The Presence of Lactose in Intestinal Tissue[1]

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ABSTRACT When lactose-1-C^{14} was injected into duodenal, jejunal, or ileal segments of the small intestine of rats, radioactivity was shown autoradiographically to be present in the mucosal and muscularis cells of all of the segments. The muscularis layer of all 3 segments appeared to contain similar amounts of the radiocarbon; the mucosal cells of the duodenum contained the least carbon-14, whereas those of the ileum contained the most. The goblet cells averaged more radioactivity than adjacent mucosal cells, but only in the jejunal region was this difference consistently significantly different. On the basis of relative accumulation of C^{14}-labeled lactose in various segments of the gastrointestinal tract of rats, it appears unlikely that the action of lactose in causing increased calcium absorption is due to the formation of a lactose-calcium complex.

Dietary lactose has long been known to stimulate the absorption of calcium from the small intestine (1). The mechanism of action, however, remains unexplained although it has been possible to question many suggested possibilities. Recently it was proposed that lactose increases calcium absorption by chelating calcium to produce a soluble form that is readily available for transport (2). The in vitro data indicated that glucose was far less effective than lactose in forming the complex and that a high molar ratio of lactose to Ca^{++} was necessary.

Animal experiments have shown that lactose may promote a slight increase of calcium absorption in the duodenum and jejunum, if at all, whereas it is highly effective in the ileum (1, 3). Recently Wasserman and Belanger (4) demonstrated both chemically and autoradiographically the absorption of lactose-1-C^{14} into the cells of the ileum of rats.

If the entry of a lactose-calcium complex into the cells was the key event in promoting the absorption of calcium, it would be expected that under similar conditions little or no lactose would be found in the mucosal cells of the duodenum or jejunum since these areas respond much less efficiently to lactose in promoting an increased absorption of calcium. This criterion was investigated in the present work.

METHODS

Nine male albino rats[3] that had been maintained with a commercial ration[4] and averaged 225 g in weight were used in this study. These rats were fasted for 24 hours and then 0.5 ml of a dosing solution was injected into a ligated segment of the small intestine of each of these rats. The duodenum was used for 3 of the rats, the jejunum for 3 other rats, and the ileum was used for the remaining rats. The dosing solution contained 20 μc of lactose-1-C^{14}, 36 mg of carrier lactose, and 6.5 mg of $CaCl_2 \cdot 2H_2O$/ml. Two hours after dosing, the animals were anesthetized and the test intestinal segment removed before the animals were killed. The middle 1.5 cm of the intestinal segment was then washed gently in warm saline and put into a fixative made of one part glacial acetic acid, 4 parts formalin, and 1.5 parts ethanol.

The autoradiographs were made using melted N.T.B.-3 nuclear emulsion[5] and then stained with methylene blue — azure — basic fuchsin according to the technique of Belanger (5). Grain counts were made as described by Johnston (6). The data

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[3] Carworth Farms, New City, New York.
[4] Rockland Rat Diet, Rockland Farms, New City, New York.
[5] Eastman Kodak Company, Rochester, New York.

were statistically analyzed using the analysis of variance (7).

RESULTS

An autoradiograph of an ileal segment is shown in figure 1; the autoradiographs for the duodenal and jejunal segments were essentially similar. All of the tissues showed the presence of C^{14} in the mucosal cells. The radioactivity appeared well scattered through the cells and not concentrated at the brush border as reported in the paper of Wasserman and Belanger (4). Grain counts made over the villi and over the muscularis areas are shown in table 1. For comparative purposes, the counts were corrected to a standard 12.7-cm length of segment and the background count was subtracted. In all segments more radiocarbon was located in the villi than in the muscularis of the segment. For all three segments, the muscularis layer contained about the same amount of radioactivity and the amount of C^{14} present in the cells of the mucosa appeared to increase as the distance down the tract increased.

TABLE 1

Grain counts over the villi and muscularis regions of microautoradiographs of intestinal segments of rats given lactose-1-C^{14}

Section	Rat	Muscularis	Villi
		Avg counts/field[1]	*Avg counts/field*[1]
Duodenum	1	24.6 ± 0.8	36.6 ± 2.8
Duodenum	4	12.6 ± 1.0	28.4 ± 2.3
Duodenum	7	13.3 ± 1.1	19.6 ± 1.6
Duodenum	Avg	16.8	28.2
Jejunum	2	14.8 ± 2.0	38.5 ± 2.5
Jejunum	5	19.7 ± 1.8	25.8 ± 1.8
Jejunum	8	14.7 ± 1.7	47.6 ± 3.3
Jejunum	Avg	16.4	37.3
Ileum	3	10.4 ± 0.9	34.7 ± 2.2
Ileum	6	12.8 ± 0.9	53.0 ± 0.6
Ileum	9	18.7 ± 0.8	62.8 ± 0.8
Ileum	Avg	13.9	50.2

[1] Average of 30 fields; corrected to a standard 12.7-cm-long segment; mean ± SE of mean.

Table 2 shows counts made of the supranuclear areas of the goblet cells compared with the average count of the mucosal cells to either side of the goblet cells. Ten such comparisons were made for each section. For all intestinal sections, the aver-

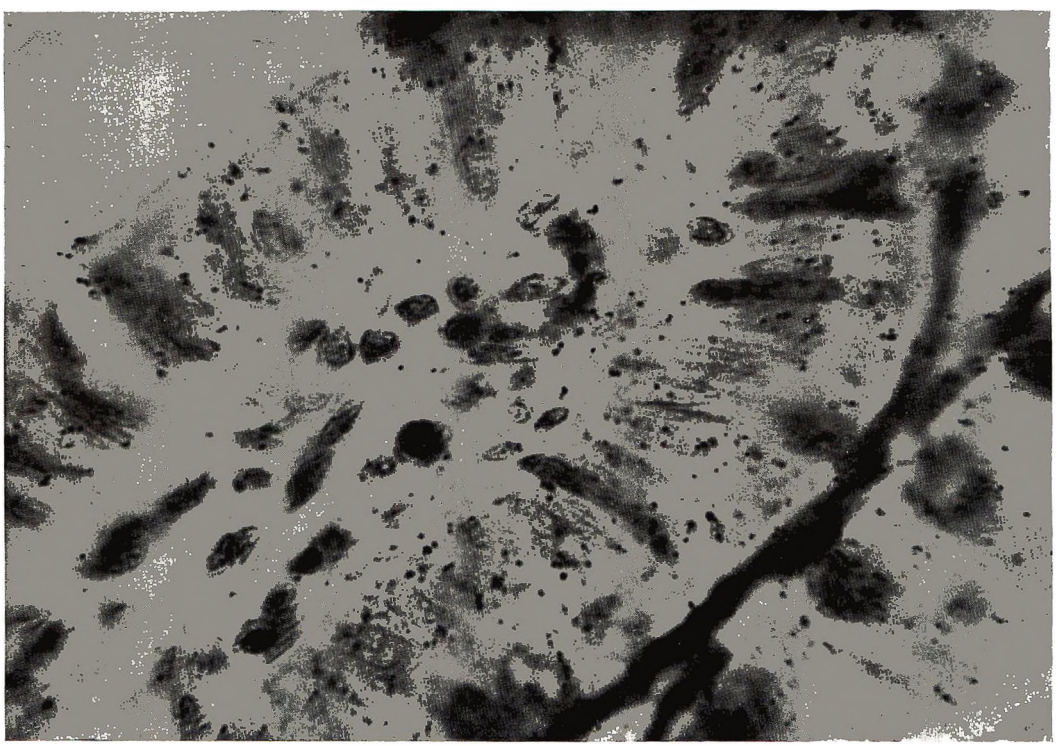

Fig. 1 Autoradiograph of a section of ileum.

TABLE 2

Grain counts over the supranuclear areas of the goblet cells and cells to either side of the goblet cells

Section	Rat	Adjacent cells	Goblet cell	$\bar{d} \pm s\bar{d}$
		Avg counts/cell	*Avg counts/cell*	
Duodenum	1	10.7	17.8	7.1 ± 2.4* [1]
Duodenum	4	8.2	11.2	3.0 ± 1.4
Duodenum	7	12.0	15.7	3.7 ± 3.1
	Avg	10.3	14.9	
Jejunum	2	20.2	27.2	7.0* ± 2.5*
Jejunum	5	14.2	17.2	3.0* ± 1.4*
Jejunum	8	21.9	29.1	7.2* ± 2.6*
	Avg	18.8	34.5	
Ileum	3	16.8	18.3	1.5 ± 1.6
Ileum	6	23.3	22.8	0.5 ± 1.4
Ileum	9	21.8	23.1	1.3 ± 2.2
	Avg	20.6	21.4	

[1] Difference between means of adjacent and goblet cells ± SE of the difference. Each average difference is composed of 10 comparisons. Astrisk indicates significance at the 5% level of probability.

age grain count of the goblet cells exceeded the counts of the adjacent mucosal cells; however, only in the jejunum was the difference consistently statistically significant ($P < 0.05$); the least difference between the cell types was observed in the ileum.

