— 1955 Lysine and cariogenicity of two experimental rat diets. Science, *122:* 557.

McCLURE, F. J., J. E. FOLK AND J. D. RUST 1956 Smooth surface caries in white rats: effects of fluoride, iodoacetate, penicillin, Crisco, butterfat and a salt mixture. J. Am. Dental Assoc., *53:* 1.

MITCHELL, D. F., E. Z. HELMAN AND D. S. CHERNAUSEK 1952 Ammoniated dentrifices and hamster caries: further studies on the effects of ingestion. Science, *116:* 537.

MORGAN, A. F., AND F. KING 1926 Changes in biological value of cereal proteins due to heat treatment. Proc. Soc. Exp. Biol. and Med., *23:* 353.

NIZEL, A. H., AND R. M. HARRIS 1955 Effects of ashed foodstuffs on dental decay in hamsters. J. Dental Res., *34:* 513.

SHAW, J. H. 1955 Effect of nutritional factors on bones and teeth. Ann. N. Y. Acad. Sci., *60:* 733.

SOBEL, A. E. 1955 Local factors in the mechanism of calcification. Ibid., *60:* 713.

SOGNNAES, R. F., AND J. H. SHAW 1954 Experimental rat caries. IV. Effect of a natural salt mixture on the caries conduciveness of an otherwise purified diet. J. Nutrition, *53:* 195.

STRALFORS, A. 1956 Inhibition of caries by phosphates. J. Swedish Dental Assoc., Jan. 20, 1956.

WYNN, W., J. HALDI, K. D. BENTLEY AND M. L. LAW 1956 Dental caries in the albino rat in relation to the chemical composition of the teeth and of the diet. II. Variations in the Ca/P ratio of the diet induced by changing the phosphorus content. J. Nutrition, *58:* 325.

———— 1957 Dental caries in the albino rat in relation to the chemical composition of the teeth and of the diets. III. Composition of incisor teeth of animals fed diets with different Ca/P ratios. Ibid., *63:* 57.

ESSENTIAL FATTY ACID DEFICIENCY

I. CONTENT OF POLYENOIC ACIDS IN TESTES AND HEART AS AN INDICATOR OF EFA STATUS [1]

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INTRODUCTION

The most common evaluation of essential fatty acid (EFA) deficiency has been either the measurement of growth or the scoring of dermal symptoms, or both. These two criteria are subject to criticism because they are not sufficiently specific. Growth is the over-all response of the animal to nutritional and environmental conditions, and dermal symptoms are influenced by factors other than EFA — humidity, for example. A chemical indicator which could be measured quantitatively might be more reliable in the assessment of EFA status because it would eliminate possible subjective errors.

That EFA deficiency is accompanied by an increase in the trienoic acid content of tissue lipides was demonstrated first by Smedley-MacLean and coworkers ('43) who isolated from deficient livers an eicosatrienoic acid as its hexabromide. This work was confirmed by qualitative spectrophotometric analysis of the polyunsaturated acids (Rieckehoff, Holman and Burr, '49; Widmer and Holman, '50), which indicated that striking increases in trienoic acid occurred in many tissues of deficient rats. The heart was the organ which showed the

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greatest change in polyunsaturated acids as a consequence of changes in dietary polyunsaturated acids. A subsequent study of the polyunsaturated acid content of lipides from various tissues revealed testes lipide to be richest in these substances (Holman and Greenberg, '53). Several studies have revealed that EFA deficiency in rats causes a severe degeneration of the spermatogenic epithelium (Evans, Lepkovsky and Murphy, '34; Panos and Finerty, '54; Aaes-Jørgensen et al., '56; Holman and Aaes-Jørgensen, '56; Funch, Aaes-Jørgensen, and Dam, '57). These histological changes might mean that changes in the content of polyenoic acids could be expected. Thus it would appear that analysis of the polyunsaturated acids of the testis or of the heart could provide an objective evaluation of an animal's EFA status.

Analysis of the unsaturated fatty acids of the testis permits two analyses or biopsies, and in some instances an animal may even be used as its own control. Such biopsy analyses are now feasible, since the development of a micromethod for the analysis of polyunsaturated acids in less than a gram of tissue (Holman and Hayes, '58). One testis provides enough tissue for histological examination and for an analysis of polyunsaturated acids by alkaline isomerization. Analyzing heart fatty acids has proved to be a good way to evaluate essential fatty acid activity, but it allows only one analysis during a nutritional study, and that at the end.

