Chromatographic Separation and Microbiologic Assay of Vitamin B_6 in Tissues from Normal and Vitamin B_6-depleted Rats [1,2]

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ABSTRACT The Toepfer and Lehman assay for pyridoxine, pyridoxal and pyridoxamine was studied to determine optimal conditions of acid hydrolysis, sample size and volume of resin bed in order to apply it to animal tissues. Subsequent studies of B_6 vitamer content and transaminase activity in tissues obtained from vitamin B_6-deficient, ad libitum-fed control and pair-fed control rats showed that (a) total vitamer content of the tissues from the vitamin B_6-depleted rats was lower than that of the pair-fed and ad libitum controls; (b) highest levels of the total vitamer content were observed in the heart and muscle of the ad libitum controls; (c) total vitamer content of liver, kidney and brain of the ad libitum controls was intermediate between that of the pair-fed and deficient group; (d) similar total results were obtained when total vitamin B_6 was determined without chromatographic separation; (e) pyridoxine was noted in the lowest concentration in the rat tissues; (f) in all tissues except muscle, pyridoxamine was the vitamer found in highest concentrations, whereas pyridoxal was highest in muscle; (g) the values for tissue transaminase activity and vitamin B_6 content of liver and kidney of the pair-fed groups were higher than those for the ad libitum controls; and (h) the vitamin B_6 content of the brain of the pair-fed groups was higher than that for the ad libitum controls, but no difference was observed in the transaminase activity.

Various methods such as rat bioassay (1, 2), chemical (3–6), fluorometric (7–9), microbiologic with yeast (10) and lactobacilli (11, 12) and paper chromatography (13–15) have been used to determine the concentration of vitamin B_6. However, some of these methods have not been used as successfully with biologic tissues of animal origin. Bioassay is extremely time-consuming and it is difficult to obtain reproducible results with these materials. Chemical procedures are not generally applicable owing to the presence of interfering substances in the tissues. At present, chromatographic separation techniques followed by microbiologic assay offer promise. The most recently reported analyses involve gas chromatography,[3] but as yet this has been used only with pure samples. However, Toepfer and Lehman (16, 17) have developed a method for the chromatographic separation of the 3 vitamers followed by microbiologic assay of the individual eluates. They have used this procedure to determine the vitamin B_6 content of various food products but primarily of plant origin (18–20). In the current study an attempt has been made to modify this procedure for use with animal tissues. Thus it was necessary to determine conditions for hydrolysis, the amount of hydrolysate to be chromatographed per volume of resin, and the volume of eluate to be collected. Correlation of the vitamin B_6 content of normal and vitamin B_6-deficient tissues with tissue transaminase activity is also presented.

MATERIALS AND METHODS

1. Animals and diets. Three groups of five male weanling rats [4] each, were used in this experiment: vitamin B_6-deficient, vitamin B_6-adequate and a vitamin B_6-adequate group which was pair-fed to the

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[3] Prosser, A. R., and A. J. Sheppard 1966 The separation of pyridoxine, pyridoxal and pyridoxamine (vitamin B_6 analogs) by gas-liquid chromatography. Federation Proc., 25: 669 (abstract).

[4] Purchased from Carworth Inc., New City, New York.

deficient group. The following diet was fed for 32 days: (in per cent) glucose, 73; casein, 18; corn oil, 4; Hegsted salt mixture, 4 (21); [5] and cod liver oil, 1. The vitamins added were: (μg/100 g of diet) thiamine chloride, 400; riboflavin, 800; pyridoxine·HCl, 400; Ca pantothenate, 2500; niacin, 4000; and choline chloride, 100,000. The vitamin B_6-deficient diet did not contain pyridoxine hydrochloride.

2. *Vitamin B_6 assay.* Reagents, resin preparation and general procedures were used as described previously (17) except as modified below. Hydrolysis: Frozen tissue samples were homogenized in acid in the proportions of 1 g tissue to 200 ml acid. Either 0.055 N or 0.44 N HCl was used. After five hours, or two hours, respectively, for each acid, of autoclaving at 120°, the hydrolysate was cooled and the pH adjusted to 4.5. Five milliliters of pH 4.5 buffer were added to each sample and each was then made up to a volume equivalent to 250 ml for a 1-g sample.

Chromatography. Ten milliliters of prepared resin were placed on a thiamine chromatography column [6] over a glass bead. This consisted of a column 160 mm long with a 2-mm bore capillary tube, an internal diameter of 11.5 mm and a reservoir of 50 ml capacity. A volume of hydrolysate equivalent to 100 to 300 mg of tissue (depending upon the specific tissue) was added to the column. Fifty milliliters of each buffer (17) were used for elution. Forty milliliters of each eluate were collected, the pH was adjusted to 5.0 and the volume made up to 50 ml. The remaining 10 ml were discarded. With each run 40 ml of acid which had been treated as the samples described above, were placed on the column to serve as a blank. Also a standard mixture consisting of 0.08 μg each of pyridoxal, pyridoxine and pyridoxamine was hydrolyzed and separated on a column for recovery calculations.

Microbiologic assay. Duplicate tubes containing 0.5, 1 and 2 ml of the column eluates were prepared. Water was added to make a total volume of 9 ml in each tube and the tubes were autoclaved for 15 minutes. One milliliter of prepared yeast basal medium was added to each tube. This consisted of a filter-sterilized vitamin-free yeast base [7] to which was added 0.5 ml of a vitamin stock solution per 100 ml of 10 X broth. The vitamin solution contained the following (in g/500 ml): biotin 0.008, Ca pantothenate, 2.5; inositol, 25; thiamine, 0.250; and nicotinic acid, 2.5.

Standard solutions. Stock solutions of the vitamers were prepared according to the method of Toepfer and Lehman (17) except that 0.055 N HCl was used. The following amounts of the 3 vitamers were used to prepare standard curves (in mμg) pyridoxal, pyridoxamine: 1.5, 3, 4, 5, 6, 9, 12, 18; and pyridoxine: 1, 2, 3, 4, 6, 7, 12.

3. *Total vitamin B_6 assay.* The procedure followed was that described above except that the hydrolyzed samples were pipetted directly without chromatographic separation. Because of the light sensitivity of vitamin B_6 all assay procedures were carried out under the illumination of gold light.[8]

4. *Transaminase activity.* Glutamic-pyruvic transaminase assays were made according to the procedure of Wroblewski and Cabaud (22).

RESULTS

The concentration of acid used for hydrolysis did not affect the liberation of the vitamin (table 1). However, increasing the time of hydrolysis resulted in more of the vitamin being released, and the greatest positive effect was observed in the pyridoxal fraction. For all subsequent assay procedures samples were hydrolyzed in 0.055 N HCl for 5 hours.

The most effective separation of the three vitamers was obtained with 100 mg of liver hydrolysate and 10 ml of resin as indicated in table 2. With larger amounts of resin the poor separation may have resulted from the use of an insufficient volume of buffer solution for the elution. Similar values were obtained when both 100 mg and 200 mg of hydrolysate were applied to the column. Therefore, 100 mg of liver sample did not exceed the capacity of the resin. Similar experiments were repeated with other tissues. The following weights of sample appeared optimal: kid-

[5] Purchased from Nutritional Biochemicals Corporation, Cleveland.
[6] Purchased from Kontes Glass Company, Vineland, New Jersey.
[7] Purchased from Difco Laboratories, Detroit, Michigan.
[8] Personal communication from E. W. Toepfer.

ney, 100 mg; heart, 300 mg; brain, 300 mg; and muscle 400 mg.

Recovery studies were made using mixtures of 2, 4, and 8 μg of each pure vitamer. These solutions were subjected to acid hydrolysis prior to chromatographic separation. As shown in table 3, the separation of the 3 vitamers became less efficient as the concentration increased. A concentration of approximately 0.1 μg of the vitamer per 50 ml of eluate was equivalent to an average amount found in a tissue sample. With each assay a standard mixture consisting of 0.08 μg each of pure pyridoxal, pyridoxine and pyridoxamine was hydrolyzed and chromatographically separated. This then served as a check on the separation of the 3 vitamers as well as on the percentage recovery or efficiency of the assay.

Reproducible results were obtained when five individual aliquots of the same liver hydrolysate were assayed. These data are shown in table 4, for the 2 tissue samples. In both instances there was close agreement between four out of five determinations of pyridoxal content. Very low amounts of pyridoxine were observed in liver with some slight variation, and the values for pyridoxamine were the most constant.

The growth curves for the three groups of rats that were used in the study of the relationship between vitamin B_6 content and tissue transaminase activity are presented in figure 1. The rate of growth of the pair-fed rats was somewhat greater than that for the deficient rats, but was markedly less than that for the ad-libitum control rats.

TABLE 1

Effect of acid concentration and time of hydrolysis on vitamin B_6 determination of liver tissue

Conc of HCl	Time	Vitamin B_6 content		
		Pyridoxal	Pyridoxine	Pyridoxamine
	hours	*μg vitamer/g tissue*		
0.055 N	2	1.00	0.33	2.00
0.44 N	2	0.95	0.35	2.00
0.055 N	5	2.20	0.50	2.55
0.44 N	5	2.65	0.45	2.45

TABLE 2

Effect of amount of hydrolysate and resin on vitamin B_6 determination of liver tissue

Wt of tissue	Vol of resin	Pyridoxal	Pyridoxine	Pyridoxamine
mg	*ml*	*μg vitamer/g tissue*		
100	10	1.60	0.45	2.90
100	20	1.00	0.60	1.25
100	30	0.03	1.60	1.50
200	10	1.82	0.55	2.95
200	20	0.02	2.30	1.75

TABLE 3

Recovery studies of pure vitamers

Amt of each added	Amount of each vitamer recovered [1]				Recovery		
	Pyridoxal	Pyridoxine	Pyridoxamine	Total	Pyridoxal	Pyridoxine	Pyridoxamine
μg	*μg*	*μg*	*μg*	*μg*	%	%	%
2	1.79 ± 0.09	2.53 ± 0.03	1.69 ± 0.06	6.00 ± 0.14	89	127	85
4	3.50 ± 0.19	4.91 ± 0.08	2.35 ± 0.19	10.96 ± 0.26	87	125	59
8	6.32 ± 0.69	6.28 ± 0.94	6.75 ± 0.52	19.35 ± 0.70	79	79	84

[1] Mean of 4 determinations and SE of mean.

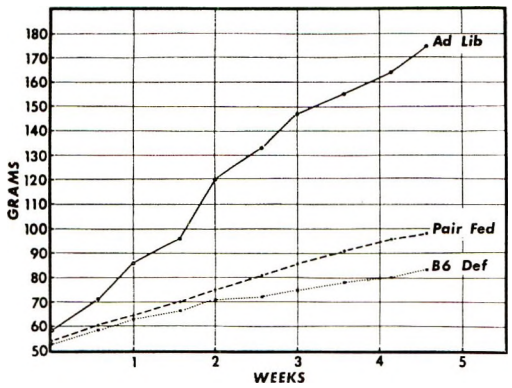

Fig. 1 Growth curves for body weights of rats maintained with vitamin B_6-deficient diets, vitamin B_6-adequate diets, and vitamin B_6-adequate diets in which the rats were pair-fed to the deficient group.

The values for tissue vitamin B_6 content which were obtained both with and without the chromatographic separation technique are shown in table 5. Whether chromatographed or not, it was clearly evident that there was less vitamin B_6 in the vitamin B_6-deficient tissues. Considering total vitamin B_6, in which the forms were chromatographed, the highest levels were in the heart and muscle of the ad libitum controls. For the same analysis in liver, kidney and brain the vitamin B_6 content of the ad libitum controls was intermediate between that of the pair-fed and the deficient group.

Very little pyridoxine was present in these tissues, the highest values being noted in the liver, brain and muscle of the ad libitum controls and in the muscle and

TABLE 4

Multiple determinations on 2 liver samples [1]

Sample	Pyridoxal	Pyridoxine	Pyridoxamine	Total
		μg vitamer/g liver		*μg/g liver*
1	4.05 ± 0.38	0.34 ± 0.08	2.87 ± 0.09	7.26 ± 0.37
2	2.76 ± 0.16	0.62 ± 0.07	2.87 ± 0.03	6.25 ± 0.10

[1] Mean of 5 determinations and SE of mean.

TABLE 5

Vitamin B_6 content of rat tissues [1]

Tissue	Group	Chromatographed				Unchromatographed
		Pyridoxine	Pyridoxal	Pyridoxamine	Total	Total
			μg vitamer/g tissue			*μg/g tissue*
Liver	vitamin B_6-deficient	0.06 ± 0.06	0.90 ± 0.20	2.26 ± 0.38	3.22	3.62 ± 0.70
	pair-fed	0.05 ± 0.02	4.04 ± 0.35**	6.31 ± 0.52**	10.40	7.60 ± 0.46**
	ad lib.-fed (controls)	0.24 ± 0.09	3.67 ± 0.34**	4.76 ± 0.39**	8.67	5.20 ± 0.80
Kidney	deficient	0.13 ± 0.04	0.15 ± 0.03	1.23 ± 0.22	1.51	1.52 ± 0.21
	pair-fed	0.08 ± 0.01	2.64 ± 0.12**	6.16 ± 1.2*	8.88	6.64 ± 0.56**
	ad lib.-fed	0.05 ± 0.02	2.28 ± 0.18**	4.03 ± 0.70**	6.36	5.80 ± 0.29**
Brain	deficient	0.09 ± 0.01	0.65 ± 0.04	1.25 ± 0.08	1.99	
	pair-fed	0.28 ± 0.02**	1.92 ± 0.08**	2.26 ± 0.06**	4.46	
	ad lib.-fed	0.23 ± 0.04*	1.28 ± 0.14**	1.96 ± 0.16**	3.47	
Muscle	deficient	0.10 ± 0.01	0.85 ± 0.06	0.54 ± 0.01	1.40	1.02 ± 0.12
	pair-fed	0.19 ± 0.03*	2.71 ± 0.33**	1.09 ± 0.05**	3.99	3.79 ± 0.43**
	ad lib.-fed	0.24 ± 0.02**	3.08 ± 0.12**	1.21 ± 0.06**	4.53	4.37 ± 0.09**
Heart	deficient	0.01 ± 0.01	0.40 ± 0.01	1.26 ± 0.11	1.67	1.47 ± 0.05
	pair-fed	0.01 ± 0.01	0.92 ± 0.10**	2.02 ± 0.26	2.95	2.80 ± 0.31**
	ad lib.-fed	0.02 ± 0.02	0.92 ± 0.04**	2.44 ± 0.12**	3.38	2.36 ± 0.18**

[1] Mean of 5 rats and SE of mean.
** Significantly different from the deficient mean, $P < 0.01$.
* Significantly different from the deficient mean, $P < 0.05$.

brain of the pair-fed group. In all of the tissues except muscle, pyridoxamine was the vitamer observed in the highest concentrations, whereas pyridoxal was the highest in muscle. Also the amount of pyridoxal and pyridoxamine was much greater in both control groups than in the vitamin B_6-deficient group in all tissues. Total vitamin B_6 content without chromatography was determined in all tissues except brain. Again in all cases, the concentration of vitamin B_6 in the pair-fed and ad libitum group was much higher than in the deficient group. However, liver and kidney values for the pair-fed group were higher than for the ad libitum control group. In the heart, the values were only slightly higher. This effect was also observed by Beaton and McHenry (23) in 1953.

Additional studies involved the determination of glutamic pyruvic transaminase activity in these tissues (table 6) as this enzyme is more sensitive to a vitamin B_6 deficiency than glutamic-oxalacetic transaminase (24–26). The increased transaminase activity which was observed in the pair-fed group was significantly different from the activity in the deficient tissues ($P < 0.01$). However, this also held true for the ad libitum controls except for the value in the kidney which was not significantly higher.

A tissue often used for the evaluation of vitamin B_6 status in man and rats is the erythrocyte (25, 26). In this study erythrocyte transaminase activity was similar in the pair-fed and ad libitum controls but markedly depressed in the deficient erythrocytes (table 6) and as previously reported (27). Evidently the erythrocyte of the pair-fed animal retained sufficient vitamin B_6 to maintain normal transaminase activity. Although transaminase activity in the plasma of pair-fed animals was somewhat less than in ad libitum controls, it was certainly more active than in the deficient plasma.

The increased transaminase activity in the livers and kidneys of pair-fed rats over that of the ad libitum controls is considered a real one, and may be explained as an adaptation of the liver and kidney enzymes to the stimulus of a semifasting state as a physiologic stress. The response of the liver transaminase enzyme and that of intestinal mucosa at least, to this kind of stress was first demonstrated by Brin et al. (28) and has been confirmed by many others (29).

A comparison of the tissue transaminase activity and the total vitamin B_6 content of liver and kidney showed that in both cases the values for the pair-fed group were higher than those for the ad libitum controls. This was also true for the vitamin B_6 content of the brains of these respective rats, although no differences were observed in the transaminase activity in this tissue.

DISCUSSION

The choice of appropriate acid concentration, length of time of autoclaving and other conditions of vitamin B_6 assay were determined empirically in this study. It was shown that the recovery of larger samples was often interfered with, possibly due to a competition between the free amino acids and the vitamin B_6 for the resin surface. Therefore, it is important not to exceed the resin capacity. The recovery studies also indicated that separation of the 3 vitamers was not absolutely

TABLE 6

Glutamic pyruvic transaminase activity [1]

Tissue	Deficient	Pair-fed	Ad libitum controls
	mg pyruvic acid/g/hour		
Liver	67 ±8.3	198 ±23.1**	112 ± 9.2**
Kidney	6.5±0.6	10.3± 0.4**	7.5± 0.5
Brain	3.7±0.3	6.5± 0.4**	6.6± 0.4**
Plasma	44 ±4.1 [2]	151 ±10.7**	203 ±19.5**
Red blood cells	72 ±3.7 [2]	343 ± 4.1**	333 ±15.6**

[1] Mean of 5 rats and SE of mean.
[2] μg pyruvic acid/ml/hour.
** Significantly different from the deficient mean, $P < 0.01$.

complete. The high recovery values for pyridoxine are probably the result of contamination with pyridoxal and pyridoxamine as a result of suboptimal elution. Much information can be obtained with this assay, nevertheless.

Pyridoxine was the vitamer observed in lowest concentrations in all of the rat tissues. In animal systems, pyridoxal and pyridoxamine are used in enzyme reactions and thus pyridoxine would be converted to these 2 vitamer forms.

The transaminase activity and the vitamin B_6 content of the livers and the kidneys of the pair-fed controls was higher than that of the ad libitum controls. Other liver enzymes have been shown to undergo adaptation to fasting (30). The increase in vitamin B_6 content along with that of transaminase activity in the pair-fed group could perhaps reflect the cellular need for the coenzyme form.

In the vitamin B_6-depleted rats the vitamin B_6 content of all vitamers decreased except for pyridoxine in the liver and kidney.

In most tissues, except muscle, pyridoxamine was present in higher amounts than pyridoxal. In the deficiency state the ratio of pyridoxamine to pyridoxal was even greater as the pyridoxamine was preserved in the cell. In muscle however, the pyridoxal content was higher than the pyridoxamine content and the reverse was true in the deficiency state. This may have been due to the fact that pyridoxal phosphate was attached to the phosphorylase enzyme which is found in large amounts in muscle and that this may be a storage form of the vitamin in this tissue (31).

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LITERATURE CITED

1. Elvehjem, C. A., C. J. Koehn, Jr. and J. Oleson 1936 New essential dietary factor. J. Biol. Chem., *115:* 707.
2. Conger, T. W., and C. A. Elvehjem 1941 The biological estimation of pyridoxine (vitamin B_6). J. Biol. Chem., *138:* 555.
3. Scudi, J. V. 1941 On the colorimetric determination of vitamin B_6. J. Biol. Chem., *139:* 707.
4. Swaminathan, M. 1940 Chemical estimation of vitamin B_6 in foods by means of the diazo reaction and the phenol reagent. Nature, *145:* 780.
5. Waisman, H. A., and C. A. Elvehjem 1941 Chemical estimation of nicotinic acid and vitamin B_6. Ind. Eng. Chem. (Anal. ed.), *13:* 221.
6. Brown, E. B., A. F. Bina and J. M. Thomas 1945 The use of diazotized *p*-aminoacetophenone in the determination of vitamin B_6 (pyridoxine). J. Biol. Chem., *158:* 455.
7. Bina, A. F., J. M. Thomas and E. B. Brown 1943 The determination of vitamin B_6 (pyridoxine) in foods. J. Biol. Chem., *148:* 111.
8. Storvick, C. A., and J. M. Peters 1964 Methods for the determination of vitamin B_6 in biological materials. Vitamins Hormones, *22:* 833.
9. Polansky, M. M., R. T. Camarra and E. W. Toepfer 1964 Pyridoxine determined fluorometrically as pyridoxal cyanide compound. J. Assn. Offic. Agr. Chem., *47:* 827.
10. Atkin, L. A., A. L. Schultz, W. L. Williams and C. N. Frey 1943 Yeast microbiological methods for determination of vitamin pyridoxine. Ind. Eng. Chem. Anal., *15:* 141.
11. Landy, M. and D. M. Dicken 1942 A microbiological assay method for six B vitamins using *Lactobacillus casei* and a medium of essentially known compounds. J. Lab. Clin. Med., *27:* 1086.
12. Rabinowitz, J. C., and E. E. Snell 1948 The vitamin B_6 group. XIV. Distribution of pyridoxal, pyridoxamine and pyridoxine in some natural products. J. Biol. Chem., *176:* 1157.
13. Winstein, W. A., and E. Eigen 1948 Paper partition chromatographic analysis and microbial growth factors. The vitamin B_6 group. Proc. Soc. Exp. Biol. Med.. *67:* 513.
14. Snyder, J. Q., and S. H. Wender 1953 The separation and determination of pyridoxine, pyridoxal and pyridoxamine by paper chromatography and microbiological assay. Arch. Biochem. Biophys., *46:* 465.
15. Hedin, P. 1963 The separation of pyridoxine, pyridoxal and pyridoxamine by a sulfonic acid ion exchange resin. J. Agr. Food Chem., *11:* 343.
16. MacArthur, M. J., and J. Lehmann 1959 Chromatographic separation and fluorometric measurement of vitamin B_6 components in aqueous solutions. J. Assn. Offic. Agr. Chem., *42:* 619.
17. Toepfer, E. W., and J. Lehmann 1961 Procedure for chromatographic separation and microbiological assay of pyridoxine, pyridoxal and pyridoxamine in food extracts. J. Assn. Offic. Agr. Chem., *44:* 426.
18. Toepfer, E. W., M. J. MacArthur and J. Lehman 1960 Chromatographic separation of vitamin B_6 components in food extracts. J. Assn. Offic. Agr. Chem., *43:* 59.
19. Toepfer, E. W., M. M. Polansky, L. R. Richardson and S. Wilkes 1963 Compari-

son of vitamin B_6 values of selected food samples by bioassay and microbiological assay. J. Agr. Food Chem., *11:* 523.

20. Polansky, M. M., E. W. Murphy and E. W. Toepfer 1964 Components of vitamin B_6 in grains and cereal products. J. Assn. Offic. Agr. Chem., *47:* 750.
21. 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.
22. Wroblewski, F., and P. Caubaud 1957 Colorimetric measurement of serum glutamic pyruvic transaminase. Amer. J. Clin. Path., *27:* 235.
23. Beaton, G. H., and E. W. McHenry 1953 Vitamin B_6 levels in rat tissues. Brit. J. Nutr., *7:* 357.
24. Brin, M., R. E. Olson and F. J. Stare 1954 Metabolism of cardiac muscle. VIII. Pyridoxine deficiency. J. Biol. Chem., *210:* 435.
25. Brin, M. 1964 Use of the erythrocyte in functional evaluation of vitamin adequacy. In: The Red Cell, eds., D. Surgenor and C. F. Bishop. Academic Press, New York, p. 451.
26. Raica, N., and H. E. Sauberlich 1964 Blood cell transaminase activity in human vitamin B_6 deficiency. Amer. J. Clin. Nutr., *15:* 67.
27. Brin, M., M. Tai, A. S. Ostashever and H. Kalinsky 1960 The relative effects of pyridoxine deficiency on two plasma transaminases in the growing and in the adult rat. J. Nutr., *71:* 416.
28. Brin, M., and R. W. McKee 1956 The effects of X-irradiation, nitrogen mustard, fasting, cortisone and adrenalectomy on transaminases in the rat. Arch. Biochem. Biophys., *61:* 384.
29. Nichol, C. A., and F. Rosen 1963 Changes in alanine transaminase activity related to corticosteroid treatment or capacity for growth. Advanc. Enzyme Regulat., *1:* 341.
30. Stielaw, W. J., R. A. Freedland and J. H. Meyer 1965 Effects of B-vitamin deficiencies and of starvation on liver enzymes of growing rats. J. Nutr., *87:* 109.
31. Krebs, E. G., and E. H. Fischer 1964 Phosphorylase and related enzymes of glycogen metabolism. Vitamins Hormones, *22:* 399.

Organ Weights and Water Levels of the Rat following Reduced Food Intake [1,2]

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ABSTRACT The effect of anorexia on the weights and water levels of body organs was determined. Ninety-two adult female albino rats of a Wistar strain were divided into groups which were subjected to various daily restrictions of food intake that resulted in a loss of body weight up to 40% at the end of 2 weeks when they were killed and autopsied. At autopsy the wet weight and water content of the following organs were measured: adrenal glands, brain, cardiac stomach, pyloric stomach, small bowel, cecum, colon, heart, kidneys, liver, lungs, muscle, ovaries, skin, spleen, salivary glands, thymus gland and residual carcass. Up to 20% loss of body weight, the effects of starvation were of a minor nature. At 30 to 40%, gastric ulcers and a stress reaction appeared, and most organs had lost considerable dry weight and gained water; but only brain showed no changes in weight.

Reduced food intake and body weight loss are common observations in toxicity studies (1). Loss of organ weight and changes in water content have been used as criteria in the assessment of drug toxicity in animals (2). The latter changes may, in turn, result from reduced food intake. Published reports on inanition from reduced food intake have been mainly concerned with biochemical and histological parameters (3, 4). The present report describes the effect of restricted food intake on the weight and water content of various organs of albino rats.

MATERIALS AND METHODS

General design. Groups of adult female albino rats of a Wistar strain bred in the animal quarters of this department were subjected to varying degrees of restricted food intake for a period of 14 days. This was accomplished by restricting the allowance of powdered laboratory ration [4] to amounts ranging between 2 and 9 g/rat. When more than 9 g ration/day were offered it was not always eaten. Controls were fed ad libitum. All animals were housed separately in metabolism cages with free access to water.

Animals. The first group of starvation experiments, comprising 23 rats of initial weight 196 ± 12 g (mean ± SD) and 9 controls (197 ± 17 g), was performed in the winter. The range of food restriction was expanded in 50 animals (195 ± 12 g) starved during the following summer and 10 rats (193 ± 10 g) served as controls. At this body weight, growth rate is slow. This facilitated comparison between starved and control animals. Numbers of animals and daily allowances of food are summarized in table 1.

Autopsy. The rats were killed with chloroform and autopsied after 14 days of reduced food intake. Animals that died before 14 days were autopsied immediately.

The organs removed and weighed [5] are listed in table 2. The contents of the gastrointestinal tract were removed by washing and milking before weighing. The organs were dried to constant weight in a forced-draft Isotemp oven [6] at 96°. Suitable aliquots were taken from skin and residual homogenized carcass. Gross pathology was recorded.

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[2] Presented in part at the annual meeting of the Society of Toxicology, Williamsburg, Virginia, 1965.
[3] Fellow, Medical Research Council of Canada.
[4] Purina Laboratory Chow Checkers, Ralston Purina Company, Limited, Woodstock, Ontario, Canada.
[5] Organs were weighed to 0.1 mg on a 1–911x3 Mettler Balance, Semi-Micro Gram-atic, except for skin and residual carcass which were weighed on a Precision Balance, Mettler K–5T. The balances were supplied by Fisher Scientific Company, Limited, Toronto, Canada.
[6] Fisher Scientific Company, Limited, Toronto, Ontario, Canada.

TABLE 1

Daily food intake, numbers of animals and loss of weight of summer and winter groups

Summer			Winter		
Daily allowance of food	No. of animals [1]	Change in body wt [2]	Daily allowance of food	No. of animals [1]	Change in body wt [2]
g		%	*g*		%
2	6(1)	−37.1±3.3	—	—	—
2.5	6(3)	−35.5	—	—	—
3	8	−32.7±4.9	—	—	—
4	6(2)	−34.9	4	9(1)	−36.5±2.3
5	8	−19.6±2.3	6	6	−25.4±2.7
7	8	−16.2±3.7			
9	8	− 9.9±3.2	8	8	−16.4±4.7
ad libitum	10	+ 1.0±2.1	ad libitum	9	+ 1.2±2.8

[1] Number of deaths in parentheses.
[2] Measured as percentage of initial body weight and expressed as mean ± SD.

TABLE 2

Weights and water levels of organs in the summer control group fed ad libitum

Organ or tissue	Weight [1]	Water level [2]
	%	%
Adrenal glands	0.0263±0.0021 [3]	71.73±1.51
Brain	0.926 ±0.066	78.18±0.23
Gastrointestinal tract		
Cardiac stomach	0.132 ±0.012	76.34±0.39
Pyloric stomach	0.390 ±0.034	76.57±0.53
Small bowel	0.990 ±0.082	79.94±1.03
Cecum	0.323 ±0.041	78.79±0.81
Colon	0.536 ±0.044	79.30±0.51
Heart	0.345 ±0.013	77.09±0.50
Kidneys	0.777 ±0.052	77.41±0.21
Liver	4.281 ±0.364	71.09±0.61
Lungs	0.655 ±0.182	78.92±0.55
Muscle (abdom. wall)	1.311 ±0.214	74.56±1.09
Ovaries	0.0285±0.0046	78.04±0.77
Skin	19.99 ±1.01	64.27±2.04
Spleen	0.359 ±0.080	76.53±0.32
Submaxillary glands	0.136 ±0.013	75.10±0.76
Thymus gland	0.0914±0.0141	78.12±1.03
Residual carcass	53.34 ±1.46	66.99±1.34

[1] Measured as % of autopsy body weight.
[2] Measured as % wet weight.
[3] Mean ± SD.

Statistical analysis. Results for winter and summer groups were computer-analyzed [7] separately. Organ weight was expressed as percentage of autopsy body weight and water content as percentage of organ wet weight. These variables were correlated with percentage loss of initial body weight as linear, hyperbolic, parabolic, semi- and double-logarithmic regressions. Standard errors, correlation coefficients and the *t* values were calculated. Results in animals that died before 14 days were compared with those in a group of starved survivors that had lost an equivalent percentage of initial body weight.

RESULTS

The number of deaths and mean weight losses are summarized in table 1. Animals fed an equivalent amount of food tended to lose more weight during the winter than during the summer.

Organ weights. Data for organ weights and water content of the summer control group are summarized in table 2. Changes in the relative weights of organs in the summer-starved groups are illustrated in figures 1, 2, and 3. Organs which resisted the effect of starvation and actually lost

[7] IBM 1620 II Digit Computer.

no weight were adrenal glands, brain, and the pyloric part of the stomach. Their weight thus became a greater percentage of autopsy body weight as the degree of starvation increased as shown in figures 1, 2, and 3. Ulcers were observed in pyloric stomach when body weight loss exceeded 30%.

The thymus gland, spleen and the liver lost weight at a rate faster than loss of body weight. Loss of weight in the remaining organs was approximately the same as that in body weight with minor differences shown in figures 1, 2, and 3.

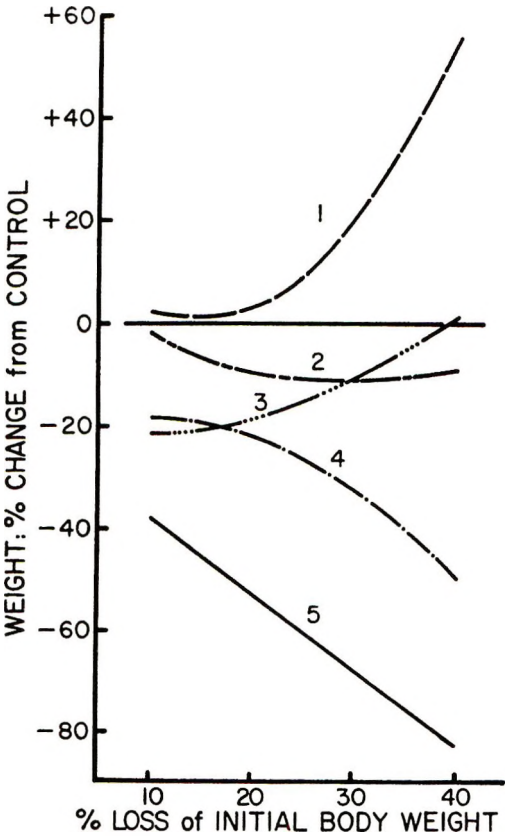

Fig. 1 Percentage change from controls (fed ad libitum) in the weight (calculated as % of autopsy body weight) of adrenal glands (1), heart (2), ovaries (3), spleen (4) and thymus (5) of rats starved during a summer season. Correlation coefficients for the original calculations of organ weight (as % of body weight, and before expressing weight as % change from controls) were, respectively, 0.80, 0.57, 0.35, 0.62 and 0.77, and were significantly different from zero correlation at $P = 0.05$ or less.

Results obtained during the winter were similar to those in the summer except in the case of heart. During the winter the relative weight of the heart increased significantly at higher degrees of starvation.

Loss of body weight of rats that died between the tenth and fourteenth days of starvation during the summer amounted to 30 to 40% of initial weight, and changes in organ weights compared with those in a group of 12 survivors starved to a similar degree are shown in table 3. The adrenal glands, cardiac stomach, heart and kidneys had lost significantly less weight and the pyloric stomach, liver, spleen and salivary glands significantly more weight than had the comparison group which survived.

Organ water content. Changes in organ water content are shown in figures 4, 5, and 6. In most organs, water levels increased at higher degrees of starvation.

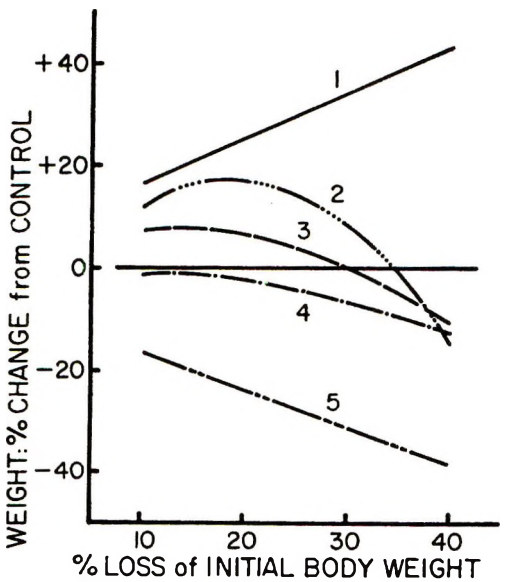

Fig. 2 Percentage change from controls (fed ad libitum) in the weight (calculated as % of autopsy body weight) of pyloric part of stomach (1), submaxillary salivary glands (2), cecum (3), colon (4) and liver (5) of rats starved during a summer season. Correlation coefficients for the original calculations of organ weight (as % of body weight, and before expressing weight as % change from controls) were, respectively, 0.59, 0.74, 0.49, 0.35 and 0.65 and were significantly different from zero correlation at $P = 0.05$ or less.

The increase sometimes followed an initial drying of the organ at lower levels of starvation, e.g., in ovaries, salivary glands, colon, heart and kidneys.

The results of the winter group were similar to those for the summer group except in the case of the adrenal glands. Water content of this organ was significantly lower in the winter controls than in the summer control animals, and in-

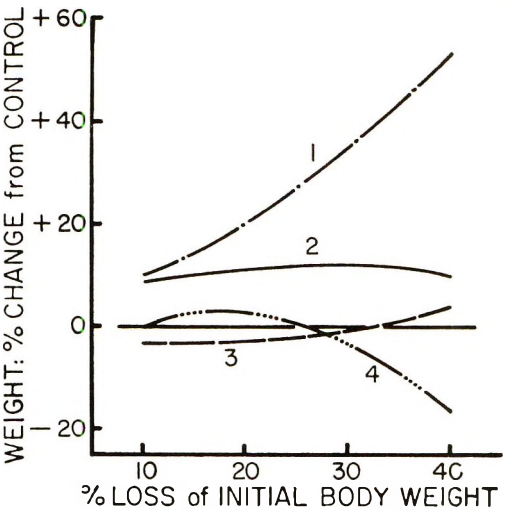

Figure 3

Fig. 3 Percentage change from controls (fed ad libitum) in the weight (calculated as % autopsy body weight) of brain (1), residual carcass (2), skin (3) and left abdominal muscle wall (4) of rats starved during a summer season. Correlation coefficients for the original calculations of organ weight (as % of body weight, and before expressing weight as % change from controls) were, respectively, 0.89, 0.43, 0.54 and 0.60 and were significantly different from zero correlation at $P = 0.05$ or less.

TABLE 3

Shifts in the weight and water level of organs and tissues of rats that died before 14 days compared with those of 12 survivors with similar loss of body weight

Organ or tissue	Weight [1,2]		Water [2,3]	
	Gain	Loss	Gain	Loss
	%	%	%	%
Adrenal glands	+42.2**		+1.54*	
Brain	+ 3.3		+0.63**	
Gastrointestinal tract				
Cardiac stomach	+ 7.3*			−0.35
Pyloric stomach		−31.7**		−2.77**
Small bowel	+ 0.1		+0.91	
Cecum	+12.1		+5.53**	
Colon	+ 3.3		+2.68**	
Heart	+12.1**		+2.75**	
Kidneys	+11.2**		+3.03**	
Liver		−24.5**	+2.23**	
Lungs	+ 0.5			−0.59
Muscle (abdom. wall)		− 9.5	+1.68**	
Ovaries		−10.6		−0.31
Skin	+ 0.4		+2.03**	
Spleen		−44.8**	+1.84**	
Submaxillary glands		−21.0**	+2.25**	
Thymus gland		−18.0	+3.77**	
Residual carcass	+ 0.7		+2.68**	

[1] Shifts are expressed as mean % change from survivors. The weight of organs is measured as % of autopsy body weight.

[2] Means for animals which died that differed at $P = 0.05$ to 0.01 from corresponding means for survivors are indicated by one asterisk, at $P \leq 0.01$ by 2 asterisks.

[3] Shifts are expressed as mean % change from survivors. The water level of organs is measured as % wet weight.

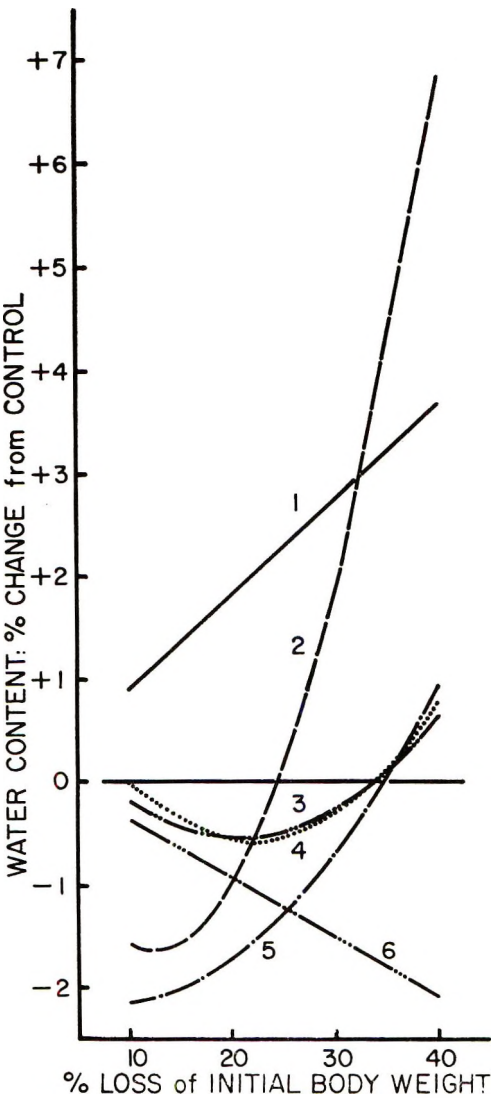

Fig. 4 Percentage change from controls (fed ad libitum) in water content (calculated as % of wet weight) of liver (1), adrenal glands (2), heart (3), kidneys (4), ovaries (5) and spleen (6) of rats starved during a summer season. Correlation coefficients for the original calculations of water content (before expressing it as % change from controls) were, respectively, 0.74, 0.81, 0.66, 0.48, 0.41 and 0.73 and were significantly different from zero correlation at $P = 0.05$ or less.

crease in the starved animals occurred at lower levels of starvation than during the summer.

Most organs of animals that died before the fourteenth day during the summer had

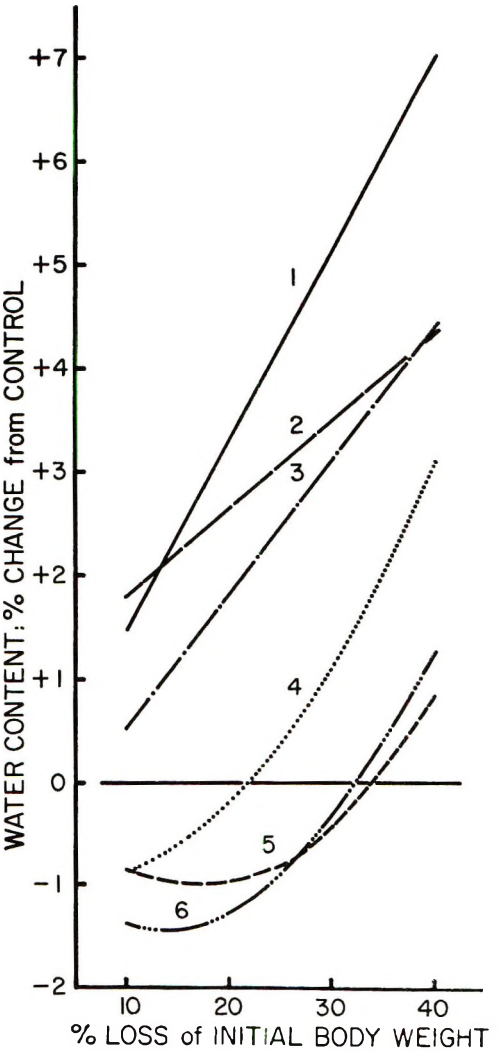

Fig. 5 Percentage change from controls (fed ad libitum) in water content (calculated as % of wet weight) of pyloric (1) and cardiac (2) parts of stomach, small bowel (3), cecum (4), colon (5) and submaxillary salivary glands (6) of rats starved during a summer season. Correlation coefficients for the original calculations of water content (before expressing it as % change from controls) were, respectively, 0.76, 0.60, 0.75, 0.72, 0.31 and 0.59 and were significantly different from zero correlation at $P = 0.05$ or less.

a marked increase in water content as shown in table 3.

DISCUSSION

It has been demonstrated in this project that as the degree of starvation and loss of body weight increases, most body organs

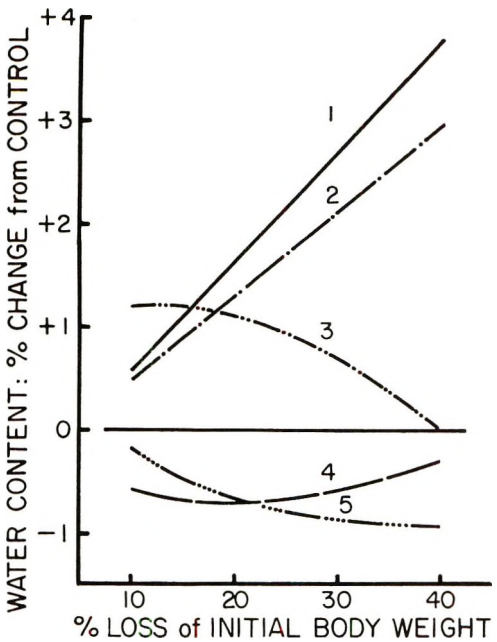

Fig. 6 Percentage change from controls (fed ad libitum) in water content (calculated as % of wet weight) of left abdominal muscle wall (1), residual carcass (2), skin (3), brain (4), and lungs (5) of rats starved during a summer season. Correlation coefficients for the original calculations of water content (before expressing it as % change from controls) were, respectively, 0.83, 0.42, 0.35, 0.53, and 0.45 and were significantly different from zero correlation at $P = 0.05$ or less.

lose fresh wet weight and gain water. A gain in water content under comparable conditions has been reported previously in the adrenal glands (5) and muscle (6) but not in liver (7). In the present work, liver water content was significantly increased in rats that survived 14 days of marked reduction in food intake (fig. 4) and the hydration was even greater in animals that died before the fourteenth day (table 3). The hydration of inanition has been found to be associated with hydropic degeneration in the adrenal glands (5) and with a relative increase in connective tissue of muscle (6).

The average water content of the organs in unstarved controls listed in table 2 is 75.5% wet weight or 308 g of water/100 g dry weight of tissue. At 40% loss of initial body weight, the average gain in water content of organs shown in figures 3 and 4 was 2.6%. This latter represents an average water content of 77.5% wet weight or 344 g of water/100 g dry weight, an increase of some 12% over the average in the controls. Thus an average loss of 40% in wet weight is equivalent to an average loss of 45% in dry weight. On the average, therefore, there was a greater percentage loss of dry weight than of wet weight.

When organ weights were calculated as dry weight, the only organ which lost no dry weight (and gained no water — see fig. 6) was brain. Brain has been reported previously to resist weight loss during starvation (4) and in the anorexia and weight loss which follow administration of lethal doses of many drugs (8). There was a slight loss of dry weight in the adrenal glands and both parts of the stomach and a considerable loss of dry weight in most other organs, especially in liver.

The appearance of ulcers in the pyloric stomach was probably associated with a stressor reaction. Loss of weight in the ovaries was probably accompanied by anestrus which has been found to occur during malnutrition (9). Beznák suggested (10) that loss of weight in the heart may be due to decreased functional demand. A similar relationship may exist for intestine, lungs, and kidneys.

Finally, it is emphasized that if administration of toxic doses of a drug produces anorexia and loss of weight and hydration of organs as reported herein, the organ changes should be related to the anorexia and not necessarily to direct effects of the drug.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. Smyth, H. F., C. S. Weil, E. M. Adams and R. L. Hollingsworth 1952 Efficiency of criteria of stress in toxicological tests. Arch. Ind. Hyg. Occupational Med., 6: 32.
2. Boyd, E. M., and L. M. Knight 1963 Postmortem shifts in the weight and water levels of body organs. Toxicol. Appl. Pharm., 5: 119.
3. Jackson, C. M. 1925 The effects of inanition and malnutrition upon growth and structure. P. Blakiston's Son and Company, Philadelphia.

4. Giese, W., and R. Hörstebrock 1962 Allgemeine Pathologie des exogenen quantitativen Nahrungsmangels. In: Handbuch der Allgemeine Pathologie XI, ed. 1. F. Roulet, Springer Verlag, Berlin, p. 446.
5. Cameron, A. T., and J. Carmichael 1946 The effect of acute starvation on the body organs of the adult white rat, with special reference to the adrenal glands. Can. J. Res. (Sec. E), *24:* 37.
6. Hines, H. M., and G. C. Knowlton 1937 Electrolyte and water changes in muscle during atrophy. Amer. J. Physiol., *120:* 719.
7. Widdowson, E. M., and R. A. McCance 1956 The effects of chronic undernutrition and of total starvation on growing and adult rats. Brit. J. Nutr., *10:* 363.
8. Boyd, E. M., K. Bereczky and I. Godi 1965 The acute toxicity of tannic acid administered intragastrically. Can. Med. Assoc. J., *92:* 1292.
9. Mulinos, M. G., and L. Pomeranz 1940 Pseudo-hypophysectomy. A condition resembling hypophysectomy produced by malnutrition. J. Nutr., *19:* 493.
10. Beznák, M. 1954 The behaviour of the weight of the heart and the blood pressure of albino rats under different conditions. J. Physiol., *124:* 44.

Functional and Allometric Descriptions of the Liver and Small Intestine in Genetically Obese Mice [1]

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ABSTRACT To assess intestinal activity in mice with the hereditary obese hyperglycemic syndrome, the small gut was studied by means of histochemistry, as well as the transport of 10^{-3} M glucose and 5×10^{-6} M L-methionine against a concentration gradient in vitro. In addition, the weight of the intestine was determined at nine and eleven weeks of age. There was no difference in the histochemical reactions of the gut between affected animals and their normal littermates. There was also no impairment of in vitro transport of the 2 nutrients. While the small gut of the obese animals weighed more than that of controls, the weight was correctly predicted by the allometric relationship on the basis of their increased body weight. The weight of the liver in the hereditary obese hyperglycemic mice was considerably greater than predicted by the allometric relationship.

Mice with the hereditary obese hyperglycemic syndrome have a number of metabolic errors (1). In view of the marked obesity of these animals, as compared with normal littermates, we sought to determine whether intestinal function was within normal limits. Three approaches were used in an effort to answer this question: histochemical observations of the intestinal mucosa, in vitro studies on transport against a concentration gradient, and comparison of the observed intestinal weights with those predicted on the basis of the allometric relationship. Since the livers of the obese mice were observed to be quite large, the allometric equation was also applied to the liver weight as a function of body weight.

METHODS

The hereditary obese hyperglycemic mice and their normal littermates (males) were obtained from the Jackson Laboratories.[3] Mice were allowed food and water ad libitum and groups of four were killed by a blow on the head at weeks 9 and 11. The animals were weighed, the livers removed and weighed after blotting, and the small intestine was weighed after washing thoroughly with pH 7.4 Krebs-bicarbonate buffer, and blotting.

Everted intestinal sacs were prepared (2) (3/mouse). The sacs were filled with 1 ml of buffer containing the compound under investigation, and incubated in 5 ml of solution of identical composition. After gassing with 95% O_2 + 5% CO_2, flasks containing the sacs and buffer were

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[3] Bar Harbor, Maine.

TABLE 1

Comparison of transport of glucose and L-methionine in 9-week-old congenital hyperglycemic obese mice and their normal littermates; transport vs. concentration gradient

10^{-3} M glucose		5×10^{-6} M L-methionine	
Control (12) [1]	Obese (12)	Control (11)	Obese (11)
serosal conc/mucosal conc		*serosal conc/mucosal conc*	
3.25 ± 0.96 [2]	3.03 ± 0.92	3.93 ± 1.55	3.24 ± 1.18
$P = 0.5$		$P > 0.2$	

[1] Number of sacs is shown in parentheses.
[2] Values shown are the final concentration ratios ± SD.

stoppered and incubated at 37° for one hour. At the end of this period, sacs were drained and weighed. Mucosal and serosal fluids were centrifuged and aliquots removed for liquid scintillation counting. Materials used were 10^{-3} M D-glucose-^{14}C and 5×10^{-6} M L-methionine-^{14}C. Since the initial condition was the same concentration on both sides of the sacs, increased radioactivity within the sacs represented movement against a concentration gradient. Results were expressed in terms of the final serosal concentration divided by the final mucosal concentration (the concentration ratio).