DISCUSSION

Chemical identification of the C^{14}-containing compound was not carried out in this study since the work of Wasserman and Belanger (4) established that the radioactivity in the mucosal cells of the ileum was present as labeled lactose. The same situation should pertain to this study particularly since the experimental period was 2 rather than 4 hours.

The autoradiographs showed that lactose is absorbed at all levels of the intestinal tract and thus the promotive effect of lactose on calcium absorption in the ileum must be explained on some other basis than a unique permeability of the ileum for a lactose-calcium complex.

The remarkable enhancement of calcium absorption in the ileum (1, 3) also tends to dispel the possibility that lactose merely serves as a metal-ion buffer to maintain Ca^{++} in a form readily available for transport (2). It appears unlikely that this physico-chemical phenomenon would occur mostly in one particular intestinal segment. Moreover, glucose, which appears to form the chelate with difficulty, does not promote calcium absorption in the duodenum or jejunum but can be as successful as lactose when placed in the ileum (2, 3). These data along with the observation that an increased absorption of calcium in the ileum can be noted when the calcium-lactose ratio is as low as 2.2:1 (8) suggest that the chelation of calcium is not important to the in vivo action of lactose.

The grain count data of this paper indicate that the concentration of lactose in the mucosal cells of the ileum exceeded that of the duodenum and jejunum. The observation could be essential to the solution of the problem particularly since Wasserman et al.[6] have produced evidence that lactose inhibited O_2 and P^{32} phosphate uptake by ileal mucosal cells.

The greater concentration of lactose in the ileal mucosal cells suggests that either the ileal cells absorb lactose more readily than the cells of the duodenum or jejunum, or that the difffusion of lactose from the mucosal cells of the ileum into the rest of the body is slower than for the other areas of the digestive tract. This study does not differentiate between these 2 possibilities. A time study of the absorption and accumulation of lactose in the cells of these regions would be more definitive.

It was observed that lactose was more readily concentrated by the goblet cells

[6] Wasserman, R. H., A. N. Taylor and C. L. Comar 1961 Lactose and the transit of calcium and strontium across the rat intestine. Federation Proc., 20: 292 (abstract).

than by the other cells of the villi. Since the most significant concentration occurred in the jejunum and the least in the ileum, areas of little and great effect of lactose, respectively, it is probable that this phenomenon is not related to the enhancement of calcium absorption. Wasserman and Belanger (4) have suggested that the material may be in the form of locally synthesized polysaccharides.

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Effect of Diet on Serum and Muscle Chloride and Muscle Moisture[1]

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ABSTRACT Twenty experimental rations, providing 9 to 36% of calories from casein, 8 to 44% of calories from lard or corn oil, and 20 to 83% of calories from sucrose, were fed to male rats from weaning to 53 weeks of age. Calculated vitamin, and mineral to calorie ratios were identical in all diets. Total muscle chloride content decreased significantly when corn oil replaced lard in the diet. When casein was substituted for sucrose, similar results were observed, but the results were not conclusive ($P = 0.1$). Serum chloride and muscle moisture were not affected by these dietary alterations. Thus, intracellular muscle moisture contents *increased* with *decreasing* total muscle chloride levels.

Interest and research have recently been directed to the study of electrolytes of physiological importance. Forbes et al. (1) reported that in man, total body chloride contents decrease with age, from 80 mEq/kg of body weight at the fifth month *in utero* to 50 mEq/kg at birth, and to 30 mEq/kg in adults. Talso et al. (2) and Barnes et al. (3) studied the normal electrolyte composition and variation of human muscle samples, with evaluations of the precision and accuracy of the techniques used. Daniel (4) reported investigations on cat, guinea pig, rabbit, and human uterine muscle electrolytes. Newman (5) and Frank and Carr (6) undertook similar studies on serum samples obtained from adult man.

Although there is a great deal of information on normal and abnormal electrolyte levels in experimental animals and man, there are no known reports of studies designed to evaluate the effects of the composition of the diet, aside from its mineral contents, on tissue and serum electrolyte levels in experimental animals. The results of the present study provide new information on relationships between the type of fat and amount of protein and carbohydrate in the diet, and serum and muscle chloride levels and muscle moisture content in adult male rats. Earlier reports from this laboratory provided data on the effects of these experimental diets on growth rates and efficiency of feed utilization (7) and serum cholesterol levels (8).

EXPERIMENTAL METHODS

Twenty-five-day-old male weanling rats[4] were distributed among 20 experimental groups of 4 animals each, of equal average weight. Food and water were provided ad libitum until individual animals achieved a body weight of 400 g. Weekly weighing and adjustment of feed intake were then used to maintain individual body weights at 400 ± 15 g until completion of the 50-week experimental period.

The composition of the experimental rations is shown as ingredient percentages by weight and by calculated calories in table 1. Purified minerals and vitamins were provided in a constant ratio to calculated calories in each diet: ration 1 was supplemented according to Lushbough et al. (9), and all others, in proportion to caloric density.

Rations 1 and 2 provided low and high levels of casein, and a low level of fat.

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TABLE 1

Composition of experimental rations[1]

Ration no.	Casein		Lard or corn oil		Sucrose		Calories/ 100 g[2]
	Weight	Calories[2]	Weight	Calories[2]	Weight	Calories[2]	
	%	%	%	%	%	%	
1	12.0	11.8	5.0	11.1	78.5	77.1	407
2	36.0	35.4	5.0	11.1	54.5	53.5	407
3	14.8	11.8	24.6	44.2	55.0	44.0	501
3C	14.8	12.0	23.6[3]	42.2[3]	55.0	45.8	492
4	44.3	35.4	24.6	44.2	25.5	20.4	501
4C	44.3	36.0	23.6[3]	42.2[3]	25.5	21.8	492
5	10.3	8.9	17.2	33.2	67.3	57.9	465
6	30.9	26.6	17.2	33.2	46.7	40.2	465
7	8.9	8.9	3.7	8.3	83.0	82.8	401
8	26.6	26.6	3.7	8.3	65.2	65.1	401

[1] Purified minerals and vitamins, and fish liver oil were provided in a constant ratio to calculated calories, to make 100%.
[2] Calculated as 4 kcal/g for casein and sucrose, 9 kcal/g for lard or corn oil.
[3] One per cent cholesterol substituted for lard or corn oil, and assumed to contribute no calories per se.

Rations 3 and 4 provided 4 times as many calories from fat as rations 1 and 2, with the same levels of casein, respectively, on a calculated calorie basis. Rations 3C and 4C contained 1% cholesterol by weight, substituted for an equal weight of fat.

Rations 5 and 6 were designed as "dilution diets": 15 parts of fat were *added* /100 parts of rations 1 and 2, respectively. Appropriate adjustments were made to maintain constant mineral- and vitamin-to-calorie ratios. Similarly, rations 7 and 8 were prepared by dilution of rations 1 and 2, with 33.75 parts of sucrose/100 parts of diet. This added sucrose was calorically equivalent to the 15 added parts of lard in rations 5 and 6.

Lard[5] and corn oil[6] were compared in each series of experimental rations, to provide information on the effects of feeding these specific natural fats on serum and muscle chloride and muscle moisture.

Serum chloride determinations for each animal were made using the VanSlyke and Hiller (10) modification of Sendroy's titrimetric method (11). For the muscle chloride determinations, a weighed sample of at least 1 g of gastrocnemius muscle from each animal was homogenized for 45 seconds[7] in 5 ml of water, containing 0.2 g $AgIO_3$. Twenty milliliters of phosphotungstic acid (10 ml conc H_3PO_4 added to 6 g H_2Wo_4 in 200 ml H_2O, and diluted to make one liter) was added to the homogenate, with further mixing for one minute. The homogenate was then filtered, and 10-ml aliquots of the filtrate used in making the muscle chloride determinations.

Muscle moisture was determined using weighed samples of at least 1 g of gastrocnemius muscle, obtained from the opposite leg of each animal, and dried at 105° for 18 hours.

RESULTS

The results observed for each group are shown in table 2, which compares the values for animals fed lard or corn oil. All determinations were made on serum and muscle samples from individual animals, and the values for standard deviations provide an index of the biological variability observed within each group.