EXPERIMENTAL

Two groups of 28 weanling male rats of the Sprague-Dawley strain were fed semi-synthetic diets containing 1% cholesterol and 5% hydrogenated coconut oil or 5% safflower oil (table 1). The dietary fats are characterized in table 2. Cholesterol was incorporated in the diets in an effort to accelerate deficiency of EFA (Peifer and Holman, '55).

Diet and water were provided ad libitum for 18 weeks. The animals were weighed and inspected weekly. Orchectomy of the right testis and epididymis was performed on a pair of animals from each group periodically during the course of

the experiment. At the beginning of the experiment, 9 of the weanling rats were killed by anesthesia and their testes and hearts were taken as initial controls. At the end of 18 weeks, testes were taken from rats previously orchectomized and from normal rats maintained on the same diet. When the rats were killed, the hearts were excised. Hearts and testes were weighed, macerated, and extracted with ethanol-ethyl ether

TABLE 1

Composition of diets

CONSTITUENT	AMOUNT
	%
Vitamin-test casein	16
Sucrose	68
α-Cellulose	4
Vitamin mixture [1]	1
Salt mixture [2]	4
Choline chloride [3]	1
Cholesterol	1
Hydrogenated coconut oil [4] or safflower oil [5]	5

[1] Ascorbic acid 500 mg, calcium pantothenate 70 mg, inositol 120 mg, 2-methyl-1,4-naphthoquinone 6 mg, niacin 60 mg, PABA 600 mg, pyridoxine hydrochloride 30 mg, riboflavin 30 mg, thiamine hydrochloride 70 mg, folic acid 11 mg, biotin 0.005 mg, and vitamin B_{12} 0.02 mg, made up to 10 gm with vitamin-test casein. Vitamin A, D_2, and E were sprayed over the diet from an ether solution, adding 5 mg, 100 mg, and 100 mg, respectively/kg diet. Diets were prepared weekly and were stored below 5°C.

[2] Wesson.

[3] 130 gm of choline chloride made up to 1 kg with vitamin-test casein.

[4] Hydrol, Durkee's Famous Foods, New York, N. Y.

[5] Pacific Vegetable Oil Co., San Francisco, Cal.

(3:1) with 5% conc. HCl, and the lipide soluble in petroleum ether was extracted and made up to a volume of 10 ml. An aliquot of each extract was analyzed for its content of poly-unsaturated acids (Holman and Hayes, '58).

RESULTS AND DISCUSSION

The growth rate of the rats fed the EFA-free diet containing 5% hydrogenated coconut oil was significantly lower than that of those fed 5% safflower oil. The average weight of

the two groups at the end of 18 weeks was 291 ± 5 and 353 ± 5 [3] gm respectively. The EFA-deficient group developed definite dermal symptoms within 6 weeks, and the average dermal score (Holman and Ener, '54) reached 4 by the 12th week and remained there until the end of the experiment. During the last 4 weeks of the experiment, many of the deficient rats developed a necrosis of the tail extending as much as 2 cm from the tip. No yellow pigmentation developed on the backs of the deficient rats. None of the rats fed safflower oil developed dermal signs of deficiency, but all developed the normal yellow pigmentation of the back (Aaes-Jørgensen, Funch and Dam, '57).

TABLE 2

Characteristics of dietary fats

	I.V.	POLYUNSATURATED ACIDS				
		Preconjugated		Nonconjugated		*Trans*
		Dienoic	Trienoic	Dienoic	Trienoic	Fatty acids
		%	%	%	%	*Moles/kg*
Hydrogenated coconut oil	2.0	0.13	0.005	0.28	0	0.22
Safflower seed oil	150	0.56	0.03	73.5	0.68	0.18

Studies of the excised testes from the EFA-deficient rats and the control rats revealed no delay in maturity of the testes, judging from the development of the spermatogenic epithelium, and from the weight of the testes. Moreover, spermatogenesis once begun was not impaired during the course of the experiment. There was no significant difference in weight between the testes of animals from the two groups. In the epididymes from rats fed hydrogenated coconut oil the number of spermatozoa appeared somewhat less than in the group fed safflower oil, but in general a normal picture was observed.