Segments of proximal, mid and distal small intestine were removed immediately after killing the animals, and immersed in liquid nitrogen; storage was at −70°. Histochemical reactions were carried out on cryostat sections cut 4 μ thick. Assays for acid phosphatase, alkaline phosphatase, nonspecific esterase, NAD-diaphorase and NADP-diaphorase were performed as previously described (3). The localized intensities of the stains were evaluated without knowledge of whether the section was from a normal or obese mouse.

To compare organ weights in normal and hyperglycemic mice with their "predicted weights," the allometric relationship was used. The equation is of the form:

$$W = AB^X \quad (1)$$

or

$$\log W = X \log B + \log A \quad (2)$$

where W is the weight of the organ, B is the body weight, and both A and X are constants descriptive of the organ's growth. For the intestine (4), we have noted that the exponent X is about 0.71; for the liver (5), it is approximately 0.78 to 0.87. Using these values, the weights of the intestine and liver of normal littermates of the obese mice were determined and used, in conjunction with their body weight, to calculate the parameter A. The equations were:

$$\log L = 0.87 \log B - 1.04 \quad (3)$$

$$\log I = 0.71 \log B - 0.70 \quad (4)$$

where L represents liver weight and I the intestinal weight. These equations were then applied to the obese mice.

TABLE 2

Comparison of the predicted weights (by equations 2 and 3) and experimentally determined weights of the liver and small intestine [1]

Group	Age	Body wt	Intestine			Liver		
			Found	Predicted	Error [2]	Found	Predicted	Error [2]
	weeks	*g*	*g*	*g*	*%*	*g*	*g*	*%*
Normal mice	9	21.5 ± 1.9 [3]	1.45 ± 0.10	1.43	− 1	1.32 ± 0.11	1.32	0
	11	24.7 ± 1.6	1.38 ± 0.24	1.59	+16	1.50 ± 0.14	1.48	− 1
Obese mice	9	34.5 ± 1.7	2.22 ± 0.35	2.00	−10	2.56 ± 0.24	1.99	−22
	11	46.0 ± 2.0	2.47 ± 0.44	2.46	0	4.33 ± 0.48	2.57	−41

[1] Four mice/group.

[2] Error $= \left(\frac{\text{Predicted} - \text{found}}{\text{Found}}\right) \times 100$.

[3] Weights (mean of the group) ± SD.

RESULTS

Table 1 compares the transport of glucose and L-methionine against a concentration gradient by the in vitro small intestine of obese and normal mice. There was no statistical difference.

Table 2 summarizes information on the predicted and experimentally determined weights of the liver and small intestine in obese mice and their normal littermates. The weight of the liver in the obese mice was underestimated by 22% at 9 weeks and 41% at 11 weeks, whereas in the normal animals there was correct prediction of the liver weight.

The histochemical studies revealed no difference in either distribution or intensity of reaction, between the intestinal segments from obese and normal mice.

DISCUSSION

When expressed in terms of transport against a concentration gradient (using comparable intestinal weights), there was no difference between the normal and obese mice. The obese mice, however, have a larger intestine and hence might tend to absorb food materials more rapidly in vivo. The lack of specific difference between the intestine of normal mice and that of their obese counterparts was reinforced by the observation of identical histochemical reactions.

Application of the allometric equations to the liver and small intestine revealed a marked difference between the normal and obese mice. The allometric equation that described the weight of the small intestine in normal mice also accuratley predicted the weight of the gut in obese mice. That is, although the weight of the small intestine in obese mice was greater than that of controls, it was no larger than predicted on the basis of the increased body weight of the obese mice. With the liver, however, this was not the case. The allometric equation, which was descriptive of the weight of the liver in normal mice, grossly underestimated the hepatic weight in the obese animals. Hence, the obese mice have a liver that is disproportionately large, even for their increased body weight. As either a causative factor or result of the disease, the enlarged liver of the hyperglycemic obese mice will likely serve as a useful starting point in obtaining data as to the nature of the disease. As data are accumulated on the extent and composition of the deposited lipids, it might be possible to calculate a lean body (or lean organ) weight, and then apply the allometric equation. In such a manner, growth of the lipid-free portion of the mice might be compared with that of the lipid components.

LITERATURE CITED

1. Mayer, J. 1966 Antagonism between alloxan and caffeine. Nature, *210:* 630.
2. Wilson, T. H., and G. Wiseman 1954 Use of sacs of everted small intestine for study of transferance of substances from mucosal to serosal surface. J. Physiol., *123:* 116.
3. Spiro, H. M., M. I. Filipe, J. S. Steward, C. C. Booth and A. G. E. Pearse 1964 Functional histochemistry of the small bowel mucosa in malabsorptive syndromes. Gut, *5:* 145.
4. Spencer, R. P., and M. J. Coulombe 1965 Quantitative approaches to intestinal growth. Growth, *29:* 323.
5. Spencer, R. P., and M. J. Coulombe 1966 Quantitation of hepatic growth and regeneration. Growth, *30:* 277.

Arginine-Lysine Antagonism in the Chick and Its Relationship to Dietary Cations [1,2]

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ABSTRACT Broiler-strain chicks were reared to four weeks of age with semi-purified diets based on protein from casein (30%), soybean (26%) or sesame meal (30%). A total of at least 2.4% arginine was required for maximal growth with the casein diet. When arginine was suboptimal, growth was stimulated by extra glycine, glutamic acid, leucine and creatine but not by ammonium acetate or citrate. Lysine depressed growth severely and this effect was largely counteracted in a competitive manner by arginine. In the presence of excess lysine, extra glycine and glutamic acid had no stimulating effect. Soybean protein supplemented with methionine supported a maximal growth rate, but addition of lysine depressed growth and produced gross symptoms of arginine deficiency. This effect was counteracted by a combination of arginine, glycine and potassium acetate. Potassium acetate supplementation of the casein diet produced a marked stimulation of growth rate with or without added lysine, but had no effect when added to the basal soybean protein diet. When added to a sesame meal diet deficient in lysine, potassium acetate appeared to depress growth. Excess arginine also depressed growth when lysine was limiting and potassium acetate was included, but not when lysine was adequate. The results suggest a metabolic antagonism between arginine and lysine.

Various explanations have been offered for the exceptionally high arginine requirement of the growing chicken fed casein as the chief source of amino acids and it appears likely that more than one mechanism is involved. Klose et al. (1) were the first to suggest that the arginine of casein is unavailable and more recent work tends to confirm this explanation if the term availability includes the concept of utilization of absorbed nutrients. By use of balance studies, O'Dell et al. (2) showed that casein nitrogen is readily digested and absorbed by the chick. The arginine requirement of chicks fed 20% protein diets is about 1.7% when the casein is the source of amino acids and about 1.1% when the protein is supplied by corn and soybean meal (3, 4). This difference has led to the suggestion that natural products contain unrecognized factors that enhance arginine utilization (4). Chicks fed diets containing 35% of casein required a total of 2.2% arginine to attain a maximal rate of gain (5). The requirement for arginine can be spared by glycine, creatine, creatinine and guanidoacetic acid (5, 6), but the use of the term "spared" has been questioned by Waterhouse and Scott (7) who reported that the favorable effect of glycine is independent of the arginine concentration in the diet. There is a wide variation in the growth rates of chicks fed a suboptimal level of arginine, and recent work (8) has shown an inverse relationship between kidney arginase and the growth rate of chicks fed a suboptimal level of arginine.

Anderson and Dobson (9) have suggested that the amino acid composition of the diet is responsible for the higher arginine requirement when casein is the source of protein. They proposed that the excess of total essential amino acids (less arginine and glycine) found in casein is responsible. The addition of nonessential amino acids such as glutamic acid, cystine, aspartic acid and alanine or nonprotein nitrogen such as diammonium citrate increased the growth rate. Lysine depressed the rate of gain, but this was largely overcome by arginine supplementation. Fisher et al. (10) have also sug-

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[2] A preliminary report has been presented: Federation Proc., 21: 8, 1962.

gested that the amino acid composition of casein accounts for the high arginine requirement. Maddy et al.[3] observed that various amino acids, including glycine, serine, glutamic acid and aspartic acid, improved the growth rate of chicks fed a casein basal diet. Leucine, an essential amino acid, stimulated growth, whereas lysine and tyrosine depressed it. Snetsinger and Scott (11) reported a growth depression when excess lysine was added to soybean and sesame meal diets. The deleterious effect was largely overcome by a combination of arginine and glycine. Jones (12) observed that the addition of lysine·HCl to a casein-gelatin diet caused growth depression which was counteracted by arginine supplementation and suggested that excess lysine induces an arginine deficiency. If arginine and lysine are metabolically competitive, it should also be possible to induce a lysine deficiency by feeding an excess of arginine.

The results of Nesheim et al. (13) indicate the importance of the proper cation-anion balance for support of maximal growth rate in chicks. Unpublished data from this laboratory show that an excess of cations is beneficial when a high level of casein is fed. Potassium and sodium supplements in the form of acetates, citrates and carbonates stimulated growth, whereas the chlorides were ineffective.

The effects of supplementing diets based on casein, soybean protein and sesame meal with an excess of various amino acids are reported here. The results suggest a competitive lysine-arginine antagonism and a physiological interaction between amino acids and the cation level of the diet.

EXPERIMENTAL

Groups of ten male Vantress × White Rock chicks were maintained from hatching to four weeks of age in electrically heated battery brooders. The dietary treatments were repeated at different times to give the numbers shown in the tables. Feed and water were supplied ad libitum and weights were recorded weekly.

The composition of the basal diets is shown in table 1.[4] Proteins of widely different amino acid compositions were used. Soybean protein, when supplemented with methionine and glycine, is an excellent source of amino acids for the chick, whereas casein is deficient in arginine, and sesame meal protein is grossly deficient in lysine. These 3 proteins were compared with respect to their adequacy for the chick when supplemented with excesses of lysine, arginine and other selected nitrogen compounds. Lysine and arginine were added as the hydrochlorides and potassium as the acetate or chloride. All supplements were added in lieu of glucose.

TABLE 1

Composition of basal diets

	Casein (A)	Soybean protein (B)	Sesame meal (C)
	%	%	%
Casein (water-washed)	35.0	—	—
Soybean protein [1]	—	30.0	—
Sesame meal [2]	—	—	58.0
DL-Methionine	0.5	0.8	—
Glycine	1.5	1.0	—
Glucose hydrate	46.8	52.0	28.8
Salts N [3]	6.0	6.0	6.0
Soybean oil	10.0	10.0	7.0
Choline·Cl	0.2	0.2	0.2
Vitamin supplement [4]	+	+	+
Calculated composition			
Crude protein	30	26	30
Arginine	1.2	1.8	2.4
Lysine	2.5	1.7	0.8

[1] The soybean protein was Promine R supplied by Central Soya Inc., Chicago.

[2] The sesame meal, donated by John Kraft Sesame Corporation, Paris, Texas, contained about 52% crude protein, 6% fat, and 9% ash.

[3] Salts N (14) supplied as percentage of the diet: $CaHPO_4$, 2.84; $CaCO_3$, 1.00; Na_2HPO_4, 0.7; NaCl, 0.4; KCl, 0.7; $MgSO_4$, 0.3; Fe citrate, 0.02; $MnSO_4 \cdot H_2O$, 0.025; KIO_3, 0.001; $ZnCO_3$, 0.013; and $CuSO_4$, 0.001.

[4] The vitamin supplement supplied per 100 g of diet: (in mg) thiamine·HCl, 1; riboflavin, 1; pyridoxine·HCl, 1; Ca pantothenate, 3; niacin, 5; inositol, 50; folacin, 0.2; biotin, 0.04; cyanocobalamin, 0.003; α-tocopheryl acetate, 2.5; menadione, 2.5; and ethoxyquin, 12.5; vitamin A, 2,000 IU and vitamin D, 430 ICU.

RESULTS

Casein based diets. In agreement with earlier studies with casein diets (5), 2.4%

[3] Maddy, K. H., L. J. Machlin and R. S. Gordon 1960 The effect of excess amino acids on the requirement for glycine. Poultry Sci., *39*: 1271 (abstract).

[4] The authors gratefully acknowledge gifts of folacin from Lederle Laboratories, Pearl River, New York; biotin from Hoffmann-LaRoche, Nutley, New Jersey; vitamin A from Distillation Products, Inc., Rochester, New York; vitamin D from Dawe's Laboratories, Chicago; and other vitamins from Merck and Company, Rahway, New Jersey. Ethoxyquin (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline) was donated by the Monsanto Company. Lysine·HCl was generously donated by Merck and Company, and arginine·HCl by General Mills, Inc., Minneapolis.

TABLE 2

Value of various nitrogen compounds as supplements to a casein diet

Diet no.	Supplements to basal diet A		No. chicks	Avg wt,[1] 4 weeks
	Arginine (as HCl)	Other compounds		
	%			g
A1	0.6	None	44	359 ± 17 [2]
A2	1.2	None	38	462 ± 11
A3	2.0	None	36	494 ± 12
A4	0.6	Glycine, 2.0 (3.5) [3]	38	452 ± 20
A5	0.6	Glycine, 3.5 (5.0)	29	412 ± 20
A6	0.6	Glutamic acid, 4.0	39	426 ± 18
A7	0.6	Leucine, 3.6	39	412 ± 21
A8	0.6	Creatine·H_2O, 1.0	25	448 ± 20
A9	0.6	NH_4 acetate, 2.1	33	368 ± 19
A10	0.6	NH_4 citrate, 3.0	35	301 ± 18
A11	0.6	Lysine·HCl, 2.0	35	228 ± 11

[1] The following diets were statistically greater than diet A1 as determined by the *t* test: A2, $P < 0.001$; A4, $P < 0.001$; A5; $P < 0.05$; A6, $P < 0.01$; A7, $P < 0.05$; A8, $P < 0.005$. A3 > A2, $P < 0.1$ but > 0.05. A11 < A1, $P < 0.001$.
[2] SE of mean.
[3] The total glycine supplement is shown in parentheses.

TABLE 3

Counteraction of lysine toxicity by supplementation of a casein diet

Diet no.	Supplements to diet A + 2% lysine·HCl		No. chicks	Avg wt,[1] 4 weeks
	Arginine (as HCl)	Other compounds		
	%	%		g
A12	0.6	None	30	217 ± 11 [2]
A13	0.6	Glycine, 2.0 (3.5)	26	211 ± 16
A14	0.6	Glycine, 3.5 (5.0)	29	213 ± 16
A15	0.6	Glutamic acid, 4.0	30	192 ± 10
A16	0.6	Leucine, 3.6	19	262 ± 17
A17	0.6	Creatine·H_2O, 1.0	25	286 ± 19
A18	1.2	None	28	391 ± 19
A19	2.0	None	28	464 ± 12

[1] The following diets were statistically greater than diet A12 as determined by the *t* test: A17, $P < 0.005$; A18, $P < 0.001$; A19 > A18, $P < 0.005$. A16 > A12, $P < 0.1$ but > 0.05.
[2] SE of mean.

TABLE 4

Counteraction of lysine toxicity by supplementation of a soybean protein diet

Diet no.	Supplements to diet B				No. chicks	Avg wt,[1] 4 weeks
	Lysine·HCl	Arginine (as HCl)	Glycine	K acetate		
	%	%	%	%		g
B	—	—	—	—	27	568 ± 11 [2]
B1	—	—	—	2.7	29	576 ± 12
B2	—	1.2	2.0(3.0)	2.7	29	579 ± 9
B3	4.0	—	—	—	29	424 ± 16
B4	4.0	—	—	2.7	30	466 ± 15
B5	4.0	1.2	—	2.7	25	500 ± 17
B6	4.0	2.4	—	2.7	27	528 ± 11
B7	4.0	—	2.0(3.0)	2.7	26	459 ± 20
B8	4.0	1.2	2.0(3.0)	2.7	30	546 ± 13
B9	4.0	1.2	2.0(3.0)	—	29	474 ± 15

[1] Statistical treatment by the *t* test showed the following significance: diet B > diet B3, $P < 0.001$; B4 > B3, $P < 0.1$ but > 0.05; B5 > B3, $P < 0.005$; B6 > B4, $P < 0.005$; B8 > B4, $P < 0.001$; B8 > B5, $P < 0.025$; B8 > B9, $P < 0.001$; B > B8, $P > 0.1$.
[2] SE of mean.

of arginine supported near the maximal growth rate (table 2). A diet that contained 0.6% of supplementary arginine, (A1, 1.8% total arginine) was improved by extra glycine and also by supplementary glutamic acid, leucine or creatine. Ammonia nitrogen was not beneficial and lysine was highly detrimental. In fact lysine accentuated the gross symptoms of arginine deficiency such as stilted gait and poor feathering.

In an attempt to counteract the toxicity of added lysine, various amino acid supplements were tested (table 3). In contrast with their beneficial effect in the absence of added lysine, glycine and glutamic acid were of no value and leucine was of questionable benefit. Creatine and arginine produced a marked growth response, but a total of more than 2.4% of arginine was required when lysine was added.

Since both lysine and arginine were added as the hydrochloride, it seemed possible that a negative cation balance may have been created. Consequently 2.7% potassium acetate was added to all diets and graded levels of arginine were supplied with and without added lysine. The results of these trials as well as those shown in table 3 are presented graphically in figure 1. The addition of potassium acetate improved the growth rate at all levels of arginine both with and without added lysine. That potassium acetate stimulation was as great in the absence of lysine·HCl as in its presence suggests that the effect was mediated by a mechanism not directly related to the extra chloride. The depressing effect of lysine·HCl was not overcome by potassium acetate, but it was partially counteracted by arginine in a step-by-step manner suggesting competitive antagonism.

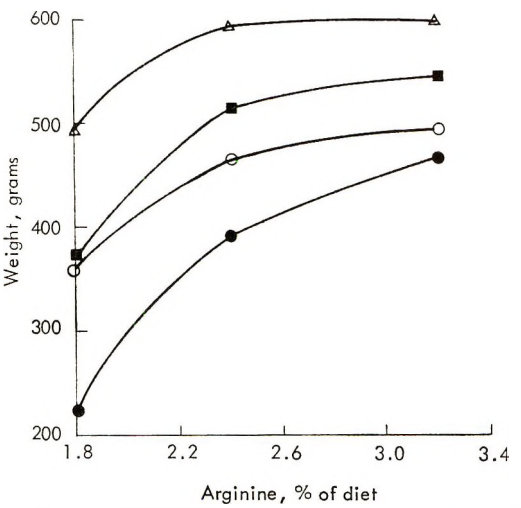

Fig. 1 Arginine-lysine antagonism as affected by potassium acetate supplementation of the casein basal diet. There were approximately 40 chicks/group among those fed potassium acetate and data for those not receiving potassium acetate are the same as in tables 2 and 3. Supplements were: ○——○, none; ●——●, 2% lysine·HCl; △——△, 2.7% K acetate; ■——■, 2.7 K acetate + 2% lysine·HCl. The arginine level shown includes the 1.2% present in the basal diet.

Soybean protein-based diets. Since lysine appeared to be antagonistic to arginine when added to a casein diet, it seemed worthwhile to test this effect in a diet with a better amino acid balance such as one based on soybean protein. The results of trials dealing with this protein are shown in table 4. In contrast with the results with casein diets, the addition of potassium acetate to the basal soybean protein diet (diet B) did not improve the growth rate nor did the further addition of arginine and glycine. The addition of 4% lysine·HCl (diet B3) significantly inhibited growth and produced symptoms

TABLE 5

Lysine requirement of chicks fed a sesame meal diet supplemented with potassium acetate or potassium chloride

Diet no.	Lysine [1]	No. chicks	Avg wt,[2] 4 weeks
	%		g
	Potassium acetate (2.7%)		
C1	0.2	29	297 ± 12 [3]
C2	0.4	38	432 ± 18
C3	0.6	29	579 ± 26
C4	0.9	28	605 ± 15
C5	1.4	40	615 ± 7
	Potassium chloride (2.0%)		
C6	0.2	27	369 ± 10
C7	0.4	37	504 ± 13
C8	0.6	30	592 ± 15
C9	0.9	30	557 ± 16
C10	1.4	40	583 ± 11

[1] Fed as the hydrochloride and expressed as the free base.

[2] Statistically diet C6 > diet C1, $P < 0.001$; C7 > C2, $P < 0.005$; C4 > C9, $P < 0.05$; C5 > C10, $P < 0.025$.

[3] SE of mean.

TABLE 6

Counteraction of arginine excess by lysine supplementation of a sesame meal diet

Diet no.	Lysine [1]	Arginine [1]	No. chicks	Avg wt,[2] 4 weeks
	%	%		g
		Potassium acetate (2.7%)		
C2	0.4	—	38	432 ± 18 [3]
C12	0.4	1.2	39	380 ± 15
C5	1.4	—	40	615 ± 7
C13	1.4	1.2	38	603 ± 11
		Potassium chloride (2.0%)		
C7	0.4	—	37	504 ± 13
C14	0.4	1.2	39	507 ± 12
C10	1.4	—	40	583 ± 11
C15	1.4	1.2	39	560 ± 17

[1] Fed as the hydrochloride and expressed as the free base.
[2] Statistically diet C2 > diet C12, $P < 0.05$.
[3] SE of mean.

typical of arginine deficiency. The further addition of potassium acetate alone (diet B4) did not improve growth significantly but both potassium acetate and arginine (diets B5 and B6) produced a highly significant growth response. However, the weights were less than that of the basal group. Glycine and potassium acetate (diet B7) were no better than potassium acetate alone, but a combination of arginine, glycine and potassium acetate (diet B8) supported a growth rate that was not significantly different from that of chicks fed the basal diet. Omission of potassium acetate (diet B9) from the combination resulted in a lower growth rate.

Sesame meal diets. An attempt was made to induce lysine deficiency by adding an excess of arginine to diets based on sesame meal. It was first necessary to determine the approximate lysine requirement under the experimental conditions. To one series of diets 2.7% potassium acetate was added and an equivalent amount of potassium chloride was added to the other series. The results obtained with graded levels of lysine are shown in table 5. Particularly striking was the difference in growth rate between those fed potassium acetate and those fed potassium chloride when lysine was limiting.

At the lower levels of lysine, growth rate was more rapid in the presence of potassium chloride than in the presence of potassium acetate. However, with the higher levels of added lysine (0.9 and 1.4%) potassium acetate supported superior gains. Near maximal growth rate was achieved with 0.6% of supplemental lysine (calculated total 1.4%).

For the purpose of studying the effect of excess arginine a suboptimal level of lysine (1.2%) was chosen. The results of adding 1.2% of arginine in the presence of potassium acetate and potassium chloride are shown in table 6. This level of arginine depressed the growth rate of chicks fed potassium acetate but had no effect on those that consumed potassium chloride. When adequate lysine was fed, excess arginine had no effect.

DISCUSSION

The results presented here are consonant with the concept presented by others (9, 10) that the amino acid composition of casein is largely responsible for the high arginine requirement of chicks fed this protein. However, it is not justifiable to attribute the effect to "essential amino acid" composition because certain essential amino acids, as for example leucine, improved growth whereas lysine had a depressing effect. The concept that glycine spares the requirement for arginine has been questioned (7) and clearly the relationship between glycine and arginine is complex. Nevertheless the addition of excess glycine to a casein diet, suboptimal in arginine, supported a rate of growth almost equal to that promoted by adequate arginine. Whether glycine spares

arginine or vice versa is a moot question, but their growth-stimulating effects are not additive (5). It seems probable that glycine and arginine serve the same or a similar function in nitrogen metabolism which is not related to protein synthesis. For example, arginine as well as glycine may be involved in uric acid biosynthesis (15). However, in this respect, it is significant that in the presence of excess lysine, extra glycine was not beneficial, whereas arginine and creatine stimulated growth. Glutamic acid and leucine also stimulated growth in the absence of added lysine but not in its presence. The mechanism by which the above amino acids stimulate growth in chicks fed casein diets is not clear. The lack of response to ammonia nitrogen suggests that they may have a function other than simply serving as a source of nitrogen.

Supplementation of both the casein and soybean protein diets with excess lysine so as to provide lysine-to-arginine ratios of 2.2:1 and 2.6:1, respectively, caused severe growth depression and produced gross symptoms of arginine deficiency. The ratio of lysine to arginine in casein protein is about 2.0, whereas in soybean protein the ratio is about 1.0. The high lysine-to-arginine ratio in casein no doubt contributes to the increased arginine requirement of the chick fed this protein as the source of amino acids. Supplementation of the casein basal diet with arginine to provide a ratio of 1.0 gave maximal growth but, when excess lysine was added, supplementation with arginine to provide a lysine-to-arginine ratio of 1.2 did not quite support the maximal rate.

The effect of excess arginine in a low lysine diet (lysine/arginine = 0.5) was not as dramatic as in the case of excess lysine, but in the presence of excess cations provided by potassium, growth was depressed by arginine (table 6). When the diet was adequate with respect to lysine, excess arginine was not detrimental. That excess arginine produces a growth depression strengthens the evidence for a true metabolic antagonism between arginine and lysine.

The growth depression caused by adding excess lysine·HCl to soybean protein was not entirely counteracted by arginine alone. Both potassium acetate and glycine were required in addition to arginine. Snetsinger and Scott (11) observed that a combination of glycine and arginine overcame lysine depression in a soybean protein diet. Part of the function of potassium acetate may be to balance the chloride ions added with the supplementary amino acids, but it appears to play another role which is not entirely clear. For example, it improved the casein diet regardless of whether lysine·HCl was added and the stimulation was not directly related to the amount of chloride ion present. However, potassium acetate was of no benefit when added to the basal soybean protein diet and tended to depress growth when added to the sesame meal diet.

LITERATURE CITED

1. Klose, A. A., E. L. R. Stokstad and H. J. Almquist 1938 The essential nature of arginine in the diet of the chick. J. Biol. Chem., *123:* 691.
2. O'Dell, B. L., O. A. Laerdal, A. M. Jeffay and J. E. Savage 1958 Arginine metabolism in the growing chick. Poultry Sci., *37:* 817.
3. Snyder, J. M., W. D. Morrison and H. M. Scott 1956 The arginine requirement of chicks fed purified and corn-soya diets. Poultry Sci., *35:* 852.
4. Krautmann, B. A., S. M. Hauge, E. T. Mertz and C. W. Carrick 1957 The arginine level for chicks as influenced by ingredients. Poultry Sci., *36:* 935.
5. Savage, J. E., and B. L. O'Dell 1960 Arginine requirement of the chick and the arginine-sparing value of related compounds. J. Nutr., *70:* 129.
6. Edwards, H. M., R. J. Young and M. B. Gillis 1958 Studies on arginine deficiency in chicks. J. Nutr., *64:* 271.
7. Waterhouse, H. N., and H. M. Scott 1961 Glycine need of the chick fed casein diets and the glycine, arginine, methionine and creatine interrelationships. J. Nutr., *73:* 266.
8. O'Dell, B. L., W. H. Amos and J. E. Savage 1965 Relation of chick kidney arginase to growth rate and dietary arginine. Proc. Soc. Exp. Biol. Med., *118:* 102.
9. Anderson, J. O., and D. C. Dobson 1959 Amino acid requirements of the chick. 2. Effect of total essential amino acid level in the diet on the arginine and lysine requirements. Poultry Sci., *38:* 1140.
10. Fisher, H., R. Shapiro and P. Griminger 1960 Further aspects of amino acid imbalance, with special reference to the high arginine requirement of chicks fed casein diets. J. Nutr., *72:* 16.
11. Snetsinger, D. C., and H. M. Scott 1961 Efficacy of glycine and arginine in allevi-

ating the stress induced by dietary excesses of single amino acids. Poultry Sci., *40:* 1675.
12. Jones, J. D. 1964 Lysine-arginine antagonism in the chick. J. Nutr., *84:* 313.
13. Nesheim, M. C., R. M. Leach, Jr., T. R. Zeigler and J. A. Serafin 1964 Interrelationships between dietary levels of sodium, chlorine and potassium. J. Nutr., *84:* 361.
14. Fox, M. R., and G. M. Briggs 1960 Salt mixtures for purified-type diets. III. An improved salt mixture for chicks. J. Nutr., 72: 243.
15. Klain, G., and B. C. Johnson 1962 Metabolism of labeled aminoethanol, glycine and arginine in the chick. J, Biol. Chem., 237: 123.

Effect of Various Mushroom Preparations on Cholesterol Levels in Rats [1]

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ABSTRACT To evaluate the effect of mushrooms on cholesterol metabolism, rats were fed, ad libitum, for 10 weeks a diet containing 5% of ground dried mushroom (*Lentinus edodes*, Kohshin) with or without exogenous cholesterol. The results indicate that the dried mushroom preparation markedly reduced plasma cholesterol levels. In further experiments, some other species of mushrooms were screened. All the caps and the stem of mushrooms used for feeding experiments were effective to various degrees in lowering the plasma cholesterol levels: *Lentinus edodes*, Donko was more effective, while *Auricularia polytricha* (Jews-ear) and *Flammulia velutipes* less effective, than *L. edodes*, Kohshin or *Agaricus bisporus* (champignon). To determine the nature of the substances responsible for the effect, mushrooms of the species *L. edodes*, Kohshin were extracted with ether, water or ethanol. The results showed that the ether-soluble fraction was ineffective, but both the water-soluble fraction and the 30% ethanol-soluble fraction were effective in lowering the plasma cholesterol.

For many years it has been generally believed in Japan that a species of mushroom, *Lentinus edodes*, is an elixir of life. Although no definite reason has been established for this legendary belief, the dried mushroom is available at the Japanese market as an important item of Japanese food. In an attempt to document the physiological value of the mushroom, the authors decided to investigate the effect of this fungus on several biochemical criteria associated with atherosclerosis and coronary heart disease. This possibility has been investigated by studying the effect of the Japanese mushroom on some aspects of cholesterol metabolism in rats.

EXPERIMENTAL

Experiment 1. Weanling albino male rats of the former University of Southern California strain were divided into groups

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TABLE 1
Composition of diets

	1	2	3	4	5
	%	%	%	%	%
Sucrose	63.26	62.01	62.01	63.26	61.51
Casein	22	22	22	22	22
Non-nutritive cellulose [1]	5	5			5
McCollum salt mix no. 185 [2]	4	4	4	4	4
Choline chloride	0.24	0.24	0.24	0.24	0.24
Vitamin mixture [3]	0.5	0.5	0.5	0.5	0.5
Cholesterol		1	1		1
Bile salts		0.25	0.25		0.25
Cottonseed oil	5	5	5	5	5
Mushroom [4]			5	5	
Ergosterol					0.5

[1] Cellu Flour, Chicago Dietetic Supply House, Chicago.
[2] McCollum, E. V., and N. Simmonds. J. Biol. Chem., 33: 55, 1918.
[3] The vitamin mixture had the following composition: (in grams) casein, 44; *p*-aminobenzoic acid, 1; inositol, 1; α-tocopheryl acetate, 1; ascorbic acid, 1; (in milligrams) Ca pantothenate, 400; niacin, 400; vitamin B_{12}, 0.3; riboflavin, 200; pyridoxine, 100; folic acid, 20; menadione, 50; biotin, 10; and vitamin A, 20,000 IU and vitamin D, 2000 IU.
[4] The cap of ground *L. edodes*, Kohshin.

TABLE 2

Plasma, liver and adrenal cholesterol levels and liver total lipids

Group	Plasma cholesterol			Liver cholesterol			Adrenal cholesterol	Total liver lipids
	Total	Free	Free	Total	Free	Free	Total	
	mg/100 ml	*mg/100 ml*	*%*	*mg/g*	*mg/g*	*%*	*mg/g*	*mg/g*
1 Cholesterol-free (CF)	58.7 ± 1.1 [1]	19.4 ± 0.9	33	1.29 ± 0.19	1.21 ± 0.19	94	14.4	26.2 ± 0.8
2 1% Cholesterol (C)	124 ± 1	27.7 ± 0.9	22	48.4 ± 0.9	3.63 ± 0.31	8	23.3	153 ± 1
3 Cholesterol + 5% ground, dried mushrooms (CM)	44.5 ± 1.0	19.7 ± 0.7	44	53.2 ± 1.0	4.54 ± 0.32	8	16.1	200 ± 1
4 Cholesterol-free + 5% ground, dried mushrooms (CFM)	45.7 ± 0.7	16.6 ± 0.4	36	2.59 ± 0.27	1.77 ± 0.13	68	9.1	56.7 ± 1.0
5 Cholesterol + 0.5% ergosterol (CE)	127 ± 2	31.7 ± 0.9	25	24.6 ± 0.9	2.16 ± 0.14	7	19.5	119 ± 1

[1] SE of mean.

of 8 each and were fed the following diets: group 1, cholesterol-free diet (CF); group 2, diet containing 1% cholesterol (C); group 3, cholesterol diet plus 5% ground dried mushroom [2] (CM); group 4, cholesterol-free diet plus 5% ground dried mushroom [3] (CFM); group 5, cholesterol diet plus 0.5% ergosterol (CE).

The complete diets are listed in table 1. The rats were fed the diets ad libitum for 10 weeks. In this experiment a group of rats was fed ergosterol because the mushroom under study is rich in ergosterol (approximately 0.3% in dry matter) and because Peterson and co-workers (1–3) reported the effect of phytosterols against plasma cholesterol in rats.

At the end of the experimental period, the animals were killed by intraperitoneal injection of pentobarbital sodium,[4] after which the blood, livers and adrenals were removed; the tissues were weighed, and frozen for later analyses.

Cholesterol analyses were performed on extracts of plasma and tissues by a modified Sperry-Schoenheimer method as described by Nieft and Deuel (4). Total liver lipids were determined gravimetrically.

Plasma, liver and adrenal cholesterol levels and liver total lipids are shown in table 2. The plasma cholesterol values of the animals given the mushroom preparation and cholesterol (group 3) were lower than those fed cholesterol without the mushroom preparation (group 2). The results indicate a markedly positive effect of the mushroom in reducing plasma cholesterol level. However, the cholesterol content in the livers and liver total lipids of the mushroom-fed animals (group 3) was higher than that of the controls (group 2). Plasma cholesterol values of rats fed the diet containing ergosterol (group 5) were comparable to those for group 2. But the liver cholesterol content of group 5 was lower than that of group 2.

Experiment 2. In this experiment, weanling male rats of the Wistar strain were divided into groups of 8 each and were

[2] The mushroom used was the cap of dried *Lentinus edodes*, Kohshin, which was ground. Analytical data of the sample: (in per cent) water, 4.2; protein, 19.9; fat, 1.9; sugars, 63.9; crude fiber, 5.7; and ash, 4.4.

[3] See footnote 2.

[4] Nembutal, Abbott Laboratories, Inc., North Chicago, Illinois.

fed the following diets: group 1, cholesterol-free diet (CF); group 2, diet containing 1% cholesterol (C); group 3, cholesterol diet plus 5% ground cap of *Lentinus edodes*, Kohshin (CLK); group 4, cholesterol diet plus 5% ground cap of *Lentinus edodes*, Donko (CLD); group 5, cholesterol diet plus 5% ground stem of *L. edodes*, Donko (CSL); group 6, cholesterol diet plus 5% ground Jews-ear (CJ); group 7, cholesterol diet plus 5% *Flammulia velutipes* (CFV); group 8, cholesterol diet plus 5% ground *Agarieus bisporus* (champignon) (CA).

The analytical data of mushroom preparations are shown in table 3. The rats were fed the diet ad libitum for 30 days. Plasma cholesterol levels are reported in table 4. All the mushrooms used were effective, to varying extents, in reducing

TABLE 3

Analytical data of dried and ground mushrooms used

	Water	Protein	Fat	Sugars and crude fiber	Ash
	%	%	%	%	%
Cap of *Lentinus edodes*, Kohshin	7.25	18.16			
Cap of *Lentinus edodes*, Donko	7.09	17.90			
Stem of *L. edodes*, Donko	11.53	8.90	1.37	80.70	7.93
Auricularia polytricha (Jews-ear)	10.56	7.80	2.20	86.98	3.02
Flammulia velutipes	7.82	23.22	3.09	66.57	7.12
Agaricus bisporus (champignon)	10.50	45.11	2.50	40.87	11.52

TABLE 4

Plasma cholesterol levels of rats fed mushrooms

	Group	Total	Free	Free
		mg/100 ml	*mg/100 ml*	%
1	Cholesterol-free (CF)	119 ±2 [1]	65.1 ± 0.7	55
2	1% Cholesterol (C)	185 ±2	85.8 ± 0.9	46
3	Cholesterol + 5% *L. edodes*, Kohshin, ground cap (CLK)	121 ±1	71.4 ± 0.8	59
4	Cholesterol + 5% *L. edodes*, Donko, ground cap (CLD)	99.6 ± 1.1	57.8 ± 0.9	58
5	Cholesterol + 5% *L. edodes*, Donko, ground stem (CSL)	131 ±2	60.8 ± 1.2	46
6	Cholesterol + 5% Jews-ear, ground (CJ)	149 ±1	67.8 ± 1.0	45
7	Cholesterol + 5% *F. leutipes* (CFV)	159 ±2	92.3 ± 1.7	58
8	Cholesterol + 5% *A. bisporus* (CA)	117 ±1	73.8 ± 0.7	63

[1] SE of mean.

TABLE 5

Analytical data of fractions separated from Lentinus edodes, Kohshin

Fraction	Yield (dry matter)	Water	Protein	Fat [1]	Sugars	Crude fiber	Ash
	%	%	%	%	%	%	%
1	2.45	—	—	100	—	—	—
2	46.87	38.00	18.44	—	71.46	—	10.10
3	9.89	34.06	17.33	—	75.97	—	6.70
4	2.61	42.10	9.57	—	86.51	—	3.92
5	39.57	72.00	22.38	—	64.99	11.17	1.46

[1] The principal fatty acids of the fat separated from *L. edodes* had the following percentage composition (determined by gas-liquid chromatography): $C_{14:0}$, 0.5; $C_{15:0}$, 0.8; $C_{16:0}$, 14.2; $C_{16:1}$, 1.1; $C_{16:2}$, 0.3; $C_{18:0}$, 0.8; $C_{18:1}$, 2.8; $C_{18:2}$, 64.8; $C_{20:1}(?)$, 1.1; $C_{20:2}(?)$, 0.7; $C_{22:1}$, 5.0; and $C_{24:0}$, 7.9.

the plasma cholesterol values, with the lowest level observed in rats fed *Lentinus edodes*, Donko (group 4). Plasma cholesterol values of the animals given either *Agaricus bisporus* (group 8) or *L. edodes*, Kohshin (group 3) were almost equal to each other and lower than in the other groups except group 4. On the other hand, the stem of *L. edodes*, Donko (group 5) is slightly less effective. Jews-ear (group 6) and *Flammulia velutipes* (group 7) had less of an effect than any of the other test fungi.

Experiment 3. Dried *L. edodes*, Kohshin was fractionated with ether, water and ethanol as indicated in figure 1. Each fraction was concentrated by using a rotary evaporator. Analytical data of the fractions are shown in table 5.

Weanling male rats of U.S.C. strain were fed each fraction to be equivalent to 10% of the diet as ground, dried mushroom. Group 1 was fed a cholesterol-free diet (CF); group 2, a diet containing 1% cholesterol (C); groups 3–7, the cholesterol diet plus fractions 1–5, respectively; group 8, cholesterol diet plus 5% ground *L. edodes*, Kohshin (CM); and group 9, cholesterol diet plus 1.5 mannitol (CMAN). The complete diets are listed in table 6. Since the mushroom contains mannitol, group 9 was included to determine whether mannitol had any effect on the criteria under investigation.

Among the fractionated constituents, the water-soluble fraction (group 4) and the 30% ethanol-soluble fraction (group 5) markedly lowered plasma cholesterol (table 7). On the other hand, the materials which were found to be ineffective were the ether-soluble fraction (group 3), and the 70% ethanol-soluble and its residue (groups 6 and 7). Mannitol (group 9) was also ineffective.

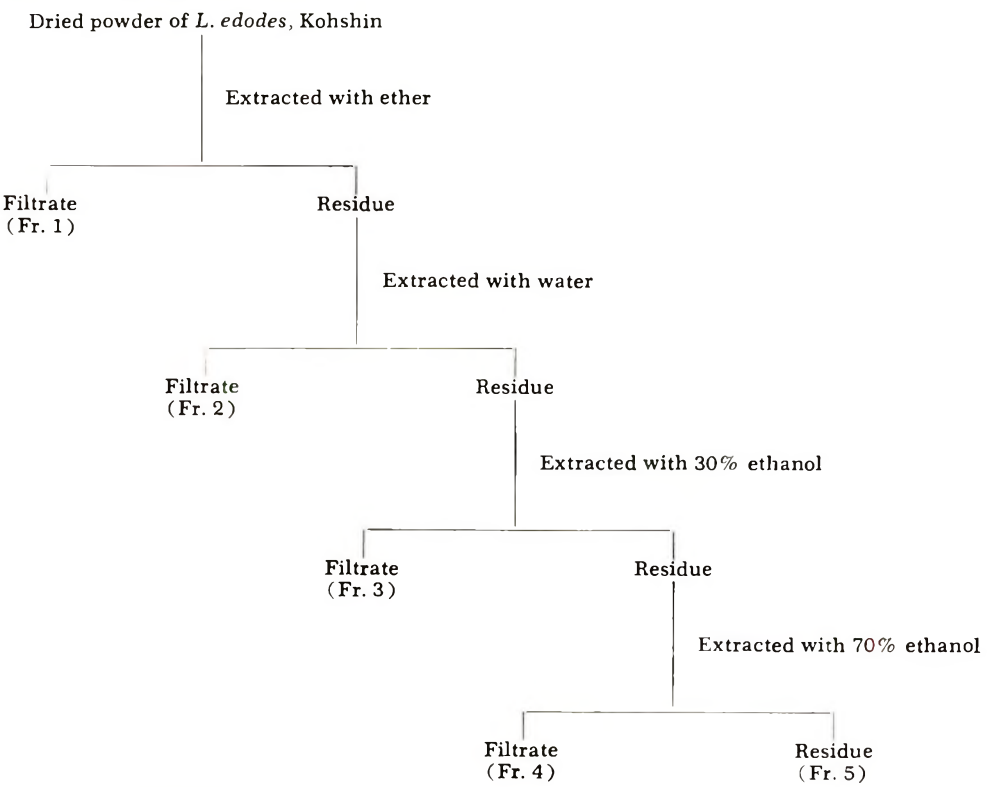

Fig. 1 Diagrammatic representation of the fractionation of constituents of *Lentinus edodes*, Kohshin.

TABLE 6

Composition of diets

	1	2	3	4	5	6	7	8	9
Sucrose	64.26	63.01	62.76	58.33	62.03	62.75	59.06	58.01	61.49
Casein	22	22	22	22	22	22	22	22	22
Non-nutritive cellulose [1]	4	4	4	4	4	4	4	4	4
Salt mixture [2]	4	4	4	4	4	4	4	4	4
Choline chloride	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24
Vitamin mixture [2]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Cholesterol	—	1	1	1	1	1	1	1	1
Bile salts	—	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Cottonseed oil	5	5	5	5	5	5	5	5	5
Fraction 1			0.25						
Fraction 2				4.68					
Fraction 3					0.98				
Fraction 4						0.26			
Fraction 5							3.95		
L. edodes, Kohshin, ground								5	
Mannitol									1.52

[1] Cellu Flour, Chicago Dietetic Supply House, Chicago.

[2] See footnotes to table 1.

TABLE 7

Plasma cholesterol levels of rats fed mushroom fractions

	Group	Total	Free	Free
		mg/100 ml	*mg/100 ml*	*%*
1	Cholesterol-free	84.4 ± 1.2 [1]	34.1 ± 0.6	40
2	1% Cholesterol	168 ± 2	80.8 ± 1.2	48
3	Cholesterol + fraction 1	177 ± 2	47.1 ± 1.0	27
4	Cholesterol + fraction 2	110 ± 1	51.7 ± 1.4	47
5	Cholesterol + fraction 3	124 ± 1	64.8 ± 0.7	52
6	Cholesterol + fraction 4	175 ± 2	75.3 ± 1.4	43
7	Cholesterol + fraction 5	166 ± 2	53.8 ± 1.1	32
8	Cholesterol + 5% *L. edodes*, Kohshin, ground cap	110 ± 1	42.8 ± 1.0	39
9	Cholesterol + 1.5% mannitol	171 ± 1	51.6 ± 0.9	33

[1] SE of the mean.

DISCUSSION

In the animals supplemented with ergosterol or mannitol without the ground mushrooms or mushroom fractions, there was no effect on the plasma cholesterol levels.

Fractionation on the cholesterol-lowering principle in the mushroom yielded 2 active fractions, one a water-soluble fraction and the other a fraction soluble in 30% ethanol.

However, although the whole ground dried mushroom preparation was effective in reducing plasma cholesterol levels, liver cholesterol levels remained high. The ergosterol supplement, however, did cause a marked decrease in hepatic cholesterol levels.

There appears to be no question that the plasma cholesterol levels in rats are lowered by presence of the mushroom in the diet under our experimental conditions.

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LITERATURE CITED

1. Peterson, D. W. 1951 Effect of soybean sterols in the diet on plasma and liver cholesterol in chicks. Proc. Soc. Exp. Biol. Med., 78: 143.

2. Peterson, D. W., C. W. Nichols, Jr. and E. A. Shneour 1952 Some relationships among dietary sterols, plasma and liver cholesterol levels, and atherosclerosis in chicks. J. Nutr., *47:* 57.
3. Peterson, D. W., E. A. Shneour, N. F. Peek and H. W. Gaffey 1953 Dietary constituents affecting plasma and liver cholesterol in cholesterol-fed chicks. J. Nutr., *50:* 191.
4. Nieft, M. L., and H. J. Deuel, Jr. 1949 Studies on cholesterol esterases. I. Enzyme systems in rat tissues. J. Biol. Chem., *177:* 143.

Effects of Vitamin B_{12} on the Weights of Certain Organs in the Rat

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ABSTRACT Comparisons were made between the tissue weight-to-body weight ratios of certain organs in vitamin B_{12}-deficient and vitamin B_{12}-sufficient littermate pairs of rats under both ad libitum and paired feeding conditions. Seminal vesicles, ovaries and uteri were smaller in young vitamin B_{12}-deficient animals, even when their vitamin B_{12}-supplemented littermates were limited to the same food intake and made the same weight gains. Mature males did not exhibit a significant difference in seminal vesicle weight. Thus the effect of the vitamin B_{12}-deficiency was to delay the maturation of the secondary sex glands rather than to prevent the eventual growth of these tissues to maturity. Enlarged thyroid glands were observed in the deficient animals, whether fed ad libitum or pair-fed. Renal and hepatic weights were also greater in such rats. The testes, the adrenals, and the heart were heavier in the deficient animals but only under ad libitum feeding conditions. No consistent differences were observed in the weights of the pituitary gland or of the spleen. No difference was observed between vitamin B_{12}-deficient and supplemented animals in their response to stilbestrol as measured by uterine weights.

Early studies of vitamin B_{12} deficiency in rats demonstrated that animals deficient in the vitamin possessed relatively larger kidneys (1–3) and livers (2, 4, 5) and smaller accessory sex glands (6). In another study (7), the heart, thyroid, kidneys and liver of a vitamin B_{12}-deficient pig weighed more and the spleen and adrenals less, per unit of body weight, than the average of 3 pigs supplemented with the vitamin. However, since a deficiency of the vitamin also decreases food consumption, the effects on the weights of these tissues may have been due either to the vitamin deficiency per se, to the concomitant inanition associated with it, or to a combination of both. In the present paper, a comparison is reported of weights of a number of organs and glands of vitamin B_{12}-deficient and vitamin B_{12}-supplemented animals under conditions of both ad libitum and pair-feeding.

EXPERIMENTAL PROCEDURE

Rats of the Wistar strain were used. They came from mothers who were transferred from a stock ration to a vitamin B_{12}-deficient ration at parturition and continued with the deficient ration during lactation. At weaning (21–25 days of age) or within 2 weeks thereafter, they were divided into sex-littermate pairs, one member of each pair being given a vitamin B_{12} supplement and the other continued with a vitamin B_{12}-deficient ration. The rats were maintained during the experimental period in individual cages furnished with raised screen floors. Distilled water was supplied ad libitum.

The vitamin B_{12}-deficient ration used during the experimental period had the following composition: (%) dextrin, 40.38; lactose, 15.0; casein (10 × hot-alcohol-extracted), 20.0; dried brewer's yeast, 10.0; salt mixture (8), 4.5; cottonseed oil, 9.85; fish liver oil, 0.15; crystalline vitamins, 0.12.[1] Vitamin B_{12}, when given, was included in the ration or fed separately by hypodermic syringe, as indicated.

At the end of the experimental period, the rats were killed by ether inhalation and the tissues removed, cleaned, blotted and weighed immediately. Tissue weights are expressed in the tables and discussed in the paper in terms of grams per 100 g of body weight.

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[1] Furnished in milligrams per 100 g of ration: thiamine·HCl, 0.5; riboflavin, 0.5; pyridoxine·HCl, 0.5; Ca pantothenate, 3.1; choline chloride, 75.0; nicotinic acid, 3.1; inositol, 3.1; p-aminobenzoic acid, 18.8; biotin, 0.006; pteroylglutamic acid, 0.06; ascorbic acid, 3.08; α-tocopheryl acetate, 6.25; and 2-methyl,1,4-naphthoquinone, 0.16.

TABLE 1

Effect of vitamin B_{12} deficiency on organ weights of male rats of different age groups fed ad libitum

Age of rats, days	56 to 57 (9 pairs) [1]			62 to 77 (10 pairs) [2]			189 to 301 (15 pairs) [3,4]		
	Without vitamin B_{12}	With vitamin B_{12} [5]	*t* value [6]	Without vitamin B_{12}	With vitamin B_{12} [7]	*t* value	Without vitamin B_{12}	With vitamin B_{12} [7]	*t* value
Avg. body wt, g	114	212	13.0 **	190	300	14.8 **	356	521	8.8 **
	g/100 g body wt			*g/100 g body wt*			*g/100 g body wt*		
Testes	1.25	1.09	2.0	1.34	1.01	6.7 **	0.78	0.68	3.0 *
Seminal vesicles	0.016	0.044	9.3 **	0.071	0.102	4.8 **	0.155	0.135	2.1
Thyroid	0.013	0.008	4.1 **	0.009	0.006	5.1 **	0.007	0.004	4.1 **
Kidneys	1.79	1.12	11.7 **	1.41	0.92	7.1 **	1.11	0.64	4.0 **
Liver	7.42	6.90	2.0	5.13	4.67	3.1 *	4.13	3.08	5.1 **
Pituitary	0.003	0.003	0.2	0.003	0.003	1.2	0.003	0.002	3.8 **
Spleen	0.295	0.376	1.5	0.231	0.218	1.5	0.168	0.147	3.2 **
Adrenals	0.014	0.010	7.7 **	0.011	0.008	4.1 **	0.007	0.005	3.2 **
Heart	0.448	0.393	3.9 **	0.427	0.354	7.9 **	0.395	0.287	4.6 **

[1] 17 pairs for body weight, testes, seminal vesicles and kidneys.
[2] Average: 67.2 days.
[3] Average: 275.6 days.
[4] 12 pairs for adrenals, 11 for thyroid and 14 for pituitary.
[5] 2 μg/day.
[6] ** adjacent to a *t* value indicates statistical significance at or less than the 1% level; * indicates significance at the 5% level or between the 5% and 1% levels; no asterisk indicates no statistically significant difference.
[7] 5 μg/10 g food.