Average values for muscle chloride ranged from 46 to 59 mEq/liter of muscle moisture, and the range for individual animals extended from 37 to 62 mEq/liter. Although average serum chloride varied within a more limited range (104 to 111 mEq/liter of serum moisture), the extreme values for individual animals were 100 and 123 mEq/liter. Muscle moisture varied less than either muscle or serum chloride. Average values for the 20 experimental groups all fell within the narrow range of 73 to 76%, the observed extremes being 68 and 81%.

The results (table 2) indicate that muscle chloride levels were consistently higher

[5] Deodorized lard, Reliable Packing Company, Chicago. Linoleic acid content, 11%.
[6] Mazola, Corn Products Company, Chicago. Linoleic acid content, 49%.
[7] Servall Omni-Mixer. Ivan Sorvall, Inc., Norwalk, Connecticut.

TABLE 2

Serum and muscle chloride and muscle moisture

Ration no.	Fat fed	Serum chloride	Muscle chloride	Muscle moisture
		mEq/liter serum moisture[1]	*mEq/liter muscle moisture*	%
1	Lard[2]	106 ± 4.6[3]	59.2 ± 4.2	73 ± 1.7
	Corn oil[2]	108 ± 3.6	51.6 ± 2.3	75 ± 0.9
2	Lard	104 ± 3.8	52.4 ± 2.0	75 ± 1.0
	Corn oil	108 ± 2.5	50.0 ± 6.4	75 ± 0.6
3	Lard	111 ± 4.4	55.2 ± 4.2	75 ± 1.5
	Corn oil	111 ± 4.7	49.3 ± 3.1	74 ± 0.0
3C	Lard	106 ± 4.1	54.1 ± 2.6	75 ± 0.8
	Corn oil	108 ± 4.1	50.7 ± 1.8	73 ± 3.5
4	Lard	105 ± 5.6	52.5 ± 3.3	75 ± 0.8
	Corn oil[2]	109 ± 7.8	49.1 ± 3.3	76 ± 3.0
4C	Lard	108 ± 2.6	51.4 ± 3.1	74 ± 1.2
	Corn oil	106 ± 5.8	50.4 ± 5.8	75 ± 0.0
5	Lard	108 ± 4.9	57.4 ± 3.5	73 ± 2.6
	Corn oil	108 ± 5.8	49.0 ± 3.5	74 ± 0.8
6	Lard	108 ± 3.1	56.0 ± 1.8	75 ± 1.5
	Corn oil	109 ± 6.0	46.6 ± 7.2	74 ± 1.0
7	Lard	108 ± 2.5	55.3 ± 2.2	74 ± 0.8
	Corn oil	106 ± 4.6	49.5 ± 2.1	74 ± 1.5
8	Lard	105 ± 4.1	52.2 ± 2.8	75 ± 0.0
	Corn oil	105 ± 4.3	46.8 ± 5.3	74 ± 1.6

[1] "Serum moisture" accounts for approximately 91% of total serum, w/w, the remainder consisting essentially of protein and electrolytes.
[2] Five animals/group.
[3] Average ± SD.

for all groups fed lard. These differences in muscle chloride attributable to the fat fed were highly significant ($P < 0.01$), when evaluated using a nested factorial model[8] for analysis of variance (table 3). The substitution of 1% cholesterol for fat had no significant effect on the muscle chloride values observed.

The substitution of casein for sucrose led to a nearly significant ($P = 0.1$) decrease in muscle chloride, as shown in table 3 (protein percentage within oil concentration).

No significant differences in serum chloride or muscle moisture were observed among the groups fed the 20 experimental rations included in this study.

We conclude from these data that the type of fat in the diet may exert a direct effect on the chloride content of muscle tissue; and more specifically, that the substitution of lard for corn oil leads to a significant increase in muscle chloride. The relative proportions of protein and carbohydrate may also have an effect on muscle chloride, but the data presented are not conclusive ($P = 0.1$).

DISCUSSION

The results observed in these studies further illustrate the complexities confronted in nutrition research. Individual variation in response to carefully controlled experimental treatment may be exceptionally wide; and the ingestion of varying kinds or amounts of a nutrient may provoke an unanticipated response in operation of the homeostatic mechanisms within the animal.

In these investigations, the type of fat and level of protein and carbohydrate in the diet exerted direct effects on total muscle chloride content, whereas no signifi-

[8] Snedecor, G. W. 1956 Statistical Methods Applied to Experiments in Agriculture and Biology, ed. 5. The Collegiate Press, Inc., Ames, Iowa.

TABLE 3

Analysis of variance, nested factorial model[1]

Source of variance	df	Sum of squares	Mean square	*F*	Estimated mean square
Between oils (types)	1	522.2	522.2	21.4**	$\sigma_e^2 + 4\sigma_{OP}^2 + 8\sigma_{CO}^2 + 40\sigma_O^2$
Oil × concentration	4	97.6	24.4	2.9	$\sigma_e^2 + 4i\sigma_{OP}^2 + 8\sigma_{CO}^2$
Among oil concentrations	4	51.1	12.8	0.5	$\sigma_e^2 + 8\sigma_P^2 + 16\sigma_C^2$
Protein percentage within oil concentrations	5	133.2	26.6	1.8*	$\sigma_e^2 + 8\sigma_P^2$
Oil type × protein concentration	5	41.9	8.4	0.6	$\sigma_e^2 + 4\sigma_{OP}^2$
Error	60	886.1	14.8		σ_e^2
	79	1732.2			

[1] Model: $X_{ijke} = \overline{X} + O_i + C_j + (CO)_{ij} + P_{k(j)} + (OP)_{ik(j)} + E_e(ijk)$,
where
O_i = oil type, i = 1,2: fixed variable
C_j = oil concentration, j = 1,2,3,4,5: random variable
$P_{k(j)}$ = protein percentage within oil concentration, k = 1,2 for all j: random variable
$(CO)_{ij}$ = oil type – oil concentration interaction
$(OP)_{ik(j)}$ = oil type – protein percentage interaction
E = error.

* P = 0.1.
** P < 0.01.

cant effects of diet on muscle moisture or serum chloride were observed. If it is assumed that the chloride concentrations in serum and in the interstitial fluid of muscle tissue are equivalent, then the effect of diet on intracellular muscle moisture may assume great and previously unrecognized importance, since an increase in total muscle chloride content was not associated with an increase in muscle moisture levels.

This implies that the moisture content of the muscle cell is directly affected by diet, viz., when muscle chloride *increased* due to lard feeding or decreased casein intake, intracellular muscle moisture *decreased*. Physiological and nutritional interrelationships of such fundamental importance warrant further detailed study.

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Changes in Succinic Dehydrogenase Activity and Fatty Acid Composition of Rat Liver Mitochondria in Essential Fatty Acid Deficiency[1]

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ABSTRACT The effects of feeding diets containing corn oil, coconut oil, tuna oil, or no fat on the sequence of development of growth inhibition and altered mitochondrial fatty acid compositions and succinic dehydrogenase activities were studied. The mitochondrial succinic dehydrogenase activities of rats fed tuna oil were higher than those of the control (corn oil) rats, but lower than those of the other EFA-deficient rats throughout the experiment. Suggestive differences between control and deficient mitochondria were noted after one week of the experiment and were marked after 2 weeks. The exposure to water, the addition of calcium ion, or the addition of snake venom elevated the mitochondrial succinic dehydrogenase activities, and eliminated or reduced the differences between the control and EFA-deficient rats. The appearance of eicosatrienoic acid and a reduction of arachidonic and linoleic acids in mitochondrial lipids from the fat-free and coconut oil groups occurred within one week after initiating the experiment. The feeding of tuna oil resulted in significant amounts of a highly polyunsaturated fatty acid of calculated carbon number 23.4, which was, perhaps, eicosapentaenoic acid, but not of eicosatrienoic acid in mitochondrial lipid. The alterations of mitochondrial fatty acid composiiton in EFA deficiency preceded slightly the appearance of elevated succinic dehydrogenase activity.

According to a previous report (1), when rats were fed a fat-free diet, certain dehydrogenase activities of intact liver mitochondria were higher than those from the control rats. After sonic disruption of the mitochondria, the activities of the 2 groups were equal, although they were higher than those of intact mitochondria. It was also reported (2) that liver mitochondria from rats fed a fat-free diet contained greater amounts of palmitoleic and eicosatrienoic acids and smaller amounts of linoleic and arachidonic acids than those from rats fed the corn oil diet.