The content of polyunsaturated acids in the testes of the two groups of rats is shown in table 3 as a function of time. In the control group, which received EFA, the content of the

[3] Standard error.

various polyunsaturated acids remained relatively constant throughout the 18-week period. An exception was hexaenoic acid, which decreased during the first interval and remained low thereafter. In the deficient group, the diene content of the testes decreased remarkably, and was consistently less than in rats which had received linoleate in the form of safflower oil. The trienoic acid content of testes of deficient rats increased strikingly, to values two to 5 times that found in the testes of rats of the same age which had received EFA. The tetraenoic and pentaenoic acids content of the testes of deficient rats exhibited no noticeable trend during the experiment, but remained high and variable. The hexaenoic acid content decreased in the first interval and remained relatively constant thereafter, but at values two to 5 times as high as in samples from rats fed EFA. The individual polyunsaturated acids in the testes of deficient rats reached a maximum near the 10th week of experiment, which is approximately the time the rat reaches sexual maturity.

The concentration of total polyunsaturated acids in the testes tissue of supplemented rats remained rather constant, whereas that in deficient rats rose to a maximum at the 10th week and thereafter decreased below that of the supplemented rats. Under the conditions of this experiment, the degeneration of the testes had not progressed far enough in 18 weeks of deficiency to be histologically apparent. These findings are in agreement with the results in previous studies (Funch, Aaes-Jørgensen, and Dam, '57).

The polyunsaturated acid content of the heart tissue of a group of initial control rats and of the two experimental groups is given in table 4. The concentration of dienoic acid increased in supplemented animals, and decreased in deficient animals. Trienoic acid increased from 42 mg/100 gm in the weanling controls to 455 in the rats kept 18 weeks on a deficient diet, a tenfold increase. The content of trienoic acid in animals supplemented with EFA decreased to 9 mg/100 gm. The content of tetraenoic and pentaenoic acids decreased in supplemented rats, but decreased even more in

TABLE 3

Average content of polyenoic acids in testes of rats

DIET CHARACTERISTICS	INITIAL CONTROLS	EFA-FREE	CONTROLS	EFA-FREE	CONTROLS	EFA-FREE	CONTROLS	EFA-FREE	CONTROLS	EFA-FREE	CONTROLS	EFA-FREE	CONTROLS
	Weeks in experiment												
	0	6		10		12		14		16		18	
	Number of animals analyzed												
	9	2	2	2	1	2	2	2	1	1	2	5	5
Diene, mg %	62	*8*	68	*43*	69	*33*	71	*8*	98	*16*	93	*32*	55
Triene, mg %	29	*88*	23	*178*	36	*153*	32	*114*	25	*99*	27	*109*	42
Tetraene, mg %	161	*109*	145	*181*	146	*147*	139	*107*	116	*88*	123	*146*	227
Pentaene, mg %	109	*122*	181	*174*	157	*155*	144	*100*	138	*78*	137	*171*	197
Hexaene, mg %	37	*13*	5	*15*	3	*12*	3	*13*	4	*14*	4	*18*	8
Total	398	*340*	422	*591*	411	*560*	389	*342*	381	*295*	384	*476*	529

TABLE 4

Average content of polyenoic acids in hearts of rats

GROUP	DIET CHARACTERISTICS	WEEKS IN EXP.	NO. OF ANIMALS	POLYENOIC ACIDS					
				Diene	Triene	Tetraene	Pentaene	Hexaene	Total
				mg %	*mg %*	*mg %*	*mg %*	*mg %*	*mg %*
	Dam's milk (weanling rats)	0	5	286	42	346	124	114	912
11	Hydrogenated coconut oil + cholesterol	18	5	69	455	134	21	11	690
12	Safflower oil + cholesterol	18	5	722	9	267	49	6	1053

deficient rats. The initially high content of pentaenoic and hexaenoic acids in the hearts of weanling rats decreased sharply with age, irrespective of the diet. The total amount of polyenoic acids in the hearts of the deficient animals was much lower than in the hearts of the weanlings or rats fed safflower oil.

The data presented here confirm the observations that the tissue content of trienoic acid increases in deficiency of essential fatty acids (Smedley-MacLean, '43; Widmer and Holman, '50; Reiser, '51; Wiese, Baughan and Hansen, '55; Dam, Engel and Nielsen, '56). This acid has been shown to be 5,8,11-eicosatrienoic acid by Mead and Slaton ('56) who isolated it from the carcasses of EFA-deficient rats. Although changes are observed in the other polyunsaturated acids of tissue, the most drastic effect of EFA deficiency seems to be upon the content of trienoic acid, which normally occurs only in low concentrations and which probably arises from the oleic acid family rather than from the linoleic and linolenic families (Montag et al., '57). It therefore is proposed that the measurement of trienoic acid content of tissue lipides is the best present chemical criterion of EFA status of the animal.