RESULTS AND DISCUSSION

As expected, when the animals were fed ad libitum, the vitamin B_{12}-supplemented rats gained considerably more weight than those maintained with the vitamin B_{12}-deficient ration. In all groups fed in this manner (tables 1 and 2), the body weights at slaughter reflect these differences. However, limiting both members of each pair to the same food intake resulted in nearly identical average body weights for vitamin B_{12}-deficient and supplemented animals at the end of the experiment (table 3). Thus, the vitamin B_{12}-fed rats in these groups served essentially as paired-weight as well as pair-fed controls. The supplemented rat in a pair was always the one whose food consumption had to be limited throughout the experiment. It tended to consume all or the greater part of its food allotment during the first few hours after feeding, whereas the deficient rat spread its food intake over a much longer period of time.

Sex organs and glands. In rats fed ad libitum, the testes appeared heavier per unit of body weight in the vitamin B_{12}-deficient animals, significantly in two of the groups and nearly so in the third group. When the rats were limited to the same food intake, however, this difference disappeared. Thus, it appears that any effect of the vitamin B_{12} deficiency upon testes weight can be explained by the concomitant inanition.

In contrast, the seminal vesicles were enlarged in the young vitamin B_{12}-supplemented rats not only when they were fed ad libitum but even when they were limited to the same food intake, although the differences were smaller in the latter case. This effect was confined to the young ani-

TABLE 2

Effect of vitamin B_{12} deficiency on organ weights of female rats [1] fed ad libitum

	Without vitamin B_{12}	With vitamin B_{12} [2]	*t* value [3]
Avg. body wt, g	90	158	18.5 **
	g/100 g body wt		
Ovaries	0.026	0.052	8.2 **
Uterus	0.044	0.156	12.7 **
Thyroid	0.013	0.010	5.4 **
Kidneys	1.72	1.12	8.1 **
Liver	7.40	6.52	3.5 **
Pituitary	0.003	0.004	4.3 **
Spleen	0.258	0.369	2.3 *
Adrenals	0.017	0.017	0.2
Heart	0.470	0.420	3.5 **

[1] 10 pairs; age: 48 to 52 days.
[2] 2 μg/day.
[3] See table 1, footnote 6.

mals, for no significant difference was detected in the older group of ad libitum-fed males, as between the vitamin deficient and supplemented animals. Thus the degree of vitamin B_{12} deficiency present here delayed the maturation of these secondary sex glands more than it delayed the general body growth, but it did not prevent the eventual growth of these tissues to maturity. The delay apparently was greater than that due to the concomitant inanition associated with the deficiency.

A similar difference was observed between young vitamin B_{12}-deficient and supplemented females with respect to the ovaries and uterus. Both weighed relatively more on the average in vitamin B_{12}-supplemented rats, either when fed ad libitum or when pair-fed. Here too the differences were considerably smaller and, in this instance, not quite statistically significant under pair-feeding conditions. However, if values in table 4 for the pair-fed females (those not given stilbestrol) are also con-

TABLE 3

Organ weights of vitamin B_{12}-supplemented and vitamin B_{12}-deficient rats limited to the same food intake

	Males 57 to 61 days of age (10 pairs) [1]			Females 48 to 50 days of age (10 pairs) [2]		
	Without vitamin B_{12}	With vitamin B_{12} [3]	t value [4]	Without vitamin B_{12}	With vitamin B_{12} [3]	t value
Avg. body wt, g	133	133	0.1	101	100	0.4
	g/100 g body wt			*g/100 g body wt*		
Testes	1.11	1.17	0.4	—	—	—
Seminal vesicles	0.014	0.025	2.3 *	—	—	—
Ovaries	—	—	—	0.026	0.032	1.9
Uterus	—	—	—	0.045	0.070	2.2 [5]
Thyroid	0.013	0.010	3.3 *	0.011	0.009	2.9 *
Kidneys	1.59	1.08	6.2 **	1.54	1.20	5.8 **
Liver	7.00	5.63	8.0 **	7.12	5.40	6.5 **
Pituitary	0.002	0.002	2.0	0.003	0.004	1.6
Spleen	0.317	0.288	0.7	0.303	0.374	1.1
Adrenals	0.015	0.018	2.2	0.018	0.019	1.8
Heart	0.439	0.409	1.7	0.433	0.420	1.0

[1] Nine pairs for spleen and adrenals; 7 pairs for pituitary; 8 pairs for thyroid.
[2] Nine pairs for pituitary.
[3] 2 μg/day.
[4] See table 1, footnote 6.
[5] $t = 2.26$ required for significance at the 5% level.

TABLE 4

Vitamin B_{12} and stilbestrol administration

Treatment	No. of litters [1]	Weight of uterus				Difference
		Without vitamin B_{12}		With vitamin B_{12}		(4 − 3) minus (2 − 1)
		No stilbestrol (1)	Stilbestrol (2)	No stilbestrol (3)	Stilbestrol (4)	
		mg	*mg*	*mg*	*mg*	*mg*
Ad libitum [2]	55	29.5	76.2	42.0	89.6	+ 0.9
Limited to same food intake [3]	5	32.5	67.3	40.0	74.5	− 0.3

[1] Weanling rats, 19 to 25 days of age.
[2] Includes 13 individual experiments; total dose of stilbestrol, 0.052 to 2.0 μg given in 1 or 4 injections (rats killed, respectively, 2 days or one day following last injection and vitamin B_{12}-supplemented rats given the vitamin, respectively, 6 to 11 days prior to stilbestrol injection or only during the injection period); vitamin B_{12} was administered in a single dose (25 μg) at the time of stilbestrol injection, in daily doses (2 μg/day) or included in the ration (0.5 μg/10 g food). None of these variations appeared to affect the results significantly.
[3] Total dose of stilbestrol, 0.083 μg, given in single dose; vitamin B_{12}-supplemented rats received the vitamin 7 to 11 days prior to injection; rats killed 2 days after injection.

sidered along with values for those shown in table 3, then both the ovaries and uterus were significantly larger in the vitamin B_{12}-supplemented rats (t ovaries = 2.3*; t uterus = 3.1**). The delay in attaining maturity of the sex organs is also reflected in a delayed vaginal potency of vitamin B_{12}-deficient animals (9).

Some evidence has also been obtained with the chick suggesting an effect of vitamin B_{12} upon the sex processes in the body. Gassner et al. (10) obtained a stimulation of testicular growth and differentiation and comb growth when the vitamin was fed to deficient cockerels. Doran and Gregory (11) noted an increase in the vitamin B_{12} content of chick blood at the onset of laying. When stilbestrol was administered to chicks, Kline (12) observed a lower response of the oviduct in vitamin B_{12}-deficient birds than in those supplemented with the vitamin.

Experiments with the rat bearing upon the latter point are summarized in table 4. The basal diet used, however, in contrast with that of Kline (12), contained ample methionine and choline. With both ad libitum-fed rats and those limited to the same food intake, uterine weights were higher when vitamin B_{12} was fed regardless of whether stilbestrol was administered. Taking this into consideration, there was clearly no difference between vitamin B_{12}-deficient and supplemented animals in their response to stilbestrol as measured by uterine weights.

Thyroid gland. The thyroid gland was enlarged significantly in all groups of vitamin B_{12}-deficient rats. Since this result was evident in pair-fed as well as in ad libitum-fed animals, it was apparently due to an effect of the vitamin deficiency beyond the associated concomitant inanition. This enlargement of the thyroid with the deficient ration is in agreement with the observations of Bertazzoli et al. (13) but somewhat in contrast with those of Meites (14), who observed no significant difference between the thyroid weights of rats fed vitamin B_{12} supplements and those of rats that received no such supplement. The basal ration used by Meites, however, although apparently not supplying a level of vitamin B_{12} sufficient for maximal growth of his animals, nonetheless did contain 20% whole milk powder; thus it could hardly have been as deficient in the vitamin as the deficient ration used in the present experiments. Ferguson et al. (15) observed an enlarged thyroid gland in vitamin B_{12}-deficient chick embryos and noted evidence suggesting that the thyroids from such embryos may be hypofunctional.

Kidneys. The kidneys were larger per unit of body weight in all groups of vitamin B_{12}-deficient animals than in their littermates receiving the vitamin. In fact, in the older group of rats that had been fed the deficient ration for a long period of time, the kidneys of the deficient animals were larger than those of the supplemented animals in actual size, as well as in relative size, despite the considerably smaller body weights of the deficient rats. This result, together with the observation that, with equalized food intake, the kidneys were also significantly larger in the vitamin-deficient animals, indicates that the enlargement was due more to the vitamin deficiency per se than to the concomitant inanition.

Kidney hypertrophy has been shown to be associated with a number of different kinds of stress (16–18). The stress put upon it by vitamin B_{12} deficiency may bear some relation to the large amount of the vitamin that it takes up when ample amounts of the vitamin are absorbed (19).

Liver. Liver weights were greater per unit of body weight in all ad libitum-fed groups of vitamin B_{12}-deficient animals, in most cases significantly so. The differences were even greater when the groups were limited to the same food intake, the size of the livers of the vitamin B_{12}-supplemented rats tending to be smaller under these conditions. A similar depression of hepatic weights in paired-weight controls was reported in a study of thiamine deficiency (17). The liver being a storage organ, this depression in relative weight may be related to the manner in which the vitamin-supplemented rats fed the limited food intake consumed their ration — in large quantities immediately after feeding with periods of comparative hunger prior to the next feeding.

Other tissues. There were no consistent differences between the weights of the pituitary gland or of the spleen in rats fed

ad libitum and no significant differences when they were pair-fed. Adrenal weights were in most cases higher in the vitamin B_{12}-deficient animals when fed ad libitum but not when pair-fed. Any apparent enlargement due to the vitamin deficiency can therefore be attributed to the smaller food intake of the deficient animals or, perhaps, since vitamin B_{12}-supplemented rats lay down relatively less fat than vitamin B_{12}-deficient rats (5), to the method of expressing the results in terms of per unit of total body weight rather than fat-free body weight. Much the same comment can be made concerning the weight of the heart, which was significantly greater in the ad libitum-fed vitamin B_{12}-deficient rats but only slightly and not significantly larger in the pair-fed animals. As with the kidney, many forms of stress (17, 18) can bring about enlargement of the adrenals and the heart.

LITERATURE CITED

1. Cary, C. A., and A. M. Hartman 1947 Unidentified nutrients. In: Yearbook of Agriculture, 1943/47. U. S. Department of Agriculture, Washington, D. C., p. 779.
2. Zucker, L. M., and T. F. Zucker 1948 Zoopherin: a nutritional factor for rats associated with animal protein sources. Arch. Biochem., *16:* 115.
3. 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.
4. Scheid, H. E., M. M. Andrews and B. S. Schweigert 1951 Liver storage of vitamin B_{12} by the rat. Proc. Soc. Exp. Biol. Med., *78:* 558.
5. Ling, C. T., and B. F. Chow 1952 Effect of vitamin B_{12} on the body composition of rats. J. Biol. Chem., *198:* 439.
6. Hartman, A. M., L. P. Dryden and C. A. Cary 1949 The role and sources of vitamin B_{12}. J. Amer. Diet. Assn., 25: 929.
7. Neumann, A. L., B. C. Johnson and J. B. Thiersch 1950 Crystalline vitamin B_{12} in the nutrition of the baby pig. J. Nutr., *40:* 403.
8. Hawk, P. B., and B. L. Oser 1931 A modification of the Osborne-Mendel salt mixture. Science, *74:* 369.
9. Dryden, L. P., A. M. Hartman and C. A. Cary 1954 Influence of vitamin B_{12} upon vaginal patency in the rat. Proc. Soc. Exp. Biol. Med., *87:* 195.
10. Gassner, F. X., A. R. Patton, H. S. Wilgus and L. W. Charkey 1950 Failure of cockerel comb and testes development on sesame meal and its prevention by vitamin B_{12}. Proc. Soc. Exp. Biol. Med., 75: 630.
11. Doran, B. M., and M. E. Gregory 1963 The influence of ovarian activity or treatment with oestradiol dipropionate on the vitamin B_{12} content of cells and plasma of the blood of the domestic fowl. Brit. J. Nutr., *17:* 583.
12. Kline, I. T. 1955 Relationship of vitamin B_{12} to stilbestrol stimulation of the chick oviduct. Endocrinology, 57: 120.
13. Bertazzoli, C., T. Chieli and E. Turolla 1959 The growth-promoting action of vitamin B_{12} in rats in relation to the composition of vitamin B_{12}-deficient diets. Ital. J. Biochem., *8:* 193.
14. Meites, J. 1950 Effects of vitamin B_{12} on normal thyroid function in rats. Proc. Soc. Exp. Biol. Med., 75: 195.
15. Ferguson, T. M., R. H. Rigdon and J. R. Couch 1957 Thyroid in B_{12}-deficient chick embryos. Endocrinology, 60: 13.
16. Addis, T., R. W. Lippman, W. Lew, L. J. Poo and W. Wong 1951 Effect of dietary protein consumption upon body growth and organ size in the rat. Amer. J. Physiol., *165:* 491.
17. Skelton, F. R. 1950 Some specific and non-specific effects of thiamine deficiency in the rat. Proc. Soc. Exp. Biol. Med., 73: 516.
18. Megel, H., H. Wozniak, L. Sun, E. Frazier and H. C. Mason 1962 Effects on rats of exposure to heat and vibration. J. Appl. Physiol., 17: 759.
19. Dryden, L. P., and A. M. Hartman 1966 Relative concentration of vitamin B_{12} in the organs of the male rat as affected by its intake of the vitamin. J. Nutr., 90: 382.

Relative Concentration of Vitamin B_{12} in the Organs of the Male Rat as Affected by its Intake of the Vitamin

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ABSTRACT The concentrations of vitamin B_{12} in certain organs of the male rat were studied under conditions where the animal was deficient in the vitamin and where it was fed ample quantities. The kidneys stored large amounts when an abundant supply was available. The pituitary and the adrenals also contained high concentrations, equal to or not far below those in the kidneys of vitamin B_{12}-deficient rats but much less than those in the kidneys of vitamin B_{12}-sufficient rats. The heart contained fair amounts when the vitamin was supplied. The other tissues studied contained much smaller concentrations, the testes and seminal vesicles having particularly low amounts when the rat was deficient in the vitamin. Rats aged 189 to 301 days differed little from those aged 62 to 77 days of age in respect to the vitamin B_{12} concentration in their tissues, except for slightly greater storage in a few of the tissues in the older group when the vitamin was administered.

Numerous workers have studied the concentration of vitamin B_{12} in and its distribution among various tissues of a number of species of animals fed a stock ration or a complete ration containing the vitamin. In some instances, they determined the vitamin B_{12} content by microbiological or other assay; in others, they followed the deposition of the radioactive vitamin. Although some investigators (1–3) found a good correlation between the deposition of radioactive vitamin B_{12} in the tissues and the amount of unlabeled vitamin already present there, others showed that the proportion taken up, by certain tissues at least, can be affected by a number of factors, one of which is the current status of the animal relative to the vitamin (4–6). Some species differences also apparently exist in the distribution of the vitamin among the tissues (1, 7).

The present paper concerns the relative concentration of the vitamin in a number of organs of the rat as affected by its vitamin B_{12} status. Such studies in the past with the rat have been confined largely to a comparison of the relative amounts in the kidney and liver (8–12). In early work using a rat assay method (13), several other tissues were also measured, but of those tested in the vitamin B_{12}-deficient rat, only the kidney gave a measurable response. Another study (6) determined the amount of radioactive vitamin B_{12} absorbed by the kidney, liver and heart, as well as by the blood, muscle, bone and gastrointestinal tract, in vitamin B_{12}-deficient and supplemented rats. One group of workers (14), comparing the vitamin B_{12} content of the tissues of 28-day-old rats obtained from mothers receiving rations unsupplemented or supplemented with a source of vitamin B_{12}, measured the amounts in the kidney, liver, spleen and heart. Salmi (15) measured microbiologically the vitamin B_{12} content of certain rat tissues — kidney, liver, alimentary canal, brain and lungs. He followed changes in the concentrations in these tissues from the fetal stage to 3 months of age and compared test rats fed a ration low but not greatly deficient in vitamin B_{12} with controls fed a ration containing 4 times this amount of the vitamin (equal to that in the stock ration). The present work compares by microbiological assay the vitamin B_{12} concentration in 9 organs and glands, obtained from rats rendered greatly deficient in the vitamin or amply supplied with it. The study includes comparisons between both immature and mature animals.

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EXPERIMENTAL PROCEDURE

Two groups of male rats, one aged 62 to 77 days and the other aged 189 to 301 days were used. Each group consisted of littermate pairs, fed ad libitum, one member of each pair having been fed a ration deficient in vitamin B_{12} and the other having received the same ration supplemented with 5 μg of the vitamin per 10 g of food. This amount of the vitamin is about 10 times that found to be required to obtain maximal growth of deficient rats in this colony within a 4-week period after weaning. Thus the rats fed vitamin B_{12} received ample amounts for their needs and even some excess. The rats and the vitamin B_{12}-deficient ration used have been described in a previous paper (16).

The animals were killed at the ages indicated and the organs and glands being studied were removed. Immediately upon removal, these tissues were cleaned, blotted, weighed and placed in buffer solution. The buffer used was a pH 5 citrate-phosphate solution with cyanide added (0.1 g NaCN/liter of buffer). At least 10 ml were used for each tissue and in no case were less than 10 ml per g of tissue added. The tissues were then ground into fine particles and the vitamin B_{12} was extracted from them by autoclaving for 20 minutes at 120°. The extracts were filtered and the residues washed with small amounts of distilled water. The filtrates were resterilized and frozen in plastic containers until assay. They were concentrated or diluted when required for the test.

The assay method used was the *Escherichia coli* tube test of Burkholder (17). While this test measures not only animal-active vitamin B_{12} but also analogues that are active for microorganisms but not for animals, such analogues are present in the tissues of animals only in very small amounts if at all (18–20). Results are expressed in the tables as micrograms of vitamin B_{12} per gram of tissue.[1]

RESULTS AND DISCUSSION

The comparative vitamin B_{12} concentrations in the organs and glands of the younger group of rats are shown in table 1, and those for the older group in table 2. As might be expected, the tissues of the vitamin B_{12}-supplemented rats were markedly higher, and in all cases significantly higher, in the vitamin than those of the vitamin B_{12}-deficient animals.

The kidneys showed the greatest increase of any of the tissues. Values for the vitamin B_{12}-supplemented rats far surpassed those for all other organs and tissues studied in the content of the vitamin per gram of tissue, exceeding their nearest values by 5 to 6 times. However, under conditions of vitamin B_{12} deprivation, the content in the kidneys decreased mark-

[1] Recovery of cyanocobalamin added to ground kidney tissue and carried through this extraction and assay procedure: 92 ± 4.9% (6 samples).

TABLE 1

Concentration of vitamin B_{12} in certain organs of rats aged 62 to 77 days [1]

Organ	Vitamin B_{12} content of organ			Vitamin B_{12}
	Rats fed no vitamin B_{12}	Rats fed vitamin B_{12}	*t* value [2]	no vitamin B_{12}
	μg/g	μg/g		
Kidneys	0.050 ± 0.0051 [3]	2.910 ± 0.3138	9.2 **	58.2
Pituitary	0.056 ± 0.0101	0.256 ± 0.0390	5.3 **	4.6
Adrenals	0.029 ± 0.0045	0.539 ± 0.0496	9.7 **	18.6
Heart	0.017 ± 0.0036	0.135 ± 0.0109	12.1 **	7.9
Liver	0.007 ± 0.0007	0.050 ± 0.0073	6.0 **	7.1
Spleen	0.009 ± 0.0013	0.052 ± 0.0037	11.8 **	5.8
Thyroid	0.009 ± 0.0016	0.081 ± 0.0150	4.8 **	9.0
Seminal vesicles	0.005 ± 0.0006	0.065 ± 0.0081	7.3 **	13.0
Testes	0.002 ± 0.0003	0.035 ± 0.0043	7.7 **	17.5

[1] Ten pairs; average age: 67.2 days. Body weight when killed: no vitamin B_{12} group, 190 g; vitamin B_{12} group, 300 g.

[2] ** adjacent to a *t* value indicates statistical significance at or less than the 1% level; * indicates significance at the 5% level or between the 5% and 1% levels; no asterisk indicates no statistically significant difference.

[3] SE of mean.

TABLE 2

Concentration of vitamin B_{12} in certain organs of rats aged 189 to 301 days [1]

Organ	Vitamin B_{12} content of organ			Vitamin B_{12}
	Rats fed no vitamin B_{12}	Rats fed vitamin B_{12}	*t* value [2]	no vitamin B_{12}
	μg/g	*μg/g*		
Kidneys	0.069 ± 0.0121 [3]	2.300 ± 0.2078	10.5 **	33.3
Pituitary	0.032 ± 0.0095	0.369 ± 0.0429	34.3 **	11.5
Adrenals	0.044 ± 0.0078	0.369 ± 0.0544	6.1 **	8.4
Heart	0.019 ± 0.0032	0.241 ± 0.0178	12.8 **	12.7
Liver	0.011 ± 0.0020	0.084 ± 0.0103	7.6 **	7.6
Spleen	0.017 ± 0.0025	0.058 ± 0.0044	12.2 **	3.4
Thyroid	0.021 ± 0.0055	0.078 ± 0.0138	4.0 **	3.7
Seminal vesicles	0.008 ± 0.0017	0.056 ± 0.0056	8.2 **	7.0
Testes	0.005 ± 0.0010	0.065 ± 0.0090	6.7 **	13.0

[1] Fifteen pairs (except 12 pairs for adrenals, 11 for thyroid and 14 for pituitary); average age: 275.6 days. Body weight when killed: no vitamin B_{12} group, 356 g; vitamin B_{12} group, 521 g.
[2] See table 1, footnote 2.
[3] SE of mean.

edly and by a much larger amount than in any of the other tissues. The concentration was then about the same or not far above that in some of the others. These results support the concepts concerning the kidney in species such as the rat as the most important organ in the body for storage of vitamin B_{12} and of a large accumulation of the vitamin in this organ as an indicator of a highly saturated state of the body with respect to this nutrient (6, 15, 21).

Under conditions of vitamin B_{12} deprivation, the present results with the younger group of rats agree with those obtained by Cooperman et al. (2), with dogs fed an undescribed but presumably stock diet, in that the pituitary contained a higher average concentration of the vitamin than any other tissues studied, including even the kidney. With the older rats, however, the kidneys, even when the animals were vitamin B_{12}-deficient, contained about twice the concentration observed in the pituitary. With both groups, when ample vitamin B_{12} was supplied, the kidneys contained far more of the vitamin per gram of tissue than did the pituitary. However, the high content of the vitamin in the pituitary relative to most of the other tissues measured, with both the deficient and the supplemented rats, is in accord with the concept that the vitamin may play an important role in the metabolism of this gland.

Another tissue of importance with respect to vitamin B_{12} concentration, equal to that of the pituitary, was the adrenal glands, and, as with the pituitary, the results suggest an importance of the adrenals with respect to the need for the vitamin in its metabolism. These observations differ somewhat from those of Cooperman et al. (2) with the dog. Although these investigators reported the adrenals to contain amounts of the vitamin relatively high compared with some of the other tissues, they noted far less there than in the pituitary. Wider et al. (22) observed that, in rats, the adrenals took up a fairly high concentration of parenterally administered ^{60}Co-labeled vitamin B_{12} and obtained evidence suggesting the importance of the vitamin to metabolism in the gland.

The heart ranked next in importance to the pituitary and adrenals with respect to vitamin B_{12} content per unit of tissue when the vitamin was supplied to the animal. This was also true with the younger group of deficient animals, but with the older group, the vitamin B_{12} content differed little from that of several of the remaining tissues studied. With this exception, the latter contained much smaller concentrations of the vitamin both in the deficient and supplemented states.

In the deficient rats, the seminal vesicles and the testes contained smaller concentrations of the vitamin than the other tissues studied but showed a relatively high rate of increase from the deficient to the sufficient stage. The possible importance of the vitamin to the development of the accessory sex glands was discussed

in a previous paper (16). Any stimulatory effect of the vitamin on the accessory sex glands might be mediated through other glands, such as the pituitary and the adrenals, where high concentrations of the vitamin were noted.

The results obtained with the older group of rats were not markedly different from those with the younger group. Tests for the significance of the differences between the 2 sets of values are summarized in table 3.

TABLE 3

Comparison of the concentration of vitamin B_{12} in certain organs of young and old rats [1]

Organ	*t* values for difference between young and old groups [2]	
	Rats fed no vitamin B_{12}	Rats fed vitamin B_{12}
Kidneys	1.4	1.6
Pituitary	1.4	1.9
Adrenals	1.7	2.3 *
Heart	0.4	5.1 **
Liver	1.9	2.7 *
Spleen	3.1 **	1.0
Thyroid	1.7	0.1
Seminal vesicles	1.8	0.9
Testes	2.7 *	3.0 **

[1] Young rats, data summarized in table 1; old rats, data summarized in table 2.
[2] See table 1, footnote 2.

Apparently the maximal depletion of the vitamin had occurred by the 62–to 77–day period. The amounts present in the tissues of the deficient animals may have been obtained from traces contained in the ration or from small amounts derived from synthesis in the alimentary tract.

With the vitamin B_{12}-supplemented rats, the older group had a higher average concentration of the vitamin in certain tissues than the younger group, whereas in other comparisons, the reverse was true. Although many organs, notably the kidneys, apparently had reached their maximal storage capacity, at least for the amount of vitamin fed, by 62 to 77 days of age, others, such as the heart, testes and liver, may have been somewhat slower in attaining this condition.

LITERATURE CITED

1. Reizenstein, P. G. 1959 Body distribution, turnover rate and radiation dose after the parenteral administration of radiovitamin B_{12}. Acta. Med. Scand., *165:* 467.
2. Cooperman, J. M., A. L. Luhby, D. N. Teller and J. F. Marley 1960 Distribution of radioactive and nonradioactive vitamin B_{12} in the dog. J. Biol. Chem., *235:* 191.
3. Gräsbeck, R., R. Ignatius, J. Järnefelt, H. Lindén, A. Mali and W. Nyberg 1961 Specific activity of radiovitamin B_{12} in organs and subcellular liver fractions after injection of Co^{58}-labeled vitamin B_{12}. Clin. Chim. Acta, *6:* 56.
4. Harte, R. A., B. F. Chow and L. Barrows 1953 Storage and elimination of vitamin B_{12} in the rat. J. Nutr., *49:* 669.
5. Rosenthal, H. L. 1959 The uptake and turnover of radioactive vitamin B_{12} in rabbit tissues. J. Nutr., *68:* 297.
6. Okuda, K. 1962 Relationship between intake of vitamin B_{12} and its storage by the kidney in the rat. J. Nutr., *77:* 131.
7. Miller, A., G. Gaull, J. F. Ross and H. M. Lemon 1956 Tissue distribution of parenteral Co^{60} vitamin B_{12} in mouse, hamster, rat and guinea pig. Proc. Soc. Exp. Biol. Med., *93:* 33.
8. Cooperman, J. M., B. Tabenkin and R. Drucker 1952 Growth response and vitamin B_{12} tissue levels in vitamin B_{12}-deficient rats and chicks fed riboflavin, 5,6-dimethylbenzimidazole and related compounds. J. Nutr., *46:* 467.
9. Jaffé, W. G., N. Indacochea and C. Embden 1956 Vitamin B_{12} in organs of experimental animals. Arch. Venezolanos Nutr., *7:* 145.
10. Jaffé, W. G., N. Indacochea and C. Embden 1957 The vitamin B_{12} content of the organs of rats with different diets. Experientia, *13:* 251.
11. Ellis, L. N., B. J. Duncan and I. B. Snow 1959 The influence of diet upon the storage of vitamin B_{12} in liver and kidney. J. Nutr., *67:* 185.
12. Okuda, K., and B. F. Chow 1961 The thyroid and absorption of vitamin B_{12} in rats. Endocrinology, *68:* 607.
13. Lewis, U. J., U. D. Register and C. A. Elvehjem 1949 Vitamin B_{12} content of various organs and tissues of the rat. Proc. Soc. Exp. Biol. Med., *71:* 509.
14. Richardson, L. R., P. W. Witten and J. R. Couch 1951 Diet of mother and vitamin B_{12} content of tissues of infant rats. Proc. Soc. Exp. Biol. Med., *76:* 265.
15. Salmi, H. A. 1963 Comparative studies on vitamin B_{12} in developing organism and placenta. Human and animal investigations with reference to the effects of low vitamin B_{12} diet on tissue vitamin B_{12} concentrations in rat. Ann. Acad. Sci. Fennicae (ser. A), 5 (no. 103): 1.
16. Dryden, L. P., and A. M. Hartman 1966 Effect of vitamin B_{12} on the weights of certain organs in the rat. J. Nutr., *90:* 377.
17. Burkholder, P. R. 1951 Determination of vitamin B_{12} with a mutant strain of *Escherichia coli.* Science, *114:* 459.

18. Ford, J. E., E. S. Holdsworth and J. W. G. Porter 1953 The occurrence of cyanocobalamin and related compounds in natural materials. Proc. Nutr. Soc., *12:* xi.
19. Berman, D., H. Yacowitz and H. H. Weiser 1956 A differential microbiological assay for vitamin B_{12} and pseudovitamin B_{12}. Appl. Microbiol., *4:* 49.
20. Emanuiloev, I., and S. Moutafchieva 1963 Vitamin B_{12} in the tissues of cattle. Compt. Rend. Acad. Bulgare Sci., *16:* 773.
21. Okuda, K. 1962 Starvation and renal uptake of vitamin B_{12}. Nature, *195:* 292.
22. Wider, S., J. A. Wider and E. P. Reineke 1958 A study of adrenal radioactivity subsequent to parenteral Co^{60}-labeled vitamin B_{12}. Endocrinology, *63:* 431.

Observations on Respiratory Decline in the Pre-necrotic Phase of Dietary Liver Necrosis [1]

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ABSTRACT The capacity to oxidize succinate was studied with liver mitochondria, homogenates or slices from rats maintained with commercial diets or with semipurified diets with or without addition of vitamin E or sodium selenite. A decline in the oxidation rate was observed with all systems in vitamin E-deficient, selenium-deficient animals, and in some instances in rats fed commercial diets. The decline was prevented by administration of large amounts of vitamin E. Dietary selenite prevented decline in mitochondria, but not in homogenates or slices. The significance of respiratory decline as a possible specific lesion of the latent phase of dietary liver necrosis is discussed.

The "respiratory decline" during the "pre-necrotic phase" of vitamin E deficiency was described originally as the inability to maintain oxygen consumption of liver slices from rats fed a necrogenic diet without vitamin E, "Factor 3" (later identified as a selenium-containing compound), with a low content of sulfur amino acids, and containing Torula yeast as the only protein source (1). This alteration was not observed in kidney or diaphragm slices from vitamin E-deficient rats, nor in the liver damaged with CCl_4 or with a choline-deficient diet.

The respiratory decline was prevented by the addition to the diet of vitamin E or of other necrosis-preventing substances, such as cystine and preparations of Factor 3, although to a variable extent in the latter case (1).

Further studies showed that various tocopherols were effective in preventing respiratory decline of liver slices when injected intraportally (2, 3) [2] or intravenously [3] and when added in vitro (4). Antioxidants also prevented the decline when injected intraportally (5) or when added in vitro (6), whereas selenium prevented almost completely the respiratory decline of liver slices when added to the diet, but not when injected intraportally shortly before death or when added in vitro (7).

The decline was not observed in liver homogenates fortified with NAD and without substrate (1), but occurred in liver homogenates without addition of NAD and with succinate as substrate, or with NAD and α-ketoglutarate as substrate (8, 9). Under these conditions the decline was prevented by some tocopherols or antioxidants given to the animals or added in vitro. Some decline was observed also with liver homogenates from rats fed a commercial diet,[4] but not from rats fed a "wheat casein diet" (8, 9).

Preparations of liver mitochondria showed respiratory decline in the oxidation of succinate. In this case α-tocopherol prevented decline when administered intraportally to the rats, but not when added in vitro. The addition of selenium to the diet was without effect on decline (10).

These observations led Schwarz (7) to conclude that respiratory decline is a biochemical lesion specific for the latent phase of dietary liver necrosis in the rat.

Dietary liver necrosis has been observed in rats fed diets without vitamin E, with 5% or 20% of casein as the protein source, which could be prevented by the addition of vitamin E or selenium to the diet (11). The aim of the experiments described in the present paper was to determine whether respiratory decline occurs also in

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[1] Research supported by a financial contribution from Consiglio Nazionale delle Ricerche, Roma.

[2] Rodnan, G. P., and K. Schwarz 1955 Dietary necrotic liver degeneration: reversal of defect in oxygen consumption by intraportal α-tocopherol. Federation Proc., *14*: 270 (abstract).

[3] Mertz, W., and K. Schwarz 1956 Dietary necrotic liver degeneration: comparison of peripheral intravenous and intraportal injection of vitamin E in respiratory decline. Federation Proc., *15*: 564 (abstract).

[4] Purina Laboratory Chow, Ralston-Purina Company, St Louis.

liver preparations from rats fed these casein-containing diets without selenium or vitamin E. Owing to the substantial difference between the necrogenic diets with casein or Torula, these experiments may give a better understanding of the relationship between vitamin E, selenium and protein intake.

EXPERIMENTAL

Diets. Two semipurified diets with 20% and 5% of casein, respectively, no vitamin E, and low selenium content as described by Bonetti and Stirpe (11) were used. The composition of the diets is shown in table 1. In some experiments the 20% casein diet was prepared with 5% of stripped lard in order to eliminate traces of vitamin E which might be present in the fats used for the original diet. Some groups of rats received α-tocopheryl acetate [5] (500 mg/kg of diet) or selenium (68 μg of selenium, as sodium selenite, per kg of diet). Control rats received the stock diet of the laboratory, composition in per cent: whole flour of mixed cereals (wheat, oats and barley), 79; crude casein, 8; half-skim milk (lipid content 12.5%), 5; dried wheat germ, 5; sodium chloride (common salt), 0.5; calcium lactate, 1.5; dried brewer's yeast, 1; raw ox liver and fresh vegetables were given once a week or a commercial diet [6] containing 20% of protein.[7] The stock diet contained 40 mg of total tocopherols/kg and 20.5 mg of α-tocopheryl acetate/kg.[8] The commercial diet contained 40 mg/kg of vitamin E.[9]

TABLE 1

Composition of semipurified diets [1]

	20% casein	5% casein
	%	%
Casein, fat-extracted [2]	20	5
Rice starch	35	40
Sucrose	35	45
Lard [3]	3	3
Salt mixture [4]	3	3
Vitamin mixture [5]	1	1
Olive oil with menadione (1 mg/g)	1	1
Cod liver oil	1	1

[1] Other diets were derived from the above, with the following modifications: 20% casein, stripped lard diet: as 20% casein, with 5% stripped lard (Distillation Products Industries, Rochester, N.Y.) instead of lard, olive oil and cod liver oil; menadione was added to the vitamin mixture, and 1.44 mg of vitamin A and 28.3 μg of vitamin D_2 were added/kg of diet as alcoholic solution; 5% casein without choline diet: as 5% casein, with choline omitted from vitamin mixture.

[2] Purchased from Laboratorio D.ri Piccioni, Brescia.

[3] Strutto (Ital.), lard that has been melted and filtered free of the solid residues after melting; supplied from S.P.A. Galbani, Melzo.

[4] Osborne and Mendel (12); mixture IV without lactose.

[5] Bonetti and Stirpe (11).

Animals. Male rats of the Wistar-Glaxo strain were used. The dietary treatment has been described previously (11). The selenium supplementation in the diet was started at birth (added to the diet given to mothers), and vitamin E was started at weaning. Rats fed the 20% casein diet were killed when 60 days old, and those fed the 5% casein diet were killed as soon as the first death due to liver necrosis occurred in each group. Chloroform intoxication was produced by injecting subcutaneously 0.2 ml of a mixture of chloroform and olive oil (1:1 v/v), 48 and 24 hours before killing.

Respiratory decline. Rats were decapitated, and the liver was quickly removed and washed in ice-cold isotonic phosphate buffer, pH 7.4. The respiratory decline was studied with slices as described by Chernick et al. (1), with homogenates (9) or with mitochondria (10). All experiments were carried out in duplicate. The values reported for oxygen uptake obtained with homogenates or mitochondria are those after subtraction of the oxygen uptake without substrate. The decline was calculated as described by the authors of the original observations.

Other determinations. Nitrogen was determined with the micro-Kjeldhal. The dialuric-acid induced hemolysis was determined as described by Rose and György (13).

RESULTS

The experiments were performed initially with liver homogenates and mitochondrial preparations, according to Corwin and Schwarz (9, 10). Succinate was used as the substrate, since these investigators reported that with this substrate decline occurs in both systems. Since the results obtained in this way were some-

[5] Ephynal Roche, generously contributed by Roche, S.P.A., Milan.

[6] Zoofarm, Padua.

[7] Manufacturer's data.

[8] Determinations kindly performed by Hoffmann-La Roche, Basle, Switzerland.

[9] See footnote 7.

what equivocal and difficult to interpret, liver slices were used in a second series of experiments.

Mitochondria and homogenates. Both determinations were carried out on the same liver of the same animals. The results obtained with mitochondria are reported in table 2. The greatest respiratory decline was observed in rats fed the stock diet or the commercial diet (− 20% and − 32%, respectively). In all other groups, including the vitamin E-deficient groups, decline was never greater than − 20% and was prevented or reduced by addition of vitamin E or selenium to the diet, with statistical significance only when selenium was added to the 20% casein diet.

The results obtained with homogenates, and with succinate as substrate, are summarized in table 3. Respiratory decline was almost nil in rats fed the stock diet, but it was − 32% in rats fed the commercial diet. In three different groups of rats fed the 20% casein diet, the decline

TABLE 2

Oxidation of succinate by rat-liver mitochondria [1]

Group [2]	Diet	Oxygen consumption [3]		Variation [4]	Statistical significance	
		0–30 min	30–60 min		P value	Reference group
				%		
1 (8)	Stock	84 ± 5.7	66 ± 3.6	−20 ± 3.45		
2 (7)	Commercial [5]	70 ± 9.7	46 ± 5.4	−32 ± 6.91		
3 (4)	Commercial [5] + vitamin E	67 ± 5.6	58 ± 2.4	−13 ± 4.1	< 0.10	1
4 (6)	Commercial [5]; chloroform-intoxicated	53 ± 4.7	50 ± 10.1	+16 ± 23.25		
5 (19)	20% Casein	47 ± 3.8	42 ± 3.5	−12 ± 4.48	< 0.30	7
6 (6)	20% Casein, stripped lard	34 ± 3.1	28 ± 6.1	−20 ± 13.70	< 0.30 < 0.10	7 8
7 (6)	20% Casein + vitamin E	61 ± 16.4	53 ± 10.1	+ 3 ± 17.00		
8 (6)	20% Casein + Se	26 ± 6.0	27 ± 4.7	+11 ± 10.75	< 0.05	5
9 (4)	20% Casein + vitamin E; chloroform-intoxicated	65 ± 0.4	65 ± 6.4	± 0 ± 9.10		
10 (6)	5% Casein	64 ± 5.8	53 ± 6.3	−18 ± 4.08	< 0.20	11
11 (7)	5% Casein + vitamin E	31 ± 5.2	31 ± 7.4	− 5 ± 8.15		
12 (6)	5% Casein + Se	54 ± 7.1	52 ± 5.3	± 0 ± 15.08	< 0.30	10
13 (4)	5% Casein + vitamin E; without choline	28 ± 6.1	24 ± 5.6	−14 ± 2.75		

[1] Each flask contained: 50 μmoles of Tris (hydroxymethyl)-aminomethane buffer, pH 7.4, 4 μmoles of $MgCl_2$, 14 μmoles of NaF, 30 μmoles of sodium succinate, 3 μmoles of NAD, and 0.8 ml of mitochondrial suspension (mitochondria from 200 mg of liver/ml) in 0.25 M sucrose, in a final volume of 3 ml. The center well contained 0.2 ml of 20% NaOH. Oxygen uptake was measured at 30°, with air as gas phase, after 10 minutes' equilibration.

[2] The numbers of rats of each group are given in parentheses.

[3] μl O_2/mitochondria from 200 mg of liver. Averages ± SE of mean.

[4] Calculated by the formula of Corwin and Schwarz (10):

$$\frac{(O_2 \text{ consumption } 0\text{–}30 \text{ min}) - (O_2 \text{ consumption } 30\text{–}60 \text{ min})}{(O_2 \text{ consumption } 0\text{–}30 \text{ min})} \times 100.$$

Averages of individual variations ± SE of mean.

[5] Zoofarm, Padua.

TABLE 3

Oxidation of succinate by rat-liver homogenates [1]

Group [2]	Diet	Oxygen consumption			Variation [3]	Statistical significance	
		0–30 min	30–60 min	60–90 min		*P* value	Reference group
		μliters O_2/mg of tissue			%		
1 (10)	Stock	95 ± 13.0 [4]	94 ± 9.5	76 ± 6.4	− 7 ± 13.7		
2 (7)	Commercial [5]	97 ± 6.0	87 ± 7.8	67 ± 4.8	−32 ± 3.38		
3 (6)	Commercial [5] + vitamin E	81 ± 8.4	83 ± 11.4	76 ± 6.5	− 2 ± 10.87	< 0.02	2
4 (6)	Commercial [5]; chloroform-intoxicated	106 ± 5.1	106 ± 6.8	82 ± 2.4	−21 ± 5.91		
5 (19)	20% Casein	93 + 5.9	88 ± 5.8	74 ± 4.6	−15 ± 6.66	< 0.20	7
6 (6)	20% Casein, stripped lard	93 ± 6.5	92 ± 5.6	86 ± 4.1	− 7 ± 3.92	< 0.30	7
7 (6)	20% Casein + vitamin E	90 ± 7.2	118 ± 6.4	96 ± 2.9	+ 6 ± 10.33		
8 (6)	20% Casein + Se	99 ± 8.9	79 ± 4.2	80 ± 4.2	−16 ± 9.16	< 0.20	7
9 (6)	20% Casein + vitamin E; chloroform-intoxicated	130 ± 6.0	117 ± 7.9	96 ± 3.7	−25 ± 5.54	< 0.02	7
10 (6)	5% Casein	124 ± 7.2	78 ± 8.0	62 ± 4.8	−50 ± 3.71	< 0.001 < 0.01	11 5
11 (7)	5% Casein + vitamin E	85 ± 7.1	90 ± 10.1	82 ± 5.9	− 2 ± 6.80		
12 (6)	5% Casein + Se	97 ± 6.7	72 ± 9.3	60 ± 11.4	−39 ± 8.75	< 0.01	11
13 (4)	5% Casein + vitamin E without choline	72 ± 1.8	69 ± 10.0	74 ± 4.1	+ 3 ± 5.80		

[1] Each flask contained: 300 μmoles of NaCl, 12 μmoles of KCl, 4 μmoles of $MgSO_4$, 40 μmoles of sodium phosphate buffer, pH 7.4, 60 μmoles of sodium succinate, and 0.5 ml of liver homogenate (1 g of liver + 9 ml of 0.25 M sucrose), in a final volume of 3 ml. Flasks were equilibrated for 15 minutes at 37° before readings. Other details as in table 2.

[2] The numbers of rats of each group are given in parentheses.

[3] Calculated by the formula of Corwin and Schwarz (9):

$$\frac{(O_2 \text{ consumption } 0\text{–}30 \text{ min}) - (O_2 \text{ consumption } 60\text{–}90 \text{ min})}{(O_2 \text{ consumption } 0\text{–}30 \text{ min})} \times 100.$$

Averages of individual variations ± SE of mean.

[4] Averages ± SE of mean.

[5] Zoofarm, Padua.

was − 41, − 4.5 and ± 0%, respectively. Mortality due to liver necrosis in the same groups was 1/14, 0/8 and 2/8, respectively; these results have been pooled in a single group in table 3. The respiratory decline was only − 7% in a group of rats fed the 20% casein diet prepared with stripped lard and consequently almost completely free of vitamin E. Mortality in this group was 0/8. The dialuric-acid hemolysis test was consistently positive in determinations made at random on some animals of these 4 groups.

Decline was marked in rats fed the 5% casein diet. The addition of selenite to the various diets was practically ineffective, whereas the addition of vitamin E prevented decline in all cases. The effect of vitamin E was statistically significant only when the differences were more marked, as in the case of the low protein diet. No decline was observed in the homogenates from rats fed the diet without choline, which had a considerably fatty liver. Chloroform intoxication caused a significant decline even in rats receiving the vitamin E-supplemented diet. There is a rough correlation between the initial oxygen uptake (i.e., the oxidation of succinate in the first 30 minutes of incubation) and respiratory decline, which appeared to be more marked in those groups showing a higher initial oxygen uptake.

Slices. The results obtained with homogenates and particularly with mitochondria were not consistent, and sometimes were not in agreement with those reported by Corwin and Schwarz (9, 10). This led to a study of respiratory decline in our animals with the slice technique of Chernick et al. (1) (table 4). Practically no decline was observed in rats fed the stock or the commercial diet. A very marked decline occurred in rats fed the 20% and 5% casein diets, which was reduced by dietary vitamin E, whereas selenite was completely ineffective. The effect of vitamin E was statistically significant in rats fed the 20% casein diet, but not in those fed the 5% casein diet, although the variation was in the expected direction in the latter case. No decline was observed in the chloroform-intoxicated rats.

DISCUSSION

The results will be discussed according to the experimental system used (mitochondria, homogenates or slices).

With mitochondria, the decline observed in preparations from rats fed the stock and commercial diets was equal to or even greater than that observed in rats fed the vitamin E-deficient diets with 20% or 5% casein. This is not in agreement with the conclusions of the Schwarz group (5, 7, 10) [10,11] that respiratory decline is specific of the pre-necrotic phase of avitaminosis E. According to these investigators (10) mitochondria from rats fed the vitamin E-supplemented Torula diet show decline of − 19%. Unfortunately they do not report data obtained with mitochondria from rats fed a normal, complete and well-balanced diet. In our experiments the addition of selenium to the 20% and 5% casein diets appeared to prevent decline in mitochondria, although the difference is not statistically significant in the case of the 5% casein diet and is at the limit of significance in the case of the 20% casein diet.

Different results were obtained with the homogenates of the same livers used for preparing mitochondria. Maximal decline was observed in rats fed the 5% casein diet. The inconsistent response of 4 groups of rats fed the 20% casein diet is rather difficult to explain, and a further complication is the lack of correlation between the extent of decline and the mortality observed in these groups. The comparison between rats fed the 20% casein diet and those fed the 5% casein diet appears to indicate that protein deficiency may at least aggravate respiratory decline in homogenates.

Respiratory decline occurs also in rats fed the nonpurified diets (commercial) [12] and in rats intoxicated with chloroform. These data, and the fact that protection is afforded by vitamin E but not by selenium, again raise the question as to whether respiratory decline of homogenates could

[10] Schwarz, K. 1961 Nutritional significance of selenium (Factor 3). Federation Proc., 20: 666 (abstract).

[11] Schwarz, K., and W. Mertz 1960 Physiological effects of trace amounts of selenium. Proc. Conference on Physiological Aspects of Water Quality, Washington, p. 79.

[12] Zoofarm.

TABLE 4

Oxygen consumption of rat-liver slices [1]

Group [2]	Diet	Oxygen consumption				Variation [3]	Statistical significance	
		0–30 min	30–60 min	60–90 min	90–120 min		*P* value	Reference group
		µliters O_2/mg N				%		
1 (8)	Stock	35 ± 1.5 [4]	30 ± 1.9	28 ± 1.9	29 ± 1.6	−14 ± 5.53		
2 (6)	Commercial [5]	27 ± 2.5	29 ± 1.5	27 ± 1.0	28 ± 1.9	+ 4 ± 7.04		
3 (4)	Commercial [5] + vitamin E	31 ± 3.5	32 ± 1.6	30 ± 2.7	31 ± 1.8	+ 2 ± 10.20		
4 (6)	Commercial [5]; chloroform-intoxicated	21 ± 1.6	25 ± 1.4	24 ± 2.6	20 ± 0.8	− 3 ± 6.54		
5 (6)	20% Casein	29 ± 1.1	22 ± 1.5	14 ± 2.9	12 ± 2.2	−59 ± 6.83	< 0.001	6
6 (6)	20% Casein + vitamin E	25 ± 1.2	24 ± 1.0	26 ± 1.8	20 ± 1.4	−21 ± 3.79		
7 (5)	20% Casein + Se	29 ± 2.6	20 ± 3.0	17 ± 2.8	12 ± 1.4	−56 ± 11.00	< 0.01	6
8 (3)	5% Casein	20 ± 2.0	12 ± 0.4	11 ± 0.9	7 ± 2.7	−61 ± 16.00	< 0.20	9
9 (6)	5% Casein + vitamin E	15 ± 1.9	11 ± 1.4	11 ± 1.7	10 ± 1.7	−27 ± 13.54		
10 (5)	5% Casein + Se	19 ± 3.5	16 ± 2.4	13 ± 2.9	8 ± 1.3	−56 ± 4.04	< 0.10	9

[1] Each flask contained about 100 mg of liver slices in 3 ml of pre-oxygenated Krebs-Ringer-phosphate buffer, pH 7.4 (Umbreit et al. (14)), containing 0.01 M glucose, and 0.2 ml of 20% NaOH in the center well. The manometers were filled with oxygen, and were equilibrated for 10 minutes at 37°, before readings.

[2] The numbers of rats of each group are given in parentheses.

[3] Calculated by the formula of Chernick et al. (1):

$$\frac{(O_2 \text{ consumption } 0\text{–}30 \text{ min}) - (O_2 \text{ consumption } 90\text{–}120 \text{ min})}{(O_2 \text{ consumption } 0\text{–}30 \text{ min})} \times 100.$$

Averages of individual variations ± SE of mean.

[4] Averages ± SE of mean.

[5] Zoofarm, Padua.

be considered as a specific phenomenon of the prenecrotic phase of avitaminosis E, as postulated by Schwarz and co-workers (5, 7, 10),[13] or whether it is an unspecific consequence of several liver-damaging causes, or even a "physiological" phenomenon, relieved only by large quantities of vitamin E. This last possibility is supported by the occurrence of decline in rats fed commercial diets, like the one used in our experiments, or the commercial laboratory ration [14] used by Schwarz, since all these diets support normal growth rate and fertility and therefore contain an amount of vitamin E which is adequate for physiological needs.

The results obtained with slices are generally in agreement with those of Chernick et al. (1). The lack of protection by selenium may indicate that the preparations of Factor 3 used by Chernick et al. (1) afforded protection possibly because they were contaminated by some substance(s) other than selenium.