On the other hand, the complete essential fatty acid (EFA) deficiency syndrome develops after a prolonged period (3). The change in the fatty acid composition of rat liver mitochondria as detected by the alkali isomerization method was found to occur about 6 weeks before the appearance of external signs of the deficiency (4). In attempting to understand the etiology of the EFA deficiency syndrome and particularly the significance of these mitochondrial alterations, it was necessary to determine the sequence of the changes in enzymatic activity and fatty acid composition of liver mitochondria from rats fed control or EFA-deficient diets.

Dietary fatty acids other than essential fatty acids affect the development of EFA deficiency signs. Saturated fatty acids in general (5–7) and certain polyunsaturated fatty acids from marine oils (8, 9) have been shown to influence EFA deficiency.

Therefore, a study of the succinic dehydrogenase activities and fatty acid compositions of liver mitochondria from rats fed fat-free diets and diets containing corn oil, hydrogenated coconut oil, or tuna oil, was carried out to understand the sequence of appearance of the manifestations of the deficiency syndrome.

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[2] Taken in part from a thesis submitted to the Faculty of the Harvard School of Public Health in partial fulfillment of the requirements for the degree of Doctor of Science in Hygiene in the Field of Nutrition, Boston, Massachusetts, April, 1963.

[3] This research was done during the tenure of an Established Investigatorship of the American Heart Association.

EXPERIMENTAL

Weanling male albino rats[4] were housed in individual cages and were divided into 4 groups and maintained with the following experimental diets:

One group was given the fat-free diet of which the percentage composition was: glucose, 77; devitaminized casein, 18; minerals, 4; and vitamin mixture, 1. In the fat-containing diets, corn oil, hydrogenated coconut oil, or tuna oil was substituted for an equal amount of glucose at the 5% level.

The vitamin mixture contained: thiamine·HCl, 200 mg; riboflavin, 200; pyridoxine·HCl, 200 mg; niacin, 600 mg; calcium pantothenate, 800 mg; choline chloride, 40 mg; biotin, 4 mg; inositol, 4 mg; folic acid, 8 mg; ascorbic acid, 2.4 g; methionine, 100 g; cyanocobalamin, 1.6 mg; vitamin A acetate (1,000,000 U/g), 200 mg; calciferol, 8 mg; α-tocopherol, 4 g; menadione, 20 mg; and glucose to make a total weight of 1,000 g.

All of the rats were weighed weekly or biweekly. After the rats had been decapitated, liver mitochondria were prepared with a 0.88 M sucrose solution as previously described (2).

Succinic dehydrogenase activities were measured by the method of Ells (10) as slightly modified. The basic system contained 1,500 μmoles sucrose, 150 μmoles potassium phosphate pH 7.4, 1.5 mg serum albumin (human, Cohn Fraction V). Two milliliters of the above assay preparation were incubated for 20 minutes at room temperature, 25 to 26°C. Then 30 μmoles KCN, 60 μmoles sodium succinate, 0.05 μmoles 2,6-dichlorophenol indophenol or water only in the blank, and 0.15 mg phenazine methosulphate were added individually and rapidly to initiate the reaction. The Beckman DU spectrophotometer was used with a Gilford automatic cuvette positioner and optical density converter, and the optical density at 600 mμ was recorded at each time setting period automatically. The temperature of the thermospacers around the cuvette carriage was maintained at 25 to 26°C.

In certain experiments, 20 μg snake venom or 2.25 μmoles $CaCl_2$ were added before the preincubation. Dried venom of the American water moccasin, *Agkistrodon piscivorus*,[5] was used as the source of lecithinase A. The dried venom was dissolved just before use.

Protein was determined by the biuret method of Gornall et al. (11).

Aliquots of the liver mitochondrial preparations were added to 5 volumes of acetone-ethanol (1:1; v/v). After heating the samples in the solvent at 40°C for one hour, precipitated protein was removed by filtration. Aliquots of the filtrate were used for the isolation of fatty acids. Fatty acid preparations were methylated by a modification of the method of Stoffel et al. (12). Analysis of the mixtures of fatty acid esters was carried out using gas-liquid chromatography. One-hundred-twenty-two-centimeter or 152-cm columns using ethylene glycol succinate as the stationary phase and an argon ionization detector were used.

RESULTS

The changes of the body weights of the rats fed hydrogenated coconut oil were similar to those of the rats fed a fat-free diet. However, the dermal syndrome of the rats fed hydrogenated coconut oil was very variable throughout the experimental period, whereas the skin lesions of the rats fed the fat-free diet were much more uniform, becoming progressively more severe throughout the experimental period. The growth of the rats fed tuna oil stopped sharply at the 15th week and the body weights decreased precipitously thereafter. Until the 15th week, the growth of the rats was less than that of the control group, but greater than that of these other 2 groups.

Succinic dehydrogenase activities for intact mitochondria are presented in figure 1. In the presence of sucrose to protect the structural integrity of the mitochondria, the succinic dehydrogenase activity of the rats fed diets without EFA was higher than that of the control rats after the second week of feeding. The mitochondrial succinic dehydrogenase activities of all of the rats in this experiment declined throughout the feeding period. In the first week of the feeding period, there was no significant difference in the en-

[4] Obtained from the Charles River Breeding Laboratories, Boston.

[5] Obtained from the Miami Serpentarium, Kendall, Florida.

zymatic activities between the control and the EFA-deficient groups. After the second week the enzymatic activities of the EFA-deficient groups were higher than those of the control group. Marked differences in the mitochondrial succinic dehydrogenase activities of the EFA-deficient groups were apparent after the fourth week of the feeding period. The rank of the mitochondrial succinic dehydrogenase activities in decreasing order among the groups fed the diets without EFA was, consistently, hydrogenated coconut oil, fat-free, and tuna oil.

In certain instances, the assay systems were supplemented with substances that would be expected to disrupt the mitochondria. The results are shown in table 1. When snake venom was added to the assay medium to hydrolyze the phospholipid in mitochondrial membrane to the lyso forms, most of the mitochondrial succinic dehydrogenase activities increased. However, differences in enzymatic activi-

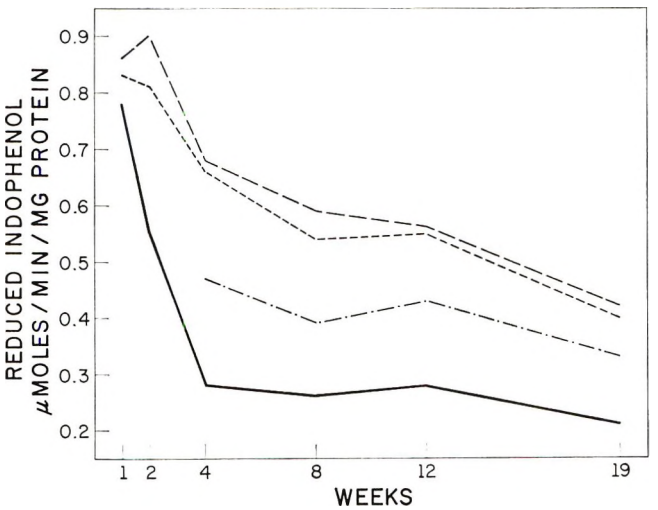

Fig. 1 Succinic dehydrogenase activities of liver mitochondria from rats fed fat-free------, corn oil ——, hydrogenated coconut oil — — —, or tuna oil —·—·—·— diets. The values at 1 and 2 weeks and of the tuna oil group for 19 weeks are the means of 2 animals. The values of the fat-free group at 19 weeks is the mean of 4 animals. All other values are the means of 3 animals.

TABLE 1

Succinic dehydrogenase activities of liver mitochondria from rats fed fat-free, corn oil, hydrogenated coconut oil or tuna oil diets (reduced indophenol μmoles/min/mg protein)

Assay system	Type of diets fed to rats	Feeding periods, weeks			
		4	8	12	19
0.5 M Sucrose and snake venom	corn oil control	0.49(3)[1]	0.46(3)	0.59(3)	0.46(3)
	fat-free diet	0.72(3)	0.60(3)	0.71(3)	0.53(4)
	hydrogenated coconut oil diet	0.67(3)	0.61(3)	0.71(3)	0.52(3)
	tuna oil diet	0.46(3)	0.54(3)	0.53(3)	0.36(2)
0.5 M Sucrose and Ca^{++}	corn oil control				0.41(3)
	fat-free diet				0.50(3)
No sucrose	corn oil control			0.90(3)	0.58(3)
	fat-free diet			0.92(3)	0.64(3)

[1] The values presented are the means for the numbers of animals shown in parentheses.

ties still persisted. Snake venom had a smaller effect on most of the mitochondrial succinic dehydrogenase activities of the rats fed diets deficient in EFA than on the control mitochondria. Addition of calcium ion increased the mitochondrial succinic dehydrogenase activities of the control and the EFA-deficient group. The assay using the hypotonic media which did not contain sucrose or calcium ion indicated that the mitochondrial succinic dehydrogenase activities of the control group and the EFA-deficient group were similar.