Heart tissue seems to be the best tissue to use for evaluating EFA status because the variations observed as a consequence of diet are the greatest in this tissue (Rieckehoff, Holman and Burr, '49; Widmer and Holman, '50). The disadvantage is that biopsies of the heart cannot be made, and the information can be gained only after the termination of the nutritional experiment. Although the magnitude of change was not as great in testis tissue, the analysis of testis lipides for their polyunsaturated acid content permits assessment of EFA status without killing the animal.

SUMMARY

Weanling male rats were fed a semi-synthetic diet containing either 5% safflower oil or 5% hydrogenated coconut oil for a period of 18 weeks. The diets were supplemented with 1% cholesterol, in an attempt to intensify EFA deficiency.

The rats fed the EFA-free diet grew significantly less than those of the control group, and developed relatively strong dermal symptoms of EFA deficiency.

Orchectomy of the right testis was performed, at intervals throughout the experiment, on a pair of rats from each group. Histological examination revealed no impairment of the spermatogenic epithelium in either group. However, analysis of the testis lipides revealed that, as the deficiency developed, dienoic acids decreased and trienoic acids increased markedly, even at a time when drastic changes of the spermatogenic tissue had not yet occurred. Analysis of testis lipide is suggested as a convenient biopsy method for evaluation of EFA status.

Analysis of the hearts of the rats at the end of the experiment revealed a very marked increase in trienoic acid content and a decrease in dienoic acid in the deficient group. Measurement of trienoic acid content of heart tissue appears to be the best criterion for the evaluation of the EFA status of an animal.

LITERATURE CITED

AAES-JØRGENSEN, E., J. P. FUNCH, P. F. ENGEL AND H. DAM 1956 The role of fat in the diet of rats. 9. Influence on growth and histological findings of diets with hydrogenated arachis oil or no fat, supplemented with linoleic acid or raw skimmilk, and of crude casein compared with vitamin-test casein. Brit. J. Nutrition, *10:* 292.

AAES-JØRGENSEN, E., J. P. FUNCH AND H. DAM 1957 The role of fat in the diet of rats. 11. Influence of a small amount of ethyl linoleate on degeneration of spermatogenic tissue caused by hydrogenated arachis oil as the sole dietary fat. Ibid., *11:* 298.

DAM, H., P. F. ENGEL AND G. K. NIELSEN 1956 Variations in the total amount of polyenoic acids in chicks and rats reared on a nearly fat-free diet. Acta Physiol. Scand., *37:* 143.

EVANS, H. M., S. LEPKOVSKY AND E. A. MURPHY 1934 Vital need of the body for certain unsaturated fatty acids. Male sterility on fat-free diets. J. Biol. Chem., *106:* 445.

FUNCH, J. P., E. AAES-JØRGENSEN AND H. DAM 1957 The role of fat in the diets of rats. 12. Effect on rats of type and quantity of dietary fat with and without linoleate supplementation. Brit. J. Nutrition, *11:* 426.

HOLMAN, R. T., AND E. AAES-JØRGENSEN 1956 Effects of *trans* fatty acid isomers upon essential fatty acid deficiency in rats. Proc. Soc. Exp. Biol. Med., *93:* 175.

HOLMAN, R. T., AND S. ENER 1954 Use of urea-inclusion compound containing essential fatty acid in an experimental diet. J. Nutrition, *53:* 461.

HOLMAN, R. T., AND S. I. GREENBERG 1953 Highly unsaturated fatty acid. I. A survey of possible animal sources. J. Am. Oil Chemists' Soc., *30:* 600.

HOLMAN, R. T., AND H. HAYES 1958 Determination of polyunsaturated acids in lipides of plasma and tissue. Anal. Chem., (in press).

KLEIN, P. D., AND R. M. JOHNSON 1954 A study of the onset of unsaturated fatty acid deficiency in subcellular particles of rat livers. Arch. Biochem. Biophys., *48:* 380.

MEAD, J. F., AND W. H. SLATON 1956 Metabolism of essential fatty acids. III. Isolation of 5,8,11-eicosatrienoic acid from fat-deficient rats. J. Biol. Chem., *219:* 705.