A comparison between the results obtained with slices, homogenates and mitochondria shows the lack of a definite correlation between the "decline" in the different preparations, even when they were obtained from the same animals, as was the case for homogenates and mitochondria. This poses the question whether "respiratory decline" of different preparations can be considered a well-defined alteration, i.e., whether the inability to maintain oxygen consumption is due to the same cause in all cases and whether the systems used for the study of the respiration are adequate, and particularly in the case of mitochondria and homogenates.

Recently Grove et al. (15) reported that respiratory decline is observable, with succinate as substrate, in liver homogenates of both vitamin E-deficient and vitamin E-supplemented rats fed a Torula diet. They observed also that decline was less severe when the concentration of succinate was higher, thus causing a higher oxygen uptake. Our results, obtained at a fixed concentration of succinate, indicate that decline is more severe when the initial oxygen uptake is higher. Although no explanation is possible at present, our results appear to indicate that decline is not correlated with absolute oxygen consumption, but rather with the oxidative capacity of the tissue.

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LITERATURE CITED

1. Chernick, S. S., J. G. Moe, G. P. Rodnan and K. Schwarz 1955 A metabolic lesion in dietary necrotic liver degeneration. J. Biol. Chem. *217:* 829.
2. Rodnan, G. P., S. S. Chernick and K. Schwarz 1956 Reversal of respiratory decline in necrotic liver degeneration by intraportal tocopherols. J. Biol. Chem., *221:* 231.
3. Green, J., E. E. Edwin, J. Bunyan and A. I. Diplock 1960 Studies on vitamin E. II. Reversal of respiratory decline in nutritional liver necrosis by intraportal administration of tocopherols and other substances. Biochem. J., *75:* 456.
4. Schwarz, K., W. Mertz and E. J. Simon 1959 In vitro effect of tocopherol metabolites on respiratory decline in dietary necrotic liver degeneration. Biochim. Biophys. Acta, *32:* 484.
5. Mertz, W., and K. Schwarz 1958 Reversal of respiratory decline in necrotic liver degeneration by intraportal antioxidants. Proc. Soc. Exp. Biol. Med., *98:* 808.
6. Mertz, W., and K. Schwarz 1959 Prevention of respiratory decline in necrotic liver degeneration by antioxidants in vitro. Proc. Soc. Exp. Biol. Med., *102:* 561.
7. Schwarz, K. 1962 Vitamin E trace elements and sulfhydryl groups in respiratory decline. Vitamins Hormones, *20:* 463.
8. Corwin, L. M., and K. Schwarz 1960 Maintenance of α-ketoglutarate and succinate oxidation in vitamin E-deficient liver homogenates by α-tocopherol, a tocopherol metabolite, menadione and diphenylphenylenediamine. Nature, *186:* 1048.
9. Corwin, L. M., and K. Schwarz 1960 Prevention of decline of α-ketoglutarate and succinate oxidation in vitamin E-deficient rat liver homogenates. J. Biol. Chem., *235:* 3387.
10. Corwin, L. M., and K. Schwarz 1959 An effect of vitamin E on the regulation of succinate oxidation in rat liver mitochondria. J. Biol. Chem., *234:* 191.
11. Bonetti, E., and F. Stirpe 1962 Conditions affecting dietary liver necrosis and liver regeneration in rats. J. Nutr., *77:* 179.
12. Osborne, T. B., and L. B. Mendel 1918 The inorganic elements in nutrition. J. Biol. Chem., *34:* 131.
13. Rose, C. S., and P. György 1952 Specificity of hemolytic reaction in vitamin E-defi-

[13] See footnotes 10 and 11.
[14] Purina.

cient erythrocytes. Amer. J. Physiol., *168:* 414.

14. Umbreit, W. W., R. H. Burris and J. F. Stauffer 1957 Manometric Techniques, ed. 3. Burgess Publishing Company, Minneapolis, p. 149.
15. Grove, J. A., M. R. Johnson and J. N. Cline 1965 Vitamin E deficiency in the rat. I. Effect of substrate concentration on respiratory decline in liver homogenates from rats fed a vitamin E-deficient diet. Arch. Biochem. Biophys., *110:* 357.

In vivo Interactions of Cadmium, Copper, Zinc and Iron in the Mouse and Rat [1,2]

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ABSTRACT Certain chemical parameters of the elements, copper, zinc, cadmium, and iron and the interactions of these elements were used to obtain insight on their absorption and utilization. Three experiments were conducted. In experiment 1, rats were subjected to a copper depletion pretreatment (pretreatment 1). Mice that received a normal diet in the pre-experimental period (pretreatment 2) were used in experiment 2, and both pretreatments were used with mice in experiment 3. Cadmium decreased gains and hemoglobin levels significantly in all experiments. With pretreatment 1, the combination of copper and zinc largely overcame the adverse effects of cadmium for rats and mice. In pretreatment 2, however, supplemental copper and zinc did not overcome the depressing effect of cadmium. Another pretreatment effect was observed with dietary cadmium. This element increased zinc concentrations in the liver and testes more markedly in pretreatment 2 than in pretreatment 1. Dietary zinc increased liver iron concentration in all cases.

In previous publications (1, 2) studies were reported of trace element interactions utilizing the chick and based on certain chemical parameters, coordination numbers, chelate configuration and isoelectronic valency shell. These concepts were utilized in the present study with the rat and mouse. The basic plan was to use interacting effects of cadmium, copper, zinc, and iron to obtain some insight on the metabolism of the essential elements involved. As in the previous report (1), in order to minimize the difficulty in interpretation encountered when excessive dietary levels of trace elements are utilized, the levels of the three essential elements in these studies were held at relatively "physiological" concentrations.

EXPERIMENTAL

Three experiments were conducted. The first experiment was conducted with rats (Holtzman strain); the second and third, with mice.[3]

In experiment 1, the rats were subjected to a pretreatment period in order to deplete their copper stores. Eighty preweaned male rats, approximately 14 days old and averaging 35 g, were distributed among 10 lactating females. Each female and its allotted 8 young were housed in individual polypropylene cages with fluffed cotton-linter cellulose bedding. During this 2-week pretreatment period, the dam and young were offered diet 1 (table 1) and fresh cow's milk ad libitum. At the end of this 2-week period, 64 of the young were weaned, divided into 8 weight groups, and the rats in each weight group were allotted at random to the 8 diets (table 1). As shown in table 2, the variables were copper, zinc, and cadmium added to the diet singly and in combination, in a 3 × 2 factorial design.

In the second experiment, 64 mice were placed on experiment directly after weaning. During the preweaning period, commercial laboratory ration [4] was available to the mice at all times. Diets and handling procedures were similar to those of experiment 1.

In experiment 3, one-half of the mice were subjected to the same pretreatment as the rats in experiment 1, and the other half were treated as the mice in experi-

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[3] Mice were obtained from W. B. Rogers at the North Carolina State Hygiene Laboratory, Raleigh, North Carolina.

[4] Purina Chow, Ralston Purina Company, St. Louis.

TABLE 1
Basal diet

	%
Casein	25.0
Glucose [1]	37.2
Starch	23.0
Hydrogenated vegetable fat [2]	4.0
$CaCO_3$	1.0
$CaHPO_4$	1.8
Vitamin mix [3]	5.0
Mineral mix [4]	3.0

[1] Crystalline glucose. Corn Products Sales Company, Norfolk, Virginia.
[2] Primex B and C (pure vegetable shortening), Procter and Gamble Company, Cincinnati.
[3] Matrone et al., J. Nutr., 86: 155, 1965.
[4] Mineral mixture for 1 kg of diet: KCl, 6.0132 g; NaCl, 5.2643 g; $MgSO_4$, 4.4933 g; glucose monohydrate (Cerelose), 13.8615 g minus adjustments for trace element variations; $MnSO_4 \cdot H_2O$, 0.0308 g; $CoCO_3$, 0.0002 g; KI, 0.0001 g; $FeSO_4 \cdot 2H_2O$, 0.3366 g (diet 1 or basal); diets 2, 5, 6 and 8, basal + 0.0251 g $CuSO_4$; diets 3, 5, 7, and 8, basal + 0.2490 g (exp. 2) and 0.4979 g (exps. 1 and 3) ZnO; diets 4, 6, 7, and 8, basal + 0.3423 g 3 $CdSO_4 \cdot 8H_2O$.

ment 2 (pretreatment 2). The 64 male mice were allotted to diets in the same manner as in experiment 1.

Diets in all experiments contained approximately 100 ppm of iron by analysis. Diet 1 (table 1), which served as the basal diet, contained by analysis approximately 2 ppm copper and 9 ppm zinc. The levels of supplemental copper used were zero and 10 ppm, and of zinc were zero and 200 in experiment 2 or 400 in experiments 1 and 3. Cadmium levels of zero and 100 ppm were used in all experiments. Each experiment extended over 5 weeks.

Body weights and feed intakes were recorded weekly; however, the feed intakes were not used because of excess scattering of the diet. Hemoglobin measurements were determined by the method of Shenk et al. (3) on blood obtained from the tail. These samples were taken at the end of each experiment and, in addition, at the fourth week in experiment 1. The animals were decapitated, and the livers excised at the end of each experiment. In experiment 3, testes were also removed. Results are expressed on a dry-weight basis. For the copper, cadmium, zinc, and iron analyses, the tissues were wet-ashed with nitric and perchloric acids, and analyses were made with a Perkin Elmer Model 303 Atomic Absorption Spectrophotometer.

All data were subjected to an analysis of variance (4). Statements of difference for overall effects in the factorial analyses are based on the F test of the analysis of variance. Least significant differences (LSD) have been included in the table for comparison of differences between 2 treatment means.

RESULTS AND DISCUSSION

Results of these studies are presented in table 2. In agreement with results obtained with chicks (1), cadmium decreased gains ($P \leq 0.01$) and hemoglobin ($P \leq 0.05$) in all experiments. In pretreatment 1, the combination of copper and zinc (diet 8, table 2) largely overcame the adverse cadmium effect for both rats and mice. These results are in accordance with those reported for chicks. However, in pretreatment 2, supplemental copper and zinc did not overcome the depressing effect of cadmium. Considering only the diets to which cadmium had been added, since cadmium is not a normal dietary constituent, it is significant to note that the pretreatments apparently affected liver cadmium concentration in an inverse manner; i.e., the rats or mice subjected to pretreatment 1, which were fed diet 8, had lower levels of liver cadmium than those fed diet 4; the reverse is true in pretreatment 2. This may offer a partial explanation for the difference in pretreatment effects on gain and hemoglobin noted above.

In rats, but not in mice, both cadmium and zinc lowered the liver copper concentration. In the mice, however, neither zinc nor cadmium alone appreciably affected liver concentrations of copper. No explanation for this discrepancy is offered except that it is presumed that species differences are involved.

As shown in table 2, the overall effect of supplemental dietary cadmium was to increase zinc concentration of the liver and testes. Analysis of the zinc data in experiment 3, however, indicated a highly significant difference ($P \leq 0.01$) between pretreatments. Cadmium increased liver and testes zinc to a greater extent in pretreatment 2 than in pretreatment 1. It is pertinent to note that in treatments where cadmium was not added to the diet the liver zinc concentration at the end of the experiment was the same regardless of pretreatment. The results showing an

TABLE 2

Results of experiments [1] *1, 2, and 3*

	Experiment 1		Experiment 2		Experiment 3			
	Pretreatment 1 [2]		Pretreatment 2 [3]		Pretreatment 1		Pretreatment 2	
	Without Cd	With Cd	Without Cd	With Cd	Without Cd	With Cd	Without Cd	With Cd
				Gains, g				
O	125.2(1)[4]	59.1(4)	14.0(1)	5.1(4)	11.5(1)	5.6(4)	10.9(1)	5.4(4)
Cu	140.2(2)	79.4(6)	15.4(2)	10.4(6)	14.9(2)	8.6(6)	14.2(2)	2.6(6)
Zn	147.9(3)	70.6(7)	11.1(3)	7.2(7)	12.9(3)	5.8(7)	9.0(3)	5.6(7)
CuZn	136.9(5)	114.5(8)	16.6(5)	7.1(8)	13.3(5)	10.6(8)	14.2(5)	6.0(8)
LSD [5]	39.4		4.3		4.0			
				Hemoglobin, g/100 ml				
O	13.8(1)	5.4(4)	13.4(1)	7.9(4)	14.5(1)	5.9(4)	13.6(1)	4.9(4)
Cu	13.9(2)	7.9(6)	14.0(2)	9.1(6)	14.2(2)	10.9(6)	14.0(2)	7.5(6)
Zn	13.4(3)	5.9(7)	13.0(3)	5.1(7)	11.2(3)	5.9(7)	8.9(3)	2.8(7)
CuZn	13.9(5)	10.9(8)	14.7(5)	7.0(8)	14.4(5)	12.4(8)	13.5(5)	9.2(8)
LSD	1.1		4.2		1.5			
				Liver copper, ppm				
O	11.0(1)	2.9(4)	12.1(1)	12.8(4)	11.1(1)	11.0(4)	8.5(1)	6.7(4)
Cu	17.7(2)	14.2(6)	13.1(2)	14.6(6)	14.2(2)	14.6(6)	13.6(2)	17.6(6)
Zn	8.4(3)	3.1(7)	12.5(3)	8.1(7)	9.1(3)	11.4(7)	5.4(3)	6.2(7)
CuZn	13.6(5)	12.6(8)	15.1(5)	15.0(8)	15.5(5)	12.9(8)	12.9(5)	18.1(8)
LSD	2.2		4.2		8.1			
				Liver zinc, ppm				
O	92.1(1)	82.7(4)	87.0(1)	196.8(4)	75.0(1)	150.7(4)	78.2(1)	114.2(4)
Cu	66.6(2)	92.6(6)	84.0(2)	160.0(6)	71.2(2)	107.0(6)	85.5(2)	191.0(6)
Zn	85.3(3)	94.6(7)	91.8(3)	194.6(7)	85.4(3)	144.0(7)	72.1(3)	193.4(7)
CuZn	66.4(5)	100.7(8)	81.7(5)	209.7(8)	71.1(5)	108.5(8)	104.9(5)	194.6(8)
LSD	36.4		23.7		68.1			
				Liver cadmium, ppm				
O	0.4(1)	77.6(4)	1.2(1)	205.6(4)	1.9(1)	152.6(4)	1.9(1)	133.0(4)
Cu	0.6(2)	101.2(6)	1.3(2)	174.5(6)	1.1(2)	124.5(6)	1.9(2)	172.8(6)
Zn	0.5(3)	74.2(7)	1.0(3)	182.0(7)	2.5(3)	105.8(7)	2.5(3)	105.9(7)
CuZn	1.8(5)	68.3(8)	0.7(5)	219.4(8)	0.7(5)	106.5(8)	1.2(5)	152.7(8)
LSD	16.6		34.0		44.1			
				Liver iron, ppm				
O	391.4(1)	244.2(4)	507.0(1)	261.6(4)	312.4(1)	476.8(4)	363.2(1)	246.4(4)
Cu	185.1(2)	109.1(6)	387.4(2)	294.4(6)	344.6(2)	116.8(6)	289.2(2)	163.2(6)
Zn	456.7(3)	275.7(7)	740.4(3)	370.2(7)	760.3(3)	502.4(7)	558.0(3)	472.9(7)
CuZn	185.6(5)	129.5(8)	420.2(5)	179.5(8)	287.3(5)	290.9(8)	282.5(5)	150.1(8)
LSD	106.7		74.4		250.4			
				Testes zinc, ppm				
O					81.5(1)	125.4(4)	139.6(1)	146.3(4)
Cu					72.8(2)	92.4(6)	139.9(2)	216.8(6)
Zn					66.4(3)	141.4(7)	154.8(3)	265.7(7)
CuZn					110.0(5)	137.4(8)	137.0(5)	150.0(8)
LSD					86.8			

[1] Experiment 1 conducted with rats and experiments 2 and 3 with mice.
[2] Animals were subjected to a depletion period in which they were fed a purified diet low in copper.
[3] Animals were fed commercial laboratory ration (Purina Chow, Ralston Purina Company, St. Louis) during the pre-experimental period.
[4] Numbers in parentheses refer to diet numbers in table 1.
[5] Least significant difference ($P \leq 0.05$) between treatment means.

overall increase in liver zinc concentration by dietary cadmium are in agreement with isotope studies showing the accumulation of ^{65}Zn in livers of rats or mice where cadmium was administered orally or by injection (5, 6). These authors offer 2 possible explanations of this phenomena. Cadmium interferes in the normal excretion of zinc (5) or the zinc turnover rate is lowered by cadmium (6). A third possibility is that cadmium may also affect the absorption of zinc through the action of cadmium on sulfhydryl groups of the intestinal tract.[5]

The most interesting effect on liver cadmium, as shown in table 2, experiment 3, is the copper-by-pretreatment interaction. In pretreatment 2, dietary copper significantly ($P \leq 0.05$) increased liver cadmium; however, in pretreatment 1 of experiment 3, copper tended to decrease liver cadmium. This pattern is similar to that shown by liver zinc in this experiment.

As shown in table 2, the overall effect of cadmium was to lower liver iron. A striking result is the effect that zinc has in increasing liver iron. Further examination of the data of all experiments, disregarding differences in species and pretreatment, show that animals fed dietary copper have a lower level of liver iron. The effect of zinc on liver iron is noteworthy since other studies on zinc toxicity report the reverse effect, that is, that zinc decreased liver iron (7–9). Since much lower levels of zinc were used in the present experiment than those reported on in the literature (200–400 ppm versus 4000–7500 ppm), these data can be interpreted that either zinc increases iron absorption at low dietary levels and decreases iron absorption at high levels, or that zinc, depending on the level in the diet, may interfere in one or more steps in the hemopoietic system. Of these 2 alternatives, the latter appears more probable. For example, if the data in table 2 on liver iron are considered, omitting those where cadmium was added to the diets, it can be rationalized that when copper was omitted from the diet and when zinc without copper was added to the diet, iron accumulated in the liver, because the copper concentration was inadequate for the normal mobilization and utilization of liver iron (10). The fact that liver iron was generally highest when zinc without copper was added to the diet can be explained by the antagonism of zinc to copper reported in zinc toxicity studies (1, 9). Explanation of the depressing effects of zinc on liver iron when toxic levels of zinc are fed, remains to be made. Recent work by Settlemire and Matrone[6] offers an explanation consistent with the hypothesis proposed herein. These investigators observed in zinc toxicity studies that the metabolic defect is a shortened red blood cell life span and a greater excretion of iron into the intestinal tract (11).[7] It is pertinent to note that in experiment 1, with rats, 400 ppm of zinc increased iron concentration in the liver but did not affect hemoglobin level, whereas, in experiment 3 with mice, 400 ppm affected both hemoglobin and liver iron accumulation, indicating a species difference. Species differences in terms of the effect of dietary levels of zinc have also been reported by Havini and Guggenheim (12).

It may be informative to evaluate the results of this study in the light of the chemical parameters discussed elsewhere (1, 2). Although the interacting effects of Cu, Cd, and Zn observed with rats and mice subjected to pretreatment 1 are similar to those reported for chicks (1, 2), discrepancies were observed with mice in pretreatment 2. Supplemental copper and zinc did not ameliorate the cadmium effect on growth as it did for rats and mice subjected to pretreatment 1, and mice fed cadmium in pretreatment 2 had a higher concentration of zinc in their liver than did those subjected to pretreatment 1. These differences between pretreatments suggest a modifying factor involving the preconditioning of the intestinal tract in relation to dietary cations. The fact that growth as well as cation concentration of the liver was affected suggests that absorption, per se, was also involved. It appears that the intestinal tracts conditioned to a normal or adequate level of essential trace elements, under the modi-

[5] Charles H. Hill 1966 Studies on the absorption and distribution of zinc in the chick. Federation Proc., 25: 483 (abstract).

[6] Settlemire, C. T., and G. Matrone 1966 Effect of high-zinc intake on life span of red blood cells and heme in the rat. Federation Proc., 25: 484 (abstract).

[7] See footnote 6.

fying influence of dietary cadmium, can absorb much more than those preconditioned to a depletion or low level of these cations.

From the chemical parameter viewpoint (1, 2) Zn^{++}, Cd^{++}, and Cu^{+} tend to form similar chelates and have similar affinities for ligands such as sulfhydryl groups, presumably present in intestinal as well as body tissues. These chelate affinities might well be the basis of mutual effects of these metal ions on liver or tissue turnover as well as on absorption. From this point of view, site of absorption must also be considered in evaluating interacting effects. For example, results with rats (13) show that copper, unlike cadmium and zinc, is absorbed to a large extent from the stomach rather than from the small intestine.

LITERATURE CITED

1. Hill, C. H., G. Matrone, W. L. Payne and C. W. Barber 1963 In vivo interactions of cadmium with copper, zinc, and iron. J. Nutr., *80:* 227.
2. Hill, C. H., B. Starcher and G. Matrone 1964 Mercury and silver interrelationship with copper. J. Nutr., *83:* 107.
3. Shenk, J. H., J. L. Hall and H. H. King 1934 Spectrophotometric characteristics of hemoglobins. I. Beef blood and muscle hemoglobins. J. Biol. Chem., *105:* 741.
4. Snedecor, G. W. 1946 Statistical Methods. The Iowa State College Press, Ames.
5. Gunn, S. A., T. C. Gould and W. A. D. Anderson 1962 Interference with fecal excretion of Zn^{65} by cadmium. Proc. Soc. Exp. Biol. Med., *111:* 559.
6. Cotzias, G. C., D. C. Borg and B. Selleck 1962 Specificity of zinc pathway through the body: turnover of Zn^{65} in the mouse. Amer. J. Physiol., 202: 359.
7. Cox, D. H., and D. L. Harris 1960 Effect of excess dietary zinc on iron and copper in the rat. J. Nutr., *70:* 514.
8. Magee, A. C., and S. Spar 1964 Effects of dietary supplements on young rats fed high levels of zinc. J. Nutr., *82:* 209.
9. Magee, A. C., and G. Matrone 1960 Studies on growth, copper metabolism of rats fed high levels of zinc. J. Nutr., 72: 233.
10. Gubler, C. J., M. E. Lahey, M. S. Chase, G. E. Cartwright and M. M. Wintrobe 1952 Studies on copper metabolism. III. The metabolism of iron in copper deficient swine. Blood, 7: 1075.
11. Carter, M. W., G. Matrone and C. Metzler 1965 Estimation of the life span of red blood cells in the growing animal in different nutritional states. J. Gen. Physiol., *49:* 57.
12. Havini, E., and K. Guggenheim 1965 Cytochrome oxidase in mice and rats maintained with a meat diet. J. Nutr., *86:* 303.
13. Van Campen, D. R. 1966 Effects of zinc, cadmium, silver, and mercury on the absorption and distribution of copper-64 in rats. J. Nutr., *88:* 125.

Changes in Rat Liver Cytochromes *b*, c_1, and c and Mitochondrial Protein in Prolonged Protein Deficiency

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ABSTRACT In protein deficiency, rat liver cytochromes *b*, c_1, and c and mitochondrial protein are lost from the cells to about 50% of normal after 8 weeks and thereafter decrease to 40% of normal after 14 weeks of depletion, at which time the animals become moribund from the deficiency. When protein is added back to the ration these components return almost linearly to normal within 8 days. That all of these components were lost at the same rate and responded at the same rate to protein repletion suggests that they are under the control of a single underlying regulator of mitochondrial metabolism. The presence of 0.3% DL-methionine in the protein-free ration appeared to protect against the loss of cytochromes *b* and c but not c_1.

In earlier papers of the present series (1–3), studies on the response of individual components of the mitochondrial electron transport system to protein depletion have been reported. In the first study (1) the response of succinic dehydrogenase, the "antimycin A-sensitive factor," which is a measure of the rate of electron transport through cytochromes *b* and c_1 using succinate as substrate, and cytochrome c were investigated. At that time no quantitative method existed for cytochrome *b* or c_1 per se. Also, it was subsequently found by us that the method used for cytochrome c which involved isolating cytochrome c and measuring the product spectrophotometrically (4), was quite inaccurate. This was due to the fact that, under the conditions used and with the minute amounts of cytochrome c observed in the necessarily small portions of liver used, cytochrome c was incompletely precipitated by trichloroacetic acid. The degree of precipitation was also found to be erratic as observed in recovery studies. With the development of a quantitative method for measuring simultaneously the cytochromes in rat liver mitochondria (5), all of the individual cytochrome components of the electron-transport system could be followed conveniently and accurately during protein depletion followed by repletion.

It has been observed in previous studies in which changes in various liver components were followed during protein depletion (2,3,6,7) that inclusion of 0.30% DL-methionine in the protein-free diet protected against the loss of succinic dehydrogenase, ubiquinone and phospholipids. However, cytochrome oxidase was not influenced by methionine under these conditions. In the present studies it was of interest to continue this investigation of the effect of methionine on the other cytochrome components of liver mitochondria to determine whether the lack of effect on cytochrome oxidase was general for mitochondrial cytochromes. This would make more complete the picture of the response of the individual components of the succinic oxidase system to prolonged protein depletion.

Thus far, with succinic dehydrogenase (1, 2) and cytochrome oxidase (3), previously reported evidence suggests that a common regulatory mechanism may exist which is involved in controlling the maintenance of mitochondrial enzyme systems under the stress of protein deficiency (3). In the present studies on cytochromes *b*, c_1 and c this speculation was further investigated.

EXPERIMENTAL METHODS

Adult male rats of the Sprague-Dawley strain, previously adjusted for 3 weeks to a complete purified diet (diet R 1) (8)

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were separated into 4 groups. Group 1 was fed the complete diet ad libitum; group 2 was pair-fed with group 4; group 3 was given diet R 1 with casein omitted; and group 4 was fed diet R 1 with both casein and methionine omitted. Briefly, diet R 1 consisted of: (in per cent) casein, 20; DL-methionine, 0.3; corn oil, 5; salts N plus molybdate (9), 6.5; glucose monohydrate, 63.5; choline chloride, 0.2; *i*-inositol, 0.02; and water-soluble vitamin mix in glucose monohydrate (1), 4.5. Fat-soluble vitamins (8) were given weekly to each rat in 2 drops of corn oil.

At various time-intervals as indicated in the figures under Results, 6 to 8 rats from groups 1, 2 and 4 were killed and liver mitochondria isolated by the method of Schneider and Hogeboom (11). Cytochromes b, c_1 and c were assayed by the method of Williams (5). Only one group of rats from group 3 was killed in these studies, after 56 days. The protein-depletion period for group 4 lasted for 100 days, at which time many of the remaining animals in group 4 became moribund from the protein deficiency. Thereupon casein was returned to the deficient ration, and more rats were killed after 1, 2, 3, 4, 8 and 13 days. Not enough rats remained in group 4 to obtain points at 13 days, however.

Liver DNA was extracted by the method of Schneider (12) and analyzed with diphenylamine (13). Mitochondrial protein was estimated by the method of Lowry et al. (14).

RESULTS

All of the results were calculated as micromoles of each cytochrome per unit weight of liver DNA. These values in turn were converted to percentage of the ad libitum-fed controls (group 1). The absolute values represented by the control value of 100% in each of the figures, respectively, were as follows: figure 1, 4.74 mμmoles of cytochrome b/mg liver DNA; figure 2, 2.97 mμmoles of cytochrome c_1/mg liver DNA; figure 3, 4.49 mμmoles of cytochrome c/mg liver DNA; figure 4, 15.2 mg of mitochondrial protein/mg liver DNA. Statistical analysis using Student's t test was used, and the results of this analysis are presented in the legend of each figure.

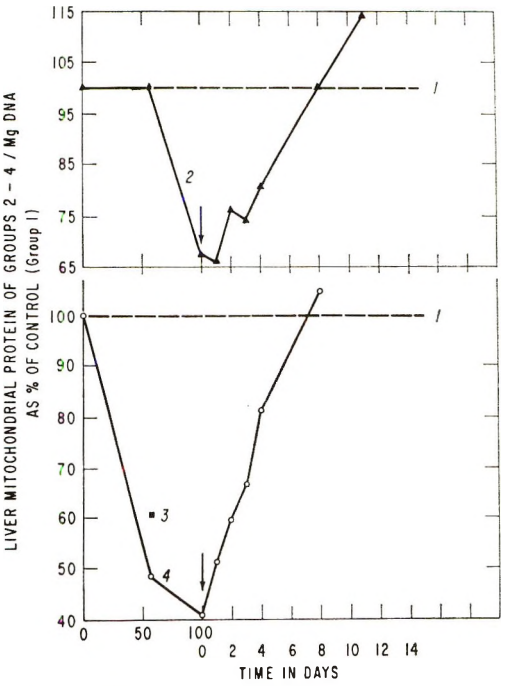

Fig. 1 The response of liver cytochrome b to protein depletion followed by repletion. Protein repletion was begun at the arrows. The horizontal dotted lines represent the ad libitum-fed controls (group 1); ▲ = group 2 (pair-fed controls); ■ = group 3 (protein-deficient rats fed 0.3% DL-methionine); ○ = group 4 (protein-deficient rats).

Statistical analysis using Student's t test indicated the following significant differences ($P < 0.01$) among the points in the figures: 56 days: 1 versus 4, 2 versus 4; 100 days: 1 versus 2, 1 versus 4, 2 versus 4; 1 day post-repletion: 1 versus 4, 1 versus 2. While $P > 0.01$ for group 3 versus 4, there was no overlapping of the limits of the standard errors of these 2 groups.

Cytochrome b in protein deficiency in the absence of added methionine (group 4) decreased to about 50% of normal after 56 days and then to 40% of normal after 100 days (fig. 1). Thus the major loss occurred over the first 8-week period. When 0.3% DL-methionine was present in the protein-free ration, some sparing action on cytochrome b was observed. The cytochrome b of the pair-fed controls did not change during the first 56-day period, but after 100 days it had decreased to about 60% of normal. A more rapid return of cytochrome b to normal occurred in the previously protein-deficient group than in the pair-fed control group. There appeared

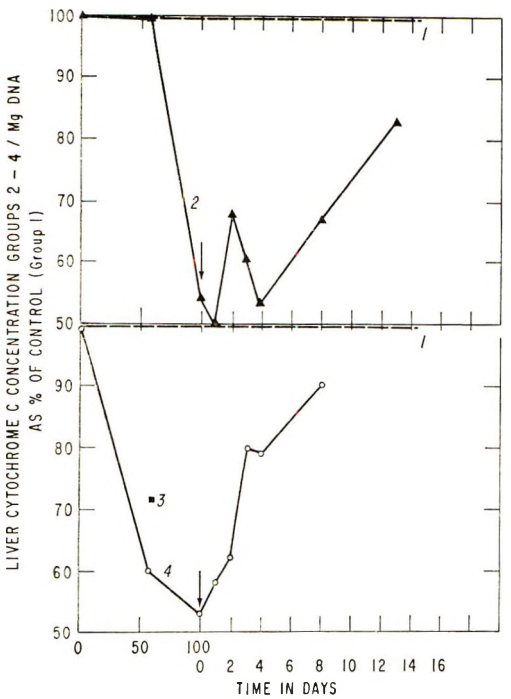

Fig. 2 The response of liver cytochrome c_1 to protein depletion followed by repletion. Protein repletion was begun at the arrows. The horizontal dotted lines represent the ad libitum-fed controls (group 1); ▲ = group 2 (pair-fed controls); ■ = group 3 (protein-deficient rats fed 0.3% DL-methionine); ○ = group 4 (protein-deficient rats).

Statistical analysis using Student's t test indicated the following significant differences ($P < 0.01$) among the points in the figures: 56 days: 1 versus 4, 2 versus 4; 100 days: 1 versus 4; 1 day post-repletion: 1 versus 4.

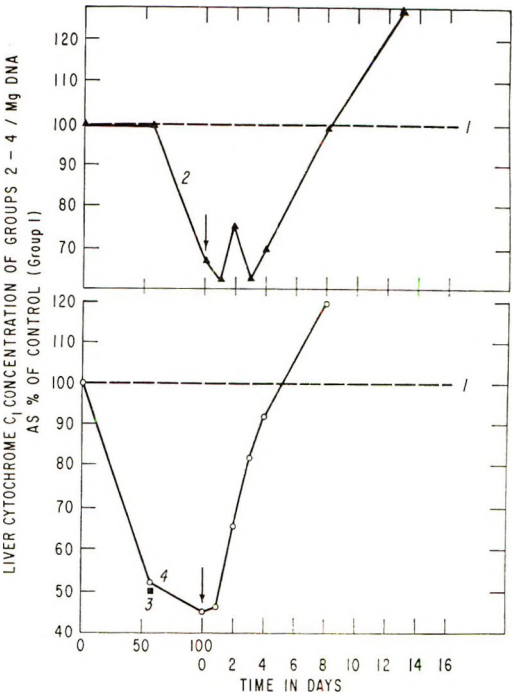

Fig. 3 The response of liver cytochrome c to protein depletion followed by repletion. Protein repletion was begun at the arrows. The horizontal dotted lines represent the ad libitum-fed controls (group 1); ▲ = group 2 (pair-fed controls); ■ = group 3 (protein-deficient rats fed 0.3% DL-methionine); ○ = group 4 (protein-deficient rats).

Statistical analysis using Student's t test indicated the following significant differences ($P < 0.01$) among the points in the figures: 56 days: 1 versus 4, 2 versus 4; 100 days: 1 versus 2, 1 versus 4; 1 day post-repletion: 1 versus 2, 1 versus 4; 2 days post-repletion: 1 versus 2, 1 versus 4; 3 days post-repletion: 1 versus 2; 4 days post-repletion: 1 versus 2.

While $P > 0.01$ for group 3 versus 4, there was no overlapping of the limits of the standard errors of these 2 groups.

to be a slight oscillation in the response of the pair-fed controls to repletion.

The results for cytochrome c_1 (fig. 2) were almost identical to those for cytochrome b. Again there was a loss in group 4 to 50% of normal after 56 days followed by a slight further loss to 45% of normal after 100 days. The pair-fed control value was unchanged after 56 days but decreased to about 65% of normal after 100 days. There was a marked lag with an oscillation in the return of the pair-fed controls to normal after beginning repletion; the previously protein-deficient group, however, returned to normal almost linearly after only a 1-day lag. There was no effect of methionine on cytochrome c_1.

With cytochrome c (fig. 3) the results for groups 2 and 4 were almost identical to those for cytochromes b and c_1. However, when methionine was present in the protein-free ration, a protective effect on cytochrome c was observed. Also the response of the pair-fed controls (group 2) to repletion followed a unique pattern in that not only a lag was observed but a pronounced oscillation occurred.

The results for mitochondrial protein (fig. 4) were almost precisely superimposable on those for cytochrome b within ex-

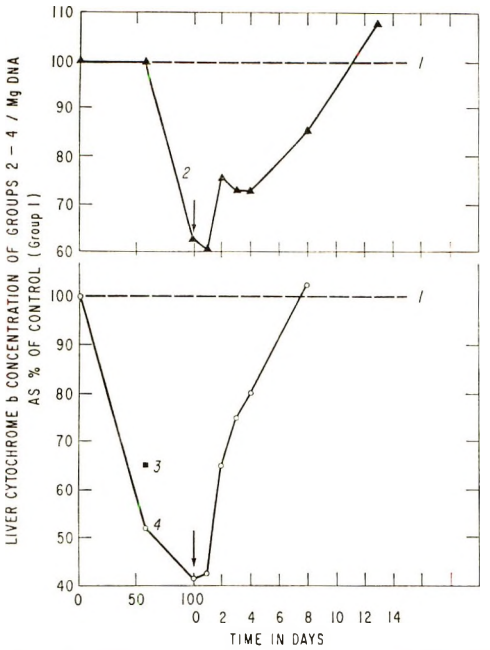

Fig. 4 The response of liver mitochondrial protein to protein depletion followed by repletion. Protein repletion was begun at the arrows. The horizontal dotted lines represent the ad libitum-fed controls (group 1); ▲ = group 2 (pair-fed controls); ■ = group 3 (protein-deficient rats fed 0.3% DL-methionine); ○ = group 4 (protein-deficient rats).

Statistical analysis using Student's t test indicated the following significant differences ($P < 0.01$) among the points in the figures: 56 days: 1 versus 4, 2 versus 4; 100 days: 1 versus 2, 1 versus 4, 2 versus 4; 1 day post-repletion: 1 versus 2, 1 versus 4; 2 days post-repletion: 1 versus 4; 3 days post-repletion: 1 versus 4. While $P > 0.01$ for group 3 versus 4, there was no overlapping of the limits of the standard errors of these 2 groups.

perimental error. There was protection of mitochondrial protein by methionine and the response of the previously protein-deficient group to repletion was considerably more rapid than that of the pair-fed controls. Again a slight oscillation in the pair-fed controls occurred during repletion.

DISCUSSION

If the net change in the number of intact mitochondria in protein deficiency is negative, then the similar losses in each of the cytochrome components would be expected. There is evidence which suggests that in the liver, mitochondria can reproduce by fission and subsequent growth (15). However, there is other evidence which suggests that if even this is the case, certain proteins (16) and lecithin (17) are synthesized outside the mitochondria and are subsequently incorporated into the mitochondrial structure. Beattie et al. (16) interpreted their studies on amino acid incorporation into mitochondrial proteins on the hypothesis that water-soluble proteins, which include cytochrome c, may be synthesized outside of the mitochondria and are subsequently incorporated into the mitochondria. Roodyn et al. (18) observed negligible labeling of soluble mitochondrial proteins as compared with insoluble mitochondrial proteins when isolated mitochondria were incubated with labeled amino acids. These types of results lead to the conclusion that insoluble proteins are synthesized in the mitochondria and other mitochondrial proteins are synthesized elsewhere and then incorporated into the mitochondria.

In the present studies, the marked similarities in the responses of cytochrome oxidase (3), cytochromes b, c_1, and c, succinic dehydrogenase (2), and mitochondrial phospholipid (3) are indicative of a common regulatory mechanism controlling the levels of these components under the stress of protein deficiency. That all of these components were lost at the same rate and responded at the same rate to protein repletion suggests that they are under the control of a single underlying regulator of enzyme maintenance in mitochondria. That cytochrome c responds almost identically to the insoluble cytochromes indicates that the underlying regulatory mechanism would control mitochondrial fission and concomitant mitochondrial synthesis of insoluble cytochromes as well as extramitochondrial synthesis of cytochrome c and incorporation into the mitochondria. Another argument that the similar responses in each of the mitochondrial enzymes to protein depletion is not due simply to a net loss of intact mitochondria is that methionine influences the maintenance of cytochromes b and c but has no effect on cytochrome c_1 or cytochrome oxidase (3). The effect of methionine is not large after 56 days of depletion and might have been shown to best advantage if that group had been killed after 100 days (cf. ref. 2). However, the effect undoubtedly

exists since it has been observed not only with cytochromes *b* and c but also with succinic dehydrogenase (2), ubiquinone (6), and phospholipids (7).

Since a lower limit of each of the enzymes studied to about 40% of normal is reached after 100 days, at which time the animals become moribund from the protein deficiency, then, in addition to the mechanism for regulating enzyme levels in mitochondria as discussed above, there must be a limiting conservation mechanism which prevents further loss even though the animals begin to die from the deficiency. It is of interest to speculate whether these "hard core" mitochondrial enzymes represent the amount of mitochondrial function which is absolutely essential for cellular survival and whether the 60% of the normal total which are lost represent a combination of excess mitochondrial enzymes plus enough enzyme concentration for normal cell maintenance and cell division.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. Williams, J. N., Jr. 1961 Response of the liver to prolonged protein depletion. II. The succinic oxidase system and its component enzymes. J. Nutr., *73:* 210.
2. Williams, J. N., Jr. 1964 Response of the liver to prolonged protein depletion. IV. Protection of succinic oxidase and succinic dehydrogenase by dietary methionine and cystine in a protein-free ration. J. Nutr., *82:* 51.
3. Williams, J. N., Jr., R. M. Jacobs and A. J. Hurlebaus 1966 Correlation of liver cytochrome oxidase activity with mitochondrial cytochrome oxidase and phospholipid concentrations in protein-deficient rats. J. Nutr., *90:* 81.
4. Rosenthal, O., and D. L. Drabkin 1943 Spectrophotometric studies. XI. The direct microspectrophotometric determination of cytochrome c. J. Biol. Chem., *149:* 437.
5. Williams, J. N., Jr., 1964 A method for the simultaneous quantitative estimation of cytochromes *a*, *b*, c_1, and c in mitochondria. Arch. Biochem. Biophys., *107:* 537.
6. Williams, J. N., Jr. 1963 Response of the liver to prolonged protein depletion. III. Coenzyme Q. Arch. Biochem. Biophysics, *101:* 512.
7. Williams, Jr., J. N., and A. J. Hurlebaus 1965 Response of the liver to prolonged protein depletion. VI. Total phospholipids and plasmalogens, and protection of phospholipids by methionine and cystine. J. Nutr., 85: 82.
8. Williams, J. N., Jr. 1961 Response of the liver to prolonged protein depletion. I. Liver weight, nitrogen and deoxyribonucleic acid. J. Nutr., *73:* 199.
9. Fox, M. R. S., and G. M. Briggs 1960 Salt mixtures for purified-type diets. III. An improved salt mixture for chicks. J. Nutr., *72:* 243.
10. Fox, M. R. S., L. O. Ortiz and G. M. Briggs 1955 Toxicity of ethionine in the young chick. Agr. Food Chem., *3:* 436.
11. Schneider, W. C., and G. H. Hogeboom 1950 Intracellular distribution of enzymes. V. Further studies on the distribution of cytochrome c in rat liver homogenates. J. Biol. Chem., *183:* 123.
12. Schneider, W. C. 1945 Phosphorus compounds in animal tissue. I. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. J. Biol. Chem., *161:* 293.
13. Davidson, J. N., and C. Waymouth 1944 Tissue nucleic acids. 3. The nucleic acid and nucleotide content of liver tissue. Biochem. J., 38: 379.
14. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall 1951 Protein measurement with the Folin phenol reagent. J. Biol. Chem., *193:* 265.
15. Bahr, G. F., and E. Zeitler 1962 Study of mitochondria in rat liver quantitative electron microscopy. J. Cell Biol., *15:* 489.
16. Beattie, D., R. Basford and S. Koritz 1966 Studies on the biosynthesis of mitochondrial protein components. Biochemistry, 5: 926.
17. Wilgram, G. F., and E. P. Kennedy 1963 Intracellular distribution of some enzyme catalyzing reactions in the biosynthesis of complex lipids. J. Biol. Chem., *238:* 2615.
18. Roodyn, D. B., J. W. Suttie and T. S. Work 1962 Protein synthesis in mitochondria. 2. Rate of incorporation of *in vitro* of radioactive amino acids into soluble proteins in the mitochondrial fraction, including catalase, malic dehydrogenase and cytochrome c. Biochem. J., *83:* 29.

Actinomycin D Inhibition of Vitamin D- and Dihydrotachysterol-induced Responses in the Chick [1]

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ABSTRACT Low doses of actinomycin D (2.0–2.5 μg/day for 14 days) administered intraperitoneally to chicks fed a rachitogenic diet did not cause any measurable deterioration of their rachitic state as assessed by serum calcium and phosphorus levels, percentage bone ash, growth, or bone weights. These observations suggest that there is a basal level of intestinal function and bone metabolism in rachitic chicks which is not inhibited by actinomycin D. The increased intestinal absorption of calcium above the basal level induced by oral antirachitic doses (2.5 μg/day) of vitamin D_3 or dihydrotachysterol$_2$ (DHT_2) was abolished by the above doses of actinomycin D. Bone ash values, however, were normal despite the actinomycin D treatment, although growth was retarded. Actinomycin D reduced the hyperstimulated calcium absorption and lowered the hypercalcemia evoked by the high dosages of vitamin D_3 or DHT_2 (250 μg/day). Various evidence suggests that certain bone effects of the sterols were relatively unaffected by actinomycin D. In birds receiving 2500 μg/day of vitamin D_3, oral ^{45}Ca was well absorbed into the blood but failed to deposit in bone; actinomycin D by inhibiting calcium absorption, caused a lowering of the hypercalcemia, but since bone uptake was negligible, the resultant serum calcium level was not as low as rachitic levels.

The antibiotic, actinomycin D, is thought to inhibit protein synthesis by binding primarily to the guanine residues in deoxyribonucleic acid (DNA) primer molecules, thus impeding DNA directed messenger-ribonucleic acid (mRNA) synthesis by obstructing the enzymic activity of RNA polymerase (1). Recently, actinomycin D has been found to inhibit parathyroid hormone-induced hypercalcemia in parathyroidectomized rats (2–4) and to inhibit the hypercalcemic effect of toxic doses of vitamin D in rats (5). Furthermore, both Norman (6) and Zull et al. (7) have indicated that vitamin D-stimulated intestinal calcium transport is inhibited by the antibiotic in chicks and rats, respectively. Harrison and Harrison (8), however, believe that the effect of actinomycin D in inhibiting intestinal calcium transport is on the transport system itself rather than only on the vitamin D-stimulated component. • Zull et al. (9) report that none of the vitamin D alterations in membrane permeability to calcium ions were affected by actinomycin D, but that vitamin D stimulation of active Ca^{++} transport in vitro and incorporation of ^{32}P into intestinal phospholipids was blocked by the antibiotic.

These various results indicate that protein synthesis may be required at some phase in the mechanism of vitamin D action. The possibilities are numerous; for example, vitamin D might initiate the production of a protein necessary for proper transport such as the calcium-binding protein studied by Wasserman (10, 11). Another possible mechanism is that synthesis of an enzyme might be necessary to activate the vitamin itself. The exact point in vitamin D and mineral metabolism in which protein synthesis is obligatory is still unresolved. The present study was conducted to examine in detail the degree of inhibition by actinomycin D of the effects in chicks of a wide dose range of vitamins D and dihydrotachysterol$_2$ (DHT_2) varying from low antirachitic levels to large toxic levels.

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METHODS

Two series of experiments were conducted with actinomycin D. Day-old male White Leghorn chicks (Mt. Hope Queens) were fed a rachitogenic diet [2] for 5 weeks with treatment occurring during the last 2 weeks of the period. In the first experimental series the crystalline sterols [3] were administered orally at the following daily dose levels: vitamin D_3, 0.25 and 100 μg (10 and 4000 IU); vitamin D_2, 2.5 and 1000 μg (100 and 40,000 IU); and dihydrotachysterol (DHT_2), 2.5 and 100 μg. There were 20 birds at each dose level, 10 receiving the sterol alone and 10 receiving the sterol plus 2.0 μg of actinomycin D [4] per day given intraperitoneally in saline. The control group for this first experimental series consisted of 10 birds maintained with the rachitogenic diet and intubated daily with 0.1 ml sesame oil and 10 birds treated the same way but in addition, injected intraperitoneally with 2.0 μg actinomycin D each day.

In the second experimental series vitamin D_2, vitamin D_3 and DHT_2 were administered at the following daily dose levels: 0.25, 2.5, 25, 250, 2500 μg (10–100,000 IU of the D vitamins). Each group given the sterols alone consisted of 8 birds; at each dose level of vitamin D_3 and DHT_2 another group of 8 birds was intubated with the sterol and injected intraperitoneally with 2.5 μg of actinomycin D. The inhibition of vitamin D_2 by the antibiotic was not studied in this second experiment but a vitamin D_2 dose-response curve was determined for comparative purposes. The control groups in this second experiment were the same as in the first except that the dose of actinomycin D was 2.5 μg instead of 2.0 μg.

Experimental procedures were essentially the same as used in previous work from this laboratory (12–14). Chicks were decapitated after the 5-week experimental period and the following parameters evaluated: serum calcium level, serum phosphorus level, percentage bone ash, extracted tibia weight, and final body weight. The data presented in the figures are given as mean values with standard errors indicated by vertical lines where applicable.

Bone analysis was performed on tibiae (taken from each chick, cleanly dissected and stored in a freezer until ready for extraction). Each tibia was broken in half and bones from each group were placed into a paper thimble and extracted in a Soxhlet apparatus for 48 hours with absolute ethanol and for an equal period of time with diethyl ether. The bones were then placed in a dessicator for 24 hours and weighed carefully. From this weight an average value was calculated for extracted tibia weight. The bones were then dried in platinum dishes at 100°, flamed over a Meker burner to burn off the major portion of volatile material and finally ashed in a muffle furnace at 750° for 16 hours. The percentage bone ash was calculated as:

$$\frac{\text{ash}}{\text{extracted}} \times 100.$$

Furthermore, in this study the appearance of ^{45}Ca in the serum and bone at different time intervals was measured following an oral dose of the isotope. ^{45}Ca (low specific activity 4.0 mCi/mg) [5] which had been previously purified chemically by oxalate precipitation was intubated orally at a concentration of 25 μCi in 0.2 ml of saline to each chick at zero time which was 24 hours prior to killing. Samples of blood (0.3 ml) were removed from different positions on each wing vein at 2 time periods following isotope administration. Due to the large number of birds being tested, the first time point ranged from 30 to 100 minutes and the second time point was 100 to 180 minutes. After centrifugation of samples, 100 μliters of serum were counted in 5 ml of scintillation fluid (15) in the Ansitron liquid scintillation counter.

In addition to these short-term measurements of blood levels of ^{45}Ca, samples

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[2] General Biochemicals Inc., Chagrin Falls, Ohio. This diet was composed of: (in %) alfalfa meal, 5.5; soybean meal, 15.4; bone meal, 4.4; yellow corn, 34.4; wheat flour middlings, 28.4; dried skim milk, 9.4; cottonseed oil, 1.0; iodized salt, 1.0; and bone charcoal, 0.5. Our lot of diet contained 1.43% calcium and 1.10% phosphorus by analysis (namely, a calcium-to-phosphorus ratio of 1:3).

[3] Crystalline vitamins D_2 and D_3 and DHT_2 were manufactured by N. V. Philips-Duphar, Weesp, Netherlands.

[4] Actinomycin D was kindly supplied by Merck, Sharp and Dohme Research Laboratories, Rahway, New Jersey.

[5] Tracerlab, Waltham, Massachusetts.

of serum were assayed as described at 24 hours after the ^{45}Ca administration. One tibia from each chick was removed at killing 24 hours after isotope administration, broken in half and extracted as described above. After ashing, the bones were ground finely in a mortar.

Fifty milligrams of ground bone ash from each tibia were weighed into a counting vial and dissolved in 0.5 ml of 1.8 N HNO_3. The samples were allowed to stand for several hours to ensure complete dissolution of the ash. Ten milliliters of counting fluid were then added to each vial and the radioactivity was assayed as above.

RESULTS

Actinomycin D (2.0 μg/day) vs. vitamin D_3, vitamin D_2 and DHT_2

Growth. Data presented in figure 1 (bottom) indicate that actinomycin D exerted growth-depressing effects on the sterol-treated chicks but not on the rachitic controls. If this decrease in final weight had been due to an action which would cause a decrease in potency of the sterol, then the actinomycin D-treated chicks given high doses of sterol would have been expected to give higher, rather than lower, weights when compared with their controls. The extracted bone weights also bear out this observation (fig. 1, middle). At every dose level, the bone weights were less in the actinomycin D-treated group than in the group treated with the sterol alone. However, no fatalities occurred which could be attributed to actinomycin D treatment.