The arachidonic acid, eicosatrienoic acid, and linoleic acid levels of liver mitochondria of rats fed the fat-free diet, or diets containing 5% fat as corn oil, hydrogenated coconut oil, or tuna oil at various periods are shown in figures 2, 3, and 4, respectively. The rats used for the mitochondrial fatty acid analysis were randomly chosen from each group. The eicosatrienoic acid was observed in the mitochondrial lipid of the rats fed the fat-free diet as early as the first week of the feeding period. The linoleic acid level was already very low within one week,

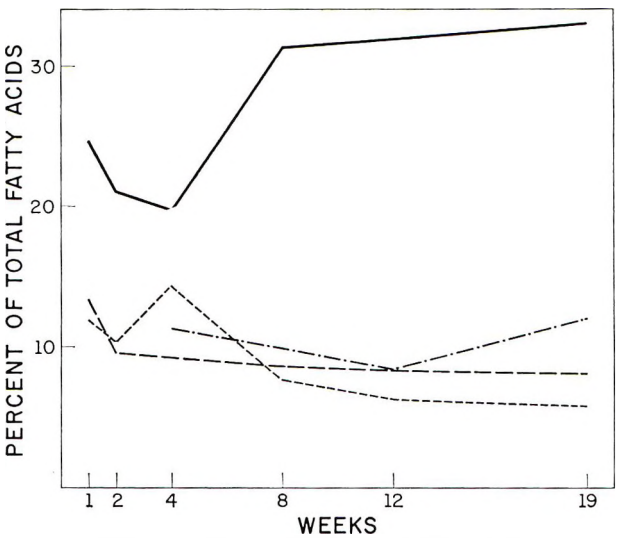

Fig. 2 Arachidonic acid level of liver mitochondrial lipid from rats fed fat free·····, corn oil ——, hydrogenated coconut oil — — — —, or tuna oil —·—·—·—· diets.

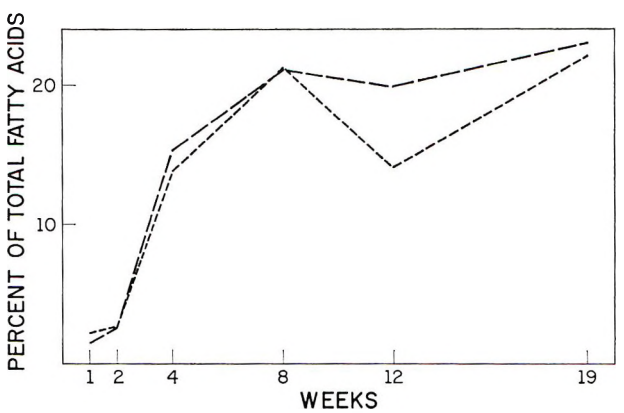

Fig. 3 Eicosatrienoic acid level of liver mitochondrial lipid from rats fed fat-free·····, or hydrogenated coconut oil — — — —, diets. The eicosatrienoic acid levels of the mitochondria from the rats fed diets containing corn oil or tuna oil were always less than 0.5% and are thus not illustrated in the figure.

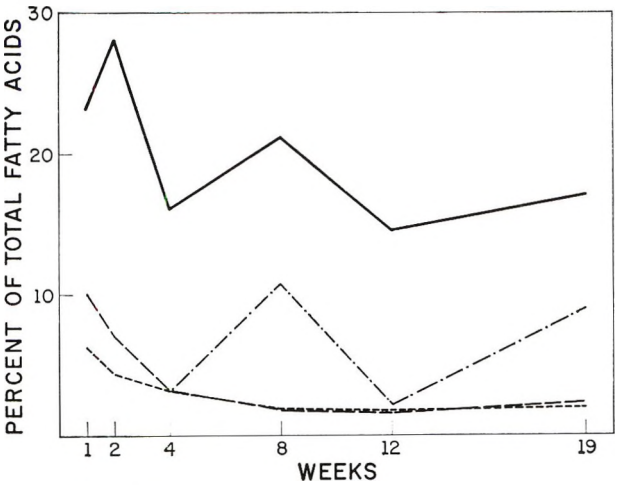

Fig. 4 Linoleic acid level of liver mitochondrial lipid from rats fed fat-free·····, corn oil ——, hydrogenated coconut oil — — — —, or tuna oil —·—·—·—· diets.

and the arachidonic acid level in the mitochondrial lipid of the rats fed the fat-free diet had decreased. The arachidonic acid level did not further decrease significantly after the eighth week. A large part of the ultimate difference in fatty acid composition between the different dietary groups existed by the fourth week of the feeding periods.

The fatty acid composition of the mitochondrial lipid from the hydrogenated coconut oil group was not significantly different from that of the fat-free group. The feeding of tuna oil as the dietary fat resulted in mitochondrial lipid fatty acid compositions which were different from those of the other groups. The linoleic acid level was low and little eicosatrienoic acid was observed. Low concentrations of 2 fatty acids with calculated carbon numbers of 21.6 and 21.9 were detected consistently. Moreover, a fatty acid with a calculated carbon number of 23.4 was obtained after 4 weeks with the tuna oil diet and was present at a concentration of approximately 10% of the total fatty acids. This latter fatty acid appeared to be an eicosapentaenoic acid, since the calculated carbon number was comparable to that obtained from the data of Farquhar et al. (13) using similar chromatographic procedures.

DISCUSSION

The addition of the completely saturated fat to the fat-free diet had little influence on the body weights and the enzyme activities determined, although it has been reported (5–7) that the substitution of saturated fatty acids in a fat-free diet accelerated the EFA deficiency syndrome. On the other hand, the result of the addition of tuna oil which has been shown (8) to be much less effective than linoleic acid for the relief of the dermal signs of EFA deficiency was somewhat different from the result of the addition of hydrogenated coconut oil. Polyunsaturated fatty acids in tuna oil appeared to have a different growth-promoting activity which originated from non-EFA polyunsaturated fatty acids.

The mitochondrial succinic dehydrogenase activities were clearly sensitive to the nutritional status with respect to EFA after the fourth week of the feeding period. Apparently the difference in the mitochondrial succinic dehydrogenase activities started to occur at the second week. Since the enzyme activities increased with the addition of snake venom or with the elimination of sucrose from the assay media, the low enzymatic activity of the intact mitochondria could be considered a manifestation of latent activity that can be released upon the disruption of mitochondria. The amount of latent activity was greater in the mitochondrial preparations from the control group than in the EFA-deficient mitochondria. The persistence of a difference in the mitochondrial succinic

dehydrogenase activities between the groups assayed with the addition of snake venom probably indicated that the alteration of phospholipid molecules within the mitochondrial membrane disrupted the mitochondria partially, but that EFA played a significant role in the structural integrity of the mitochondria even when part of the phospholipids were in the lyso form. The addition of calcium ion exhibited a similar enzymatic activity-enhancing effect for all groups. Calcium ion has been considered (14) to destroy the permeability barrier of mitochondria and then to permit free penetration of the reactants. In this assay system the reactant with the limiting rate of penetration was phenazine methosulphate, rather than succinate (14). It appeared that the elimination of the permeability barrier was less effective for revealing the complete enzymatic activity than structural disintegration by the exposure to a hypotonic medium. The succinic dehydrogenase activities of the control and EFA-deficient mitochondria were essentially equal when the mitochondria were suspended in a sucrose free medium. Deoxycholate has also been reported (14) to enhance the rate of the succinate-phenazine methosulfate reaction in the same way that calcium ion did.

A composite presentation of the changes in fatty acid composition, enzyme activity, and body weight for the rats fed a fat-free diet are presented in figure 5. The decrease of arachidonic acid in liver mitochondrial lipid was detected as early as one week after initiation of the experiment. The eicosatrienoic acid level was measurable even at the first week and continued to increase until the fourth week and reached a plateau corresponding with the time of stabilization of arachidonic acid. On the other hand, mitochondrial succinic dehydrogenase activities of EFA-deficient rats became markedly higher than those from control rats at the fourth week. The differences between the body weights of the control and deficient groups became distinct at about the same time as did the abnormalities in the enzyme studies. The dermal syndrome in the deficient rats first appeared at about the fourth week and progressed more slowly than the changes described above.