MONTAG, W., E. KLENK, H. HAYES AND R. T. HOLMAN 1957 The eicosapolyenoic acids occurring in the glycerophosphatides of beef liver. Ibid., *227:* 53.

PANOS, T. C., AND J. C. FINERTY 1954 Effects of a fat-free diet on growing male rats with special reference to the endocrine system. J. Nutrition, *54:* 315.

PEIFER, J. J., AND R. T. HOLMAN 1955 Essential fatty acids, diabetes and cholesterol. Arch. Biochem. Biophys., *57:* 520.

REISER, R. 1951 The synthesis and interconversions of polyunsaturated fatty acids by the laying hen. J. Nutrition, *44:* 159.

RIECKEHOFF, I. G., R. T. HOLMAN AND G. O. BURR 1949 Polyethenoic fatty acid metabolism. Effect of dietary fat on polyethenoic fatty acids of rat tissues. Arch. Biochem., *20:* 331.

SMEDLEY-MACLEAN, I. 1943 The Metabolism of Fat. Methuen, London, p. 39.

WIDMER, C., AND R. T. HOLMAN 1950 Polyethenoic fatty acid metabolism. II. Deposition of polyunsaturated fatty acids in fat-deficient rats upon single fatty acid supplementation. Arch. Biochem., *25:* 1.

WIESE, H. F., M. A. BAUGHAN AND A. E. HANSEN 1955 Influence of fat in diet on distribution of unsaturated fatty acids in serum of dogs. Federation Proc., *14:* 453.

THE VITAMIN B_{12} CONTENT OF AZOTOBACTER VINELANDII [1]

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(Received for publication March 7, 1958)

A dried azotobacter product has been proposed as a possible food supplement, primarily because of its high protein content (Almon, Kilgore and Gieger, '57). Because protein foods are depended on to furnish accessary as well as primary food factors, the vitamin content of this product becomes of interest. Some vitamins of the B group have been found by Lee and Burris ('43) to compare well in quantity in azotobacter with those in protein foods of animal origin. But a good quantitative assay for vitamin B_{12} has not been reported. The work of Burton and Lochhead ('51), revealing low vitamin B_{12} activity for *Lactobacillus leichmannii* 7830 in 8 strains of azotobacter (representing three species) was done on liquid culture suspensions of unrecorded density.

EXPERIMENTAL

Microbiological assays

The study here reported was an investigation of the vitamin B_{12} content of dried *Azotobacter vinelandii,* strain O, grown in three media. For comparison, two series of determinations were done also on the freshly harvested, undried organisms. The media were (a) the sucrose-salts medium described earlier (Almon, Kilgore and Gieger, '57); (b) the same medium with 0.5 mg of cobalt (as $CoSO_4$) per liter, and (c)

[1] Published with the approval of the Director, Mississippi Agricultural Experiment Station as Journal Paper no. 703, 1958.

a citrus molasses medium prepared by diluting 80 ml of citrus molasses to 400 ml with tap water, adjusting the pH to 7.3, filtering through paper, making the volume of the filtrate up to 4 liters with tap water and adding 1.5 gm of K_2HPO_4. Sterilization was at 120°C for 25 minutes in the autoclave. Aeration during growth was accomplished with porous stone diffusers. Harvesting and drying proceeded according to our previous report (Almon, Kilgore and Gieger, '57).

Vitamin B_{12} assays were carried out according to the U.S. Pharmocopeia method ('51 and '54), using Difco media, and titrating acidity after incubation for 72 hours instead of reading turbidity after 6 to 24 hours. The batches which were analyzed without drying were weighed directly from the supercentrifuge cylinder, two samples into vials for extraction of of vitamin B_{12}, and larger samples into moisture dishes for determination of water content. For vitamin B_{12} determination sufficient quantities were weighed to permit a 1:50 dilution of the extract since the extracting fluid recommended by the U.S.P. method is inhibitory to *L. leichmannii* unless diluted 1:20 or more.

TABLE 1

Vitamin B_{12} content of Azotobacter vinelandii as determined by the U.S.P. method

MEDIUM	TREATMENT OF HARVESTED CELLS	VITAMIN B_{12} MILLIMICROGRAMS PER GRAM, DRY BASIS
Sucrose-salts (no added cobalt)	None; fresh paste	13.55 ± 2.9 [3]
Sucrose-salts (no added cobalt)	Dried at 95°C	11.0 ± 0.68 [1]
Sucrose-salts plus 0.5 mg Co per liter	None; fresh paste	29.8 ± 3.5 [2]
Sucrose-salts plus 0.5 mg Co per liter	Dried at 95°C	19.9 ± 3.2 [2]
Citrus molasses	Dried at 95°C	17.3 ± 3.1 [3]

[1] Mean — 6 analyses, mixture from 20 harvests.