Serum calcium levels. At each dose level of sterol, actinomycin D treatment resulted in a lowered serum calcium level (fig. 2, bottom). Actinomycin D inhibited the sterol-induced increase in the serum calcium at antirachitic dose levels of

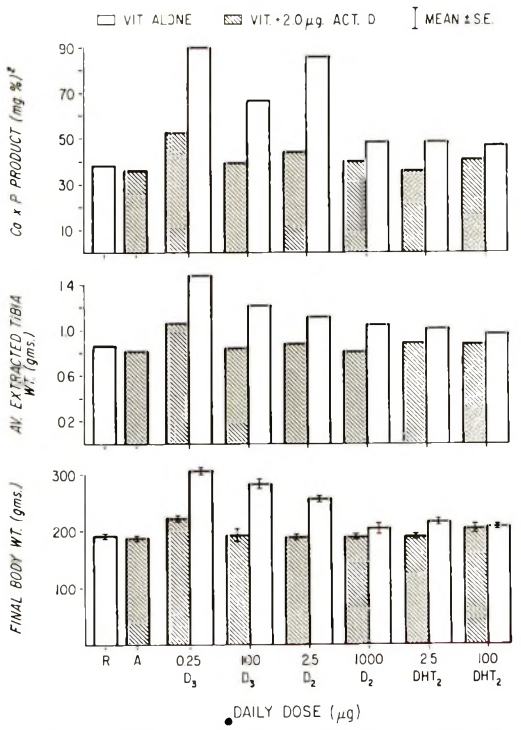

Fig. 1 Effect of actinomycin D on response of rachitic chicks to vitamins D_2, D_3, and DHT_2. *Top:* serum Ca × P products, values as mg/100 ml. *Middle:* extracted tibia weights. *Bottom:* final body weights. Column labeled *R* refers to rachitic controls, and column labeled *A* indicates actinomycin D controls.

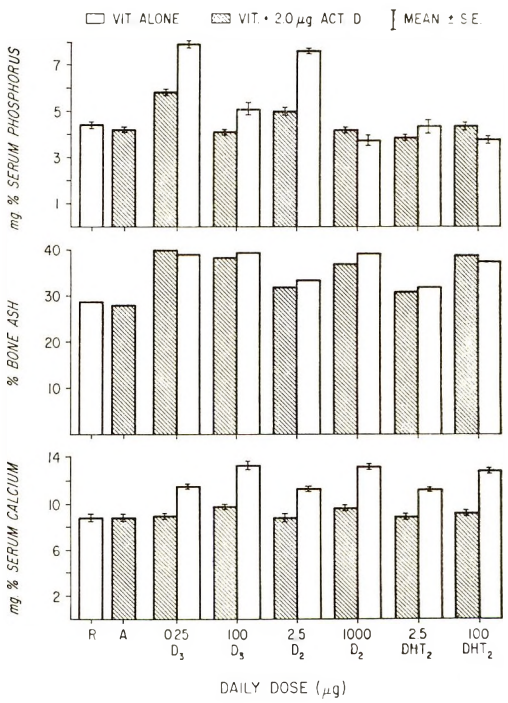

Fig. 2 Effect of actinomycin D on response of rachitic chicks to vitamins D_2, D_3, and DHT_2. *Top:* serum phosphorus level in mg/100 ml. *Middle:* % bone ash. *Bottom:* serum calcium level in mg/100 ml. Column labeled *R* refers to rachitic controls, and column labeled *A* indicates actinomycin D controls.

sterol. For example, with 0.25 μg/day (10 IU) vitamin D_3 the serum calcium was 11.4 mg/100 ml for the sterol alone and 9.0 mg/100 ml for the sterol plus 2.0 μg of actinomycin D. The inhibitor also blocked the hypercalcemia associated with the large doses of the sterols, although in this experiment the hypercalcemia in control groups was not as marked as usual; for example, in figure 2 (bottom) 100 μg/day vitamin D_3 resulted in a serum calcium level of 13.2 mg/100 ml.

Bone ash. Figure 2 (middle) illustrates that the percentage bone ash values were essentially the same whether the sterol was administered alone or given with actinomycin D. However, since the extracted tibia weights in each group treated with the inhibitor were greatly diminished, actinomycin D resulted in a decreased total mineral content in the bones.

Serum phosphorus level. At the low doses of sterol, the serum phosphorus level was reduced in the actinomycin D-treated groups (fig. 2, top). An example of this is the group receiving 2.5 μg/day vitamin D_2 which had a serum phosphorus value of 7.6 mg/100 ml, whereas the same dose of sterol resulted in a value of 5.0 mg/100 ml when administered simultaneously with the antibiotic. At high doses of the sterols, hypophosphatemia was present in both the controls and actinomycin D-treated groups.

Ca × P product. The Ca × P product, representing a measure of the "calcifying" property of the serum, reflected changes in the serum calcium and phosphorus levels responses (fig. 1, top). The actinomycin D treatment resulted in lowered products at every dose level; in some cases values were below the rachitic control value.

Actinomycin D effect on rachitic chicks. Based on the values of the various measured parameters, actinomycin D had little deleterious effect on the rachitic state (figs. 1 and 2). Of most importance, however, the serum calcium level was the same in the rachitic group (8.8 mg/100 ml) and the group injected intraperitoneally with 2.0 μg of actinomycin D (8.8 mg/100 ml).

Actinomycin D (2.5 μg/day) vs. vitamin D_3 and DHT_2

Sterol lethality. Survival curves for groups of chicks given 2,500 μg/day of vitamins D_2 and D_3 and DHT_2 and for the 2 groups given vitamin D_3 or DHT_2 with simultaneous intraperitoneal injections of 2.5 μg of actinomycin D are shown in figure 3. From this figure it is evident that DHT_2 was much more toxic than either vitamin D_2 or D_3. Vitamin D_3 at this dose level appeared slightly more toxic than

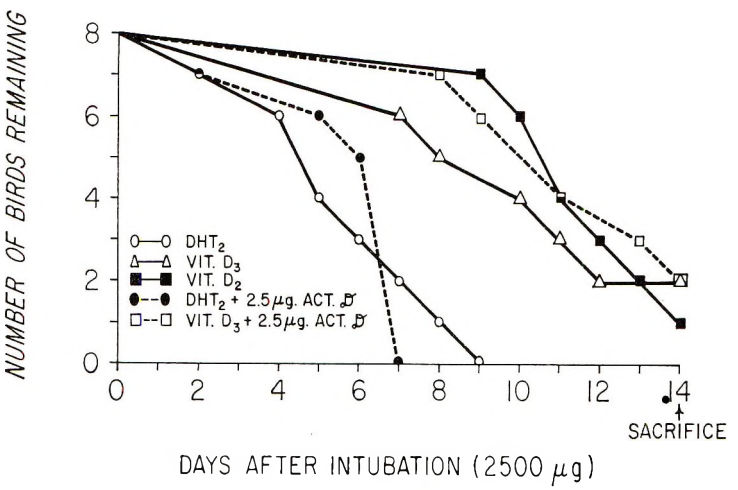

Fig. 3 Survival curves of chicks treated with vitamin D_2 or with vitamin D_3 and DHT_2 with or without actinomycin D.

vitamin D_2. These observations, plus those given elsewhere indicating that DHT_3 is slightly more toxic than DHT_2 (14), establish that in the chick, DHT_3 is the most toxic of the 4 sterols, followed in decreasing order by DHT_2, vitamin D_3 and vitamin D_2. This order is identical to that observed in rats (16).

Actinomycin D had little additional effect on the number of fatalities in each group; few deaths were directly attributable to the inhibitor treatment even though toxic manifestations of the antibiotic were evident in the other parameters.

Growth. Sterol-stimulated growth, as measured by final body weights, was inhibited at all dose levels by actinomycin D (fig. 4, bottom). However, no inhibition was noted in chicks given only the rachitogenic diet, e.g., rachitic chicks weighed 171 g, and actinomycin D chicks weighed 184 g. It is well known that vitamin D_3 is an order of magnitude more potent than vitamin D_2 at both antirachitic and toxic dose levels (12, 13). The dihydrotachysterol$_2$ results indicated a greater stimulation of growth by the 0.25 μg/day level than was produced by the same dose of vitamin D_2. Also, the data in figure 4 confirm that the therapeutic range of DHT_2 is very narrow, since the final body weight started decreasing immediately above the 0.25 μg/day dose; each of the parameters studied in this experiment bears out the closeness between optimal and toxic dose levels of DHT.

Tibia weight. Intraperitoneal injection of 2.5 μg of actinomycin D resulted in a decreased extracted tibia weight at each level studied (fig. 4, middle). No significant difference was noted between the values of the 2 control groups (rachitic, 0.74 g; actinomycin D treated, 0.79 g), but the values for the inhibitor-treated groups at the highest dose level of each sterol fell below these control values (2500 μg vitamin D_3, 0.65 g; 250 ug DHT, 0.63 g). Vitamin D_2 proved to be less potent than either vitamin D_3 or DHT_2.

Percentage bone ash. The values for the percentage bone ash for each group are graphed versus the log-dose of sterol in figure 5 (middle). Except for the 2 control groups which resulted in essentially the same responses (rachitic, 30.1%; actinomycin D-treated, 29.4%) and the 0.25 μg/day (10 IU) vitamin D_2 group which was predictably low (31.7%), all of the other groups exhibited bone ash values in the normal range. No deleterious effects resulted from administration of the antibiotic. This was the only parameter studied on which actinomycin D had little inhibitory effect.

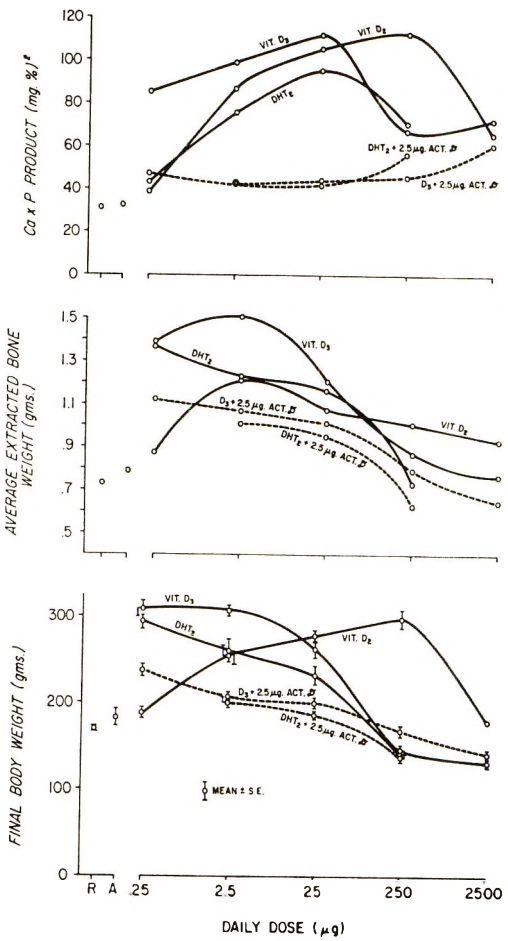

Fig. 4 Effect of actinomycin D on response of rachitic chicks to vitamin D_3 and DHT_2. *Top:* serum Ca × P products, values as mg/100 ml. *Middle:* extracted tibia weights. *Bottom:* final body weights. *R* indicate rachitic controls. *A* indicates actinomycin D controls.

Serum phosphorus level. Actinomycin D markedly reduced the serum phosphorus level at the dosages where the sterols alone produced normal serum phosphorus levels. At high dose levels where the effect of the sterol was to produce hypophosphatemia,

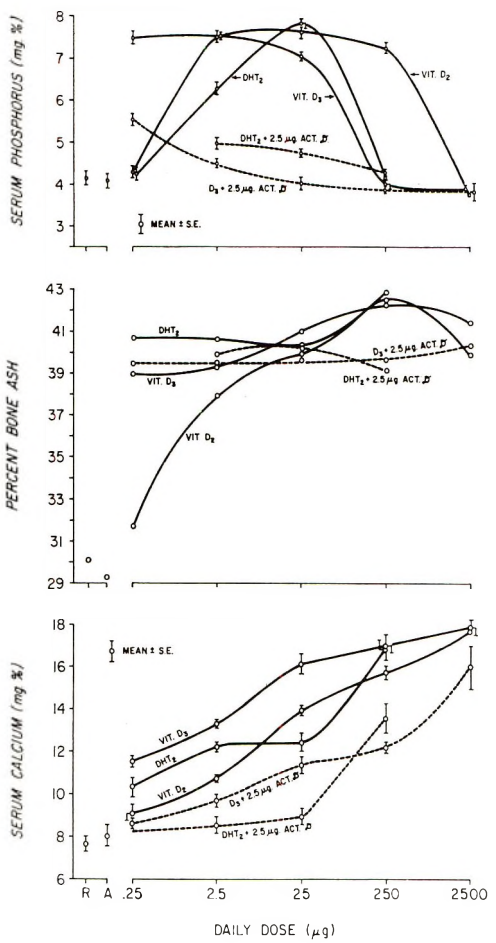

Fig. 5 Effect of actinomycin D on response of rachitic chicks to vitamin D_3 and DHT_2. *Top:* serum phosphorus level in mg/100 ml. *Middle:* % bone ash. *Bottom:* serum calcium level in mg/100 ml. *R* indicates rachitic controls. *A* refers to actinomycin D controls.

the actinomycin D did not further aggravate this condition (figs. 2 and 5, top). Again, the 2 control groups gave essentially equivalent responses (rachitic, 4.1 mg/100 ml; actinomycin D-treated, 4.1 mg/100 ml). On this parameter, DHT_2 was less potent than vitamin D_2 at low dose levels but much more toxic than either vitamin D_2 or D_3 at the higher dose levels. The very narrow range for optimal response to DHT_2 is again evident.

Serum calcium level. Both control groups exhibited essentially equivalent serum calcium levels as shown in figure 5 (bottom) (rachitic, 7.6 mg/100 ml; actinomycin D-treated, 8.0 mg/100 ml), but actinomycin D administration caused a lowered serum calcium level with the sterols at each dose level.

Vitamin D_3 and vitamin D_2 both followed smoothly rising dose-response curves, whereas DHT_2 followed a plateau over a wide dose range followed by a sudden rise as shown in figure 5 (bottom). The 2 vitamin D dose-response curves illustrate the potency difference between vitamins D_2 and D_3 with flattening out at the higher dose levels (13).

Ca × P product. Figure 4 (top) indicates the serum Ca × P products for the actinomycin D-treated groups were well below the respective control group products at each dose level studied. No difference was evident between the value for the rachitic group [31 (in mg/100 ml)^2] and that for the actinomycin D treated group [33 (in mg/100 ml)^2]. Both of the curves resulting from concurrent actinomycin D treatment were near the rachitic control value over the entire dose range studied. The curve for DHT_2 did not reach as high a value as that obtained with either vitamin D_2 or D_3.

Bone deposition of ^{45}Ca. In figure 6 (bottom) the counts per minute of ^{45}Ca in 50 mg of bone ash 24 hours after an oral dose of ^{45}Ca for each group are plotted logarithmically against the log-dose of sterol. The values for the control groups were essentially the same (rachitic, 453,000 count/min; actinomycin D treated, 421,000 count/min). Very little difference was noted between the curves obtained for the sterols with or without the simultaneous inhibitor treatment. In fact, almost all the treated groups gave essentially the same values at a given dose level, usually between 200,000 and 400,000 count/min per 50 mg bone ash. The rachitic group with or without the 2.5 μg/day of actinomycin D gave higher uptakes of ^{45}Ca than any of the treated groups. At the high toxic doses of vitamin D_3 and DHT_2, the amount of ^{45}Ca deposited in the bone diminished and was negligible at the highest dose of vitamin D_3 (2,500 μg/day), due most likely to the fact that the cellular components of bone were essentially

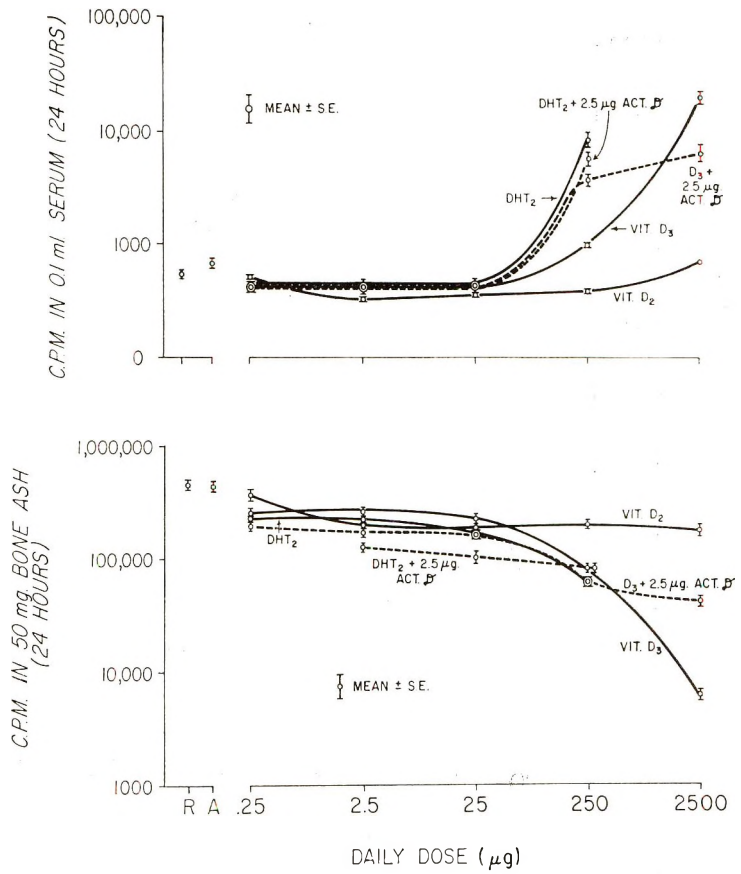

Fig. 6 Concentrations of ^{45}Ca in serum and bone following oral administration to rachitic chicks treated with vitamin D_3 or DHT_2 with or without actinomycin D. *Top:* serum level of ^{45}Ca. *Bottom:* bone level of ^{45}Ca. *R* indicates rachitic controls. *A* refers to actinomycin D controls.

dead (17). It appears that actinomycin D had little effect on the specific activity of the ^{45}Ca in the bone at 24 hours after an oral dose.

Twenty-four-hour serum ^{45}Ca level. Figure 6 (top) graphically illustrates that the serum level of ^{45}Ca at 24 hours complemented the bone data presented in figure 6 (bottom). The 2 control groups resulted in similar values (rachitic, 542 count/min; rachitic plus actinomycin D, 679 counts/min) in the number of counts per minute in 0.1 ml serum, and all 5 curves were essentially equivalent in the dose range between 0.25 and 25 µg/day. At the high levels of administered sterols, elevated ^{45}Ca concentrations were present in serum (e.g., the 2500 µg/day vitamin D_3 value was 19,700 count/min). This comparatively huge excess of ^{45}Ca in the serum was anticipated, since the ^{45}Ca was not being deposited in bone, and therefore was not being removed from the serum as rapidly as in the other groups. As in the 24-hour deposition of ^{45}Ca, actinomycin D treatment had little effect on the amount of ^{45}Ca in the serum at 24 hours; each sterol gave essentially the same value whether administered alone or simultaneously with actinomycin D.

Short-term appearance of ^{45}Ca. In figures 7 and 8 are presented the results of short-term monitoring of serum ^{45}Ca levels from the wing vein of the chick after an oral ^{45}Ca dose. The results obtained with

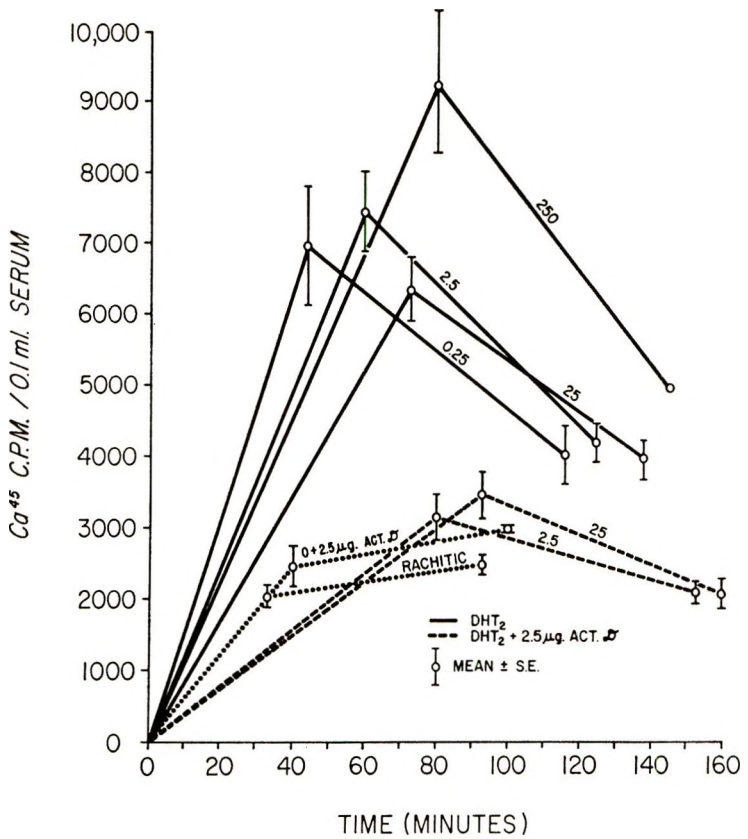

Fig. 7 Effect of actinomycin D on the appearance in serum of ^{45}Ca administered orally to rachitic or DHT_2-treated chicks.

vitamin D^2 are comparable to those with vitamin D_3 and therefore are not separately presented.

Figure 7 indicates that in the rachitic and the actinomycin D-treated control groups, the rates at which the ^{45}Ca appeared in the serum were essentially the same. A similar curve was exhibited by the group given 0.25 µg/day of vitamin D_2 (not shown). Thus, in the 3 groups in which the serum calcium values were in the rachitic range (fig. 5, bottom) a relatively low basal rate of ^{45}Ca appearance in the serum was observed. Increasing doses of vitamin D_2 above 0.25 µg/day resulted in increased rates of appearance of the ^{45}Ca in the serum.

Figure 7 also illustrates the data obtained with DHT_2. Increasing doses of DHT_2 resulted in a more rapid appearance of ^{45}Ca in the serum. In all instances the groups given actinomycin D injections simultaneously with the DHT_2 treatment displayed curves in the basal range. The curves indicated by the dashed lines in figure 7, representing inhibitor treatment, are completely different from and much lower than those illustrated with solid lines which represent treatment with DHT_2 alone. None of the antibiotic-treated groups exhibited a counting rate equal to the minimal rate observed in the groups treated with DHT_2 alone.

The results for the groups given vitamin D_3 treatment are graphed in figure 8. Except for the 2500 µg/day plus inhibitor group, each of the actinomycin D groups resulted in a curve that was quite similar to the rachitic curve. Increasing doses of vitamin D_3 resulted in increased rates of

appearance of the ^{45}Ca. The results with the 2500 μg/day vitamin D_3 were especially interesting, though based on only one surviving chick (all others in the group had died due to toxicity of the sterol); therefore no standard error could be determined. At this dose level the appearance of ^{45}Ca in the serum was extremely rapid and not completely blocked by the actinomycin D.

In each of the ^{45}Ca experiments, the effect of actinomycin D was to block the sterol-induced increase in the rate of appearance of the ^{45}Ca in the serum. This obstruction of the sterol-induced response was complete, and as a result the antibiotic-treated groups showed basal values. Although this technique has been represented erroneously as *measuring* intestinal absorption of calcium (6), it does give an indication of such absorption. Since deposition of ^{45}Ca was essentially the same at each dose level at least at 24 hours (fig. 6, bottom), with or without inhibitor treatment, the data in figures 7 and 8 probably measure to a first approximation the relative differences in the degree of intestinal absorption of calcium. With this reservation, the data may be interpreted to mean that actinomycin D inhibited the sterol-induced stimulation of calcium movement across the intestine. Furthermore, an important point is that actinomycin D did not affect the basal calcium movement across the intestine in the rachitic chicks.

DISCUSSION

The major point of the present study is that with chronic administration to chicks of a low dose level of actinomycin D, it was possible to inhibit a number of vitamin D- and dihydrotachysterol-induced responses in sterol-treated chicks, but not produce any measurable effects in vitamin D-deficient chicks at the same dose of

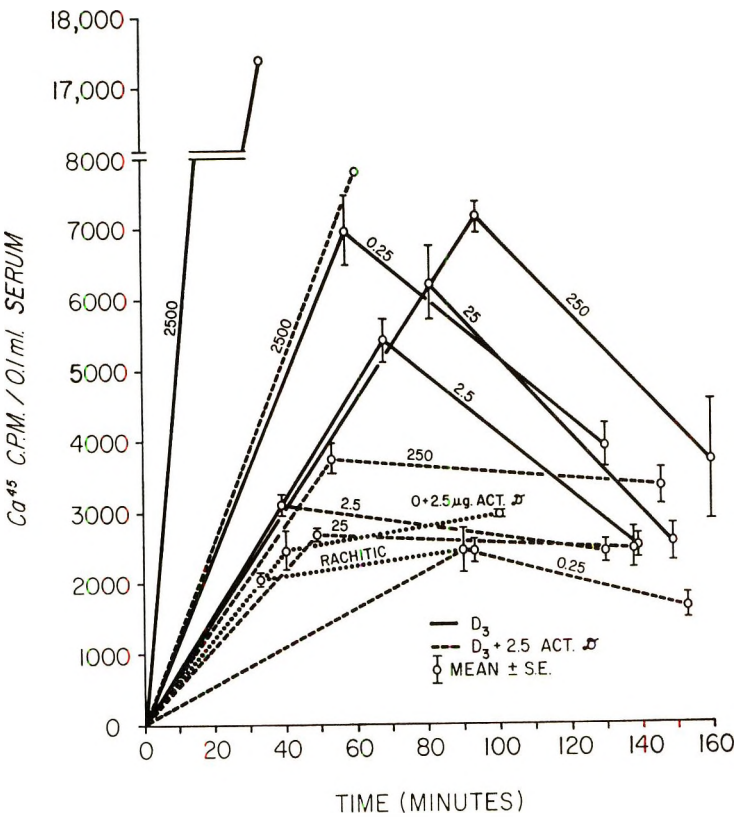

Fig. 8 Effect of actinomycin D on the appearance in serum of ^{45}Ca administered orally to rachitic or vitamin D_3-treated chicks.

actinomycin. From these data, it appears that in the rachitic chick some basal level of calcium and bone metabolism functions by processes which are relatively insensitive to various inhibitors, such as actinomycin D or o,p'-DDD (18) capable of blocking certain vitamin D-induced responses; these processes may not be directly dependent on protein synthesis and might be regulated largely by physicochemical rather than metabolic phenomena.

Sensitivity of various protein-synthetic pathways to actinomycin D inhibition must vary considerably, since the low dose used in this study was well below lethal levels, yet was sufficiently high to greatly inhibit most of the changes in the measured parameters that were induced by the calcemic sterols.

Actinomycin D treatment resulted in values for the serum Ca × P products which were lower in each instance than the respective controls (figs. 1 and 4) and were quite close to the rachitic values. However, in each of the inhibitor-treated groups the tibial percentage bone ash (figs. 2 and 5) was almost normal. Thus, it appears that in the actinomycin D-treated birds, relatively normal bone mineralization was maintained at least to the the extent of having a normal percentage bone ash despite a serum Ca × P product at or near rachitic levels. This suggests that an effect of the calcemic sterols on bone was not being blocked by actinomycin. Perhaps this actinomycin-insensitive bone effect of the sterols was responsible for the increased serum calcium level observed at the highest doses of vitamin D_3 or DHT_2 given despite actinomycin treatment (see fig. 5, bottom), and the fact that deposition of ^{45}Ca in bone 24 hours after oral administration was relatively unaffected by actinomycin.

Strict comparison of the results of the present study with those of previous investigators is difficult because of dose or species differences. Thus, for example, the report of an actinomycin D effect on calcium metabolism in vitamin D-deficient rats (8) is at variance with our results in chicks. Is this a species difference or a difference due to the much larger actinomycin dose used in the rat experiments? The total daily doses of actinomycin D used in this study are equivalent to single doses of 28 μg in the case of the first experimental series and 35 μg in the second series. It has been shown experimentally (19), and is consistent with the postulated biochemical mechanism of action of actinomycin D that the effects of the antibiotic are dependent on the total dose administered. The total doses of 28 or 35 μg represent dose levels of between 10 and 20 μg/100 g body weight and are well below the reported short-term LD_{50}'s (19). Most previous investigators (3, 5, 7), except for Norman (6), have utilized doses (generally 1 μg/g body weight) of actinomycin D close to or exceeding the lethal dose. In addition the usual procedure has been to give a single dose of vitamin D and evaluate the results shortly thereafter. The present experiment is unique in that a small effective dose of actinomycin D was given chronically to chicks treated with a wide dose range of sterols, also administered chronically.

The greater hypercalcemic and toxic potency of the reduced derivative, dihydrotachysterol, compared with vitamin D, has raised the question of whether the latter must undergo such a reduction in the body prior to exerting its hypercalcemic action. The more rapid hypercalcemic action of DHT than vitamin D would be in line with such a reaction.

Sallis and Holdsworth (20) reported that while vitamin D_2 had little activity in stimulating in vivo Ca absorption by chick intestine, the corresponding DHT_2 (with same side chain) was much more potent, suggesting that the stereochemical changes in ring B had "activated" the vitamin D_2 molecule. Unfortunately for their argument, vitamin D_3 with the same structure in ring B as vitamin D_2 was more potent even than DHT_2 or DHT_3. The effect of actinomycin D in blocking DHT_2 as well as vitamin D_2 or D_3 action also argues, at least, that the sensitive step is not one between a vitamin D and a DHT-like reduced derivative.

LITERATURE CITED

1. Goldberg, I. H., and E. Reich 1964 Actinomycin inhibition of RNA synthesis directed by DNA. Federation Proc., 23: 958

2. Tashjian, A. H., Jr., D. A. Onties and T. L. Goodfriend 1964 Mechanism of parathyroid hormone action: Effects of actinomycin D on hormone-stimulated ion movements in vivo and in vitro. Biochem. Biophys. Res. Commun., *16:* 209.
3. Rasmussen, H., C. Arnaud and C. Hawker 1964 Actinomycin D and the response to parathyroid hormone. Science, *144:* 1019.
4. Talmage, R. V., C. W. Cooper and J. Nevenschwander 1965 The effect of actinomycin D on parathyroid-induced changes in bone and kidney. Gen. Comp. Endocrinol., *5:* 475.
5. Eisenstein, R., and M. Passavoy 1964 Actinomycin D inhibits parathyroid hormone and vitamin D activity. Proc. Soc. Exp. Biol. Med., *117:* 77.
6. Norman, A. W. 1965 Actinomycin D and the response to vitamin D. Science, *149:* 184.
7. Zull, J. E., E. Czarnowska-Misztal and H. F. DeLuca 1965 Actinomycin D inhibition of vitamin D action. Science, *149:* 182.
8. Harrison, H. E., and H. C. Harrison 1966 Actinomycin D inhibition of intestinal transport of calcium and of vitamin D action. Proc. Soc. Exp. Biol. Med., *121:* 312.
9. Zull, J. E., E. Czarnowska-Misztal and H. F. DeLuca 1966 On the relationship between vitamin D action and actinomycin-sensitive processes. Proc. Nat. Acad. Sci., 55: 177.
10. Taylor, A. N., and R. H. Wasserman 1965 A vitamin D_3-dependent factor influencing calcium binding by homogenates of chick intestinal mucosa. Nature, *205:* 248.
11. Wasserman, R. H., and A. N. Taylor 1966 Vitamin D_3-induced calcium-binding protein in chick intestinal mucosa. Science, *152:* 791.
12. Chen, P. S., Jr., and H. B. Bosmann 1964 Effect of vitamin D_2 and D_3 on serum calcium and phosphorus in rachitic chicks. J. Nutr., *83:* 133.
13. Chen, P. S., Jr., and H. B. Bosmann 1965 Comparison of the hypercalcemic action of vitamins D_2 and D_3 in chicks and the effect on tetracycline fixation by bone. J. Nutr., *87:* 148.
14. Bosmann, H. B., and P. S. Chen, Jr. 1966 Comparative effects of dihydrotachysterol$_2$ and dihydrotachysterol$_3$ in chicks. J. Nutr., *90:* 141.
15. Bray, G. A. 1960 A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem., *1:* 279.
16. Roborgh, J. R., and T. J. DeMan 1960 The hypercalcemic activity of dihydrotachysterol$_2$ and dihydrotachysterol$_3$ and of vitamins D_2 and D_3 after intravenous injection of the aqueous preparations. II. Comparative experiments on rats. Biochem. Pharmacol., *3:* 277.
17. Yuile, C. L., P. S. Chen, Jr., and H. B. Bosmann. Rachitic bone lesions in chicks after multiple graded doses of vitamins D and dihydrotachysterols. Arch. Path., in press.
18. Chen, P. S., Jr., and H. B. Bosmann. The inhibitor o,p′-DDD and vitamin D and dihydrotachysterol function in the chick. Amer. J. Physiol., in press.
19. Philips, F. S., H. S. Schwartz, S. S. Sternberg and C. T. C. Tan 1960 The toxicity of actinomycin D. Ann. N. Y. Acad. Sci., *89:* 348.
20. Sallis, J. D., and E. S. Holdsworth 1962 Influence of vitamin D on calcium absorption in the chick. Amer. J. Physiol., 203: 497.

Determination of the Minimum Dietary Essential Amino Acid-to-Total Nitrogen Ratio for Beef Protein Fed to Young Men [1,2]

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ABSTRACT The extent to which beef protein could be isonitrogenously replaced with nonspecific nitrogen without influencing the efficiency of dietary nitrogen utilization was studied in 9 male college students. They were fed at constant nitrogen intakes, equivalent to 0.39 g protein/kg body weight, and daily urinary nitrogen was measured. Strained beef protein furnished 90% of the nitrogen of the basal diet, with oatmeal and tomato juice the remainder. When the nitrogen of the basal diet was isonitrogenously replaced by a nonspecific nitrogen source, a mixture of glycine and diammonium citrate in which each furnished equal amounts of nitrogen, a 20% replacement caused a barely significant increase in urinary nitrogen excretion in one of the five healthy subjects studied. A 25% replacement caused no significant increase in urinary nitrogen excretion in three of four healthy subjects studied, but a 30% replacement was tolerated by only two of six subjects. A 20% replacement gives an E/T_N ratio of 2.16 and 26.4% of the total nitrogen furnished by essential amino acids; 25% replacement results in an E/T_N ratio of 1.89 and 24.3% of the nitrogen from essential amino acids. Sulfur-amino acids and tryptophan may be limiting in the 30% diluted diet. One subject receiving a 20% dilution showed a markedly increased nitrogen excretion during an acute febrile infection despite the low protein intake.

The 1957 report of the Food and Agricultural Organization (FAO) and World Health Organization (WHO) Committee on Protein Requirements proposed a provisional pattern of essential amino acids as a guide towards further studies of protein requirements (1). A significant and largely ignored feature of this reference protein was that by expressing the essential amino acids per gram of total protein nitrogen (E/T_N ratio), the proportion of essential amino acids to total nitrogen was thus fixed.

A more recent FAO/WHO report (2) takes cognizance of this and emphasizes the 2 components of man's protein requirement: 1) the amounts and proportion of essential amino acids, and 2) the total nitrogen required for body protein synthesis and other nitrogen-containing compounds. The essential amino acid pattern of whole egg protein, expressed as proportions of the essential amino acids to the total essential amino acid concentration, was proposed as the new reference protein. This had the advantage of encouraging separate investigations of the effects of changes in the ratio of essential amino acids to one another and also of changes in the relationship between the essential amino acids and the total nitrogen required.

Whole egg protein has a higher E/T_N ratio (3.22) than the 1957 FAO reference protein (2.02) but protein scores based on the latter agreed reasonably well with biological assays (2–3). Expressed in another way, the essential amino acids in whole egg supply 37.6% of the total protein nitrogen, whereas the comparable figure is 23.6% in the 1957 FAO reference protein. Furthermore, the data obtained by Rose and Wixom (4) from 2 adult subjects suggests that as little as 14 to 23% of the total dietary nitrogen from es-

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[3] On leave from the Department of Biochemistry, National Taiwan University College of Medicine, Taiwan, supported by NIH Fellowship no. I FO5-TW-853-01.

sential amino acids is enough to maintain minimum nitrogen balance.

It appears, therefore, that the E/T_N ratio of whole egg protein is higher than that required for maintenance of nitrogen equilibrium in adult subjects. Investigations are underway in our laboratories to determine the extent to which the E/T_N ratios of various proteins may be reduced, by isonitrogenous substitution of the protein with a nonspecific nitrogen source, without influencing the nutritive value of the protein. Using diets in which whole egg protein furnished 90% of the total dietary protein, we found that the E/T_N ratio of the basal diet (3.08) could be reduced to between 2.18 and 1.85 in most young adults without changing the nutritive value of the diet (5). At these ratios essential amino acids furnish between 21 and 25% of the total dietary nitrogen.

Since beef protein is known to be of high nutritive value, it appeared probable that the E/T_N ratio of a diet in which this protein substitutes for the whole egg protein of the diet as used in our earlier studies, could also be reduced significantly without influencing the efficiency of dietary nitrogen utilization. A comparison of the essential amino acid concentrations and E/T_N ratios of the whole egg protein, the beef protein diets and the 1957 FAO reference protein is given in table 1. On the basis of our previous observations with whole egg, it can be predicted that the total nitrogen of a beef formula diet could be isonitrogenously replaced with nonspecific nitrogen to the extent of approximately 20 to 30% without affecting its nutritive value for young adults. At these levels of substitution the final dietary E/T_N ratios would be from 2.16 to 1.89. The experiments reported here test this prediction.

EXPERIMENTAL

Subjects. Nine male college students, 19 to 26 years old (table 2), were allowed to continue their normal activities. All were in good health as determined by medical history and examination. Subjects were required to maintain the same level of daily physical activity throughout the experiment.

Diet. The composition of the liquid formula diet, as calculated for a 70-kg subject, is shown in table 3, the amount received depending upon the weight of the individual subject. Beef protein supplied 90% of the dietary protein, with oatmeal [4]

[4] Buckeye Rolled Oats, homogenized. Specially prepared in standardized form through the courtesy of the Quaker Oats Company, Barrington, Illinois.

TABLE 1

Proportions of essential amino acids to total nitrogen [1] in the experimental diets, whole egg protein diet and the 1957 FAO reference protein

Amino acid	Basal egg formula [2]	Basal beef formula	Diluted beef formula,[3] %			1957 FAO reference protein
			20	25	30	
	mg/g N	*mg/g N*	*mg/g N*	*mg/g N*	*mg/g N*	*mg/g N*
Isoleucine	398	318	254	239	223	270
Leucine	530	495	396	371	347	306
Lysine	384	522	418	392	365	270
Methionine + cystine	321	223	179	167	156	270
Phenylalanine + tyrosine	608	462	370	347	323	360
Threonine	300	268	214	201	188	180
Tryptophan	99	72	58	54	50	90
Valine	444	338	270	254	237	270
Total essential amino acids	3084	2698	2159	2025	1889	2016
EAAN,[4] %	35.7	32.9	26.3	24.7	23.0	23.6
E/T_N ratio [5]	3.08	2.70	2.16	2.03	1.89	2.02

[1] Calculated from Orr, M. L., and B. K. Watt 1957 Amino Acid Content of Foods. Home Econ. Res. Rep. no. 4, USDA, Washington, D. C.
[2] Whole egg protein diet used in previous studies by Scrimshaw et al. (5).
[3] % isonitrogenous replacement of dietary nitrogen with glycine and diammonium citrate mixture supplying equal amounts of nitrogen.
[4] % of total nitrogen contributed by essential amino acids.
[5] Grams of total essential amino acids/g total dietary nitrogen.

TABLE 2

Age, weight and daily intake of calories by young men studied for the metabolic effects of dietary protein dilution with glycine and diammonium citrate

Subject	Age	Weight		Calculated caloric intake
		Initial	Final	
	years	*kg*	*kg*	*kcal/kg/ body wt*
DK	22	66.3	65.4	44
KS	19	65.1	65.2	49
WT	19	83.4	82.9	48
WV	20	76.3	76.3	46
YH	26	62.2	61.5	47
DQ	24	69.5	69.7	47
BW	20	74.9	74.5	46
BN	22	75.9	76.0	43
DP	22	73.1	72.8	43

TABLE 3

Basal beef formula diet used during experimental periods

	g/100 g formula	*g/day* [1]
Strained beef	20.1	181.9
Oatmeal	6.5	59.2
Corn oil	15.9	145.0
Dextrins and maltose [2]	13.6	123.0
Lemon juice	0.8	7.0
Salt	0.2	1.5
Methyl cellulose	0.9	8.0
K_2HPO_4	0.5	4.1
$Ca_{10}(OH)_2(PO_4)_6$	0.3	2.4
H_2O	41.3	375
Total	100	907.1
Tomato juice	—	158
Iron [3]	—	—
Vitamins [4]	—	—
Cornstarch, cookies and desert	—	variable [5]
Carbonated beverage	—	variable [5]

[1] For a 70-kg subject.
[2] Dexin, Burroughs Wellcome and Company, Tuckahoe, New York. Approximate composition: dextrins, 75%; maltose, 24%.
[3] Given daily as a tablet supplying 14 mg Fe. Furnished through the courtesy of Smith, Kline and French, Philadelphia.
[4] Unicap multivitamin capsule, Upjohn Company, Kalamazoo, Michigan.
[5] Intake varied among subjects in order to adjust diet to individual caloric needs but was constant for any one subject.

and tomato juice each supplying 5%. The beef protein used was a strained beef product.[5]

Caloric intake of the subjects was based upon an average of previous intakes as estimated from individual dietary histories and the calculation based upon food nomogram. Further changes were found unnecessary in view of the stable body weight of the subjects throughout the experimental period (table 2).

"Dilution" of dietary protein. As in the previous studies (5) the protein nitrogen in the formula diet was "diluted" by substituting isonitrogenously part of the dietary nitrogen with a mixture of glycine and diammonium citrate, in which each compound supplied equal amounts of nitrogen. Table 1 summarizes the concentration of essential amino acids per gram of dietary nitrogen, the E/T_N ratios and percentages of total nitrogen contributed by the essential amino acids in experimental diets. The pattern of the basal egg diet used in our previous studies (5) and the 1957 FAO reference protein are given for comparison.

Experimental sequence. Subjects were fed initially 0.39 g protein/kg/day, a level found to supply nitrogen at or below the minimal nitrogen requirement. The basis for use of a low protein diet in these studies has been described previously in detail (5). The subjects were fed the basal or undiluted beef formula for 12 to 16 days depending upon the time required to reach a relatively steady state of urinary nitrogen secretion. The sequence in which dilutions of the nitrogen of the basal diet were made and the length of each dilution phase are given with the results.

Handling and analysis of samples. Urine was collected for complete 24-hour periods. Total nitrogen, urea nitrogen and creatinine were analyzed in fresh urine as described previously (5). Alpha-amino nitrogen was determined by the method of Albanese and Irby (6).

Statistical analysis. Total urinary nitrogen excretion was the principal indicator of dietary nitrogen utilization. The approach and its limitations have been discussed in an earlier publication (5). Student's *t* test was used to determine whether urinary nitrogen excretion during a dilution period differed significantly from that for the corresponding baseline period. The average of daily values, after a relatively steady state of urinary nitrogen excretion was achieved by subjects fed the basal beef formula, was taken as the baseline.

[5] Gerber Strained Beef, Gerber Baby Foods, Fremont, Michigan. Prepared by grinding, cooking and sterilization at approximately 115° for 75 minutes. N.P.U. was 70 by rat assay compared with 63 for casein controls.

RESULTS

The data from DK are shown in figure 1 to illustrate the method of daily evaluation. A summary of results for total urinary nitrogen excretion during the experimental periods for all subjects is given in table 4. Results obtained from subject DP will be considered separately since this subject contracted an acute infection during the experiment.

Four of the healthy subjects studied at the 20% dilution level failed to show a statistically significant increase in urinary nitrogen excretion. The increase in urinary nitrogen excretion shown by subject BN at this level of dilution just reached statistical significance ($P < 0.05$). A 30% level of dilution was found too high for most subjects; only two of the six subjects studied tolerated this level of dilution. No significant increase in nitrogen excretion was observed in the 3 of the 4 healthy subjects studied at the 25% level of dilution. These results suggest that

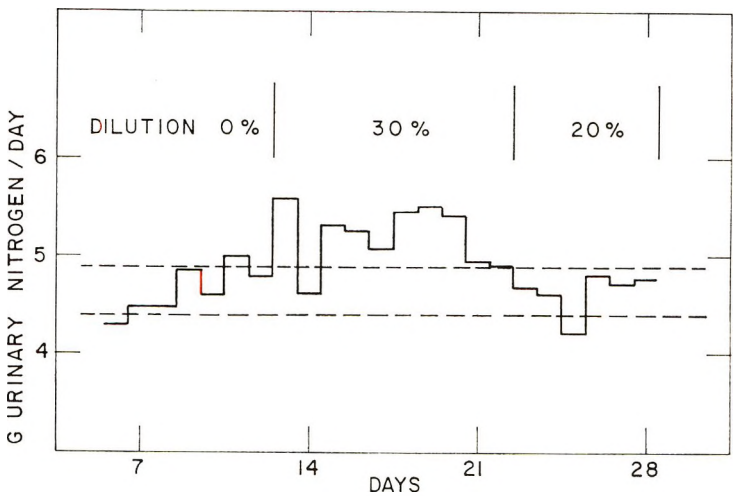

Fig. 1 Urinary nitrogen excretion per day in subject DK, fed the basal beef formula diet with 30 and 20% replacement of nitrogen with glycine and diammonium citrate. Broken lines indicate mean baseline nitrogen excretion ± one SD.

TABLE 4

Effects of dilution of beef protein formula with glycine and diammonium citrate on urinary nitrogen excretion of young men

Subject	Dietary intake	Dilution of dietary protein, %			
		0	20	25	30
	g N/day	*g urinary N/day*	*g urinary N/day*	*g urinary N/day*	*g urinary N/day*
DK [1]	4.10	4.64 ± 0.24 [2](7) [3]	4.63 ± 0.23(6)	—	5.20 ± 0.28*(10) [4]
KS [1]	4.02	3.36 ± 0.14 (3)	3.41 ± 0.19(6)	—	3.98 ± 0.26*(10) [4]
WT [1]	5.12	5.64 ± 0.31 (7)	—	—	5.50 ± 0.35 (16) [4]
WV [1]	4.64	4.34 ± 0.26 (6)	4.59 ± 0.41(6)	—	5.10 ± 0.42*(10) [4]
YH [5]	3.93	3.60 ± 0.14 (6)	—	3.56 ± 0.29 (9) [4]	3.83 ± 0.51 (11)
DQ [5]	4.39	4.26 ± 0.35 (7)	—	4.40 ± 0.29 (9) [4]	5.03 ± 0.39*(4) [6]
BW [5]	4.64	4.12 ± 0.51 (11)	4.09 ± 0.31(11) [4]	4.52 ± 0.52*(14)	—
BN [5]	4.74	4.10 ± 0.28 (11)	4.41 ± 0.28*(11) [4]	4.37 ± 0.30 (8)	—
DP [5]	4.56	3.97 ± 0.15 (7)	5.45 ± 0.51*(8) [7]	4.62 ± 0.32*(8) [4,7]	—

[1] Subjects fed undiluted basal diet for 12 days prior to dilution.
[2] Mean ± SD.
[3] Numbers in parentheses are numbers of days observed with the diet.
[4] Dilution tested first after baseline period.
[5] Subjects fed undiluted basal diet for 16 days prior to dilution.
[6] Last 4 days of the total 9 days of this dilution. Overall value for 9-day period 4.53 ± 0.64.
[7] Subject contracted a febrile condition during the experiment.
* Significantly greater than baseline period (0%) at 0.05 level.

TABLE 5

Effects of dilution of beef protein formula with glycine and diammonium citrate on urea nitrogen excretion of young men

Subject	Dietary intake	Dilution of dietary protein, %			
		0	20	25	30
	g N/day	*g urea N/day*	*g urea N/day*	*g urea N/day*	*g urea N/day*
DK [1]	4.10	3.08 ± 0.25 [2]	3.23 ± 0.27	—	3.90 ± 0.20* [3]
KS [1]	4.02	2.13 ± 0.45	2.19 ± 0.34	—	2.66 ± 0.32* [3]
WT [1]	5.12	3.67 ± 0.31	—	—	3.61 ± 0.35 [3]
WV [1]	4.64	2.69 ± 0.37	2.74 ± 0.74	—	3.35 ± 0.27* [3]
YH [4]	3.93	2.18 ± 0.14	—	2.20 ± 0.26 [3]	2.36 ± 0.44
DQ [4]	4.39	2.71 ± 0.22	—	3.04 ± 0.28* [3]	3.30 ± 0.28* [5]
BW [4]	4.64	2.60 ± 0.38	2.55 ± 0.44 [3]	2.78 ± 0.46	—
BN [4]	4.74	2.53 ± 0.19	2.89 ± 0.31* [3]	2.71 ± 0.15*	—
DP [4]	4.56	2.48 ± 0.18	3.45 ± 0.64* [6]	3.22 ± 0.43* [3,6]	—

[1] Subjects fed undiluted basal diet for 12 days prior to dilution.
[2] Mean ± SD for periods shown in table 4.
[3] Dilution tested first after baseline period.
[4] Subjects fed undiluted basal diet for 16 days prior to dilution.
[5] Value for entire 9-day period.
[6] Subject contracted a febrile condition during the experiment.
* Significantly greater than baseline period (0%) at 0.05 level.

most normal, young adults can tolerate a 20 to 25% dilution of the beef formula diet. After the last period shown in table 4, subject BN was returned to the 20% level of dilution and again showed an increased rate of urinary nitrogen excretion ($P < 0.05$). The daily excretion value for this period was 4.45 ± 0.35 g N.

Urea nitrogen excretion during the experimental periods is summarized in table 5. The results parallel those described earlier for total urinary nitrogen excretion and indicate that the increased urinary nitrogen excretion is largely accounted for by an increased urea excretion.

Table 6 shows results for α-amino nitrogen excretion during the various periods. Although the changes were not wholly consistent, α-amino nitrogen excretion tended to be slightly higher at higher dilution levels.

Urinary creatinine excretion during the study is shown in table 7. The completeness of the 24-hour collections is confirmed by the small variation in creatinine excretion during any one experimental period for most subjects. The mean coefficient of variation during the baseline period was 4.6%.

Subject DP developed a febrile condition, owing to an upper respiratory infection, during the latter part of the 25% dilution period. Urinary nitrogen excretion during this period (fig. 2) was significantly higher than during the corresponding baseline period. An even greater increase in urinary nitrogen excretion occurred during the 20% dilution which followed. Although fever was not recorded at this time, it appears probable that the markedly higher rate of nitrogen excretion was associated with the infection. In this subject urea nitrogen excretion also paralleled the change in total nitrogen excretion.