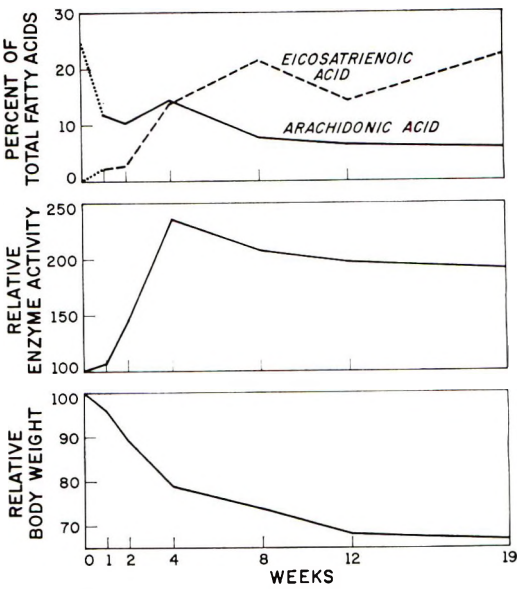

Fig. 5 The development of essential fatty acid deficiency as indicated by fatty acid composition, relative enzyme activity, and relative body weight. The concentrations of eicosatrienoic and arachidonic acids were determined on lipid extracts of liver mitochondria from rats fed a fat-free diet. The fatty acid levels at the starting time were equivalent to the values observed in the controls fed corn oil containing diets. The relative enzyme activity is the factor obtained by dividing the mean succinic dehydrogenase activity (× 100) of liver mitochondria from rats fed fat-free diet by the value for rats fed the corn oil containing control diet at each time of measurement. The relative body weight is the relative mean body weight of rats fed the fat-free diet compared with those of rats fed the corn oil control diet at each time of measurement.

The very early appearance of the altered fatty acid composition of the mitochondrial lipid and of the differences in succinic dehydrogenase activities of carefully isolated mitochondria suggested that the sequence of development of the increased metabolic rate and growth inhibition in rats fed diets deficient in EFA was as follows:

Altered fatty acid composition of mitochondrial lipid → structural alteration of mitochondria → increased activity of certain enzymes in the electron transport chain with inefficient coupling of oxidative phosphorylation (15). It must be remembered that the causal relationship between the structural alteration of mitochondria in EFA deficiency and the ineffi-

ciency of oxidative phosphorylation has not been definitely established. Fletcher and Sanadi (16) have presented evidence that rat liver mitochondria were turned over as a unit with a half-life of 10.3 days. This observation was consistent with the observations in the present report that the fatty acid compositions and enzyme activities of rat liver mitochondria were altered rapidly when EFA-deficient diets were fed. The observation that the mitochondrial enzyme activities were altered before the mitochondrial fatty acid pattern had been completely converted to that noted in long term EFA deficiency suggests the possibility that feeding diets relatively low in EFA (but sufficient to prevent the skin lesions) for long periods might also result in alterations of mitochondrial enzyme activity.

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Action of Linoleic and Arachidonic Acids upon the Eicosatrienoic Acid Level in Rat Heart and Liver

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ABSTRACT The effects of dietary linoleate and arachidonate on eicosa-5,8,11-trienoic acid concentration in heart and liver lipids of essential fatty acid-deficient rats were investigated. Pyridoxine deficiency and 4-deoxypyridoxine·HCl addition to the diet were also investigated to reduce the conversion of linoleic into arachidonic acid and thus differentiate the effects produced by both acids. The addition of 4-deoxypyridoxine was observed not to impair the conversion of oleic into eicosa-5,8,11-trienoic acid, but it appeared to reduce slightly the conversion of linoleic into arachidonic acid. Arachidonate was shown to be a specific regulator of eicosa-5,8,11-trienoic acid percentage in heart and liver lipids, whereas linoleate appeared to require its previous conversion into arachidonic acid. Arachidonic acid effect on eicosa-5,8,11-trienoic concentration discards the competence of both synthesis: linoleate into arachidonate and oleate into eicosatrienoate as the possible regulating mechanism.

Linoleic and arachidonic acids are considered essential fatty acids (EFA), but there are other acids with double bonds in the 6- and 9- position counted from the methyl end of the acid which also have EFA activity (1). Nevertheless, it has not yet been proved, whether the principal activity of these acids is due to their structure, per se, or to their conversion into arachidonic acid. However, it has been proved that linoleic (2), γ-linolenic (octadeca-6,9,12-trienoic acid) (3) and eicosa-8,11,14-trienoic (4) acids, which are transformed into arachidonic acid in the animal body, show the highest EFA activity (1). In addition, γ-linolenic and eicosa-8,11,14-trienoic acids have a very short life when injected into the animals and are very quickly transformed into arachidonic acid. All these results suggest that the main EFA activity of these acids, or at least some of its effects, may be due to their conversion into arachidonic acid.

To confirm this theory, a study of the comparative effects of linoleic and arachidonic acids upon the eicosa-5,8,11-trienoic acid concentrations was attempted. Eicosa-5,8,11-trienoic acid is synthesized from oleic acid (5) by rats deprived of essential fatty acids, and its appearance in the heart muscle is a sensitive assay system for EFA deficiency (6). The addition of linoleic or arachidonic acid to the diet of EFA-deficient rats, reduces the eicosatrienoic acid concentration to normal levels. Linoleic acid may be an active EFA in addition to its known conversion into arachidonic acid. Accordingly, the effect of linoleic acid on the appearance of eicosa-5,8,11-trienoic acid was studied under conditions in which the conversion of linoleic acid into arachidonic acid was limited by 4-deoxypyridoxine·HCl (DP) addition to the diet and pyridoxine deficiency. Pyridoxine deficiency was provoked in the experimental animals, since according to reports of Witten and Holman (7) and Dam et al. (8), the lack of this vitamin may impair the conversion of linoleic into arachidonic acid.

The function of pyridoxine in fat metabolism has been studied by several investigators and reviewed by Sakuragi (9). Later works as those of Swell et al. (10) and Johnston et al. (11) consider that pyridoxine may act indirectly in the biosynthetic scheme from linoleate to arachidonate, and according to Kirshman and Coniglio (12) this vitamin would affect the metabolism of fatty acids in general and would not be specifically involved in the conversion of linoleic into arachidonic acid. Wakil (13) indicated in addition, that pyridoxal phosphate might be in-

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volved in the elongation of long-chain fatty acids in a mitochondrial enzyme system.

MATERIALS AND METHODS

Methyl linoleate. Methyl linoleate (95% pure) was obtained from sunflower seed oil[2] by the method of urea adducts (14) and vacuum distillation.

Methyl arachidonate. Methyl arachidonate (79% pure) was obtained from hog liver by saponification (15), fractional crystallization (16) and vacuum distillation. It contained only 2.2% of methyl linoleate estimated by gas liquid chromatography.

Experimental. Two feeding trials were made with weaned male albino rats from the strain of the Institute, under the conditions detailed in tables 1 and 2. In both trials the effect of 1% methyl arachidonate and 1% methyl linoleate addition to the EFA-deficient diet of the rats, was studied. To impair the conversion of linoleic into arachidonic acid and to study its own particular effect, the rats were deprived of pyridoxine. In the first trial pyridoxine was not added to the diet, whereas in the second, pyridoxine was omitted, but 450 mg 4 deoxypridoxine·HCl[3]/kg of food were added. The effect observed in the fatty acid composition of heart and liver was compared with those of other groups of rats fed a control diet containing methyl linoleate, EFA-deficient diet, pyridoxine-deficient diet and EFA- and pyridoxine-deficient diet. The mortality was very high in the animals deprived of pyridoxine, and they showed marked symptoms of acrodynia. Hearts and livers of the various groups were pooled separately immediately after the animals were killed. The lipids were extracted with chloroform-methanol mixture (17) and transesterified with methanol by the procedure of Stoffel et al. (18). The fatty acid composition was determined by gas liquid chromatography. A Pye apparatus with ionization detector and argon flow was used. The columns were 122-cm long and 4 mm in diameter and were packed with 10% polyethylene-glycol-adipate. The original samples were run at 180° and 200°C and the composition, calculated from the surface of the peaks, was tabulated (tables 3 and 4). It was expressed in moles per 100 moles of total fatty acids.

Peaks of fatty acids were identified by comparison of their retention times relative to stearate with those of known standards and by determining the carbon numbers as described by Woodford and van Gent (19). The identity was confirmed by running some samples in 10% Apiezon N, and by hydrogenation and rechromatog-

[2] Supply of sunflower seed oil was kindly furnished by Molinos Río de la Plata, Argentina.
[3] A Grade, California Company for Biochemical Research, Los Angeles.