[2] Mean — 8 analyses, 4 separate harvests.

[3] Mean — 6 analyses, three separate harvests.

Results appear in the table. The vitamin B_{12} activity shown is very low when compared with that of some other microorganisms, notably *Bacillus megaterium* which yields 6 to 10.3 μg per gram (Garibaldi et al., '53) as contrasted with the millimicrograms of azotobacter, and *Serratia marcescens* which has yielded as much as 13.1 μg per gram (Hill and Branion, '53). The lower amounts in the dried preparations as compared with the fresh organisms may be due to two causes: (a) the fresh organisms were not washed, and small amounts of extracellular vitamin may have been included in the determination; (b) no attempt to stabilize the vitamin during drying was made since the introduction of cyanide for stabilization would have made the product unfit for the animal feeding experiment conducted later.

Rat feeding trials

Since some microorganisms are known to contain pseudovitamin B_{12}, inactive for mammals (Ford et al., '53), it was considered advisable to confirm the identity of the factor demonstrated in the *L. leichmannii* assay by a rat growth experiment. Accordingly, weanling male Sprague-Dawley rats were depleted of vitamin B_{12} stores by feeding a vitamin B_{12}-deficient test diet,[2] vegetable protein base, for two weeks. The rats were then divided into 4 groups of 4 each. One group, the negative controls, continued to receive the deficient ration alone, with a supervised supplement of 60 mg of mannitol. A second group, the positive controls, received 60 mμg of cobalamin in 60 mg of mannitol per day, fed as a separate supplement with ingestion likewise supervised. A third group received 5.5 gm per day of dried organisms grown in sucrose-salts medium without cobalt, containing the calculated equivalent of 60.5 mμg of vitamin B_{12}. The 4th group received 3 gm of dried organisms grown in sucrose-salts medium with cobalt, providing the calculated equivalent of 59.7 mμg per day. Ingestion of these rather large amounts of dried azotobacter

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

was ensured by mixing the weighed amount with a small amount of the deficient ration and offering it to the animal as its only food until it was eaten. After ingestion of the dose, the deficient ration was provided ad libitum for the rest of the 24 hour period. Water was provided ad libitum for all animals.

Weight gains during the two weeks' experimental period for the 4 groups of animals averaged as follows: negative controls 43.8 gm; positive controls, 57 gm; organisms grown without cobalt, 58 gm; organisms grown with cobalt 60.8 gm. The interpretation of these results is difficult since the inclusion of as much as 5.5 gm of dried azotobacter in the ration of one group of rats and 3 gm in a second group made their nutritional intake with regard to proportions of carbohydrate, proteins, minerals, and vitamins markedly different from that of the other two groups. However, there is no evidence from this trial or from our previous experience with mice (Almon, Kilgore and Gieger, '57), to indicate that the dried azotobacter preparations are either stimulatory or inhibitory in comparison with rations which are nutritionally adequate for growth. Moreover, the two azotobacter preparations used in the rat feeding reported here were fed at different levels, because of their differing vitamin B_{12} assays, but supported approximately equal gains in weight. These gains were compatible with the vitamin B_{12} content as compared with the positive controls. Also, the raising of the protein content of the diet by means of the azotobacter meal (which is approximately 75% protein) would be expected to inhibit rather than stimulate growth in the absence of an adequate supply of vitamin B_{12} (Hartman, Dryden and Cary, '49). The conclusion therefore seems reasonable that the growth-promoting property of the dried azotobacter added to the vitamin B_{12}-deficient ration was due to quantitative substitution for the lack of vitamin B_{12} in the ration.