TABLE 6

Effects of dilution of beef protein formula with glycine on α-amino nitrogen excretion of young men [1]

Subject	Dilution of dietary protein, %			
	0	20	25	30
	mg/day	*mg/day*	*mg/day*	*mg/day*
DK	226	260	—	267
KS	232	224	—	246
WT	252	—	—	317
WV	280	328	—	323
YH	179	—	177	229
DQ	228	—	235	254
BW	209	233	276	—
BN	213	214	207	—
DP	225	251	243	—

[1] Analysis performed on a composite urine sample prepared for each period as indicated in footnotes of table 4.

TABLE 7

Urinary creatinine excretion by young men studied for the effects of dilution of a beef protein formula diet

Subject	Dilution of dietary protein, %			
	0	20	25	30
	g/day	*g/day*	*g/day*	*g/day*
DK	1.80 ± 0.03 [1]	1.75 ± 0.03	—	1.78 ± 0.05
KS	1.89 ± 0.03	1.79 ± 0.07	—	1.83 ± 0.05
WT	2.47 ± 0.05	—	—	2.37 ± 0.05
WV	2.00 ± 0.11	1.97 ± 0.04	—	1.98 ± 0.06
YH	1.68 ± 0.05	—	1.63 ± 0.04	1.67 ± 0.04
DQ	1.92 ± 0.03	—	1.89 ± 0.06	1.93 ± 0.03
BW	1.89 ± 0.22	1.78 ± 0.17	1.93 ± 0.18	—
BN	1.99 ± 0.20	1.99 ± 0.06	2.06 ± 0.07	—
DP	1.91 ± 0.08	1.92 ± 0.03	1.86 ± 0.08	—

[1] Mean ± SD for periods indicated in table 4. None of the differences among dilutions are statistically significant, except for WT ($P < 0.01$).

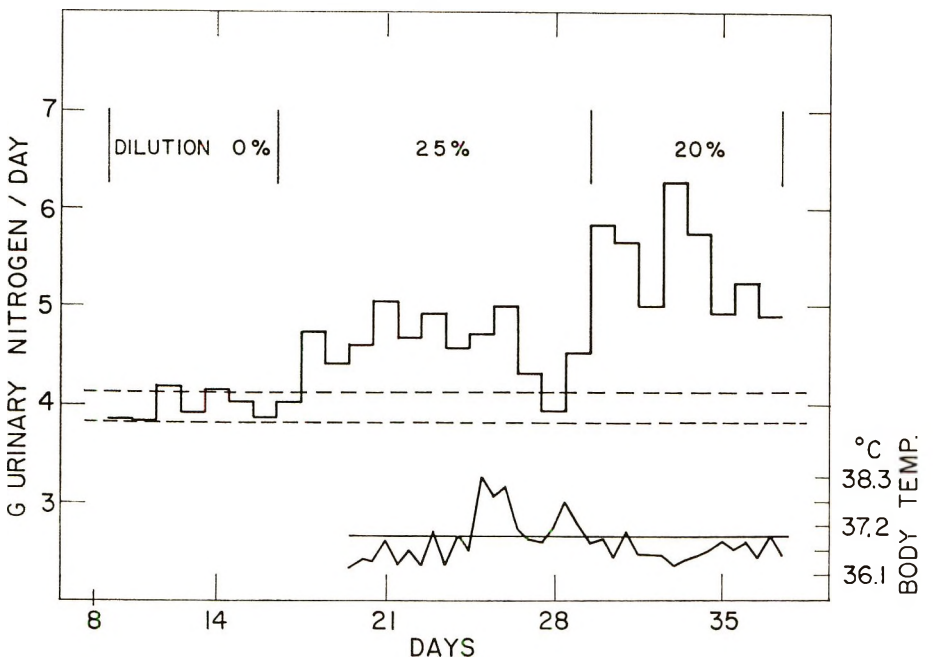

Fig. 2 Urinary nitrogen excretion per day in subject DP fed the basal beef formula diet with 30 and 20% replacement of nitrogen with glycine and diammonium citrate, who contracted an acute infection during the experiment. Broken lines indicate mean baseline nitrogen excretion ± one SD.

DISCUSSION

The results suggest that a diet in which beef protein supplies 90% of the total protein intake, with the remainder from oatmeal and tomato juice, can be diluted to the extent of 20 to 25% without a decrease in the efficiency of dietary nitrogen utilization by most young men. The dietary E/T_N ratios within this range of dilution lie between 2.16 to 2.02, the essential amino acids contributing 24.7 to 26.3% of the total dietary nitrogen (table 1).

From our previous studies with whole egg protein diets (5) the prediction was made that the basal beef formula could be

diluted to the extent of 20 to 30% (E/T_N ratio 2.16 to 1.89) without significantly decreasing dietary nitrogen utilization in most subjects. The results of the present experiment support this prediction with the qualification that a 30% dilution appears to be too high in the case of most young adults. An E/T_N ratio of 1.89 for beef essential amino acids appears to lie outside of the normal range. This may be due to the fact that methionine and cystine are the limiting amino acids in beef protein when compared with the amino acid pattern of whole egg protein (2).

The FAO/WHO Expert Group on Protein Requirements (2) suggested 190 to 220 mg methionine plus cystine/g nitrogen to be necessary for adequate human nutrition. The 30% diluted formula in the present study supplied approximately 156 mg methionine plus cystine/g nitrogen. Tryptophan may also be limiting in our beef formula diet since a 30% diluted diet supplies 50 mg/g nitrogen as compared with the 60 to 70 mg tryptophan suggested by the FAO/WHO Expert Group as being adequate for human nutrition. The influence of sulfur amino acids or tryptophan supplementation, or both, on the ability of subjects to tolerate a 30% dilution should be studied. These considerations emphasize that minimum dietary E/T_N ratios will depend upon the adequacy of the essential amino acid pattern of the dietary protein tested.

The results from subject DP are of special interest since he developed a febrile condition during the study. On the basis of the results obtained with the other subjects it appears probable that his increased rate of urinary nitrogen excretion during the latter part of the study was due to this fact. An increased nitrogen excretion at this low level of protein intake is noteworthy since it has been shown in experimental animals that the catabolic response to stress, as measured by urinary nitrogen excretion, is reduced or even abolished at low levels of protein intake (7–8). It is possible, therefore, that infection or trauma may increase the proportion of essential amino acids to total nitrogen required. We intend to investigate this possibility.

As stated previously (5), the present experiments are relatively short-term ones; long-term studies will be required before minimum E/T_N ratios for various proteins or combinations of proteins can be recommended as satisfactory for the continued well-being of population groups. Furthermore, it is recognized that minimum dietary E/T_N ratios for infants and possibly for elderly persons may be higher than those determined for the young adults in the present experiments. This is also under study.

ACKNOWLEDGMENT

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LITERATURE CITED

1. Food and Agriculture Organization 1957 Protein Requirements. FAO Nutritional Studies no. 16 Food and Agriculture Organization of the United Nations, Rome.
2. Food and Agriculture Organization 1965 Protein Requirements. Report of a Joint FAO/WHO Expert Group. FAO Nutrition Meetings Report Series no. 37. Food and Agriculture Organization of the United Nations, Rome.
3. Howe, E. E., E. W. Gilfillan and J. B. Allison 1960 Efficacy of the FAO amino acid reference standard for growth of the weanling rat. J. Nutr., 70: 385.
4. Rose, W. C., and R. L. Wixom 1955 The amino acid requirements of man. XVL. The role of the nitrogen intake. J. Biol. Chem., 217: 997.
5. Scrimshaw, N. S., V. R. Young, R. Schwartz, M. L. Piché and J. B. Das 1966 Minimum dietary essential amino acid-to-total nitrogen ratio for whole egg protein fed to young men. J. Nutr., 89: 9.
6. Albanese, A. A., and V. Irby 1944 Determination of urinary amino nitrogen by the copper method. J. Biol. Chem., 153: 583.
7. Munro, H. N., and M. I. Chalmers 1945 Fracture metabolism at different levels of protein intake. British J. Exp. Pathol., 26: 396.
8. Madden, S. C., and W. A. Clay 1945 Protein metabolism and protein reserves during acute sterile inflammation. High protein intake compensates for increased catabolism. J. Exp. Med., 82: 65.

Effect of Phytate on Iron Absorption in the Rat

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ABSTRACT Hemoglobin regeneration was studied in groups of anemic rats fed purified diets containing 10 or 20 ppm of iron in which either 45 or 75% of the phosphorus was replaced with phytate phosphorus. The results were compared with those obtained with control animals receiving phytate-free diets. In the groups receiving 20 ppm of iron, hemoglobin regeneration was more rapid than in the groups given 10 ppm. However, at either level of iron, dietary phytate had no effect on iron absorption as measured by total hemoglobin regeneration.

A great deal of confusion exists concerning the role of phytate in nutrition (1), and, in particular, its effect on iron absorption remains a controversial issue. Much of this controversey has arisen because various investigators have reported conclusions based on different types of experimental designs, techniques and experimental subjects.

Balance studies, performed on human subjects by Walker et al. (2), and by Foy et al. (3), showed no consistent effect of dietary phytate on iron absorption. However, Hussain and Patwardhan (4) showed that, when phytate phosphorus was increased from 8 to 40% of the total dietary phosphorus, iron absorption was reduced from 11 to 3%. In contrast, Apte and Venkatachalam (5), using a diet and techniques similar to those of the latter workers, observed iron absorption to be 30% instead of 3%.

Sharpe et al. (6), in studies with radioactive iron, noted no correlation between the phytate content of foods and reduction in iron absorption in humans; however, they reported that added sodium phytate reduced iron absorption considerably. Using iron utilization for red cell production in normal human adults, Turnbull et al. (7) showed that the addition of sodium phytate to 5 mg of ferrous ascorbate resulted in the reduction of iron absorption by around 50%. In contrast, the addition of sodium phytate to hemoglobin iron, if anything, enhanced iron absorption.

Sathe and Krishnamurthy (8) studied hemoglobin regeneration in rats, and concluded that phytin phosphorus inhibited the absorption of iron. However, they presented neither the phytate nor the iron content of the diets used, and the differences in values for hemoglobin regeneration among their groups of rats were not statistically significant.

The present work was initiated after previous rat studies[4] failed to show a correlation between the phytate content of certain cereals and legumes and the physiological availability of iron in these foods. Although red cell utilization of radioactive iron is the most accurate technique for measurement of iron absorption, its use in rats poses technical difficulties. Moreover, iron balance studies suffer from severe limitations of inaccuracy. Hemoglobin regeneration in anemic rats has been shown to be a suitable criterion for the measurement of iron absorption (9, 10) and appeared to be best suited for this investigation.

EXPERIMENTAL

Weanling rats of the Sprague-Dawley strain were maintained with a low iron (< 5 ppm) basal diet (table 1) for 3 to 5 weeks, or until their hemoglobin levels were below 7 g/100 cm^3.

The desired levels of 10 or 20 ppm of iron (10 or 20 mg/kg) in the experimental

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[4] Esfahani, M., and J. W. Cowan, unpublished data.

TABLE 1

Composition of basal low iron diet

	g/kg
Vitamin-free casein [1]	200
Reagent grade sucrose	600
Non-nutritive cellulose [2]	50
Corn oil [3]	100
Vitamin mixture [4]	10
Mineral mixture (iron-free) [5]	40

[1] Obtained from General Biochemicals Inc., Chagrin Falls, Ohio.

[2] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

[3] Mazola (Corn Products Company, Argo, Illinois) in glass containers.

[4] Each kilogram of mixture contained the following vitamins, triturated in dextrose: (in grams) vitamin A conc (200,000 units/g), 4.5; vitamin D conc (400,000 units/g), 0.25; α-tocopherol, 5.0; ascorbic acid, 45.0; inositol, 5.0; choline chloride, 75.0; riboflavin, 1.0; menadione, 2.25; p-aminobenzoic acid, 5.0; niacin, 4.5; pyridoxine·HCl, 1.0; thiamine·HCl, 1.0; Ca pantothenate, 3.0; and (in micrograms) biotin, 20; folic acid, 90; and vitamin B_{12}, 1.35 (Vitamin Diet Fortification Mixture, Nutritional Biochemicals Corporation).

[5] Contained the following: (g/100 g) $CaCO_3$, 26.000; $CaH_4(PO_4)_2 \cdot H_2O$, 10.500; $CoCl_2$, 0.005; $CuSO_4$, 0.030; $MgSO_4$, 10.200; $MnSO_4$, 0.510; KI, 0.080; K_2HPO_4, 41.200; NaCl, 11.450; and $ZnCl_2$, 0.025.

diets were attained by mixing thoroughly the appropriate amount of ferrous sulfate with the basal diet. For the phytate diets, sodium phytate [5] was added at the expense of K_2HPO_4 at one of 2 levels: 0.7 or 1.2% by weight. These amounts represented 45 and 75% of the total dietary phosphorus respectively. All experimental diets were isocaloric.

During the experiment, the animals were housed individually in stainless steel cages; the diets and deionized water were supplied ad libitum. Eight anemic rats (4 males and 4 females) were assigned to each of 6 diets according to a randomized block design based on hemoglobin concentration (11). Diet groups 1, 2 and 3 were fed the diet containing 10 ppm of iron as ferrous sulfate; the other 3 groups received 20 ppm. In diets 2 and 5, 45% of the die-

[5] Obtained from Sigma Chemical Company, St. Louis.

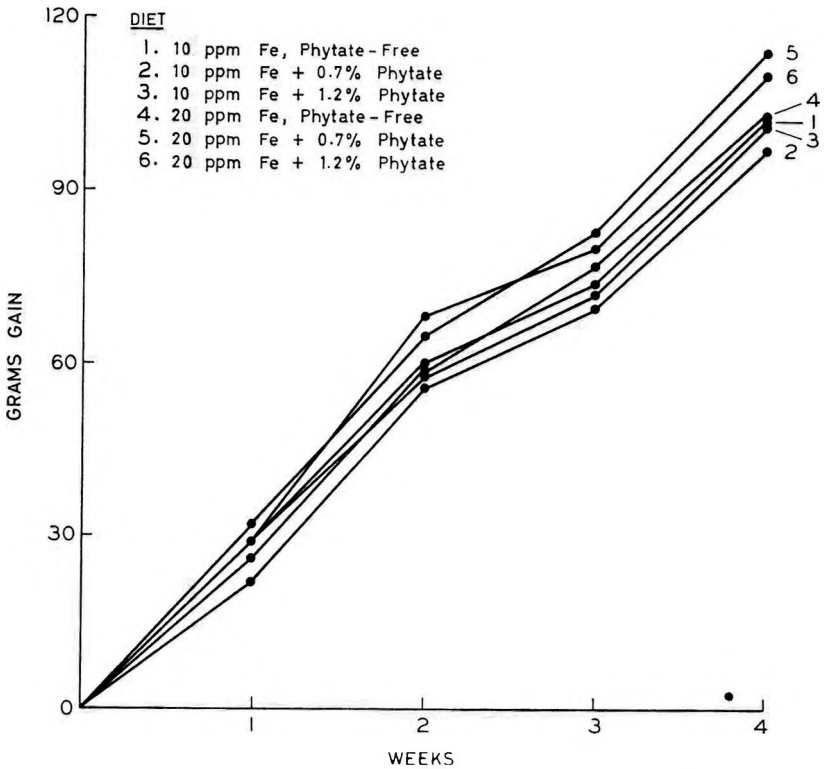

Fig. 1 Growth curves for 6 groups of anemic rats fed for 4 weeks purified diets containing various levels of iron and sodium phytate. Each point represents the average of 8 values.

tary phosphorus was replaced by phytate phosphorus, whereas in diets 3 and 6, phytate represented 75% of the total dietary phosphorus. Diets 1 and 4 were phytate-free and served as controls.

Feed intake and weight gains were recorded weekly; the experiment was terminated at the end of 4 weeks. Hemoglobin was determined on each animal weekly using the cyanomethomoglobin method (12). Total hemoglobin was calculated on the basis of 6.7 ml of blood/100 g body weight (9).

RESULTS AND DISCUSSION

The growth curves for the 6 dietary groups are shown in figure 1; growth was rapid and average gains were similar among all the groups (range, 97–113 g in the 4 weeks).

A level of 20 ppm of dietary iron is considered marginal, yet adequate, for normal hemoglobin regeneration in the anemic rat (13). However, in planning the present study, it was speculated that at this level of iron, there may be sufficient iron left over for hemoglobin regeneration after the chelating potential of the added phytate is satisfied. Thus, to accentuate any effect of dietary phytate, the presumed submarginal level of iron (10 ppm) was used in addition to the higher level of 20 ppm.

Figure 2 shows the hemoglobin regeneration curves (expressed as gain in hemoglobin concentration) for the 2 control groups fed the phytate-free diets. The rate of gain in the group which received 20 ppm was much more rapid than in the group receiving the lower level, and at the end of 4 weeks, average hemoglobin concentrations in the former group approached normal (13.84 g/100 cm^3). These results are in agree-

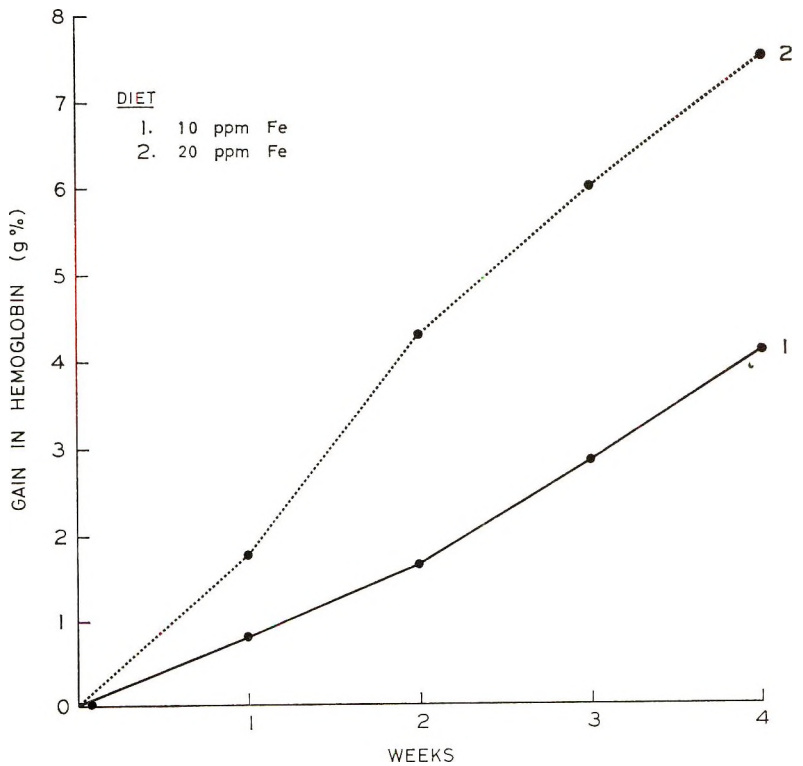

Fig. 2 Hemoglobin regeneration curves for groups of anemic rats fed for 4 weeks diets containing 10 and 20 ppm of iron from ferrous sulfate. Each point represents the average of 8 values.

TABLE 2

Average iron intake and hemoglobin regeneration in anemic rats fed various levels of iron and sodium phytate [1]

Level of dietary phytate	Iron intake	Hb conc		Total Hb [2]		P value	Iron intake	Hb conc		Total Hb		P value
		Initial	Gain	Gain	Gain/mg Fe			Initial	Gain	Gain	Gain/mg Fe	
%	*mg*	*g/100 cm³*	*g/100 cm³*	*g*	*g*		*mg*	*g/100 cm³*	*g/100 cm³*	*g*	*g*	
			2 weeks						2 weeks			
0.0	1.61	6.28	1.68	0.444	0.276		3.20	6.33	4.38	0.758	0.237	
0.7	1.46	6.13	1.83	0.444	0.304	ns [3]	2.77	5.94	4.17	0.775	0.280	ns
1.2	1.61	6.33	1.96	0.467	0.290	ns	3.25	6.21	3.51	0.704	0.217	ns
			3 weeks						3 weeks			
0.0	2.46	6.28	2.84	0.661	0.269		4.90	6.33	6.09	1.090	0.240	
0.7	2.25	6.13	2.02	0.589	0.262	ns	4.94	5.94	5.21	1.032	0.209	ns
1.2	2.48	6.33	3.25	0.704	0.284	ns	5.02	6.21	5.23	0.995	0.198	ns
			4 weeks						4 weeks			
0.0	3.43	6.28	4.08	1.009	0.294		6.88	6.33	7.51	1.509	0.219	
0.7	3.20	6.13	3.04	0.878	0.274	ns	6.94	5.94	7.03	1.537	0.221	ns
1.2	3.47	6.33	3.80	0.968	0.279	ns	7.04	6.21	6.44	1.402	0.226	ns

[1] Eight rats/group; 4 males and 4 females.
[2] Calculated on the basis of 6.7 ml blood/100 g body weight (9).
[3] Not significantly different from the control values ($P > 0.05$) as determined by analysis of variance (11).

ment with those of Raven and Thompson (13) who reported that the hemoglobin levels of growing anemic rats fed diets containing 20 ppm of inorganic iron returned to normal or near normal in 4 weeks. The curve for hemoglobin regeneration on 10 ppm (fig. 1) shows clearly that this was a submarginal level and was not sufficient to promote normal hemoglobin regeneration. After this diet had been fed for 4 weeks the average hemoglobin concentration increased to only 10.36 g/100 cm³.

The data in table 2 are the average values for iron intake and hemoglobin regeneration (gain in grams per 100 cubic centimeters and gain in total hemoglobin) in the 6 diet groups at the end of 2, 3 and 4 weeks. The first week was considered one of adjustment to the diets and the results were excluded from the table. The values for iron intake indicate that food consumption in all groups was similar; at 10 ppm, the iron intake was about one-half in all instances compared with that of the groups receiving 20 ppm.

The hemoglobin values in table 2 show that, even at the submarginal level of 10 ppm of iron, the rate of hemoglobin regeneration was not affected by the presence of either level of dietary phytate. When expressed on the basis of milligrams of iron consumed, there were no significant differences, at either level of iron, between control values and those of the diets containing phytate.

It was concluded from the results of this work that high levels of phytate have no effect on iron absorption in the rat. Therefore, the presence of phytic acid in foods fed to rats should not be expected to decrease the physiological availability of the iron in such foods.

LITERATURE CITED

1. Taylor, T. G. 1965 The availability of calcium and phosphorus of plant materials for animals. Proc. Nutr. Soc., *24:* 105.
2. Walker, A. R. D., F. W. Fox and J. T. Irving 1948 Studies in human mineral metabolism. I. The effect of bread rich in phytin phosphorus on the metabolism of certain mineral salts with special reference to calcium. Biochim. J., *42:* 452.
3. Foy, H., A. Kondi and W. H. Austin 1959 Effect of dietary phytate on fecal absorption of radioactive ferric chloride. Nature, *183:* 691.

4. Hussain, R., and V. N. Patwardhan 1959 The influence of phytate on the absorption of iron. Indian J. Med. Res., *47:* 676.
5. Apte, S. V., and P. S. Venkatachalam 1964 The influence of dietary calcium on absorption of iron. Indian J. Med. Res., *52:* 213.
6. Sharpe, L. M., M. C. Peacock, R. Cooke and R. S. Harris 1950 The effect of phytate and other food factors on iron absorption. J. Nutr., *41:* 433.
7. Turnbull, A., F. Cleton and C. A. Finch 1962 Iron absorption. IV. The absorption of hemoglobin iron. J. Clin. Invest., *41:* 1897.
8. Sathe, V., and K. Krishnamurthy 1953 Phytic acid and absorption of iron. Indian J. Med. Res., *41:* 453.
9. Thompson, A., and A. M. Raven 1959 The availability of iron in certain grass, clover and herb species. 1. Perenial ryegrass, cocksfoot and timothy. J. Agr. Sci., *52:* 177.
10. Whipple, G. H., and F. S. Robscheit-Robbins 1936 Iron and its utilization in experimental anemia. Amer. J. Med. Sci., *191:* 11.
11. Snedecor, G. W. 1962 Statistical Methods, ed 5. The Iowa State College Press, Ames.
12. Hainline, A. 1958 Hemoglobin In: Standard Methods of Clinical Chemistry, vol. 2. Academic Press, New York.
13. Raven, A. M., and A. Thompson 1959 The availability of iron in certain grass, clover and herb species. 2. Alsike, broad red clover, Kent wild white clover, trefoil and lucerne. J. Agr. Sci., *53:* 224.

Linoleic Acid Requirement of the Chick

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ABSTRACT The linoleic acid requirement of young male chicks was determined during 4- to 6-week periods on the basis of growth response and also the triene-to-tetraene ratio of fatty acids in liver and erythrocytes. Dietary fat levels in different experiments ranged from 3 to 8% and the dietary content of linoleic acid varied from 0.18 to 4.3% of calories. Maximal body weight was obtained with 0.9 to 1.1% of calories as linoleic acid, and these supplements also resulted in triene-to-tetraene ratios of 0.4 or less in liver. In erythrocytes, 1.3 to 1.8% of dietary calories as linoleic acid were required to produce triene-to-tetraene ratios of 0.4. The latter criterion indicates a maximal linoleic acid requirement under the conditions of these experiments of 1.8% of calories, whereas the criteria of growth and liver triene-to-tetraene ratio gave a requirement of 1%.

It is well established that the chick requires a dietary source of essential fatty acid (1–3); however, the quantitative requirement has not been ascertained as thoroughly as it has for other species. Recently, Hill (4) estimated the linoleic acid requirement of the chick on the basis of the liver fatty acid composition. We have used a similar approach while varying the type and amount of dietary fat. In addition to determining the effect on erythrocyte and liver fatty acids, we have also recorded the effect of dietary linoleic acid on growth.

Linoleic acid deficiency results in alterations in the distribution of tissue fatty acids which are remarkably similar for all species that have been studied. The most prominent fatty acid changes are decreases in linoleic and arachidonic acids (and also of other acids derived from linoleic acid) and increases in oleic and 5,8,11-eicosatrienoic acids, the latter being derived from oleic acid (5). The proposal by Holman (6) of using the ratio of 5,8,11-eicosatrienoic acid to arachidonic acid (the triene-to-tetraene ratio), as developed for the rat, has also been used satisfactorily with other species (7, 8). A dietary linoleate level which results in a triene-to-tetraene ratio in tissues of about 0.4 or less has been shown to supply the physiological requirement for essential fatty acid as determined by growth rate and prevention of dermal symptoms.

EXPERIMENTAL

One-day-old male Arbor Acres chicks, eight to a group, were kept in electrically heated brooders with wire-mesh flooring. They were fed a casein-gelatin diet (table 1) in which the amount of fat and also the composition was varied in each experiment. Food and water were available ad libitum and lighting was continuous. In experiments where growth was used as a response, duplicate groups of 8 chicks were selected with similar average weights. A few small and large chicks were removed from all groups after the second week.

The linoleic acid content of the dietary fats as determined by gas chromatography was as follows: (as per cent of total fatty acids) trilaurin,[1] 0; triolein,[2] 3.1; hydrogenated coconut oil,[3] 0; safflower oil,[4] 77.1; olive oil,[5] 5.4. No linolenic acid was detected in any of the fats. The mixtures of these fats used in various experiments were also analyzed to verify the linoleic acid content of each diet. The triolein, safflower oil and olive oil were stabilized by the addition of 0.01% each of propyl gallate and citric acid, and were stored at 4° as were also the complete diets.

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[1] Drew Chemical Corporation, Boonton, New Jersey.

[2] See footnote 1.

[3] Hydrol, Durkee Famous Foods, Chicago.

[4] Pacific Vegetable Oil Corporation, Richmond, California.

[5] Progresso, Uddo and Taornina Corporation, Brooklyn, New York.

TABLE 1

Composition of chick diet C73

	%
Vitamin-free casein [1]	20
Gelatin	8
Salt mix N [2]	6
Vitamin mix [3]	2
Stabilized vitamin A [4]	1
L-Arginine	0.8
DL-Methionine	0.3
Ethoxyquin [5]	0.02
Fat [6]	3–8
Glucose monohydrate	to make 100

[1] Nutritional Biochemicals Corporation, Cleveland.
[2] Fox, M. R. S., and G. M. Briggs 1960 J. Nutrition, 72: 243.
[3] Provided in mg/kg of diet: thiamine·HCl, 8; riboflavin, 8; Ca pantothenate, 20; choline chloride, 2000; niacin, 100; pyridoxine·HCl, 8; D-biotin, 0.3; folic acid, 3; and vitamin B_{12}, 0.1. Added in ethanol solutions: (mg/kg diet) vitamin D_3, 0.02; 2-methyl-1,4-naphthoquinone, 1; *dl*,*α*-tocopheryl acetate, 100.
[4] Nopcay 20, Nopco Chemical Company, Newark, New Jersey, provided 0.7 mg retinyl palmitate.
[5] Santoquin, Monsanto Company, St. Louis.
[6] Amount for each experiment given in the tables. Fats are described in the text.

Total lipids were extracted from liver and washed erythrocytes and the total fatty acids methylated and analyzed by gas liquid chromatography as described previously (9). A 4 mm × 180 cm column of 15% ethylene glycol succinate on 80–100 mesh Gas-Chrom P was operated at 180°. Peak areas were determined by triangulation and the results expressed as area percentage. The positional isomers of eicosatrienoic acid separated distinctly and are designated as 20:3ω6 (8,11,14 isomer) or 20:3ω9 (5,8,11 isomer).

RESULTS

Detailed results of fatty acid analyses of liver and erythrocytes are given for only one study (table 2). In this 6-week experiment, a dietary fat level of 4.5% was composed of 2% hydrogenated coconut oil with the remainder made up of varying

TABLE 2

Effect of varying linoleic acid intakes on weight gain and on the fatty acid composition of liver and erythrocytes [1]

Dietary fat, %	0	4.5	4.5	4.5	4.5	4.5	4.5
Calories as linoleate, %	0	0.40	0.55	0.87	1.15	1.71	2.77
Average wt, g	626 ± 19 [2]	793 ± 19 [3]	780 ± 14	822 ± 14	776 ± 18	795 ± 23	831 ± 25
Feed efficiency [4]	0.520	0.612	0.683	0.595	0.601	0.605	0.611
RBC fatty acids, % of total fatty acids							
16 ald.	0.4 ± 0.2 [2]	0.8 ± 0.2	1.4 ± 0.3	0.9 ± 0.1	0.5 ± 0.1	1.1 ± 0.3	1.4 ± 0.2
16:0	17.6 ± 0.8	18.2 ± 0.6	18.1 ± 0.4	18.4 ± 0.2	16.7 ± 0.4	19.4 ± 0.4	19.1 ± 0.5
16:1	4.9 ± 0.6	2.5 ± 0.2	2.2 ± 0.3	2.2 ± 0.1	1.7 ± 0.2	1.8 ± 0.2	1.7 ± 0.1
18:0	9.5 ± 0.8	13.2 ± 0.3	12.9 ± 0.5	13.6 ± 0.6	14.4 ± 0.5	13.0 ± 0.4	14.6 ± 0.2
18:1	60.9 ± 0.9	47.2 ± 0.8	44.9 ± 0.3	42.6 ± 1.6	37.6 ± 0.8	33.4 ± 0.5	24.2 ± 0.8
18:2	1.2 ± 0.1	10.4 ± 0.4	12.6 ± 0.4	15.7 ± 1.2	18.3 ± 0.7	20.1 ± 0.8	26.3 ± 0.8
20:3ω9	4.3 ± 0.2	3.0 ± 0.3	2.7 ± 0.1	2.2 ± 0.1	2.1 ± 0.1	1.8 ± 0.1	1.3 ± 0.1
20:3ω6	tr [5]	0.8 ± 0.1	0.9 ± 0.1	1.0 ± 0.0	1.4 ± 0.1	1.4 ± 0.0	1.6 ± 0.1
20:4	0.7 ± 0.1	2.3 ± 0.3	2.6 ± 0.1	2.5 ± 0.1	4.5 ± 0.3	4.7 ± 0.1	6.3 ± 0.4
22:4	0	tr	tr	tr	0.7 ± 0.0	0.8 ± 0.1	1.1 ± 0.1
20:3ω9/20:4	6.2	1.3	1.0	0.85	0.48	0.39	0.20
Liver fatty acids, % of total fatty acids							
16:0	21.9 ± 1.0	24.1 ± 2.1	24.6 ± 1.1	22.9 ± 1.1	25.3 ± 1.1	19.9 ± 2.6	22.7 ± 2.2
16:1	5.2 ± 0.5	4.9 ± 1.0	4.5 ± 0.4	4.6 ± 0.3	5.4 ± 0.7	3.5 ± 0.9	3.6 ± 0.6
18:0	14.6 ± 1.9	18.6 ± 2.0	20.5 ± 1.0	20.8 ± 0.6	18.7 ± 1.5	23.2 ± 2.1	23.5 ± 1.6
18:1	50.3 ± 1.8	39.1 ± 2.2	35.7 ± 1.6	33.3 ± 2.1	35.7 ± 3.4	31.6 ± 2.2	28.9 ± 2.4
18:2	0.5 ± 0.1	4.2 ± 0.7	5.1 ± 0.4	7.8 ± 1.2	6.2 ± 1.1	9.0 ± 0.9	9.5 ± 1.2
18:3	0.8 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.7 ± 0.1	0.5 ± 0.0
20:3ω9	4.9 ± 0.8	3.3 ± 0.6	3.2 ± 0.5	2.4 ± 0.5	2.0 ± 0.5	2.8 ± 0.1	2.0 ± 0.4
20:3ω6	tr	1.1 ± 0.2	1.3 ± 0.1	1.7 ± 0.1	1.4 ± 0.9	2.2 ± 0.2	2.1 ± 0.3
20:4	1.3 ± 0.2	3.5 ± 0.6	4.0 ± 0.3	5.5 ± 0.5	4.2 ± 0.8	6.8 ± 0.8	6.9 ± 1.0
20:3ω9/20:4	3.7	0.94	0.80	0.43	0.48	0.41	0.29

[1] 13 to 15 male chicks/group; 6-week period. Dietary fat contained 2% hydrogenated coconut oil and 2.5% of a variable mixture of olive oil and safflower oil to provide the amounts of linoleic acid indicated. Diet calculated to have 398 kcal/100 g.
[2] Mean ± SE.
[3] Significantly greater than the fat-free group ($P < 0.001$).
[4] Weight gain (g)/feed consumed (g).
[5] Trace, less than 0.3%.

TABLE 3

Weight gains and the triene-to-tetraene ratios of erythrocyte and liver fatty acids of chicks fed varying dietary levels of linoleic acid [1]

Calories as 18:2	Weight	Feed efficiency	20:3ω9/20:4	
			RBC	Liver
%	g	g gain/g feed		
0.0 [2]	529 ± 11 [3]	0.585	7.64	4.92
0.0 [4]	568 ± 17	0.589	—	—
0.18	643 ± 10 [5]	0.618	3.26	2.31
0.56	640 ± 15	0.616	1.36	0.92
0.84	658 ± 13	0.620	0.77	0.51
1.13	685 ± 15 [6]	0.664	0.58	0.34
1.61	686 ± 12	0.679	0.30	0.14
4.34	686 ± 16	0.633	0.04	0.02

[1] 13 to 15 male chicks/group; 5-week period; 4.5% dietary fat (2% trilaurin, 0 to 2.5% triolein and 0 to 2.5% safflower oil).
[2] Fat-free diet.
[3] Mean ± SE.
[4] Fat-free diet + 2% glycerol.
[5] Significantly greater than the fat-free group ($P < 0.001$).
[6] Significantly greater than the 0.56% group ($P < 0.001$), but not different from the 0.84% group ($P > 0.1$).

proportions of olive oil and safflower oil to give the designated amounts of linoleic acid. The triene-to-tetraene ratio in the red cells approached 0.4 when the dietary linoleate constituted 1.15 to 1.71% of calories, whereas in the liver this ratio was achieved at 0.87% of calories. With respect to body weight, a highly significant increase over the fat-free group was achieved with the lowest amount of linoleate, 0.40% of calories. Higher dietary levels of linoleic acid produced a somewhat erratic response, in that the weights with both the 0.87 and 2.77% levels were significantly greater ($P < 0.01$) than the weights achieved at the other levels of linoleate supplementation. However, the average weight of even the lightest group (1.15% of calories, 776 g) is close to maximal growth for this strain of chicks. All levels of linoleate improved feed efficiency over that of the fat-free group.

In a second experiment of 5 weeks' duration, the dietary fat level of 4.5% was composed of 2% trilaurin, and the remainder was made up of varying proportions of triolein and safflower oil (table 3). In the erythrocytes, a triene-to-tetraene ratio < 0.4 was produced by 1.61% of calories as linoleate, whereas in the liver 1.13% of calories was sufficient. Two per cent of glycerol in the fat-free diet may have improved body weight ($P < 0.05$), but the lowest level of dietary linoleate, 0.18% of calories, gave a highly significant increase ($P < 0.001$). Maximal growth was attained with 0.84 to 1.13% of calories as linoleate. Feed efficiency was improved only at the higher levels of linoleate intake.

TABLE 4

Triene-to-tetraene ratios of erythrocyte fatty acids of chicks fed varying dietary levels of linoleic acid

Calories as 18:2	20:3ω9/20:4
%	
Series 253 [1]	
0.0	2.26
0.67	0.71
1.34	0.40
2.00	0.20
Series 244 [2]	
0.0	1.31
0.18	1.02
0.36	0.76
0.89	0.47
1.76	0.27
2.65	0.14

[1] Six chicks/group; 4-week period; 8% dietary fat (6.8–8.0% hydrogenated coconut oil and 0–1.2% safflower oil).
[2] Five male chicks/group; 4-week period; 3% dietary fat (1.5–3% hydrogenated coconut oil and 0–1.5% safflower oil).

In two additional studies of 4 weeks' duration, the dietary fat levels were 3 and 8%, provided by mixtures of hydrogenated coconut oil and safflower oil. The number of birds in the experiments was small and hence growth data were not meaningful. The triene-to-tetraene ratios of the erythrocytes (table 4) show that 1.34% of cal-

ories as linoleate was adequate in the diet with 8% total fat, whereas with 3% dietary fat 0.89 to 1.76% of calories produced a ratio of about 0.4.

DISCUSSION

Available criteria for evaluating the essential fatty acid requirements of young animals over a short period are limited to either the growth response or the changes in tissue fatty acid composition. In the case of the chick, although the typical essential fatty acid deficiency pattern in tissues is readily demonstrable in 4 weeks, growth effects in this time are not consistently observed. Differences in results reported by different investigators can probably be attributed to the variable linoleic acid stores in newly hatched chicks and also to the type and amount of dietary fat. In our earlier studies with a slower growing strain of chicks (1), an effect of a fat-free diet on growth was not apparent until the sixth week. With the faster growing Arbor Acres chick, a growth difference has been noticeable after only 4 weeks. If, however, instead of a fat-free diet, a diet containing fat low in linoleic acid is used, then growth is not significantly retarded even though the triene-to-tetraene ratio is considerably greater than 0.4. Thus, in the 2 growth experiments (tables 2 and 3) the lowest dietary levels of linoleic acid, 0.18 and 0.40% of calories, produced body weights equal to levels of 1% or higher. It should be noted that in the diet containing 0.18% of calories as linoleate (table 3) all of the linoleic acid was contributed by the linoleic acid contained in the 2.5% triolein, i.e., there was no added safflower oil. This result strongly suggests that the growth response was due to unsaturated fat per se and not entirely to its linoleic acid content. Hill (4), using a similar casein-gelatin diet with a mixture of hydrogenated coconut oil and corn oil fed for 4- or 7-week periods, obtained maximal body weights with 0.25 and 0.5% of calories as linoleate but not with 0.13%. One difference in these experiments was that we kept the amount of saturated fat in the diet constant and substituted the linoleate source (safflower oil) for an oil poor in linoleate (olive oil or triolein), whereas Hill substituted corn oil for the saturated fat. A strict comparison of the response to dietary oils which vary in linoleic acid content (and without more highly unsaturated fatty acids) does not appear to have been made previously with the chick.

A comparison of the triene-to-tetraene ratios in liver and erythrocytes (tables 2 and 3) indicates that liver may be more sensitive to dietary linoleate, i.e., the triene-to-tetraene ratio is lower in liver than in erythrocytes, particularly at lower dietary levels of linoleate. If the liver ratio is used to estimate linoleate requirement (a ratio about 0.4 or less), then in these 2 experiments the values would be 0.87 and 1.13% of calories, whereas the erythrocyte ratios indicate a requirement of 1.15 to 1.61% of calories as linoleate. In the other 2 experiments in which only erythrocytes were analyzed, linoleate requirements of 1.34% and 1.76% of calories are indicated. The range of caloric requirement for linoleic acid from these studies thus is 0.9 to 1.8%. This is similar to the range of 1 to 2% estimated by Hill (4) from a plot of the triene-to-tetraene ratio versus the dietary linoleate content. Of special interest is a comparison of the liver triene-to-tetraene ratios at similar linoleate intakes obtained by Hill with those reported here. In the following summary, the first 2 ratios are our values and the third is that of Hill:

0.50–0.56% of calories:	0.80, 0.92, 0.73
0.75–0.87% of calories:	0.43, 0.51, 0.42
1.00–1.15% of calories:	0.48, 0.34, 0.33

There was thus very good agreement between the 2 studies.

Although the triene-to-tetraene ratios from liver and erythrocyte analyses indicate a linoleic acid requirement of the growing male chick of 0.9 to 1.8% of calories, it would be desirable to have a correlation of this biochemical parameter with physiological observations such as was done in the rat where the body weight and dermal score were used (5). Dermal symptoms in the chick do occur (1) but are not amenable to quantitative evaluation. As noted by Hill (4), the growth of young chicks in experiments of this type tends to be irregular; however, meaningful information should be obtainable under properly controlled conditions. In both of the experiments reported here in which

body weight was measured, although all dietary levels of linoleate improved growth over that of the fat-free groups, the response to increasing amounts of linoleic acid was not consistent. Further experiments in which dietary oils low in linoleic acid are fed with oils high in the acid should be able to clarify the young chick's linoleate requirement for growth.

LITERATURE CITED

1. Bieri, J. G., G. M. Briggs, M. R. Spivey Fox, C. J. Pollard and L. O. Ortiz 1956 Essential fatty acids in the chick. I. Development of fat deficiency. Proc. Soc. Exp. Biol. Med., *93:* 237.
2. Machlin, L. J., and R. S. Gordon 1961 Effect of dietary fatty acids and cholesterol on growth and fatty acid composition of the chicken. J. Nutr., *75:* 157.
3. Fisher, H., A. S. Feigenbaum and H. S. Weiss 1961 Requirement of essential fatty acids and avian atherosclerosis. Nature, *192:* 1310.
4. Hill, E. G. 1966 Effect of dietary linoleate on chick liver fatty acids: Dietary linoleate requirement. J. Nutr., *89:* 465.
5. Fulco, A. J., and J. F. Mead 1959 Metabolism of essential fatty acids. VIII. Origin of 5,8,11-eicosatrienoic acid in the fat-deficient rat. J. Biol. Chem., *234:* 1411.
6. Holman, R. T. 1960 The ratio of trienoic: tetraenoic acids in tissue lipids as a measure of essential fatty acid requirement. J. Nutr., *70:* 405.
7. Hill, E. G., E. L. Warmanen, C. L. Silbernick and R. T. Holman 1961 Essential fatty acid nutrition in swine. I. Linoleate requirement estimated from triene : tetraene ratio of tissue lipids. J. Nutr., *74:* 335.
8. Reid, M. E., J. G. Bieri, P. A. Plack and E. L. Andrews 1964 Nutritional studies with the guinea pig. X. Determination of the linoleic acid requirement. J. Nutr., *82:* 401.
9. Bieri, J. G., and E. L. Andrews 1963 Metabolic effects of selenium as related to vitamin E. J. Amer. Oil Chem. Soc., *40:* 365.

Influence of the Type of Dietary Saturated Fatty Acid on Lipemia, Coagulation and the Production of Thrombosis in the Rat [1]

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ABSTRACT The degree of saturation of a dietary fat in a low protein diet and the resulting hypercholesterolemia or hypertriglyceridemia could not be correlated, in the rat, with the incidence of phlebothrombosis as initiated by a *Salmonella typhosa* endotoxin, nor to the recalcification plasma clotting time. To determine whether in a fat, it is the type of the saturated fatty acid rather than its degree of saturation that predisposes to thrombosis, various saturated fatty acids or their methyl esters were added to lard in the diet of the rat. Only the palmitic or stearic acid feeding resulted in a high incidence of thrombosis and a shortened clotting time, although the triglyceride levels in serum were approximately the same whichever fatty acid was fed. Under the present conditions, it appears therefore, that the thrombogenic capacity of a dietary fat depends primarily on the type of saturated fatty acids it contains. This thrombogenicity does not appear to be related to the concentration of cholesterol or triglycerides in serum, but possibly to the type of proteins these parameters were bound to. The plasma clotting time gave a good indication of the thrombotic tendency on a group basis.

Epidemiologic studies have shown that the high incidence of coronary heart disease (1, 2) and of venous thrombosis (3–5) in certain countries is usually associated with a high intake of saturated fats. A relationship has also been found to exist between the cholesterol level and the incidence of coronary heart disease in several (6, 7), but not all (8), populations studied. Although it appears that saturated fats raise the serum cholesterol in man (9–11) as well as in animals (12–14), studies performed on chicks and rabbits indicated that there is no relationship between the degree of atherosclerosis and the level of cholesterol in the blood (15–18). It has been concluded (15) that the development of experimental atherosclerosis is dependent, not only on the concentration of cholesterol in the serum, but on other pathogenetic factors as well.

Several investigators have reported that long-chain saturated fatty acids accelerate the clotting of blood (19–21). In addition, it has been demonstrated that only these long-chain saturated fatty acids can induce thrombotic phenomena when injected intravenously into animals (22, 23). The aim of the present experiments was to determine whether it is the type of the dietary saturated fatty acid rather than the degree of saturation that predisposes to thrombosis. For this purpose, rats were fed for several weeks high fat, low protein diets differing only in the type of fat or of fatty acid they contained. After removal of blood for determination of the lipemic and coagulation changes, an endotoxin was injected intravenously to initiate the formation of large hepatic vein thrombosis as described previously (24–26).

EXPERIMENTAL

Male rats of the Holtzman strain, with an initial body weight of 140 to 160 g, were used for these studies. At the beginning of each experiment, each group comprised 6 rats. Most of the experiments were repeated once or several times, and the results reported in the tables were, therefore, obtained from pooled data. In

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the course of the studies, several animals were eliminated for various reasons; the final number of animals retained in each group is indicated in the corresponding table.

The rats were housed 6 per cage in a constant-temperature environment and given, ad libitum, tap water and the hyperlipemic diets. All the diets contained: (in per cent) [4] casein, 11; cholesterol, 5; salt mixture (27), 4; sodium cholate, 2; sucrose, 23; vitamin mixture, 2; and cellulose as reported in detail elsewhere (25). In addition, in experiment 1 (table 1) isocaloric amounts of coconut oil (group 1), butter (group 2), or arachis oil (group 3) were added to these basic ingredients. In all the other experiments (3 to 5), water (7%) was included in the diet and the fat consisted of lard alone (31%) (tables 2, 3 and 4, group 1), a fatty acid (8%) plus lard (tables 2, 4 and 5), or a methyl ester (10%) plus lard (21%) (table 3).

At the end of the feeding periods indicated in the corresponding tables and after 17 hours' fasting, altogether 3 ml of blood were removed in 2 ml siliconized syringes, using 20-gauge needles, from the jugular vein of all the rats, by a clean venipuncture, under ether anesthesia. A few drops of blood were first removed with one syringe, and 1 ml of blood was then collected in another syringe containing 0.1 ml of sodium citrate (3.8%) and thoroughly mixed immediately. The first syringe was again connected to the needle and blood was removed to 2 ml for chemical determination. Immediately after withdrawal of blood for the laboratory tests, the rats were given, by the same needle, 1 ml/100 g body weight of physiologic saline containing a *Salmonella typhosa* (0901, Boivin type) lipopolysaccharide [5] at the dosage indicated in the tables.

All the glassware and needles were given at least 6 coats of silicon, according to the technique reported by Bauer (28). For the plasma clotting time, the citrated blood was transferred to a centrifuge tube kept at 30°, preliminary experiments having shown that the clotting time of plasma kept at this temperature remains practically constant for several hours. After 6 minutes' centrifugation at 2000 rpm, the plasma was removed and also kept at 30° until the test was performed. One-tenth milliliter of plasma was incubated for 10 minutes at 37° before the addition of 0.2 ml of M/100 calcium chloride solution (0.11 g anhydrous calcium chloride, 0.42 g sodium chloride, distilled water 100 ml), which was also kept at 37°. After one minute, the tube was tilted gently every 10 seconds, until a firm clot formed. The test was performed in duplicate. In experiments 1 and 3, the tubes were of plas-

[4] The salt mixture, the vitamin mixture (Vitamin Diet Fortification Mixture), the cellulose (Alphacel) and the fatty acids were obtained from Nutritional Biochemicals Corporation, Cleveland. The vitamin mixture contained/kg: vitamin A, 900,000 units; vitamin D, 100,000 units; and (in g) α-tocopherol, 5; ascorbic acid, 45; inositol, 5; choline chloride, 75; menadione, 2.25; p-aminobenzoic acid, 5; niacin, 4.5; riboflavin, 1; pyridoxine·HCl, 1; thiamine·HCl, 1; Ca pantothenate, 3; and (in mg) biotin, 20; folic acid, 90; vitamin B_{12}, 1.35; and dextrose to make 1 kg.

[5] Difco Laboratories, Detroit.

TABLE 1

Influence of the dietary fat on the production of thrombosis,[1] coagulation and lipemic changes (feeding period, 11 weeks) [2]

Dietary fat, wt %	Coconut oil, 32	Butter, 38	Arachis oil, 32
Group	1	2	3
No. of animals	18	17	15
Cholesterol, mg/100 ml	1222 ±284 [3]	978 ±72	297 ± 12
Triglycerides, mg/100 ml	299 ± 85	176 ±25	59 ± 5
α-Lipoproteins, %	12.5± 1.4	10.2± 0.9	0.3± 0.3
Plasma clotting time, (plastic tubes) sec	191 ± 18	171 ±14	175 ± 10
Thrombosis, (incidence) %	33	94	57
Survival, min	142 ± 21	189 ±15	1091 ±135
Mortality, %	100	100	33

[1] Thrombosis was initiated by the intravenous injection of *S. typhosa* lipopolysaccharide (0.6 mg/kg).

[2] Diet composition in addition to the fats listed in the table: (wt %) casein, 11; cholesterol, 5; salt mixture, 4; sodium cholate, 2; sucrose, 23; vitamin mixture, 2; and cellulose to make 100.

[3] Mean ± SE.