TABLE 1

Experimental conditions of the first trial with rats deprived of pyridoxine

	Control[1]	EFA-deficient[2]	Vitamin B_6-deficient[3]	EFA- and vitamin B_6-deficient	EFA- and vitamin B_6-deficient + linoleate	EFA- and vitamin B_6-deficient + arachidonate
No. of rats, initial	10	10	10	10	10	10
No. of rats, final	9	7	4	3	3	3
Age, days	23	23	23	23	23	23
Deficient-diet period, days	—	86	—	86	66	66
1% Linoleate-supplemented diet period, days	86	—	86	—	20	—
1% Arachidonate-supplemented diet period, days	—	—	—	—	—	20

[1] Animals received the EFA-deficient diet plus 1% methyl linoleate.
[2] EFA-deficient diet: (in grams) fat-free casein, 19; sucrose, 77; McCollum-Davis salt mixture no. 4 (31), 4; and vitamins. Each kilogram of diet contained the following vitamins: (in milligrams) thiamine, 10; riboflavin, 10; pyridoxine, 10; Ca pantothenate, 50; niacin, 25; inositol, 1000; biotin, 2.0; *p*-aminobenzoic acid, 10; folic acid, 2.0; choline chloride, 1000; vitamin B_{12}, 0.1; α-tocopherol, 30; menadione, 50; vitamin A, 3000 IU; and vitamin D, 800 IU.
[3] Animals received the EFA-deficient diet minus pyridoxine, plus 1% methyl linoleate.

TABLE 2

Experimental conditions of the second trial with rats deprived of pyridoxine and fed diets supplemented with 4-deoxypyridine·HCl (DP)

	Control[1]	EFA-deficient[2]	EFA-deficient + DP	EFA-deficient + DP + linoleate	EFA-deficient + DP + arachidonate
No. of rats, initial	3	10	10	10	10
No. of rats, final	3	7	3	3	3
Age, days	21	21	21	21	21
EFA-deficient period, days	—	114	97	97	97
EFA- and vitamin B_6-deficient period, days	—	—	7	7	7
EFA- and vitamin B_6-deficient + DP period, days	—	—	10	3	3
EFA- and vitamin B_6-deficient + DP + 1% linoleate period, days	—	—	—	7	—
EFA- and vitamin B_6-deficient + DP + 1% arachidonate period, days	—	—	—	—	7

[1] Diet as in table 1.
[2] Diet as in table 1.

raphy in 10% polyethylene-glycol-adipate. Eicosatrienoic acid was also recognized by separation in a preparative column, isomerization and spectrophotometry in UV (20). Eicosatrienoic acid from EFA-deficient rats is mainly constituted by eicosa-5, 8,11-trienoic acid (21, 22), and small amounts of eicosa-7,10,13-trienoic acid (22).

RESULTS AND DISCUSSION

The results from the first trial (table 3) showed that pyridoxine deficiency caused only a moderate decrease in the molar percentage of arachidonic acid in the heart (table 3, column 1 vs 3 and 5) when the rat received linoleic acid in the diet. However, this effect was not noted in the liver (column 7 vs 9 and 11). These results suggest little or no influence of pyridoxine on the rate of conversion of linoleic to arachidonic acid.

On the other hand, the concentration of eicosatrienoic acid was not increased either in liver or in heart by pyridoxine deficiency (table 3, columns 1 vs 3 and 7 vs 9), as it was by EFA deficiency. However, the simultaneous deficiency of EFA and pyridoxine produced higher percentages of eicosatrienoic acid both in heart and liver (table 3, columns 4 and 10). These results suggest that the synthesis of eicosa-5,8, 11-trienoic acid was not impaired by pyridoxine deficiency under the present experimental conditions. However, other investigators such as Williams and Scheier (23) observed that rats receiving a fat-free diet without vitamin B_6 contained lower amounts of eicosatrienoic acid in the liver than those animals receiving a similar diet supplemented with pyridoxine. However, the experimental conditions were rather different, and these investigators obtained a slight decrease in essential fatty acids.

When the rats previously receiving a diet depleted of both EFA and pyridoxine were administered linoleate and arachidonate, a decrease in the level of eicosatrienoic acid in heart and liver was noticed (table 3). The arachidonate effect may be easily interpreted as a direct influence of the acid on eicosa-5,8,11-trienoic acid synthesis and it would agree with Bozian and Coniglio's[4] suggestion that arachidonate exerts a regulatory function in the conversion of oleic acid into eicosa-5,8,11-trienoic acid. Linoleate cannot be considered responsible by itself for the same effect, since dietary pyridoxine deficiency ap-

[4] Bozian, R. C., and J. G. Coniglio 1962 The effect of ethyl arachidonate upon the metabolism of oleic acid. Federation Proc., *21*: 186.

TABLE 3

Comparative heart and liver fatty acid composition of EFA- and pyridoxine-deficient rats fed diets supplemented with linoleic and arachidonic acids (trial 1)

Fatty acids[1]	Heart						Liver					
	1	2	3	4	5	6	7	8	9	10	11	12
	Control	EFA-deficient	Vitamin B_6-deficient	EFA- and vitamin B_6-deficient	EFA- and vitamin B_6-deficient + linoleate	EFA- and vitamin B_6-deficient + arachidonate	Control	EFA-deficient	Vitamin B_6-deficient	EFA- and vitamin B_6-deficient	EFA- and vitamin B_6-deficient + linoleate	EFA- and vitamin B_6-deficient + arachidonate
	mole %	*mole %*	*mole %*	*mole %*	*mole %*	*mole %*	*mole %*	*mole %*	*mole %*	*mole %*	*mole %*	*mole %*
16	19.9	20.3	16.1	14.9	13.3	17.9	23.9	23.3	22.4	18.4	19.7	25.4
16:1	4.8	10.2	5.0	7.0	3.2	7.8	9.2	17.4	7.1	8.6	5.3	9.3
18	18.3	14.3	19.4	23.0	23.0	24.7	14.6	10.8	17.5	14.8	20.6	16.3
18:1	21.1	31.0	27.4	30.0	30.7	26.4	29.8	37.7	33.3	37.1	32.1	26.0
18:2	9.0	2.2	9.0	2.2	13.5	2.4	4.5	2.1	3.6	2.1	5.1	1.9
20:3	0.8	8.4	0.7	11.8	1.8	0.3	0.9	3.4	1.0	9.2	0.3	0.8
20:4	19.2	6.8	10.9	6.1	8.9	12.5	9.9	0.8	9.2	3.0	8.1	14.6

[1] Only the major fatty acids are represented; the number before the colon denotes chain length, and the number after the colon, number of double bonds.

peared to be ineffective in producing a total blocking of linoleate conversion into arachidonic acid (table 3) as deduced from the relatively high concentration of this acid. Therefore, the decrease of eicosatrienoic percentage may be due as much to the linoleate molecule as to the arachidonic acid synthesized from linoleate.

The second experiment was conducted in view of the results of the first trial — that pyridoxine deficiency in the diet was not able to produce a complete elimination of the pyridoxine in animals or at least a level that resulted in an effective blocking of linoleate conversion into arachidonic acid. Therefore, 4-deoxypridoxine was fed to pyridoxine-deficient rats. The amount added (450 mg/kg) was the same as that used by Holman (24), to reduce the content of polyunsaturated acids in rats. Ott (25) demonstrated that in animals deoxypyridoxine competes with pyridoxine, whereas Holman (24) observed that when rats receiving EFA were given 30 mg/kg of pyridoxine and 450 mg/kg 4-deoxypyridoxine, the content of polyunsaturated acids was restored to the normal level.

The results shown in table 4 (columns 2 vs 3, and 7 vs 8) showed 1) that pyridoxine deprival and DP feeding produced higher percentages of eicosatrienoic acid in liver and heart lipids of EFA-deficient rats. These data suggest that in our experimental conditions, eicosa-5,8,11-trienoic acid synthesis was not prevented by the deoxypyridoxine addition to the diet. Holman (26) in a preliminary study suggested that eicosatrienoic acid synthesis appeared to be impaired when deoxypridoxine was fed.