DISCUSSION

Some of the implications of the low amounts of the vitamin here reported seem worthy of comment. In the amounts of

a dried azotobacter product which might conceivably be used as human food to improve the protein content of a diet, the small amount of vitamin B_{12} included as an incidental adjunct may not be important. However, as a biological phenomenon having general implications for the field of nutrition as a whole the low levels present in these azotobacter cells are very interesting. Whereas other vitamins of the B group occur in unusually large amounts in azotobacter (cf. Lee and Burris, '43) the amounts of vitamin B_{12} found in the assays here reported are among the lowest which have been reported for any organism. If the widely held belief is valid that microorganisms either synthesize vitamin B_{12} or require it preformed in their milieu because it is needed for their metabolism, then azotobacter is an organism which can satisfy its needs with very low amounts — how low has not been determined, for even the lowest amounts here reported (in fresh cells) are accompanied by optimal rates of growth and maximal yields. If organisms do, indeed, require vitamin B_{12} for some fundamental processes such as phosphorus metabolism and synthesis of nucleic acid, the question arises as to how higher plants generally lacking demonstrable amounts of cobalamin perform these functions. It is conceivable that amounts too small to be assayed conveniently would suffice.

The importance, in this connection, of the recent work of Gray, Speirs and Matrone ('57) is considerable, for this work shows that turnip plants, at least, do sometimes contain — either in or on their leaves — sufficient of a vitamin B_{12}-active substance to be assayed by conventional methods. Earlier work by Robbins, Hervey and Stebbins ('50), had given similar indications. Further investigation of the source and role of this vitamin in plant metabolism may require more delicate methods of assay than those now in use, although the possible implication of an analogue not detected at all by the usual methods must not be overlooked.

SUMMARY

Analyses of *Azotobacter vinelandii* for vitamin B_{12} by the U.S.P. method, using *Lactobacillus leichmannii* 7830, gave

values of approximately 14 and 30 mμg per gram in freshly harvested organisms, calculated to an air-dry basis. The difference in level depended on whether or not cobalt was added to the medium for the growth of the azotobacter. Because stabilizing with cyanide was purposely avoided, washing the organisms and drying them at 95°C resulted in losses of from one-fifth to one-third of the vitamin present in the fresh paste. Addition of dried azotobacter to a standard vitamin B_{12}-deficient ration permitted rats to gain weight to the same degree as a control group fed the same ration with a vitamin B_{12} supplement amounting to the level calculated to be contained in the azotobacter.

ACKNOWLEDGMENT

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LITERATURE CITED

ALMON, L., L. KILGORE AND M. GIEGER 1957 Nutritive qualities of *Azotobacter vinelandii.* J. Am. Dietetic Assoc., *33:* 481–484.

BURTON, M. O., AND A. G. LOCHHEAD 1951 Studies on the production of vitamin B_{12}-active substances by micro-organisms. Canad. J. Bot., *29:* 352–359.

FORD, J. E., E. S. HOLDSWORTH, S. K. KON AND J. W. G. PORTER 1953 Occurrence of the various vitamin B_{12} active compounds. Nature, *171:* 150–151.

GARIBALDI, J. A., K. IJICHI, N. S. SNELL AND J. C. LEWIS 1953 Bacillus megatherium for biosynthesis of cobalamin. Ind. Eng. Chem., *45:* 838–846.

GRAY, L. F., M. SPEIRS AND G. MATRONE 1957 The nutritive value of several foods grown at different locations. J. Nutrition, *63:* 345–359.

HARTMAN, A. M., L. P. DRYDEN AND C. A. CARY 1949 A role of vitamin B_{12} in the normal mammal. Arch. Biochem., *23:* 165–168.

HILL, D. C., AND H. D. BRANION 1953 Chick growth studies with dried cells of a strain of *Serratia marcescens.* Poultry Sci., *32:* 367–368.

LEE, S. B., AND R. H. BURRIS 1943 Large-scale production of Azotobacter. Ind. Eng. Chem., *35:* 354–357.

ROBBINS, W. J., A. HERVEY AND M. E. STEBBINS 1950 Studies on Euglena and vitamin B_{12}. Bull. Torrey Bot. Club, *77:* 423–441.

U.S. Pharmocopeia XIV. Vitamin B_{12} activity assay. Third supplement (1951) 15–18; Fourth supplement (1954), 10–14.

INVITATIONS FOR NOMINATIONS FOR 1959 AMERICAN INSTITUTE OF NUTRITION AWARDS

Nominations are requested for the 1959 annual awards administered by the American Institute of Nutrition to be presented at the next annual meeting. Nominations may be made by anyone, including members of the Nominating Committees and non-members of the Institute.

The following information must be submitted: (1) Name of the award for which the candidate is proposed and (2) a statement as convincing as possible as to the basis for the nomination, stating the eligibility of the candidate (this may include the pertinent bibliography of the most appropriate and significant recent papers on which the nomination is based, but such bibliography is not necessary unless later requested by the Nominating Committee). Reprints are not required, nor are seconding statements. *Five copies of all documents* must be sent to the chairman of the appropriate Nominating Committee *before December 1, 1958,* to be considered for the 1959 awards.