TABLE 2

Influence of saturated fatty acids on the production of thrombosis,[1] coagulation and lipemic changes (feeding period, 6 weeks) [2,3]

Dietary fatty acid (no. carbon atoms)	None	Caprylic C_8	Capric C_{10}	Palmitic C_{16}	Stearic C_{18}	Arachidic C_{20}	Behenic C_{22}
Group	1	2	3	4	5	6	7
No. of animals	24	17	12	17	35	6	18
Cholesterol, mg/100 ml	417 ± 28 [4]	1035 ± 100	796 ± 72	637 ± 55	936 ± 75	1067 ± 87	734 ± 56
Triglycerides, mg/100 ml	62 ± 4	159 ± 17	136 ± 14	148 ± 18	125 ± 8	141 ± 13	150 ± 10
α-Lipoproteins, %	1.0 ± 0.2	53.7 ± 10	43.6 ± 14.0	7.5 ± 1.4	3.9 ± 0.8	44.8	70.3 ± 0.9
Gain body wt, g	57 ± 4	27 ± 3	17 ± 4	53 ± 4	48 ± 4	61 ± 9	73 ± 3
Plasma clotting time, (siliconized tubes) sec	267 ± 10	238 ± 7	240 ± 11	200 ± 9	209 ± 7	240 ± 14	268 ± 22
Thrombosis, incidence %	4	8	16	70	51	16	17
Thrombosis, severity 0–3	0.1	0.2	0.2	1.6	1.4	0.2	0.3
Mortality, %	13	66	33	70	69	33	50

[1] Thrombosis was initiated by the intravenous injection of *S. typhosa* lipopolysaccharide (1 mg/kg).
[2] Diet composition: (wt %) casein, 11; cellulose, 15; cholesterol, 5; lard, 31; salt mixture, 4; sodium cholate, 2; sucrose, 23; vitamin mixture, 2; water, 7; fatty acids were added at the expense of lard (8%).
[3] Group comparison, triglycerides: group 5 vs. groups 2 to 7, $P > 0.05$; group 1 vs. groups 2 to 7, $P < 0.001$; plasma clotting time: groups 4 and 5 vs. groups 1, 2, 3, 7, $P < 0.02$; group 5 vs. group 6, $P < 0.1$.
[4] Mean ± SE.

tic (cellulose nitrate, no. 654) [6] instead of siliconized glass.

In experiments 1, 2 and 3 (tables 1, 2 and 3), the determinations of the lipemic parameters were performed on individual or pooled sera of 2 animals, according to the following techniques: the total serum cholesterol by the Trinder (29) method; the lipoproteins, by prestained paper electrophoresis (30); the triglycerides, by the Van Handel method (31). In experiments 4 and 5, the lipoprotein electrophoretic fractionation was carried out according to the following technique. The paper electrophoresis cells, the power supply and the scanning apparatus used were all from Beckman Instruments Inc. The paper strips used were Beckman no. 319 328, with a standard barbital buffer of pH 8.6 and an ionic strength of 0.05 (1.84 g diethyl barbituric acid, 10.27 g sodium diethyl barbiturate, distilled water 1000 ml). The techniques used were generally those recommended by the manufacturer, except for the following modifications. For lipoproteins, 0.04 ml of serum was applied in 0.01 ml amounts and a constant potential of 120 v was passed through the paper for 17 hours. The strips were then heated for 15 minutes in an oven at 120 to 130° and, as reported by other workers (32), stained for 1 hour in a fat red 7B [7] solution (saturated solution of the dye in absolute ethyl alcohol 60 ml, diluted before use with distilled water 40 ml and then filtered twice). The strips were decolorized for approximately 2 minutes with constant agitation in a 2% acetic acid solution to which was added 0.02 ml of a commercial sodium hypochlorite (12%) per 100 ml and then quickly immersed, still under agitation, in a 2% solution of acetic acid. Three additional baths of the 2% acetic acid were used to complete the staining and the strips were dried for 15 minutes in an oven at 120°. The scanning was performed on a RB model Analytrol [8] with two 500 mμ interference filters and a B-5 cam. The proteins were stained with the bromophenol blue dye in alcoholic solution, according to the technique recommended by the manufacturer.[9]

[6] International Equipment Company, Boston.
[7] Ciba Company, Dorval, P. Qué., Canada.
[8] See footnote 5.
[9] Beckman Instruments Inc., Fullerton, California, Technical bulletin 6095 A.

TABLE 3

Influence of methyl caprylate and methyl palmitate on the production of thrombosis,[1] coagulation and lipemic changes (feeding period, 8 weeks)[2]

Dietary fat, wt %	Lard, 31	Lard, 21 + methyl caprylate, 10	Lard, 21 + methyl palmitate, 10
Group	1	2	3
No. of animals	11	12	11
Cholesterol, mg/100 ml	415 ±58[3]	1101 ±87	985 ±54
Triglycerides, mg/100 ml	79 ± 3.4	167 ±13	211 ±22
α-Lipoproteins, %	1.8± 0.3	69.0± 2.0	14.0± 0.5
Plasma clotting time, (plastic tubes) sec	185 ± 6	160 ±10	140 ±13
Thrombosis, incidence, %	18	8	90
Thrombosis, severity, 0–3	0.36	0.08	2.7
Mortality, %	36	75	100

[1] Thrombosis was initiated by the intravenous injection of *S. typhosa* lipopolysaccharide (0.6 mg/kg).
[2] In addition to the fats listed in the table, the diet was composed of: (wt %) casein, 11; cellulose, 15; cholesterol, 5; sodium cholate, 2; salt mixture, 4; sucrose, 23; vitamin mixture, 2; and water, 7.
[3] Mean ± SE.

TABLE 4

Influence of saturated fatty acids on the lipoprotein electrophoretic fractions and the production of thrombosis[1] (feeding period, 6 weeks)[2]

Dietary fatty acid	None	Capric	Behenic
Group[3]	1	2	3
Lipoprotein fractions, %			
α-	5.3	12.0	14.2
α_1-	31.3	26.9	20.2
α- + α_1-	36.6	38.9	34.4
α_2-	16.7	18.7	20.7
β-	31.9	28.8	33.1
origin	14.8	13.6	11.8
Triglycerides, mg/100 ml	68	81	83
Thrombosis, incidence, %	18	0	33
Thrombosis, severity, 0–3	0.3	0	0.8

[1] Thrombosis was initiated by the intravenous injection of *S. typhosa* lipopolysaccharide (1 mg/kg).
[2] In addition to the fatty acids listed in the table, included at the expense of lard (8%), the diet was composed of: (wt %) casein, 11; cellulose, 15; cholesterol, 5; lard, 31; salt mixture, 4; sodium cholate, 2; sucrose, 23; vitamin mixture, 2; and water, 7.
[3] Six rats/group.

TABLE 5

Influence of saturated fatty acids on the production of thrombosis,[1] coagulation and lipemic changes (feeding period, 10 weeks)[2]

Dietary fatty acid (no. carbon atoms)	Capric C_{10}	Stearic C_{18}	Behenic C_{22}
Group	1	2	3
No. of animals	10	10	11
Triglycerides, mg/100 ml	94 ±14[3]	146 ±11	164 ±22
α- + α_1-lipoproteins, %	31.1± 1.8	27.9± 1.3	26.6± 2.0
Gain in body wt, g	35 ± 5	76 ± 4	81 ± 7
Plasma clotting time, (siliconized tubes) sec	252 ±12	221 ±16	232 ±19
Thrombosis, incidence, %	30	70	64
Thrombosis, severity, 0–3	0.7	1.6	1.2
Mortality, %	30	60	54

[1] Thrombosis was initiated by the intravenous injection of *S. typhosa* lipopolysaccharide (0.6 mg/kg).
[2] Diet composition: (wt %) casein, 11; cellulose, 15; cholesterol, 5; fatty acid, 8; lard, 23; salt mixture, 4; sodium cholate, 2; sucrose, 23; vitamin mixture, 2; and water, 7.
[3] Mean ± SE.

After the intravenous injection of the endotoxin some or most of the animals, depending on the experiment, died in 3 to 18 hours. The survivors were killed 24 hours after the injection, autopsy was performed on every animal, and the red hepatic infarcts were evaluated macroscopically.

The macroscopic readings were verified by histologic examination of the left hepatic lobe. As reported previously (24, 26), the incidence of hepatic infarcts corresponds exactly to the incidence of occlusive thrombosis. The severity of the lesions was graded in terms of an arbitrary scale of zero to 3.

RESULTS

Experiment 1, (table 1). The intravenous endotoxin injection in the rats fed the diets for 11 weeks resulted in a 100% mortality rate in groups 1 and 2 fed coconut oil and butter, respectively, whereas it was only 33% in group 3 fed arachis oil. Survival time was shortest (142 minutes) in group 1, longer in group 2 (189 minutes), and longest in group 3 (1091 minutes). In contrast with this, the incidence of thrombosis was 33% in group 1, 94% in group 2, and 57% in group 3. The type of thrombosis observed here is the one described in detail previously, occurring mostly in the large hepatic veins (phlebothrombosis) and giving rise to multiple, large red infarcts (24, 26).

The plasma clotting time was lowest in group 2, although this result was not statistically significant. The cholesterol and triglyceride levels in serum were highest in group 1 fed coconut oil, which was also the most saturated among the 3 fats used here. The cholesterol and triglyceride levels were lower with butter and lowest with arachis oil, the least saturated fat. The percentage of α-lipoproteins was highest in group 1 and lowest in group 3.

Experiment 2 (table 2). Addition to the diet, at a given level (8%), of any of the saturated fatty acids used (groups 2 to 7) significantly increased ($P < 0.001$) the triglycerides in serum from 62 mg/100 ml (group 1) to between 125 (group 5) and 159 (group 2). On the other hand, the differences in the triglyceride levels between each of the groups receiving the added fatty acids (groups 2 to 7) were not significant. The total serum cholesterol was also increased by the addition of the saturated fatty acid to the diet, but this parameter presented more variations among the different groups than did the triglycerides. The percentage of the α-lipoproteins was markedly lower in group 1 fed lard alone, and in groups 4 and 5 fed palmitic and stearic acid, respectively. (Lard contains only 2 saturated fatty acids, i.e., palmitic and stearic acid.) Except for group 6 as compared with group 5, the plasma clotting time was significantly lower in groups 4 and 5 than in the other groups. The incidence and severity of thrombosis resulting from the injection of the endotoxin was by far the highest in groups 4 and 5.

The lowest body-weight gains (27 and 17 g, respectively) were observed in animals fed caprylic and capric acid, whereas the highest gain (73 g) was recorded with behenic acid, the fatty acid with the longest chain in the present series.

Experiment 3 (table 3). Addition to the diet of 10% of either methyl caprylate (group 2) or methyl palmitate (group 3), at the expense of lard, also resulted in a marked increase in the serum cholesterol and triglyceride levels as compared with lard alone (group 1), ($P < 0.001$). In contrast with this, there was no significant difference between groups 2 and 3 with respect to the cholesterol ($P > 0.2$) or the triglyceride ($P > 0.05$) levels. However, the percentage of α-lipoproteins was markedly more elevated in group 2 than in group 3. Group 1, fed lard alone, presented the lowest percentage of α-lipoproteins. The plasma clotting time, performed here in plastic tubes, was shortest in group 3, although the difference between groups 2 and 3 was not significant. However, in group 3, the incidence and severity of thrombosis was by far the highest.

Experiment 4 (table 4, fig. 1). The purpose of this experiment was to investigate which of the lipoprotein electrophoretic fractions, as determined by poststaining with fat red 7B, could be related to the incidence of thrombosis. Three groups from experiment 2, i.e., those fed lard alone (group 1), capric (group 2) and behenic acids (group 3), were repeated

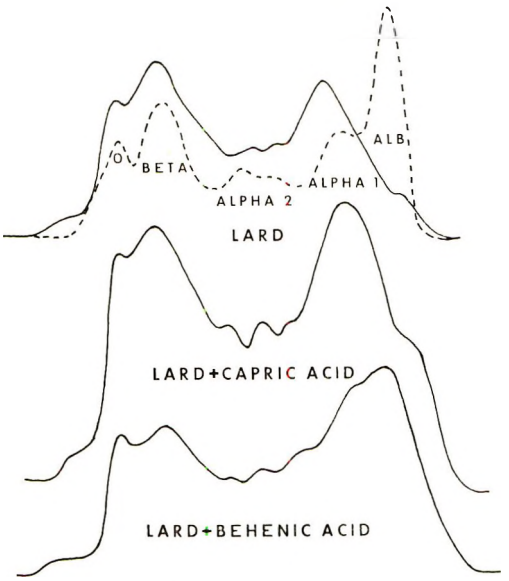

Fig. 1 Typical lipoprotein electrophoretic patterns obtained with fat red 7B staining.

here. With the staining technique used in the present experiment, the percentage of the lipoprotein fractions (fig. 1) could easily be determined with accuracy. As with prestaining (experiment 2), the lowest percentage of α-lipoproteins was found in group 1 fed lard alone. However, if fraction α_1- was added to the α-fraction, the resulting percentage was 34.4 for group 3 fed behenic acid and presenting an incidence of thrombosis of 33%. It was 36.6% in group 1, where the incidence of thrombosis was 18%, and 38.9% in group 2, where no thrombosis was observed. Therefore, the percentage of α- + α_1-lipoprotein appears to be more inversely related to the incidence of thrombosis than does the α-value alone. Among the other fractions, the percentage of β-lipoproteins appeared to be directly related to the incidence of thrombosis (table 4).

Experiment 5 (*table* 5). The purpose of this experiment was to investigate to what extent capric and behenic acids were less thrombogenic than stearic acid by maintaining the animals with the diets for a longer period. After 10 weeks of dietary feeding, group 2 (stearic acid) presented an incidence of thrombosis of 70%; but in group 3 (behenic acid), the incidence was 64%. The incidence of thrombosis was only 30%, in group 1 fed capric acid. The plasma clotting time was lowest in group 2, although not significantly so, and the percentage of α- + α_1-lipoprotein was highest in group 1. The gain in body weight was still highest in group 3, fed behenic acid, and lowest in group 1, fed capric acid.

DISCUSSION

As reported previously (25), in most experiments with endotoxin it appears that there is little relationship between the mortality rate and the incidence of thrombosis. The animals appear to die largely from shock, and the mortality rate expresses the susceptibility to shock rather than the severity of the thrombotic phenomenon. In experiment 1, the survival time was roughly inversely related to the level of cholesterol or of triglycerides in the serum and, finally, to the degree of saturation of the dietary fat. In contrast, neither the incidence of thrombosis nor the plasma clotting time could be correlated with the cholesterol or triglyceride levels, or therefore with the degree of saturation of the fat. In the chick, it has also been reported that the severity of cholesterol deposition in the aorta is not related either to the plasma level of cholesterol or to the saturation of the fat used (14, 18). The low thrombogenicity of coconut oil feeding observed under the present experimental conditions is also concordant with studies carried out in coconut-eating Polynesians (10). Although the serum cholesterol of this population is elevated, ischemic heart disease does not appear to be common.

Coconut oil, butter, and arachis oil differ markedly in both their degree of saturation and also in the type of saturated fatty acid they contain. For example, the 2 saturated fatty acids present in the largest quantities in coconut oil are lauric and myristic acid, whereas palmitic and stearic acid predominate in butter and arachis oil. It was, therefore, for the purpose of eliminating one variable, namely the degree of saturation, that the present experiments concerning the addition of various fatty acids to the diet were undertaken. It was observed that when only the type of saturated acid was changed, and not the degree of saturation, the triglycerides did not

vary significantly from one group to another. However, the incidence of thrombosis and the plasma clotting time differed markedly according to the fatty acid used. Particularly at 6 weeks, a high incidence of thrombosis and a short clotting time were recorded only in groups fed palmitic and stearic acids.

The addition to the diet of free fatty acids does not seem to be factitious, since similar results were obtained when methyl esters of these same acids were added; in fact, the low thrombogenicity of coconut oil could not be explained in any way other than by its content of short-chain saturated fatty acids. Preliminary experiments have shown that lauric and myristic acids do not appear to be more thrombogenic than capric acid. In addition, each group of animals fed the fats tested here (coconut oil, butter, arachis oil) were characterized by a different lipoprotein electrophoretic pattern. The pattern of coconut oil could be superposed upon that of lard plus capric acid; the pattern of butter upon that of lard plus palmitic or stearic acid; and arachis oil, which contains some very long-chain fatty acids, presented a pattern which could be superposed upon that of behenic acid. Although many more experiments are needed to verify these preliminary results, it appears that the lipoprotein electrophoretic pattern induced by a dietary fat is due, at least in part, to its content of saturated fatty acids.

Therefore, with respect to the effect on the lipoprotein pattern as well as on the production of thrombosis, it appears to be immaterial whether free fatty acids themselves, their esters, or fats containing these acids, are added to the food.

Among the lipemic parameters examined here in the groups fed a fatty acid, the only one that was found to bear some relationship with the incidence of thrombosis was the percentage of α-lipoproteins. We previously reported the value of this parameter for evaluating a thrombotic tendency in the rat (33), and its usefulness has also been reported in man as an index of atherosclerosis (34, 30). Nevertheless, in animals fed lard alone and presenting a very low incidence of thrombosis, the percentage of the α-lipoproteins was the lowest of all the groups. To further evaluate the relationship between certain lipoprotein fractions and the production of thrombosis, modifications of the electrophoretic technique were introduced, with poststaining of the strips by fat red 7B. The various fractions could then be evaluated more accurately and yielded results comparable to those reported in man (32). Nevertheless, even with this method, the percentage of α-lipoproteins was still the lowest in the group fed lard alone (exp. 4). In contrast, the α_1-fraction in this group was higher than in the other groups and, although these experiments have to be repeated and extended on a much larger scale, it is probable that this fraction is no more thrombogenic than the α-fraction. Therefore, the sum of these 2 fractions has to be considered for more accurate evaluation of the thrombotic tendency and could conduce to more reliable results than just the sole percentage of α-lipoproteins, as in experiments 1 and 2.

After 10 weeks of feeding, the incidence of thrombosis was much higher in the group receiving behenic acid than in the group receiving capric acid. Behenic acid, therefore, appears to be more thrombogenic than capric acid, although less so than palmitic or stearic acid. However, since lard, which of itself contains palmitic and stearic acids, is added to all these diets, complete evaluation of the thrombogenicity of the different fatty acids could be achieved only by feeding pure triglycerides as the sole fat. The present experiments indicate, however, that palmitic and stearic acids could be the most thrombogenic fatty acids, since they predispose the animals to thrombosis in the shortest period of time.

These results are similar to those obtained by the addition of fatty acids to blood to shorten the in vitro thrombus-formation time (20). While short-chain saturated fatty acids were almost ineffective in this respect, stearic acid was the most efficient. However, behenic and arachidic acid were reported to be more thrombogenic in vitro than palmitic acid (20) and all these long-chain fatty acids, but not the short-chain, were able to induce similar massive thrombosis by infusion in the dog (21).

LITERATURE CITED

1. Page, I. H., E. V. Allen, F. L. Chamberlain, A. Keys, J. Stamler and F. J. Stare 1961 Dietary fat and its relation to heart attacks and strokes. Circulation, 33: 133.
2. Joliffe, N., and M. Archer 1959 Statistical association between international coronary disease death rates and certain environmental factors. J. Chron. Dis., 9: 636.
3. Thomas, W. A., J. N. P. Davies, R. M. O'Neal and A. A. Dimakulangan 1960 Incidence of myocardial infarction correlated with venous and pulmonary thrombosis and embolism. Amer. J. Cardiol., 5: 41.
4. Gore, I., A. E. Hirst and K. Tanaka 1964 Myocardial infarction and thrombo-embolism: A comparative study in Boston and Kyushu, Japan. Arch. Int. Med., *113:* 323.
5. Hirst, A. E., I. Gore, K. Tanaka, I. Samuel and I. Krishtumukti 1965 Myocardial infarction and pulmonary embolism. Arch. Path., *80:* 365.
6. Keys, A. 1957 Epidemiologic aspects of coronary artery disease. J. Chron. Dis., 6: 552.
7. Keys, A., and F. Fidanza 1960 Serum cholesterol and relative body weight of coronary patients in different populations. Circulation, 22: 1091.
8. Lowenstein, F. W. 1964 Epidemiology investigations in relation to diet in groups who show little atherosclerosis and are almost free of coronary ischemic heart disease. Amer. J. Clin. Nutr., *15:* 175.
9. Beveridge, J. M. R., W. F. Connel and G. A. Mayer 1957 The nature of the substances in dietary fat affecting the level of plasma cholesterol in humans. Can. J. Biochem. Physiol., 35: 257.
10. Hunter, J. D. 1962 Diet, body build, blood pressure, and serum cholesterol levels in coconut-eating Polynesians. Federation Proc., 21: 36.
11. Bronte-Stewart, B., A. Antonis, L. Eales and J. F. Brock 1956 Effects of feeding different fats on serum-cholesterol level. Lancet, *1:* 521.
12. Tidwell, H. C., J. C. McPherson and W. W. Burr 1962 Effect of the saturation of fats upon the deposition of ingested cholesterol. Amer. J. Clin. Nutr., *11:* 108.
13. Wigand, G. 1959 Production of hypercholesterolemia and atherosclerosis in rabbits by feeding different fats without supplementary cholesterol. Acta Med. Scand., *166:* (suppl. 351) 1.
14. Leveille, G. A., and E. Sauberlich 1963 Lipid changes in plasma, alpha lipoproteins, liver and aorta of chicks fed different fats. Proc. Soc. Exp. Biol. Med., *112:* 300.
15. McMillan, G. C., B. I. Weigensberg and A. C. Ritchie 1960 Effects of dietary fats in rabbits fed cholesterol. Arch. Path., 70: 220.
16. Funch, J. P., G. Kristensen and H. Dam 1962 Effects of various dietary fats on serum cholesterol, liver lipids and tissue pathology in rabbits. Brit. J. Nutr., *16:* 497.
17. Kritchevsky, D., A. W. Moyer, W. C. Tesar, R. F. J. McCandless, J. B. Logan, R. A. Brown and M. E. Englert 1956 Cholesterol vehicle in experimental atherosclerosis. II. Influence of unsaturation. Amer. J. Physiol., *185:* 279.
18. Banerye, S., P. Narasimha and S. K. Ghosh 1965 Biochemical and histochemical changes in aorta of chicks fed vegetable oils and cholesterol. Proc. Soc. Exp. Biol. Med., *119:* 1081.
19. Pilkington, T. R. E. 1957 The effect of fatty acids and detergents on the calcium clotting time of human plasma. Clin. Sci., *16:* 269.
20. Connor, W. E., and J. C. F. Poole 1961 The effect of fatty acids on the formation of thrombi. Quart. J. Exp. Physiol., *46:* 1.
21. Connor, W. E., J. C. Hoak and E. D. Warner 1965 The effects of fatty acids on blood coagulation and thrombosis. In: Genetics and the Interaction of Blood Clotting Factors. Thromb. Diath. Haemorrh., *Suppl. 17:* 89.
22. Day, H. J., and L. A. Soloff 1963 Thrombosis and coagulation effects produced by long chain saturated fatty acids in dogs. Clin. Res., *11:* 192.
23. Hoak, J. C. 1963 Structure of thrombi produced by injections of fatty acids. J. Lab. Clin. Med., 62: 884.
24. Renaud, S. 1963 Thrombosis and hepatic infarct elicited in hyperlipemic rats by bacterial endotoxin. Lab. Invest., *12:* 1078.
25. Renaud, S., and C. Allard 1965 Influence of dietary factors on certain blood parameters and the production of endotoxin-initiated phlebothrombosis in rat. Can. J. Physiol. Pharmacol., *43:* 359.
26. Renaud, S. 1965 Endotoxin-induced hepatic vein thrombosis in hyperlipemic rats. Sequence of events. Lab. Invest., *14:* 424.
27. Wesson, L. G. 1932 A modification of the Osborne-Mendel salt mixture containing only inorganic constituents. Science, 75: 339.
28. Bauer, J. D. 1963 Coagulation of blood and hemorrhagic diseases. In: Gradwohl's Clinical Laboratory Methods and Diagnosis. C. V. Mosby Company, p. 1194.
29. Trinder, P. 1952 Determination of cholesterol in serum. Analyst, 77: 321.
30. Allard, C., and G. Choquette 1962 Percentage of alpha-lipoproteins, total cholesterol and atherosclerosis. Can. J. Biochem. Physiol., 40: 1515.
31. Van Handel, E. 1961 Suggested modification of the micro-determination of triglycerides. Clin. Chem., 7: 249.
32. Straus, R., and M. Wurm 1958 A new staining procedure and a method for quantitation of serum lipoproteins separated by paper electrophoresis. Amer. J. Clin. Path., 29: 581.
33. Renaud, S., and C. Allard 1962 Thrombosis in connection with serum lipidic changes in the rat. Circulation. Res., *11:* 388.
34. Wurm, M., R. Kositchek and R. Straus 1960 Lipoproteins quantitated by paper electrophoresis as an index of atherosclerosis. Circulation, 21: 526.

Effects of Mineral and Vitamin Supplementation on Swimming Times and Other Parameters Related to Performance of Rats on a Low Calorie Regimen [1]

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ABSTRACT The effect of mineral and vitamin supplementation was studied on certain performance characteristics of rats on a low calorie, semipurified diet regimen. Male, albino, rats of the Charles River strain, 6 months old, were used. At a one-third ad libitum caloric level or less, the rats at nonsupplemented mineral-vitamin levels, on the whole, swam 26% longer than the groups receiving additional minerals and vitamins. This trend was the same with a low fat diet with corn oil and a relatively high fat diet with butter as the fat source. Lifespan was not affected at the 5% probability level by the mineral-vitamin supplementation used. A significant difference in blood glucose levels between rats receiving mineral-vitamin supplements versus those receiving none was found only after 10 days with one-third ad libitum caloric intake plus 9 days of complete caloric deprivation. However, the pooled results of blood glucose measurements during the whole period of caloric restriction showed no significant effect of the mineral-vitamin supplementation used. Likewise, water consumption was not significantly affected by mineral-vitamin supplementation. The same was true for weight loss.

The studies reported here were conducted to document the degree to which physical performance, among other factors, in rats can be affected by supplements of vitamins and minerals to certain low calorie diets already containing these vitamins and minerals. This information is valuable in refuting assertions relative to alleged military ration design inadequacies.

Keys and Henschel (1) state that in neither brief periods of extreme exercise nor in prolonged severe exercise and semi-starvation were there indications of any effects of supplementation with thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid and ascorbic acid on muscular ability, endurance, resistance to fatigue or recovery from exertion in healthy young men. The effect of water-soluble vitamins, fed in small amounts to fasted albino rats, is both reported to be negligible (2), and to enhance survival time (3). Chevul et al. (4) state that fasted rabbits survived longer when given small doses of vitamin A or E daily. Rate of weight loss in fasted rats is reported to be both unaltered by administration of water-soluble vitamins (3), and to be increased (5). A number of investigators have called attention to the absence of vitamin deficiencies in humans undergoing starvation during World War II both in Europe and in the Orient (6–9). The riboflavin-to-N ratio in the urine was markedly increased, however, in normal human subjects during a 24- to 37-hour fast (10). In fasting guinea pigs, the tissue content of ascorbic acid was diminished (11).

Kleiber [2] observed that rats given salts and vitamins died as early as rats given water only. Rats given salts and vitamins in addition to glucose (one-fifth of caloric maintenance) died as early as rats given water and glucose only. The use of a salt mixture, containing the amounts of Na, Cl, K and P in a 1200-kcal diet, did not decrease

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[1] This paper reports research undertaken at the U. S. Army Natick (Mass.) Laboratories and has been assigned no. TP 70 in the series of papers approved for publication. The findings in this report are not to be construed as an Official Department of the Army Position.

[2] Kleiber, M. 1945 Efficiency of rate of energy conversion (power) in starvation. Federation Proc., 4(1): 158 (abstract).

loss of N or weight during a 4-day fast of 2 obese male patients, as compared with a 4-day starvation period with water only (12).

It may be held that a decreased concentration of certain minerals in blood or other tissues can constitute evidence of increased requirements. An increase in the percentage of Na, Cl, and N in the body of rats during starvation with distilled water, but a decrease in the percentage of K has been reported by McCance and Morrison (13). Keys et al. (6), however, report no significant change in the percentage composition of the fat-free dry substance of the skeleton of starved rabbits. The concentration of K (14) and Na and Cl (15) in muscle is reported to be decreased during fasting in rabbits and rats, respectively. During starvation or semistarvation, decreased levels have been reported in blood and serum of Na, K, Ca, Mg, and Cl (6, 9, 16–21), as well as unchanged levels of Na, K, Ca, and Cl (6, 16, 17, 20), and increased levels of Na, Ca, and Cl (6, 13, 15). However, with respect to increased levels of certain minerals, there are indications that dehydration occurs during fasting (16, 20). In a young girl, according to Gamble et al. (16), the plasma Na decreased through the sixth day of the fast and then started to increase; on the fifteenth day of the fast, the plasma Na was back to normal. According to Mellinghoff (17), serum Ca of humans subjected to semistarvation during World War II was at first abnormally low, but soon increased to normal. It appears, however, that in many cases blood and serum Cl concentration decreases progressively with increasing severity of starvation (6, 16, 20, 21). Such declines in blood and serum inorganic ion levels in certain cases could indicate a beneficial effect on performance of an increased concentration in the diet of such ions during a period of reduced food intake.

METHODS

Male, albino rats, randomly bred, and Caesarian-derived from Charles River strain SD were used. They were housed individually at 21 ± 1° in stainless steel cages with wire-mesh bottoms. At the start of the experiment these rats were 3 months old and weighed 353 ± 30 g (mean ± SD).

After the rats had been fed a commercial laboratory ration [3] for a brief period, they were divided into 2 groups of 34 animals each. One group received: (in %) vitamin-free casein, 20.0;[4] corn oil, 6.0 (14% of calories, "low" fat diet); cellulose, 4.0;[5] mineral mixture, 4.2; vitamin mixture, 0.4; and the remainder cornstarch.[6] The other group received the same diet, but with 24.8% saltless butter (41% of calories, "high" fat diet) replacing the corn oil and part of the cornstarch. The group given 41% of calories from butter was included to determine whether an amount and kind of fat approximating that used in combat rations would bring about a different reaction to the mineral-vitamin levels tested than a level of corn oil frequently fed to rats. The mineral mixture, per 100 g of diet, consisted of: $CaCO_3$, 0.66 g; $CaHPO_4$, 1.50 g; Na_2HPO_4, 0.72 g; KCl, 0.69 g; $MgSO_4$, 0.26 g; $MnSO_4 \cdot H_2O$, 18.5 mg; $ZnCO_3$, 4.7 mg; $CuSO_4$, 5.0 mg; Fe citrate (16.7% Fe), 61 mg; KIO_3, 255 µg; Cr acetate, 2.1 mg (22). The vitamin mixture, per 100 g of diet, consisted of: vitamin A (as palmitate), 600 IU; vitamin D_2, 300 IU; α-tocopherol, 8 mg; menadione, 0.03 mg; thiamine·HCl, 0.6 mg; riboflavin, 0.5 mg; pyridoxine·HCl, 0.3 mg; niacin, 3.0 mg; Ca pantothenate, 1.6 mg; vitamin B_{12} + intrinsic factor, 0.075 USP units (0.38 g); inositol, 1.7 mg; *p*-aminobenzoic acid, 1.7 mg; choline dihydrogen citrate, 350 mg; and ascorbic acid, 6 mg. These amounts of minerals and vitamins are based primarily on the recommendations for the rat of the National Research Council (23). The intrinsic factor was added because in calorically restricted rats, the intrinsic factor may not be as active as in rats fed ad libitum. Ascorbic acid was included to minimize the destruction of thiamine (24). Dibasic sodium phosphate rather than K_2HPO_4 was used, again, to reduce thiamine losses (25, 26). The diets were kept refrigerated. Tests conducted in

[3] Purina Laboratory Chow, Ralston Purina Company, St. Louis.
[4] General Biochemicals, Inc., Chagrin Falls, Ohio.
[5] Alphacel, Nutritional Biochemicals Corporation, Cleveland.
[6] Buffalo Starch no. 3401, Corn Products Company, New York.

our laboratory have indicated that, when ratios of vitamins and minerals, as used in these diets, were mixed and kept at 0°, the loss of thiamine per week did not exceed 15%. Unmodified cornstarch can be expected to produce better weight gains in rats than unmodified potato starch, dextrose, or commercial preparations of corn dextrin (27). The diets described were fed ad libitum for 10 weeks. The rats were then 6 months old. After this period, the high and the low fat group were each subdivided into 3 groups[7] of about equal size: (a) a group fed ad libitum; (b) a group fed an amount (9.0 g/rat/day) of food approximately one-third of the average previous ad libitum consumption; and (c) a group that had a similarly restricted caloric intake but in addition received an amount (0.62 g/rat/day) of mineral-vitamin mixture, of the same percentage composition as used in the complete diets, that that increased the intake of these minerals and vitamins to approximately that of the rats fed ad libitum. The period during which the caloric intake for part of the rats was thus restricted (i.e., to about one-third that of the animals fed ad-libitum) lasted 10 days. Thereafter, the restricted groups were subjected to complete caloric deprivation. During this latter period, the groups (c) received an amount (1.0 g/rat/day) of mineral-vitamin mixture, bringing the intake again to approximately the level consumed by the rats fed ad libitum. To make the rats consume this mixture, 1.25 g of sodium cyclamate and 125 mg of sodium saccharin[8] per 100 g of mineral-viamin mixture were added. About 90% of the rats at all times consumed this sweetened mineral-vitamin mixture completely; the remainder always consumed most of it. The mineral-vitamin mixture was prepared each week and kept refrigerated. Water was supplied ad libitum. During the periods with the semipurified diets, the water was all-glass distilled. The resistance of this water was more than 500,000 ohms/cm^3 at 22°.

During the ad libitum and restricted feeding periods, swimming trials were conducted. The animals were conditioned to swimming during ad libitum feeding. The trial periods were gradually increased until they were conducted to exhaustion. The point of exhaustion was considered to have been achieved when the rat did not reach the surface for 10 seconds, or if signs of disorientation were observed, whichever came earlier. No animal was lost due to drowning. Equal weights were attached to the base of the tail of all rats for the swimming tests. These weights were gradually increased during the ad libitum conditioning feeding period, and then reduced again in one step for both the ad libitum-fed and restricted groups in the course of the restricted feeding period for the latter; these weights constituted 4 to 5% of the body weights of the rats. The animals were tested, one per tank, in 121-liter plastic cans, filled to a height of at least 60 cm with water. The temperature of the water was kept at about 28°. (A direct relationship has been reported to exist between swimming time and water temperature (28).) The rats were always tested between 1 and 4 PM to limit possible diurnal effects on performance. A total of 10 swimming tests for all rats was performed, each about 6 days apart.

Postabsorptive (overnight fasting) blood glucose determinations were carried out once a week using the "glucostat" micromethod,[9] on a 0.3- to 0.4-ml sample from the tail.

RESULTS

Swimming times. During the training period with semipurified diet, before restriction of food intake and the use of mineral and vitamin supplements, the swimming times were not significantly different between any of the future subgroups (i.e., within the low fat, respectively "high" fat group). For example, 7 days before the first swimming test of the actual experiment (period 1, table 1), the swimming times for what were to become the 6 different groups 4 days later were: LF,[10] 6.47 ± 4.21 (mean ± SD); HF, 9.69 ± 4.54; LF + MV, 5.40 ± 2.77; HF + MV, 7.18 ±

[7] The following abbreviations will be used henceforth: LF = low fat, reduced food intake, with corn oil as the fat source; HF = "high" fat, reduced food intake, with butter as the fat source; + MV = plus additional minerals and vitamins.

[8] As powdered tablets of Sucaryl, Abbott Laboratories, North Chicago, Illinois.

[9] Worthington Biochemical Corporation, Freehold, New Jersey.

[10] See footnote 7.

TABLE 1

Effect of different diet regimens on swimming times, measured at different periods during diet treatment

Treatment [1]	Swimming time		
	Period 1 [2]	Period 2	Period 3
	minutes	*minutes*	*minutes*
LF	7.53 ± 4.75 [3]	10.62 ± 4.22	8.27 ± 4.38
HF	8.83 ± 4.96	10.69 ± 4.60	8.28 ± 3.67
LF + MV	4.43 ± 1.84	8.05 ± 4.18	6.12 ± 4.29
HF + MV	6.66 ± 3.69	11.59 ± 4.61	7.37 ± 3.39
LF ad libitum	4.93 ± 2.73	8.48 ± 4.60	8.94 ± 3.96
HF ad libitum	7.87 ± 4.58	12.35 ± 4.28	11.73 ± 4.82

[1] LF = low fat, reduced food intake, with corn oil as the fat source; HF = "high" fat, reduced food intake, with butter as the fat source; +MV = plus additional minerals and vitamins.
[2] Period 1, after feeding 3 days at one-third ad libitum caloric level for the 4 restricted groups; weights of 26.5 g at the base of the tail of all rats; 9 to 12 rats/group. Period 2, after feeding 10 days at one-third ad libitum caloric level for the 4 restricted groups; weights of 20 g used for all rats; 9 to 11 rats/group. Period 3, after feeding 10 days at one-third ad libitum caloric level plus 7 days of complete caloric deprivation for the 4 restricted groups; 20-g weights used for all rats; 9 to 11 rats/group. Each rat was tested once in period 1, once in period 2, etc.
[3] Mean ± SD.

4.42; LF ad libitum, 5.78 ± 3.86; and HF ad libitum, 6.95 ± 4.33 minutes, respectively. The swimming times at this date were for the low fat group as a whole 5.83 ± 3.47 minutes, for the "high" fat group 7.96 ± 4.47 minutes. According to Student's t test, the latter difference is significant at the 5% probability level. This difference did not exist during the period with the commercial ration [11] for what were going to become the low fat and "high" fat groups (4.74 ± 1.37, respectively, 4.59 ± 1.47 minutes). For the experimental period, the swimming times for the rat groups at different times are shown in table 1. Analysis of variance (29) indicated that the nonsupplemented groups swam 48% longer ($P < 0.05$) than the groups supplemented with the mineral-vitamin mixture. When the effect of the factors, fat level and caloric level, was analyzed, it became evident that at periods 1 and 2, the rats fed the high fat butter diet swam significantly longer ($P < 0.05$); only at period 3 the rats fed ad libitum swam significantly longer ($P < 0.05$) than the other groups. In the analyses of variance, all values were adjusted for an average number of replications.

To make possible an anlysis of the effects of fat level and kind of fat, and mineral-vitamin level for the pooled results of periods 1, 2 and 3, results obtained on different dates were multiplied by different factors, so that the products of the sum of the means for all groups and the particular factors used equaled each other for the different periods; the results for the different periods were then multiplied by the factors thus obtained. An analysis of variance of the results thus pooled indicated that, for the whole period studied, overall swimming times were 26% longer ($P < 0.025$) at the nonsupplemented than at the supplemented mineral-vitamin levels. When only the results obtained with groups supplemented with the mineral-vitamin mixture were compared, the rats fed the high fat butter diet swam significantly longer ($P < 0.025$) than the animals fed the low fat corn oil diet.

Lifespan. The lifespan in days, from the start of restriction of the calorie level for the different groups [12] was as follows (mean ± SD):

LF	36.9 ± 9.0	HF	35.2 ± 9.1
LF + MV	31.0 ± 6.5	HF + MV	40.3 ± 6.6

Analysis of variance showed a significant ($P < 0.05$) interaction of the factors, fat level and kind of fat, with the mineral-vitamin level. When only the results at the supplemented mineral-vitamin level were compared, the lifespan was found to be 30% longer ($P < 0.005$) for the rats fed the high fat, butter diet. None of the other differences between groups was significant at the $P < 0.05$ level as determined by analysis of variance and Student's t test.

[11] See footnote 3.
[12] See footnote 7.

TABLE 2

Effect of different diet regimens on postabsorptive or fasting blood glucose levels, measured at different periods during diet treatment

Treatment [1]	Blood glucose levels		
	Period 1 [2]	Period 2	Period 3
	mg/100 ml	*mg/100 ml*	*mg/100 ml*
LF	74.0± 9.4 [3]	49.2± 7.2	43.0± 6.0
HF	73.1± 4.2	45.3±10.1	37.2± 4.5
LF+MV	70.3± 6.0	44.6± 7.8	50.3± 7.5
HF+MV	70.1± 6.7	50.1± 9.0	47.4±11.1
LF ad libitum	70.5± 6.2	81.4± 3.3	61.8±13.2
HF ad libitum	75.4±10.3	75.3± 9.6	66.5±14.5

[1] LF = low fat, reduced food intake, with corn oil as the fat source; HF = "high" fat, reduced food intake, with butter as the fat source; +MV = plus additional minerals and vitamins.
[2] Period 1, after feeding 5 days at one-third ad libitum caloric level for the 4 restricted groups; 8 to 11 rats/group. Period 2, after feeding 10 days at one-third ad libitum caloric level plus 2 days of complete caloric deprivation for the 4 restricted groups; 7 to 10 rats/group. Period 3, after feeding 10 days at one-third ad libitum caloric level plus 9 days of complete caloric deprivation for the 4 restricted groups; 6 to 11 rats/group.
[3] Mean ± SD.

TABLE 3

Effect of different diet regimens on water consumption, measured at different periods during diet treatment

Treatment [1]	Water consumption		
	Period 1 [2]	Period 2	Period 3
	ml/day	*ml/day*	*ml/day*
LF	13.0±6.3 [3]	12.2±6.9	12.6±4.9
HF	13.1±6.4	15.9±7.7	14.2±4.6
LF+MV	15.5±5.7	16.0±8.8	16.0±6.1
HF+MV	10.3±3.6	14.2±8.2	14.5±5.9
LF ad libitum	19.5±5.9	19.3±5.8	16.3±4.2
HF ad libitum	16.6±7.1	13.8±6.1	15.4±3.4

[1] LF = low fat, reduced food intake, with corn oil as the fat source; HF = "high" fat, reduced food intake, with butter as the fat source; +MV = plus additional minerals and vitamins.
[2] Period 1, after feeding 7 and 8 days at one-third ad libitum caloric level for the 4 restricted groups, average of 2 days; 9 to 12 rats/group. Period 2, after feeding 10 days at one-third ad libitum caloric level plus 2 and 3 days of complete caloric deprivation for the 4 restricted groups; 9 to 12 rats/group. Period 3, after feeding 10 days at one-third ad libitum caloric level plus 9 and 10 days of complete caloric deprivation for the 4 restricted groups; 8 to 12 rats/group.
[3] Mean ± SD.

Postabsorptive or fasting blood glucose levels. The blood glucose values for the rat groups at different periods are shown in table 2. Analysis of variance showed that only at periods 2 and 3 were blood glucose levels significantly higher ($P < 0.005$) for the rats fed ad libitum than for those on restricted caloric levels, namely, 66% higher at period 2 and 44% at period 3. Only at period 3 were glucose levels higher (22%; $P < 0.005$) in groups receiving the mineral-vitamin supplement compared with nonsupplemented groups. In the analyses of variance, all values, again, were adjusted for an average number of replications. Again, to make possible an analysis of the effects of fat level and kind of fat, and mineral-vitamin level for the pooled results of periods 1, 2 and 3, results obtained on different dates were multiplied by different factors as described previously. However, no significant ($P < 0.05$) differences for the factors fat level and mineral-vitamin level were found.

Water consumption. Water consumption is shown in table 3. When the values at the supplemented mineral-vitamin level were compared, intake during period 1 was 50% higher ($P < 0.05$) with the low fat, corn oil diet than with the high fat, butter diet. Water consumption during period 1 was 37% higher ($P < 0.025$) ad libitum than at the calorically restricted levels. Statistical analysis, as outlined previously, of the pooled results of periods 1,

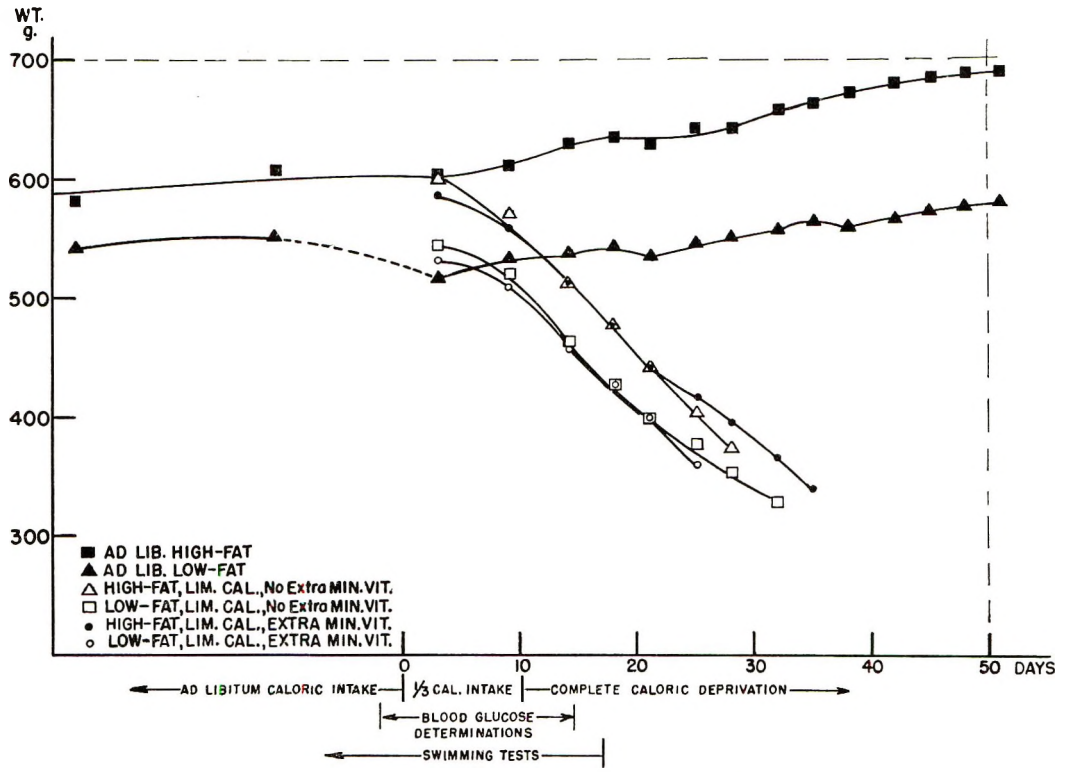

Fig. 1 Average weights for each rat group determined at different times during the course of the experiment.

2 and 3 showed a significant ($P < 0.05$) interaction of the factors fat level and kind of fat, with mineral-vitamin level.

Weights. Average weights are shown in figure 1. The average weights for the rats fed the high fat diet at all times were higher than those for the animals fed the low-fat diet. This was true both for the rats fed ad libitum and for those on a calorically restricted intake. At the same fat levels, there was no significant difference in weight, from the start of complete caloric deprivation until the eleventh day of this period, between the groups with and without additional minerals and vitamins.

Autopsy results. Upon expiration, the majority of the calorically deprived rats showed symptoms of enteritis, hemorrhage of the kidneys and mottling of the lungs. For each of these symptoms, no striking difference was noticeable in percentage occurrence between groups. However, for both enteritis and mottling of the lungs, and for the pooled incidence of the 3 symptoms mentioned, the percentages for the groups without additional minerals and vitamins, both LF and HF,[13] were lower than for the groups receiving additional minerals and vitamins. Of 12 rats, 6 "low" fat and 6 "high" fat, fed ad libitum throughout the experiment, none showed any disease symptoms or abnormality of organs except fatty infiltration of the liver of all rats fed the high fat, butter diet.

DISCUSSION

Literature cited in the introduction indicated that the concentration of a number of minerals and vitamins in blood and parts of the body can be decreased and excretion increased during certain phases of fasting. However, this in itself does not necessarily constitute proof of an increase in requirement of the total body under these conditions for such minerals and vitamins. Different tissues are being cata-

[13] See footnote 7.

bolized at different rates during caloric restriction. Also, the requirement of different soft tissues for minerals and vitamins may differ, both normally or during caloric restriction. Though not constituting any proof in this respect, it is worthwhile noting that, for instance, the iron content of the liver was observed by Kojima (30) to increase with the duration of a starvation period in rabbits, but in the spleen, the iron content decreased. Starvation of rabbits resulted in an increase of vitamin B_{12} concentration in the kidney, liver, heart, and urine, but plasma and spleen concentrations of vitamin B_{12} remained normal, according to Rosenthal and Cravitz (31). Also, there may be some available "base depots" in the body, for instance of Na in "unessential" body water. However, Saks (32) observed in dogs that, in general, the rate of K excretion during inanition parallels that of N. Further, Aikawa (33) reported that the decrease in total body K during starvation could be accounted for on the basis of tissue catabolism.

During the period at one-third ad libitum caloric level, the rats fed the high fat diets (all 3 groups pooled) swam significantly longer than those fed the low fat diets. This may have been because of a thicker layer of subcutaneous fat in the rats fed the high fat diets, as was evident at autopsy of animals fed ad libitum. If greater buoyancy was the only reason for the better performance, this difference should not show up if similar rats, under otherwise identical conditions, were run on a treadmill. It is striking that calorie level did not exert a significant effect (at the 5% probability level) on swimming times until after 10 days at one-third of ad libitum caloric intake plus a period of complete caloric deprivation. Also, it took more than 5 days at one-third of ad libitum caloric level for the blood glucose level to become significantly lower than in the rats fed ad libitum. The significantly higher blood glucose levels at period 3 in the rats given mineral-vitamin supplementation, as compared with the groups without this addition, were not expressed in better swimming performance at this phase in the experiment, or in longer lifespan.

At no time was a significantly higher ($P < 0.05$) water consumption observed for the calorically restricted groups receiving additional minerals and vitamins when compared with nonsupplemented groups. This addition of minerals was 0.62 g/rat/day.

Weight loss, in grams per day, was about equal for all groups, up to about 20 days after the start of caloric restriction. The weight curves give no indication of water retention as caused by additional intake of minerals during this period.

The pooled results indicate that, during a period of caloric restriction, the unsupplemented rats swam 26% longer ($P < 0.025$) than the rats given the mineral-vitamin mixture. Fat level and kinds of fat, as used, had no effect on the general trend of these results. Lifespan was not significantly affected (5% probability level) by the additional mineral-vitamin levels used, either with the high fat butter diet or with the low fat corn oil regimen.

Under the conditions of this experiment, additional minerals plus vitamins proved not to be beneficial with respect to the performance parameters measured.