General Regulations for A.I.N. Awards. An individual who has received one Institute award is ineligible to receive another Institute award unless it is for outstanding research subsequent to or not covered by the first award.[1] A Jury of Award composed of A.I.N. members, which makes final selection and remains anonymous, may recommend that an award be omitted in any given year if in its opinion the work of the candidates nominated does not warrant the award. An award is usually given to one person but, if circumstances and justice so dictate, a Jury of Award may recommend that any par-

[1] Including recipients of the former Mead-Johnson award. These are listed at the end of this notice.

ticular award be divided between two or more collaborators in a given research.

Membership in the American Institute of Nutrition is not a requirement for eligibility for an award and there is no limitation as to age. Presentation of awards will be made at the annual dinner at the annual meeting and time will be allotted on the scientific program for a brief review by the recipient of the work upon which the award has been based.

1959 Borden Award in Nutrition

The Borden Award in Nutrition, consisting of $1,000 and a gold medal, is made available by the Borden Company Foundation, Inc. The award is given in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of milk or its components. 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. Employees of the Borden Company are not eligible for this award nor are individuals who have received a Borden Award from another administering association unless the new award be for outstanding research on a different subject or for specific accomplishment subsequent to the first award.

Former recipients of this award are: 1944 — E. V. McCollum; 1945 — H. H. Mitchell; 1946 — P. C. Jeans and Genevieve Stearns; 1947 — L. A. Maynard; 1948 — C. A. Cary; 1949 — H. J. Deuel, Jr.; 1950 — H. C. Sherman; 1951 — P. György; 1952 — M. Kleiber; 1953 — H. H. Williams; 1954 — Agnes Fay Morgan and A. H. Smith; 1955 — A. G. Hogan; 1956 — F. M. Strong; 1957 — no award; 1958 — L. D. Wright.

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1959 Osborne and Mendel Award

The Osborne and Mendel Award of $1,000 and an inscribed scroll has been established by the Nutrition Foundation, Inc., for the recognition of outstanding recent basic research 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 approximately the calendar year preceding the annual meeting of the Institute, or who has published recently a series of papers of outstanding significance. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration.

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Former recipients of the Mead-Johnson Award presented by A.I.N. are: 1939 — C. A. Elvehjem; 1940 — W. H. Sebrell, Jr., J. C. Keresztesy, J. R. Stevens, S. A. Harris, E. T. Stiller, and K. Folkers; 1941 — R. J. Williams; 1942 — G. R. Cowgill; 1943 — V. du Vigneaud; 1944 — A. G. Hogan; 1945 — D. W. Woolley; 1946 — E. E. Snell; 1947 — W. J. Darby, P. L. Day, and E. L. R. Stokstad; 1948 — F. Lipmann; 1949 — Mary S. Shorb and K. A. Folkers; 1950 — W. B. Castle; 1951 — no award; 1952 — H. E. Sauberlich.

INVITATION FOR NOMINATIONS FOR FELLOWS

The Fellows Committee of the American Institute of Nutrition invites nominations for Fellows in the Society. Eligible candidates are active or retired members of the Society who have passed their sixty-fifth birthday (by the time of the annual meeting) and who have had distinguished careers in nutrition. Up to three Fellows will be chosen each year.

Nominations may be made to the Chairman of the Fellows Committee by any member of the Society.

Nominations (in 5 copies) are due by January 1. A supporting statement giving the reason for the nomination is desirable but not necessary.

Final selection will be made by the Fellows Committee and a suitable citation will be presented at the Annual Dinner in April. The following persons have been elected previously as Fellows of the Society:

Thorne M. Carpenter	Elmer V. McCollum
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THE JOURNAL OF NUTRITION is the official organ of the American Institute of Nutrition. The officers of the Institute for 1958-59 are: William J. Darby, President; D. Wayne Woolley, President-Elect; George M. Briggs, Secretary; J. B. Brown, Treasurer; E. W. McHenry, Paul György, J. H. Roe, and R. R. Williams, Councillors.

Preliminary notices, or papers already published or in press elsewhere, will not be accepted. Unusually long papers that would take a disproportionate part of a single issue can be considered only if published as a supplement, the entire cost of which is assumed by the author.

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