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LITERATURE CITED

1. Keys, A., and A. F. Henschel 1942 Vitamin supplementation of U.S. Army rations in relation to fatigue and the ability to do muscular work. J. Nutr., 23: 259.
2. Sakurai, Y., Y. Aoyagi and M. Seimiya 1944 Effect of vitamins on restricted feeding. III. The work effect on fasting animals. Bull. Inst. Phys. Chem. Res. (Tokyo) (Chem. ed.), 23: 202 (Chem. Abstr., 43: 8022b, 1944).
3. Ligori, M. G. 1955 Vitamins and fasting albino rats. Congr. Inter. Biochem., Résumés Commun., 3rd Congr., Brussels: p. 115 (cited in Chem. Abstr., 51: 3769g, 1957).
4. Chevul, M. L., M. Beltan and M. Cormier 1951 Inanition in the rabbit; influence of

vitamins A and E. Compt. Rend. Soc. Biol., *145:* 1633.
5. Giacalone, O. 1955 Action of aneurine on limit of resistance to starvation and on percentage daily loss of body weight. Arch. Fisiol., *55:* 68 (cited in Chem. Abstr., *49:* 16112e, 1955).
6. Keys, A., J. Brožek, A. Henschel, O. Mickelsen and H. L. Taylor 1950 The Biology of Human Starvation, University of Minnesota Press, Minneapolis.
7. Leyton, G. B. 1946 Effects of slow starvation. Lancet, *2:* 73.
8. French, C. E., and F. J. Stare 1947 Nutritional surveys in Western Holland: Rotterdam, 1945. J. Nutr., *33:* 649.
9. Walters, J. H., R. J. Rossiter and H. Lehmann 1947 Malnutrition in Indian prisoners-of-war in the Far East. Lancet, *1:* 205.
10. Windmueller, H. G., A. A. Anderson and O. Mickelsen 1964 Elevated riboflavin levels in urine of fasting human subjects. Amer. J. Clin. Nutr., *15:* 73.
11. Martini, E., and A. Bonsignore 1935 Behavior of ascorbic acid of inanition tissues. Arch. Sci. Biol. (Italy), *21:* 167 (cited in Chem. Abstr., *30:* 141b, 1936).
12. Duncan, L. E., Jr., R. J. Meyer, J. E. Howard, D. Wagner and H. Eisenberg 1948 Mineral balance during brief starvation. The effect on serum electrolytes and mineral balance of maintaining the intake of certain mineral constituents. J. Clin. Invest., *27:* 389.
13. McCance, R. A., and A. B. Morrison 1956 The effect of equal and limited rations of water and of 1, 2, and 3 percent solutions of sodium chloride on partially nephrectomized and normal rats. Quart. J. Exp. Physiol., *41:* 365.
14. Gambaro, G. C., G. Ghigliotti and G. Lamedica 1955 Protein and hydro-mineral picture of rabbit white muscle in various stages of fasting. Arch. "E. Maragliano" Patol. Clin., *11:* 207 (cited in Chem. Abstr., *50:* 4370f, 1956).
15. Woodbury, D. M., L. A. Marti and M. S. Naisbitt 1953 Extrarenal effects of deoxycorticosterone, adrenocortical extract, and adrenocorticotropic hormone on plasma and tissue electrolytes in fed and fasted rats. Amer. J. Physiol., *174:* 1.
16. Gamble, J. L., G. S. Rose and F. F. Tisdall 1923 The metabolism of fixed base during fasting. J. Biol. Chem., *57:* 633.
17. Mellinghoff, K. 1949 Potassium and calcium content of human blood during prolonged fasting. Deut. Arch. Klin. Med., *194:* 277.
18. White, R. R., K. R. Christian and V. J. Williams 1956 Blood chemistry and hematology in sheep on decreasing levels of feed intake followed by starvation. New Zealand J. Sci. Technol., *38A:* 440.
19. Cheynol, J., and A. Quinquad 1932 Changes in the blood calcium of dogs in a state of inanition. Bull. Soc. Chim. Biol., *14:* 1048.
20. Sunderman, F. W. 1947 Studies in serum electrolytes. XIV. Changes in blood and body fluids in prolonged fasting. Amer. J. Clin. Pathol., *17:* 169.
21. Morgulis, S., and A. C. Edwards 1924 Chemical changes in the blood during fasting and subsequent feeding. Amer. J. Physiol., 68*:* 477.
22. Schroeder, H. A., W. H. Vinton, Jr. and J. J. Balassa 1963 Effects of chromium, cadmium and lead on the growth and survival of rats. J. Nutr., *80:* 48.
23. National Academy of Sciences, Committee on Animal Nutrition 1962 Nutrient requirements of laboratory animals, publ. 990. National Academy of Sciences — National Research Council, Washington, D. C.
24. Kandutsch, A. A., and C. A. Baumann 1953 Factors affecting the stability of thiamine in a typical laboratory diet. J. Nutr., *49:* 209.
25. Waibel, P. E., H. R. Bird and C. A. Baumann 1954 Effects of salts on the instability of thiamine in purified chick diets. J. Nutr., *52:* 273.
26. Rombouts, J. E. 1953 The destruction of B vitamins by certain salt mixtures in purified rations. J. Nutr., *50:* 255.
27. Reussner, G., Jr., J. Andros and R. Thiessen, Jr. 1963 Studies on the utilization of various starches and sugars in the rat. J. Nutr., *80:* 291.
28. Richter, C. P. 1957 On the phenomenon of sudden death in animals and man. Psychosom. Med., *19:* 191.
29. Steel, R. G. D., and J. H. Torrie 1960 Principles and Procedures of Statistics with Special Reference to the Biological Sciences. McGraw-Hill Book Company, New York.
30. Kojima, K. 1931 Iron in normal and pathological tissues and its biological interpretation. VI. Distribution of iron in various organs of starvation rabbits. Nagoya J. Med. Sci., *5:* 94 (cited in Chem. Abstr., *25:* 5702, 1931).
31. Rosenthal, H. L., and L. Cravitz 1958 Organ, urine, and feces vitamin B_{12} content of normal and starved rabbits. J. Nutr., *64:* 281.
32. Saks, S. 1930 Mineral metabolism of dogs during inanition. Acta Biol. Exp. (Warsaw), *5:* 225 (cited in Chem. Abstr., *25:* 3698, 1931).
33. Aikawa, J. K. 1951 Effect of starvation on exchangeable potassium and tissue potassium^{42} content in rabbits. Proc. Soc. Exp. Biol. Med., 78*:* 524.

Glycogen Metabolism in Meal-fed Rats and Chicks and the Time Sequence of Lipogenic and Enzymatic Adaptive Changes [1]

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ABSTRACT The time course of the lipogenic and enzymatic adaptations to meal-feeding (access to food for a single daily 2-hour period) in rat adipose tissue and in rat and chick liver were investigated. The incorporation of acetate-1-^{14}C into fatty acid had increased in rat adipose tissue after 5 to 7 days of meal-feeding, and in chick liver after 7 days. The activities of glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and NADP-malic dehydrogenase did not increase in rat adipose until after 9 days of meal-feeding and did not increase over control values in liver of meal-fed chicks. The data are interpreted as demonstrating that the hyperlipogenesis induced by meal-feeding is not dependent upon increased activities of the dehydrogenase enzymes studied. These data are discussed in relation to the NADPH requirements for the support of lipogenesis. Glycogen metabolism was studied in liver of meal-fed rats and chicks and in rat diaphragm and adipose tissue. In both rats and chicks, the level of glycogen in liver of meal-fed animals was depleted to a lesser extent by 22 hours of fasting than that in liver of nibbling (ad libitum-fed) animals. In chicks, liver glycogen increased to twice normal levels in meal-fed animals killed after the daily 2-hour meal. In meal-fed rats, liver glycogen was not increased by the 2-hour meal, and values remained at about 50% of those found in fed control animals. However, four hours after the initiation of the meal period, hepatic glycogen levels in meal-fed animals were equivalent to those observed in livers of fed control rats. Glycogen in diaphragm and adipose tissue of meal-fed or nibbling rats fasted for 22 hours was similar, but glycogen increased more rapidly and reached a higher level in tissues of meal-fed rats in a 6-hour period following refeeding. The possible physiological significance of these observations is discussed.

Significant metabolic adaptations are induced by limiting an animal's access to food or meal-feeding. Tepperman and Tepperman (1) first showed that liver slices of meal-fed rats incorporated more acetate-^{14}C and glucose-^{14}C into lipids than did comparable tissue preparations from ad libitum-fed animals. Hollifield and Parson (2), Stevenson et al. (3) and Leveille and Hanson (4) demonstrated that meal-feeding also stimulated the incorporation of radioactive precursors into fatty acids by isolated adipose tissue. Accompanying the increased rate of lipogenesis in liver and adipose tissue is an increase in pentose cycle enzymes, glucose 6-phosphate (G-6-*P*) dehydrogenase and 6-phosphogluconate dehydrogenase (2, 5, 6) and in NADP-malic dehydrogenase (5, 6). Leveille and Hanson (6) have also demonstrated increased citrate cleavage enzyme activity in adipose tissue of meal-fed rats. The increase in enzyme activity appears to be related to the production of NADPH for fatty acid synthesis. The enzymes of the pentose cycle in adipose supply substantial quantities of NADPH (7), and a role for malic enzyme in the supply of reduced coenzyme has also been proposed (4, 8–10). However, it is not clear whether the increased enzyme activity, and hence supply of NADPH, stimulates increased lipogenesis or perhaps the enhanced lipid synthesis might stimulate increased enzyme activity (11, 12).

Meal-feeding has also been shown to influence glycogen metabolism. Tepperman and Tepperman (1) and Hollifield and Parson (2) observed that liver glycogen of meal-fed rats was more resistant to depletion by fasting than that of control

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[1] The principles of laboratory animal care as promulgated by the National Society for Medical Research were observed.

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rats, and that glycogen accumulation in liver proceeds at an elevated rate upon refeeding. Similar increases in tissue glycogen have been observed in liver (1) and in adipose tissue (13) of fasted-refed rats.

The studies to be reported were designed to investigate the relative changes in lipogenesis and enzyme activity with respect to time in tissues of meal-fed rats and chicks and to evaluate the effect of meal-feeding on glycogen metabolism.

MATERIALS AND METHODS

Experimental animals

Rats. Male rats of the Holtzman strain, weighing 200 to 300 g were used for all studies. The animals were housed individually in stainless steel cages having raised wire floors, and maintained in a temperature and humidity controlled room (21° and 50% relative humidity). Commercial laboratory ration [3] was fed in all of the studies to be described; the pellets were ground to a powdered form in order to obtain accurate food consumption data. Meal-fed rats were allowed access to food from 8 to 10 AM only, whereas control animals were fed ad libitum. Water was available at all times. Body weight and food consumption, determined at weekly intervals, followed the pattern described previously (4).

Chicks. Male Hyline White Leghorn chicks were fed commercial chick starting ration [4] from day-old until they reached a body weight of approximately 600 g. At this time, the chicks were divided into 2 groups; one was fed ad libitum (control) and the other was allowed access to food from 8 to 10 AM only (meal-eaters). The chick starting ration was used as diet throughout the study. The chicks were housed in heated cages having raised wire floors and had access to water at all times. Body weight and food consumption were determined weekly and followed a pattern similar to that described previously (14).

Metabolic studies

Rats were killed by decapitation and chicks by cervical dislocation. The tissues to be used, epididymal adipose tissue and liver for the rat studies and liver tissue in chick studies, were rapidly excised and chilled. Pieces of the thin peripheral portion of the epididymal fat pads weighing about 100 mg and liver slices weighing approximately 150 to 200 mg and prepared from the left lateral lobe were used for the in vitro study of lipogenesis from acetate-1-^{14}C. The tissues were incubated in 2.0 ml of Ca^{++}-free Krebs-Ringer bicarbonate buffer (15), pH 7.4, at 38° for 3 hours under 95% O_2 to 5% CO_2 in a shaking water bath (90 strokes/minute). The buffers contained 10 μmoles and 0.167 μCi acetate-1-^{14}C per ml and glucose and insulin [5] as indicated in the tables of results. Incubations were carried out in flasks stoppered with rubber serum stoppers and fitted with a hanging well containing a 2 × 2 cm piece of Whatman no. 1 filter paper. $^{14}CO_2$ was determined essentially as described by Buhler (16). At the end of the 3-hour incubation period, the filter paper in the center well was saturated with 0.1 ml of 25% KOH, and 0.5 ml of 0.2 N H_2SO_4 was added to the incubation medium to liberate CO_2. Shaking was continued for 20 to 30 minutes to ensure complete liberation and trapping of the CO_2. The filter paper containing radioactive CO_2 was transferred to a counting vial and allowed to air-dry. When dry, the paper was flattened in the bottom of the vial and 10 ml of scintillation solution were added. The tissues were rinsed several times in saline and transferred to tubes containing 5% ethanolic KOH. The tissues were saponified by refluxing at 85°. The KOH solutions were diluted with water, and the nonsaponifiable lipid fraction was extracted with three 5-ml portions of petroleum ether (bp 30–60°). The aqueous phase was acidified with HCl and the fatty acids were removed by 3 successive 5-ml extractions with petroleum ether. The combined ether extracts were backwashed with water, and the ether fraction was transferred to liquid scintillation vials. The ether was evaporated under a stream of O_2-free nitrogen and the fatty acids dissolved in 10 ml of scintillation solution. When cholestero-

[3] Purina Rat Chow, Ralston Purina Company, St. Louis.
[4] Purina Chick Starter, Ralston Purina Company.
[5] The insulin used in these studies was generously supplied by Dr. W. Bromer, Eli Lilly and Company, Indianapolis, Indiana.

genesis was studied, cholesterol was isolated from the nonsaponifiable fraction as the digitonide and counted (17).

Glycogen determination

Portions of liver and diaphragm were quickly transferred to tared tubes containing hot 30% KOH. Tissue weights were determined, and the tubes were placed in a boiling water bath until digested. Adipose tissue was extracted with chloroform: methanol (2:1, v/v) and the defatted tissue was digested in hot 30% KOH. The glycogen was co-precipitated from the KOH solution with Na_2SO_4 as described by Van Handel (18). The glycogen was dissolved in water and appropriate aliquots were taken for quantitation by the anthrone reaction (19), or for counting by suspending in Bray's scintillation medium (20).

Enzyme studies

Adipose tissue or liver tissue was homogenized in 0.15 M KCl, and the homogenates were centrifuged at 1000 × *g* at 5° for 15 minutes. The clear intermediate layer of the adipose tissue homogenate was used for assay. The supernatants from the liver homogenates were recentrifuged at 15000 × *g* for 30 minutes at 5°, and the clear supernatant was used for assay. Glucose 6-phosphate dehydrogenase (G-6-*P*) (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) were assayed separately in adipose tissue by the procedure of Horecker and Smyrniotis (21). In liver, the combined activity of G-6-P dehydrogenase and 6-phosphogluconate dehydrogenase was determined using both glucose 6-phosphate and 6-phosphogluconate as substrates (22). NADP-malic dehydrogenase (malic enzyme) (EC 1.1.1.40) was assayed spectrophotometrically by the method of Ochoa (23). Nitrogen content of the homogenates was determined by micro-Kjeldahl digestion followed by Nesslerization (24). The activity of the enzymes was expressed as units per milligram of nitrogen, where a unit is defined as the transformation of 1 μmole of substrate/minute at 30°.

The scintillation solution used contained per liter: 4 g 2,5-diphenyloxazole (PPO), 0.015 g 1,4-bis-2-(5-phenyloxalolyl) benzene (POPOP), 230 ml ethanol and toluene to 1 liter. The radioactivity was determined in a Nuclear-Chicago Model 722 ambient temperature liquid scintillation spectrometer. Data were statistically evaluated where applicable by means of the *t* test.

RESULTS

Time course of adaptive changes in adipose tissue of meal-fed rats

The data presented in figure 1 are the result of an attempt to delineate the relative time sequence of the increased lipogenesis and enzymatic activity in epididymal adipose tissue of meal-fed rats. Rats with an average body weight of 230 g were divided into 2 groups; one was meal-fed and the other allowed to eat ad libitum. Five rats per group were killed on the days indicated, and the ability of isolated adipose tissue to incorporate acetate-1-^{14}C into fatty acids and the activities of G-6-P dehydrogenase, 6-phosphogluconate dehydrogenase and NADP-malic dehydrogenase were determined. Control nibbling animals were fasted 22 hours on the day preceding the experiment and refed for 2 hours prior to killing; meal-fed rats were killed immediately after the daily meal.

The data in figure 1 show that after 5 days of meal-eating, lipogenesis was identical in adipose tissue of meal-fed and control rats, and the activity of the enzymes studied was 50 to 95% of control values. After 7 days of meal-feeding, lipogenesis was markedly enhanced in tissue of meal-fed rats, whereas enzyme activity was still equivalent to or below that of control animals. Enzyme activity was not significantly elevated in tissue of meal-fed rats until day 9. NADP-malic dehydrogenase and G-6-*P* dehydrogenase remained significantly higher in tissue of meal-fed rats throughout the remainder of the study, whereas 6-phosphogluconate dehydrogenase activity was not as markedly, if at all, increased. 6-Phosphogluconate dehydrogenase does not appear to be adaptive in adipose tissue of meal-fed rats (6). These data suggested that the increase in lipogenesis resulting from meal-feeding is not the result of increased enzyme activity, since it occurred 2 days before any change in enzyme activity was noted.

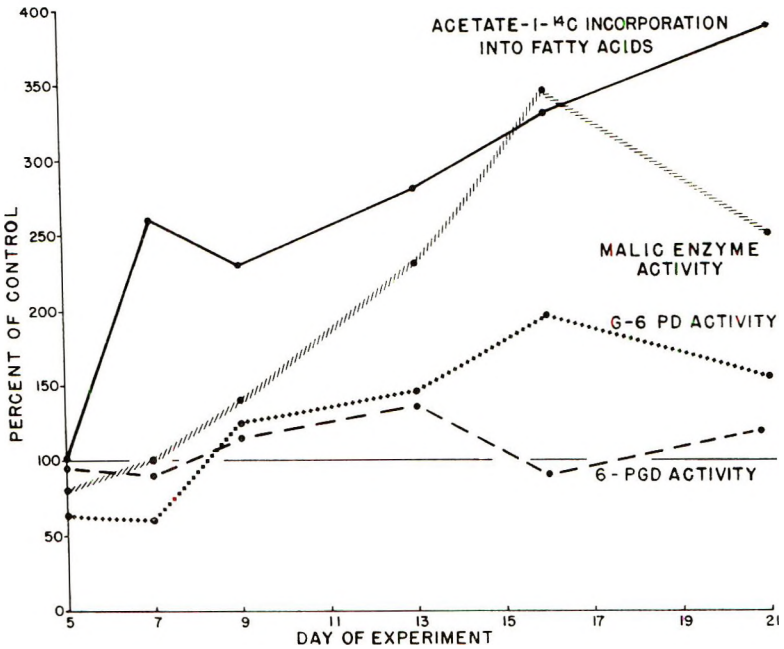

Fig. 1 Acetate-1-^{14}C incorporation into fatty acids by isolated adipose tissue and the activities of glucose 6-phosphate, 6-phosphogluconate and NADP malate dehydrogenases in adipose tissue of meal-fed and nibbling rats. Pieces of adipose tissue weighing approximately 100 mg were incubated in Ca^{++}-free Krebs-Ringer bicarbonate buffer containing per ml: 10 μmoles acetate, 5 μmoles glucose, 0.1 unit insulin and 0.167 μCi acetate-1-^{14}C. Each point represents the average for 5 meal-fed compared with 5 nibbling rats.

To verify these observations, this experiment was repeated, killing animals at more frequent intervals, and the results of this study are shown in figure 2. In this study, acetate-1-^{14}C incorporation into fatty acids was enhanced by day 5 of meal-feeding, although, as previously observed (fig. 1), the activity of the enzymes studied was only 45 to 90% of control values. Lipogenesis remained significantly higher in adipose tissue of meal-fed animals throughout the study and, again, enzyme activities were not increased significantly until the ninth day of meal-feeding. The results of these 2 studies demonstrate that the ability of adipose tissue from meal-fed rats to incorporate acetate-1-^{14}C into fatty acids increases 2 to 4 days before any significant increase in NADP-malic dehydrogenase, G-6-*P* dehydrogenase and 6-phosphogluconate dehydrogenase occurs. A further increase in lipogenesis does appear to accompany the increase in enzyme activity, but the initial lipogenic response to meal-feeding is not dependent upon the increased dehydrogenase activity.

Time course of changes in liver of meal-fed chicks

Chicks weighing approximately 600 g were divided into 2 groups; one was meal-fed and the other was allowed to eat ad libitum. Three chicks per group were killed on the days indicated (fig. 3) and hepatic fatty acid synthesis from acetate-1-^{14}C, NADP-malic dehydrogenase and combined G-6-*P* dehydrogenase plus 6-phosphogluconate dehydrogenase activities were determined. Meal-fed chicks were killed immediately following their daily meal, whereas control chicks were allowed access to food until the time of killing. The results of this experiment, shown in figure 3, demonstrate that lipogenesis had increased above control values after 7 days of meal-feeding and remained elevated throughout the study. However, enzyme activity was not increased signifi-

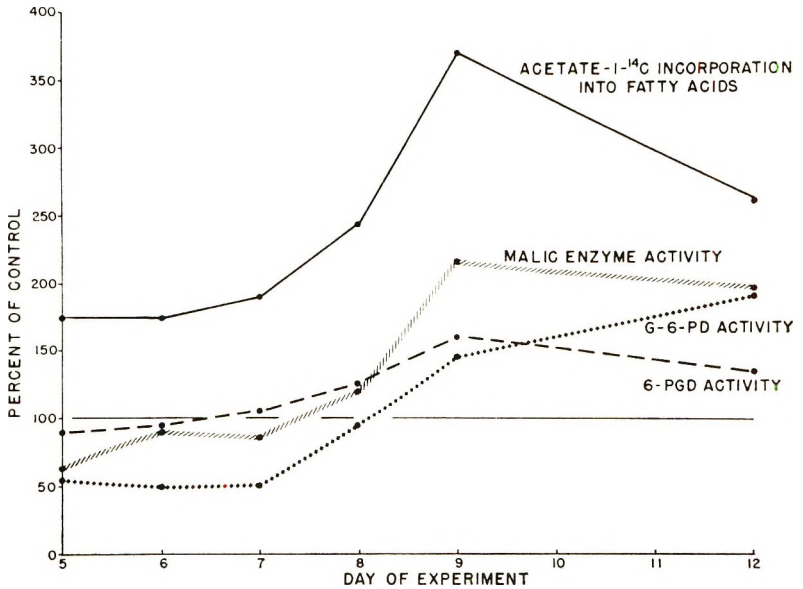

Fig. 2 Acetate-1-^{14}C incorporation into fatty acids by isolated adipose tissue and the activities of glucose 6-phosphate, 6-phosphogluconate and NADP malate dehydrogenases in adipose tissue of meal-fed and nibbling rats. Incubation conditions as described for figure 1. Each point represents the average for 5 meal-fed compared with 5 nibbling rats.

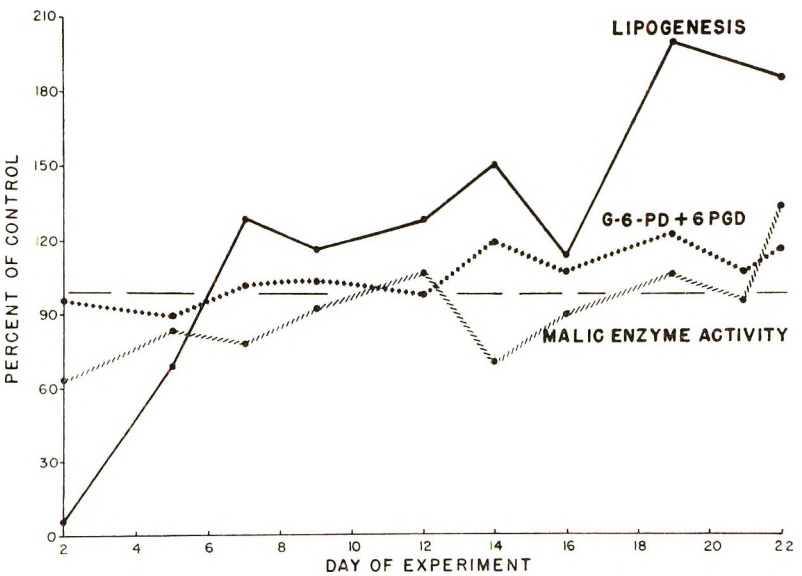

Fig. 3 Acetate-1-^{14}C incorporation into fatty acids by liver slices and the hepatic activities of glucose 6-phosphate, 6-phosphogluconate and NADP malate dehydrogenases of meal-eating and nibbling chicks. Incubation conditions as described for figure 1 except that insulin was omitted. Each point represents the average for 3 meal-fed chicks compared with 3 nibbling chicks.

cantly in liver of meal-fed chicks. There is a suggestion of increased enzyme activity, but this is not very great. These data implied, as did the results of the rat studies, that enhanced lipogenesis is not dependent upon a concomitant increase in dehydrogenase activity.

Liver glycogen levels of meal-fed chicks and in vitro acetate-1-^{14}C utilization

To study the effect of dietary status in more detail, the experiment described in table 1 was carried out. Chicks weighing 600 g were meal-fed or allowed access to food ad libitum for 4 weeks. Some nibbling and meal-eating chicks were killed after having been fasted for 22 hours or fasted 22 hours and refed for 2 hours. Another group of control chicks was allowed access to food until the time of killing. Tissues were incubated in a bicarbonate buffer containing acetate-1-^{14}C with or without added unlabeled glucose. Lipogenesis was virtually abolished by a 22-hour fast in nibbling chicks and was still reduced after 2 hours of refeeding. Lipogenesis in tissue from fasted meal-fed chicks was slightly higher than in tissue of nonfasted control chicks, but not significantly so. Refeeding for 2 hours increased acetate incorporation approximately twofold in liver slices of meal-fed animals, to a value significantly greater than that observed for liver slices of fed control chicks. The oxidation of acetate-1-^{14}C to $^{14}CO_2$ was not markedly altered by the various treatments studied. Cholesterogenesis was depressed by fasting and stimulated by refeeding. The addition of unlabeled glucose to the incubation medium consistently stimulated acetate incorporation into fatty acids but not into cholesterol, nor was acetate oxidation influenced.

Hepatic glycogen content in these chicks was of particular interest (table 1). Fasting for 22 hours depleted liver glycogen almost completely in nibbling chicks, whereas the level of glycogen in the liver of the meal-eaters after a similar fast was equivalent to that of fed control chicks. Refeeding for 2 hours returned liver glycogen levels to normal in nibbling chicks and to about twice normal levels in meal-fed animals.

Glycogen metabolism and lipogenesis in rat liver and adipose tissue

An experiment similar to that described above was conducted with rats. Rats weighing approximately 270 g were allotted to a meal-feeding or nibbling regimen and were used after 8 weeks. The results of this study are shown in table 2. Hepatic lipogenesis was lower in tissue slices of fasted or fasted-refed meal-eaters and

TABLE 1

Effect of fasting and refeeding on liver glycogen levels and in vitro utilization of acetate-1-^{14}C by liver slices of meal-eating and nibbling chicks

Treatment [1]	Liver glycogen	Acetate-1-^{14}C utilization [2]		
		$^{14}CO_2$	Fatty acid	Cholesterol
	mg/g liver	*mμmoles utilized/100 mg tissue/3 hours*		
		Buffer A, no glucose added		
Nibbler, fed	12.9 ± 4.2 [3]	6107 ± 1128	1038 ± 259	102.6 ± 8.0
Nibbler, fasted	0.1 ± 0.05	4782 ± 204	8 ± 2	2.6 ± 0.9
Nibbler, refed	12.8 ± 3.7	7033 ± 988	145 ± 45	38.5 ± 8.2
Meal-eater, fasted	15.0 ± 5.2	6522 ± 673	1380 ± 420	48.2 ± 13.0
Meal-eater, refed	25.2 ± 6.3	7052 ± 642	2416 ± 315	65.8 ± 15.6
		Buffer B, 5 mM glucose added		
Nibbler, fed	—	5899 ± 857	1254 ± 336	94.7 ± 7.2
Nibbler, fasted	—	6085 ± 292	20 ± 5	8.6 ± 0.4
Nibbler, refed	—	7145 ± 756	179 ± 48	37.6 ± 7.1
Meal-eater, fasted	—	7108 ± 444	1645 ± 418	50.8 ± 8.4
Meal-eater, refed	—	6448 ± 770	2768 ± 294	69.9 ± 11.6

[1] Fasted chicks were maintained for 22 hours without food prior to killing; refed chicks were fasted 22 hours and refed for 2 hours immediately prior to killing; fed chicks had access to food until killed.
[2] Buffer contained per ml: 10 μmoles acetate, 0.167 μCi acetate-1-^{14}C and, where indicated, glucose 5 μmoles.
[3] Mean for 4 chicks ± SEM.

TABLE 2

Effect of fasting and refeeding on adipose tissue and liver glycogen levels and in vitro utilization of acetate-1-^{14}C by adipose tissue and liver slices from meal-eating and nibbling rats

Treatment [1]	Glycogen/g tissue		Acetate-1-^{14}C utilization [2]				
			Adipose tissue		Liver slices		
	Liver	Adipose tissue	CO_2	Fatty acid	CO_2	Fatty acid	Cholesterol
	mg	*μg*	*mμmoles utilized/100 mg tissue/3 hours*				
Nibbler, fed	60.2 ± 1.9 [3]	36.5 ± 3.7	757 ± 52	276 ± 34	3333 ± 188	270.2 ± 83.3	23.4 ± 3.4
Nibbler, fasted	7.7 ± 1.9	48.2 ± 10.6	877 ± 88	142 ± 21	3071 ± 177	2.3 ± 0.6	2.8 ± 0.4
Nibbler, refed	20.4 ± 1.8	46.2 ± 7.4	782 ± 57	214 ± 24	3476 ± 286	4.8 ± 2.1	4.2 ± 1.8
Meal-eater, fasted	29.7 ± 1.8	43.1 ± 2.5	866 ± 81	683 ± 104	4204 ± 316	46.6 ± 8.8	51.5 ± 18.3
Meal-eater, refed	29.2 ± 2.7	442.0 ± 115.2	834 ± 76	1308 ± 226	3324 ± 183	42.9 ± 11.6	35.6 ± 5.3

[1] Fasted rats were maintained for 22 hours without food prior to killing; refed rats were fasted 22 hours and refed for 2 hours immediately prior to killing; fed rats had access to food until killed.

[2] Buffers contained per ml: acetate, 10 μmoles; acetate-1-^{14}C, 0.167 μCi; glucose, 5 μmoles; and for adipose tissue 0.1 unit insulin.

[3] Mean for 8 rats ± SEM.

nibblers than in tissue of fed rats. Refeeding did not significantly increase hepatic lipogenesis, but tissue from fasted or refed meal-eaters did incorporate more acetate into fatty acid than did liver slices from nibbling animals fasted and refed on the day of experiment, in a manner analogous to the treatment of meal-eaters. Cholesterol synthesis by liver slices was highest in tissue of fasted meal-eaters and decreased somewhat upon refeeding. Cholesterogenesis was markedly reduced in liver slices of nibbling rats by fasting and was not significantly influenced by refeeding. The oxidation of acetate by liver slices and adipose tissue was similar for all groups studied.

In rats, alterations in fatty acid synthesis due to the treatments used were quite different for adipose tissue than for liver slices. In adipose tissue from nibbling rats, fatty acid synthesis was reduced by fasting to about 50% and returned to 78% of normal after 2 hours of refeeding, whereas tissue of fasted meal-eaters incorporated approximately 150% more acetate into fatty acids than did tissue of nibbling rats which had not been fasted. When the meal-fed rats were refed for 2 hours, their adipose tissue incorporated about 4.7 times as much acetate into fatty acids as comparable tissue from fed controls. The glycogen content of liver was reduced to about 10% of normal in nibbling animals as a consequence of a 22-hour fast; refeeding for 2 hours resulted in an increase to approximately 33% of normal. Liver of meal-fed rats fasted for 22 hours contained 50% of the amount of glycogen observed in livers of normal ad libitum-fed rats, and this level was not altered by refeeding for 2 hours. Adipose tissue glycogen was relatively uninfluenced by fasting or refeeding in nibbling animals. In fasted meal-fed rats adipose tissue glycogen levels were similar to those in tissue of nibbling rats, but after 2 hours of refeeding the level had increased by about tenfold. To study the influence of time after the initiation of the daily meal on glycogen level in adipose tissue, meal-fed rats were killed 4 hours after the initiation of the 2-hour meal. The results of this experiment are shown in table 3. Lipogenesis from ace-

TABLE 3

Liver and adipose tissue glycogen levels and lipogenesis by isolated adipose tissue of meal-fed rats as influenced by refeeding [1]

Treatment	Lipogenesis [2]	Glycogen/g tissue	
		Liver	Adipose tissue
		mg	*μg*
Nibblers	349 ± 71 [3]	59 ± 3	55 ± 3
Meal-eaters	1559 ± 315	52 ± 5	2236 ± 771

[1] Meal-fed rats were killed 4 hours after the initiation of the daily 2-hour meal; nibblers had access to food until killing.
[2] For adipose tissue incubated in Ca++-free Krebs-Ringer bicarbonate buffer containing per ml: 10 μmoles acetate, 0.167 μCi acetate-1-^{14}C, 5 μmoles glucose and 0.1 unit insulin.
[3] Mean for 3 rats ± SEM.

tate-1-^{14}C was 4.5-fold higher in adipose tissue of meal-fed as compared with tissue from fed nibbling animals. Liver glycogen levels were now equivalent for both groups, whereas 2 hours after the initiation of the meal period. the level of glycogen in liver of meal-fed rats was only 50% of control values (table 2). Adipose tissue glycogen, which was tenfold higher than control 2 hours after the start of the meal (table 2), increased to over 40 times the level in tissue of fed nibblers 4 hours after initiation of the meal (table 3).

The effect of time after refeeding on glycogen metabolism in meal-eating and nibbling rats was studied further, as shown in table 4. Both meal-eating and nibbling animals were fasted for 22 hours prior to initiation of the experiment. To obviate any differences due to the amount of food consumed, the rats were force-fed 10 ml of a suspension containing 25% glucose and 25% starch and 4.80 μCi of glucose-U-^{14}C. Animals were killed before feeding and 2, 4 and 6 hours after feeding, and the glycogen content and radioactivity in the isolated glycogen were determined in liver, diaphragm and epididymal adipose tissue. The adipose tissue glycogen content of nibbling rats did not change appreciably during the first 4 hours and showed only a slight increase between the fourth and sixth hour after feeding. In tissue from meal-fed rats, the glycogen content increased linearly over the 6-hour period, showing approximately an 80-fold increase 6 hours after feeding. In diaphragm muscle, glycogen levels were similar in tissue of meal-fed and nibbling

TABLE 4

Effect of refeeding on glycogen levels and glycogen synthesis in liver, diaphragm and adipose tissue of meal-fed and nibbling rats [1]

Regimen	Time after feeding	Glycogen/g tissue			Glucose-U-^{14}C incorporation		
		Adipose	Liver	Diaphragm	Adipose	Liver	Diaphragm
	hour	*μg*	*mg*	*mg*	*dpm × 10^3/g tissue*		
Nibbler	0	46 ± 7 [2]	2.7 ± 0.1	1.6 ± 0.2	—	—	—
	2	45 ± 7	13.2 ± 2.1	5.1 ± 1.3	0.15 ± 0.03	7.6 ± 0.8	5.2 ± 1.9
	4	42 ± 4	28.0 ± 4.2	4.0 ± 0.9	0.13 ± 0.02	19.2 ± 3.0	4.5 ± 1.2
	6	73 ± 15	36.5 ± 1.7	8.1 ± 1.2	0.28 ± 0.12	30.1 ± 5.3	11.4 ± 2.3
Meal-eater	0	41 ± 2	16.2 ± 7.0	1.2 ± 0.5	—	—	—
	2	500 ± 110	26.0 ± 2.8	8.4 ± 1.1	2.1 ± 0.4	9.4 ± 1.7	10.5 ± 1.7
	4	1268 ± 62	39.4 ± 2.9	11.7 ± 1.2	5.6 ± 0.2	26.5 ± 1.1	21.7 ± 2.6
	6	3196 ± 1996	46.0 ± 4.2	16.9 ± 4.0	13.9 ± 9.1	37.5 ± 6.8	29.0 ± 6.6

[1] Rats were fasted 22 hours and force-fed 10 ml of a suspension containing 25% glucose, 25% starch and 4.80 μCi glucose-U-^{14}C, then killed as indicated. The animals were subjected to their respective dietary regimen for 6 weeks prior to use.
[2] Mean for 3 rats ± SEM.

animals following a 22-hour fast and increased during the 6-hour period after feeding. The increase was more rapid and reached a higher value in tissue of meal-fed animals than in that of nibblers. In diaphragm of nibbling animals, glycogen increased fivefold, as contrasted with a 14-fold increase in tissue of meal-fed animals. In liver tissue, the values for both meal-fed and nibbling rats increased during the 6-hour period after feeding; values were higher in livers of meal-fed rats but the overall increase was similar for both groups. In nibbling rats, liver glycogen increased by 34 mg/g, as compared with 30 mg/g for meal-fed rats during the 6 hours following force-feeding. Data in table 4 show that the radioactivity in glycogen paralleled the changes in glycogen level, demonstrating that the increased glycogen in these 3 tissues was derived from the fed glucose.

DISCUSSION

The stimulation of lipogenesis resulting from meal-feeding has been well-documented for both rat (1) and chick (14) liver and rat adipose tissue (2–4). Yet, this adaptive phenomenon is poorly understood, and the triggering mechanism involved remains to be demonstrated. Tepperman and Tepperman (1) have investigated the "superlipogenic" response induced in liver of animals fed meals or refed after a 48-hour fast. These authors demonstrated an elevated fasting glycogen level in liver of meal-fed rats, an observation confirmed by Hollifield and Parson (2) and in the present study. Tepperman and Tepperman also demonstrated an increase in G-6-*P* dehydrogenase and 6-phosphogluconate dehydrogenase (5) and NADP-malic dehydrogenase (12) activities in liver of meal-fed rats. A similar increase in the activity of these enzymes has been shown in epididymal adipose tissue of meal-fed rats (2, 6).

Tepperman and Tepperman (5), as a result of their investigations, proposed a mechanism for the control of lipogenesis dependent upon the production of NADPH for reductive lipogenesis. They proposed that the availability of NADPH limited fatty acid synthesis and that "supernormal" lipogenesis was dependent upon an increased pentose cycle activity to generate adequate reducing equivalents. In a more recent report (12), this position has been modified and they have considered the difficulties in determining whether enhanced lipogenesis is the result of increased pentose cycle activity or whether the increased $NADP^+$ resulting from lipogenesis stimulates dehydrogenase activity. The arguments for and against this mechanism have recently been considered in detail (11), and presently it appears more likely that increased enzyme activities are a result of enhanced lipogenesis. The data presented in this report demonstrate that the increase in fatty acid synthesis observed in adipose tissue of meal-fed rats and in liver of meal-eating chicks is not dependent upon increased activities of G-6-*P* dehydrogenase, 6-phosphogluconate dehydrogenase or NADP-malic dehydrogenase.

The higher activities of G-6-*P* dehydrogenase, 6-phosphogluconate dehydrogenase and NADP-malic dehydrogenase, although not essential for the initial increase in fatty acid synthesis, undoubtedly reflect the increased demand for NADPH to support fatty acid synthesis. Recent studies in this laboratory have shown that 24% of the glucose utilized by isolated adipose tissue of meal-fed rats is metabolized via the pentose pathway, as contrasted with 16% for tissue of nibbling rats. The important contribution of the pentose pathway to the overall NADPH requirement for lipogenesis is even more apparent when it is considered that 10 to 15 times more glucose is utilized by adipose tissue of meal-fed as compared with ad libitum-fed rats. However, the amount of reduced coenzyme produced via the pentose pathway appears to be inadequate to meet the demands for rapid lipogenesis in rat adipose tissue. Flatt and Ball (7) and Rognstad and Katz (25) have shown that under conditions favoring lipogenesis in adipose tissue, when pentose pathway activity is high, only about 50 to 60% of the required reducing equivalents are generated by this pathway. Another source of reducing power appears to be the coupling of malic dehydrogenase and NADP-malic dehydrogenase (6, 8–10). These enzymes would catalyze the reaction oxaloacetate→

malate → pyruvate and, in the process, oxidize 1 mole of NADH and reduce 1 mole of NADP, in essence, effecting a transhydrogenation from NADH to NADP. This mechanism could account for the adaptability of malic enzyme in conditions promoting rapid lipogenesis.

The metabolism of glycogen in adipose tissue has recently been extensively reviewed (26). Although normal glycogen levels are low in adipose tissue, glycogen metabolism appears to be dynamic. The influence of meal-feeding on adipose tissue glycogen metabolism has received very little attention. Tuerkischer and Wertheimer (13) demonstrated that refeeding after a fast results in an elevation in adipose tissue glycogen. Wertheimer (27) has suggested that glycogen formation in adipose tissue precedes fatty acid synthesis. Chari-Briton et al. (28) reported that adipose tissue of meal-fed rats accumulated glycogen and incorporated ^{14}C-labeled substrates into glycogen when fed following a fast. Leveille and Hanson (4, 6) have demonstrated that adipose tissue of meal-fed rats converts more glucose-^{14}C to glycogen in vitro than does tissue of control ad libitum-fed rats. The significance of the accumulation of glycogen in tissue of meal-fed rats observed in this study is not clear. However, it seems unlikely that the suggestion of Wertheimer (27), that glycogen deposition is necessary for lipogenesis to occur, is applicable. Reports from this laboratory (29)[6] have shown that lipogenesis from acetate-^{14}C can be stimulated in tissue from fasting rats by the addition of unlabeled pyruvate to the medium. Similarly, Herrera et al. (31) have reported that the reduced lipogenesis in adipose tissue of rats fasted for 96 hours can be returned toward normal by preincubation in a medium containing pyruvate. These studies, when considered in light of the fact that virtually no gluconeogenesis can occur from pyruvate in adipose tissue because of the absence of fructose-1, 6-diphosphatase (31), rule out an obligatory role of glycogen formation in lipogenesis. The observations cited do not, however, rule out the possibility that in the intact organism glycogen formation in adipose tissue may be related to lipogenesis. One other possibility which should be considered is the requirement for α-glycerophosphate formation, particularly in the period of fast between meals. Since adipose tissue cannot reutilize glycerol resulting from lypolysis for esterification of fatty acids (32), α-glycerophosphate must be formed from dihydroxyacetone phosphate. Pyruvate and presumably lactate can serve as precursors of α-glycerophosphate (29),[7] but glucose would probably be the precursor of choice. It is therefore conceivable that part of the overall adaptive response to meal-eating in the rat involves the increased ability of tissues, including adipose tissue to store abnormally large amounts of glycogen during the meal period to be used judiciously during the period of fast between meals for those reactions requiring or preferentially utilizing glucose. Such reactions would include the supply of glucose as an energy source for nervous tissue and α-glycerophosphate formation in adipose tissue. This possibility is being pursued.

The higher level of glycogen in livers of fasted meal-eating rats and chicks, as compared with fasted nibbling animals, observed in this study is similar to the results reported for rats by Tepperman and Tepperman (1) and by Hollifield and Parson (2). It is not possible to determine whether the higher glycogen levels in tissue of meal-fed rats is due to an increased rate of synthesis or a decrease in utilization. The similarity of the glycogen specific activities in tissues of meal-fed and nibbling rats (table 4) suggests that following refeeding the increased glycogen levels are due to an increased rate of synthesis in the meal-fed animal rather than a differential rate of utilization. Such a conclusion is in accord with the observations of Whitney and Roberts (33) in rats fed a high fat diet and implies that gluconeogenesis and glycogenesis are greater in tissues of meal-fed animals or those fed a high fat diet. Consistent with this concept is the observation of Krebs et al. (35) that gluconeogenesis is higher in tissues of rats fed a high fat diet.

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[6] Leveille, G. A. 1966 Lipogenesis in adipose tissue of meal-fed rats. A possible regulatory role of α-glycerophosphate formation (manuscript in preparation).

[7] See footnote 6.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. Tepperman, J., and H. M. Tepperman 1958 Effects of antecedent food intake pattern on hepatic lipogenesis. Amer. J. Physiol., *193:* 55.
2. Hollifield, G., and W. Parson 1962 Metabolic adaptations to a "stuff and starve" feeding program. I. Studies of adipose tissue and liver glycogen in rats limited to a short daily feeding period. J. Clin. Invest., *41:* 245.
3. Stevenson, J. A. F., V. Feleki, A. Sylavko and J. R. Beaton 1964 Food restriction and lipogenesis in the rat. Proc. Soc. Exp. Biol. Med., *116:* 178.
4. Leveille, G. A., and R. W. Hanson 1965 Influence of periodicity of eating on adipose tissue metabolism in the rat. Can. J. Physiol. Pharmacol., *43:* 857.
5. Tepperman, H. M., and J. Tepperman 1958 The hexosemonophosphate shunt and adaptive hyperlipogenesis. Diabetes, *7:* 478.
6. Leveille, G. A., and R. W. Hanson 1966 Adaptive changes in enzyme activity and metabolic pathways in adipose tissue from meal-fed rats. J. Lipid Res., *7:* 46.
7. Flatt, J. P., and E. G. Ball 1964 Studies on the metabolism of adipose tissue. XV. An evaluation of the major pathways of glucose catabolism as influenced by insulin and epinephrine. J. Biol. Chem., *239:* 675.
8. Young, J. W., E. Shrago and H. A. Lardy 1964 Metabolic control of enzymes involved in lipogenesis and gluconeogenesis. Biochemistry, *3:* 1687.
9. Wise, E. M., Jr., and E. G. Ball 1964 Malic enzyme and lipogenesis. Proc. Nat. Acad. Sci., *52:* 1255.
10. Pande, S. V., R. P. Khan and T. A. Venkitasubramanian 1964 Nicotinamide adenine dinucleotide phosphate-specific dehydrogenases in relation to lipogenesis. Biochim. Biophys. Acta, *84:* 239.
11. Masoro, E. J. 1962 Biochemical mechanisms related to the homeostatic regulation of lipogenesis in animals. J. Lipid Res., *3:* 149.
12. Tepperman, J., and H. M. Tepperman 1961 Metabolism of glucose-1-C^{14} and glucose-6-C^{14} by liver slices of refed rats. Amer. J. Physiol., *200:* 1069.
13. Tuerkischer, E., and E. Wertheimer 1942 Glycogen and adipose tissue. J. Physiol., *100:* 385.
14. Leveille, G. A., and R. W. Hanson 1964 Influence of periodicity of eating in the chicken. Amer. J. Physiol., *209:* 153.
15. Umbreit, W. W., R. H. Harris and J. F. Stauffer 1964 Manometric Techniques, ed. 4. Burgess Publishing Company, Minneapolis, p. 132.
16. Buhler, D. R. 1962 A simple scintillation counting technique for assaying $C^{14}O_2$ in a Warburg flask. Anal. Biochem., *4:* 413.
17. Kabara, J. J. 1957 A quantitative micromethod for the isolation and liquid scintillation assay of radioactive free and ester cholesterol. J. Lab. Clin. Med., *50:* 146.
18. Van Handel, E. 1965 Estimation of glycogen in small amounts of tissue. Anal. Biochem., *11:* 256.
19. Hassid, W. Z., and S. Abraham 1957 In: Methods in Enzymology, vol. 3, eds., S. P. Colowick and N. O. Kaplan. Academic Press, New York, p. 34.
20. Bray, G. A. 1960 A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem., *1:* 279.
21. Horecker, B. L., and P. Z. Smyrniotis 1955 In: Methods in Enzymology, vol. 1, eds., S. P. Colowick and N. O. Kaplan. Academic Press, New York, p. 323.
22. Glock, G. E., and P. McLean 1953 Further studies on the properties and assay of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of rat liver. Biochem. J., *55:* 400.
23. Ochoa, S. 1955 In: Methods in Enzymology, vol. 1, eds., S. P. Colowick and N. O. Kaplan. Academic Press, New York, p. 699.
24. Johnson, M. J. 1941 Isolation and properties of a pure yeast polypeptidase. J. Biol. Chem., *137:* 575.
25. Rognstad, R., and J. Katz 1966 The balance of pyridine nucleotides and ATP in adipose tissue. Proc. Nat. Acad. Sci., *55:* 1148.
26. Shafrir, E., B. Shapiro and E. Wertheimer 1965 Handbook of Physiology, Section 5: Adipose Tissue, eds., A. E. Renold and G. F. Cahill, Jr. American Physiological Society, Washington, D. C.
27. Wertheimer, E. 1945 Glycogen in adipose tissue. J. Physiol., *103:* 359.
28. Chari-Briton, A., S. Lepkovsky, R. M. Lemmon and M. K. Dimick 1960 Conversion of glucose to glycogen after ingestion of a high-carbohydrate diet. Amer. J. Physiol., *198:* 787.
29. Leveille, G. A. 1966 In vitro lipogenesis in adipose tissue of fed and fasting rats: mechanism of pyruvate stimulation. Life Sci., *5:* 421.
30. Herrera, M. G., G. R. Phillips and A. E. Renold 1965 Stimulation of metabolic activity of adipose tissue from fasted rats by prolonged incubation in vitro. I. Requirement for glucose and insulin. Biochim. Biophys. Acta, *106:* 221.

31. Weber, G., H. J. Hird, N. B. Stamm and D. S. Wagle 1965 Enzymes involved in carbohydrate metabolism in adipose tissue. In: Handbook of Physiology, Section 5: Adipose Tissue, eds., A. E. Renold and G. F. Cahill, Jr. American Physiological Society, Washington, D. C., p. 225.
32. Vaughan, M. 1961 The metabolism of adipose tissue in vitro. J. Lipid Res., 2: 293.
33. Whitney, J. E., and S. Roberts 1955 Influence of previous diet on hepatic glycogenesis and lipogenesis. Amer. J. Physiol., *181:* 446.
34. Krebs, H. A., D. A. H. Bennett, P. De Gasquet, T. Gascoyne and T. Yoshida 1963 Renal gluconeogenesis. The effect of diet on the gluconeogenic capacity of rat kidney-cortex slices. Biochem. J., *86:* 22.

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2. Brown, Q. R., V. A. Ham and I. V. Long 1971 Effects of dietary fat on cholesterol metabolism. J. Nutr., *94:* 625.

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counts per minute	count/min
cubic centimeter(s)	cm^3
cubic millimeter	mm^3
degree(s)	°
degrees of freedom	df (*in tables*)
gram(s)	g
international unit(s)	IU (*to be used only when weight can not be given*)
kilogram(s)	kg
liter(s)	(spell out)
meter(s)	m
microgram(s)	μg (not γ)
micromicrogram(s)	μμg
microcurie(s)	μCi
micron(s)	μ
micromicron(s)	μμ
micromolar (unit of concn)	μM
micromole (unit of mass)	μmole
milligram(s)	mg
milligrams %	(*never use*)
milliliter(s)	ml
millimeter(s)	mm
millimicrogram(s)	mμg
millimicron(s)	mμ
millimole(s)	mmole
molar (mole per liter)	M
parts per million	ppm
per cent	%
probability (in statistics)	*P*
square centimeter	cm^2

[1] Style Manual for Biological Journals 1960 American Institute of Biological Sciences, 2000 P street, N. W., Washington 6, D. C.

square meter	m^2
square millimeter	mm^2
standard deviation	SD
standard error	SE
t (Fisher's test)	*t*
weight (in tables)	wt

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[2] See footnote 1.

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