2) It was observed that although the linoleate added to an EFA- and pyridoxine-deficient diet supplemented with DP (table 4) was absorbed and stored in heart (11.5%) and liver (7.0%), reaching values even higher than those of the control rats (8.7 and 3.7%, respectively), the arachidonic acid percentage (9.4% in heart and 5.5% in liver) slightly surpassed the values of those rats fed an EFA- and vitamin B_6-deficient diet supplemented with DP (8.1 and 2.9%, respectively). Meanwhile, the proportion of arachidonic acid in control rats was very high: 23.6% in heart and 15.9% in liver. The molar composition tab-

TABLE 4

Effect of linoleic and arachidonic acids on heart and liver fatty acid composition of EFA- and pyridoxine-deficient rats fed diets supplemented with 4-deoxypyridine·HCl (DP) (trial 2)

Fatty acids[1]	Heart					Liver				
	1 Control	2 EFA-deficient	3 EFA- and vitamin B_6-deficient	4 EFA- and vitamin B_6-deficient + linoleate	5 EFA- and vitamin B_6-deficient + arachidonate	6 Control	7 EFA-deficient	8 EFA- and vitamin B_6-deficient	9 EFA- and vitamin B_6-deficient + linoleate	10 EFA- and vitamin B_6-deficient + arachidonate
	mole %	*mole %*	*mole %*	*mole %*	*mole %*	*mole %*	*mole %*	*mole %*	*mole %*	*mole %*
16	13.4	23.0	11.7	9.6	12.2	25.5	31.2	20.7	20.3	23.2
16:1	2.3	7.3	4.8	3.0	2.3	4.3	14.4	5.7	3.3	2.4
18	22.8	19.9	21.2	20.5	28.3	20.1	16.6	13.7	19.8	18.6
18:1	19.5	25.0	28.4	23.1	17.9	21.1	27.0	41.0	26.5	22.5
18:2	8.7	2.4	2.7	11.5	2.7	3.7	0.6	1.6	7.0	2.0
20:3	0.5	7.6	18.7	15.3	6.4	2.0	3.8	9.2	5.7	1.2
20:4	23.6	7.6	8.1	9.4	23.9	15.9	1.3	2.9	5.5	21.6

[1] Only the major fatty acids are represented; the number before the colon denotes chain length, and the number after the colon, number of double bonds.

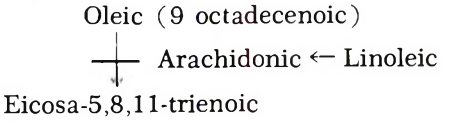
ulated corresponds with the actual content of the acids compared, since the percentage of lipids and the weight of the organs were very similar (for instance, the lipid content of the liver was 2.65% for the pyridoxine-deficient group given the DP supplement and 2.11% for the same group given the linoleate supplement. The comparatively low percentages of arachidonic acid attained in the presence of DP may then be attributed to the slight conversion of linoleate into arachidonic acid. This slight conversion might be due to DP or to the short term (one week) of the linoleate addition to the diet, which would not allow the reaching of higher levels of arachidonic acid. This second possibility may be only partially discarded, considering that the conversion of linoleate into arachidonic acid is rather fast in EFA-deficient rats receiving pyridoxine (27).

3) The addition of linoleate to the diet of EFA- and pyridoxine-deficient rats supplemented with DP produced a slight decrease of eicosatrienoic acid percentage in heart and liver (table 4, column 4 vs 3, and 9 vs 8) and a scarcely important increase of arachidonic acid, despite the high percentages of linoleic acid stored. However, when the addition of linoleate to EFA-deficient rats, whether given the pyridoxine supplement[5] or not (table 3 columns 5 and 11), produced high amounts of arachidonic acid, percentage of the eicosatrienoic acid in heart and liver decreased to levels equal or very approximate to those of the control animals. As eicosatrienoic acid is decreased only when high amounts of arachidonic acid are present, this effect can be attributed either to a particular property of arachidonic acid or to a competence between the conversions of linoleate into arachidonic acid, and oleic into eicosa-5,8,11-trienoic acid. Dhopeshwarkar and Mead (28) have proposed that oleic acid competes with linoleic acid as a substrate for the enzymes involved in arachidonic acid formation.

The addition of arachidonate to animals fed an EFA- and pyridoxine-deficient diet, supplemented with DP or not, provoked a

[5] Mercuri, O., and R. R. Brenner 1961 Efecto del ácido nonanoico en el metabolismo graso de ratas alimentadas con dieta carente en ácidos grasos esenciales. IV. Reuniao Associaçao Latino-Americana de Ciéncias Fisiológicas, Riberao Prêto, Brasil, p. 12.

remarkable decrease of eicosa-5,8,11-trienoic acid in heart and liver, together with an important storage of arachidonic acid, but no increase of linoleic acid (table 4, columns 5 and 10; table 3, columns 6 and 12). This result compared with the previous one demonstrates undoubtedly a specific effect of arachidonic acid in the regulation of eicosa-5,8,11-trienoic acid concentration in liver and heart. This regulation may occur directly via the conversion of oleic into eicosa-5,8,11-trienoic acid as shown graphically in the following diagram:



or indirectly, for instance, by displacement of eicosatrienoic acid from phospholipids. This second hypothesis is formulated since De Tomás et al. (29) demonstrated that eicosatrienoic, as well as arachidonic acid, esterify the β-position of phosphatidylethanolamine and phosphatidylcholine, and Collins (30) suggested greater stability for phospholipid molecules containing arachidonic acid.

The decrease of eicosatrienoic acid concentration, by direct addition of arachidonate to the diet, discards in this case, the competence of both synthesis: linoleate into arachidonate and oleate into eicosatrienoate as the possible regulating mechanism. Also linoleate did not appear to have much influence upon the conversion of oleic into eicosa-5,8,11-trienoic acid; for that purpose it would especially require a previous conversion into arachidonic acid.

The effect produced by acids of the linolenic family on the eicosatrienoic acid synthesis is now being studied.

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Some Relationships between Caloric Restriction and Body Weight in the Rat

IV. FECAL NITROGEN, FAT, ENERGY, AND ASH AND URINARY CREATININE[1]

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ABSTRACT The relationship between the caloric restriction adaptation and fecal excretion of nutrients was investigated in ad libitum and caloric-restricted rats. Both ad libitum controls and restricted animals excreted approximately 4 to 6% as much nitrogen in the feces as ingested in the diet, indicating similar efficiencies of absorption. The fecal fat was about 5% of the total fat intake in both groups, and the fecal excretion of I^{131} following intragastric administration of I^{131}-labeled triolein was also about 5% of that administered. Feces of both groups contained approximately 5% as much energy, measured by combustion in a bomb calorimeter, as was ingested in the diet. However, feces of restricted rats contained about 30% as much mineral ash as did the ingested diet, whereas, feces from rats fed ad libitum contained only about 20% of the ingested mineral. Urinary creatinine excretion by restricted animals was constant over the 44 days of caloric restriction but gradually increased in the ad libitum controls.

The gradual decrease in caloric requirements exhibited by rats fed sufficient food to just maintain body weight has been described by Quimby (1), Kaunitz et al. (2), and by Lee and Lucia (3). This adaptation is not accompanied by changes in gross body composition, by changes in the weights of heart, liver, or kidneys, or by changes in the lipid content of the liver. A gradual decrease in the utilization of C^{14}-labeled glucose by way of the hexose monophosphate pathway has been observed, but there is an increase in the activity of glucose-6-phosphate dehydrogenase in the liver (4). No persistent significant alterations in the concentrations of oxidized or reduced pyridine nucleotides (DPN, DPNH, TPN, and TPNH) or in the in vivo or in vitro incorporation of acetate-1-C^{14} into carcass and liver lipids has been demonstrated (5).

This caloric requirement adaptation may be the result of alterations in any one of a number of different mechanisms, such as fecal energy excretion, urinary energy excretion, total or volitional activity, and others. The studies reported here were limited to an investigation of one of these factors, fecal excretion. The fecal excretion of fat, nitrogen, energy, and ash by rats fed ad libitum and a caloric restricted diet was measured.

METHODS

Male rats, of the Long-Evans strain, were obtained from an established commercial dealer. The rats weighed either 150 to 160 g, or 190 to 210 g, depending on the experiment. All animals were housed in individual cages with wire bottoms and with facilities for the collection of feces and urine. The diet had the following composition: (g/100 g of diet) casein, 18; cottonseed oil, 8; salts (USP XIV), 4; powdered sucrose, 66.9; cellulose, 3; and choline, 0.1. The details of the diet composition, the mineral mixture, and vitamin supplements have been described elsewhere (3). Animals designated as controls were fed this diet ad libitum and had access to it at all times. Restricted animals were fed weighed quantities of food every second day. The weight of food was calculated to be just sufficient to maintain

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the body weight of the animal at either 160 or 200 g (depending on the particular experiment). All animals were offered water ad libitum and vitamins every fourth day. All animals were weighed every second day.

Fecal nitrogen excretion. Animals that had been fed the diet ad libitum or had been restricted for 4 weeks were used for these experiments. Restricted animals were maintained at 200 g. For a period of 6 days all food offered was weighed and the amount uneaten at the end of this time was determined. Loss through spilling was negligible. The weight of food eaten by each rat was calculated by difference. All feces were collected during the 6 days. The nitrogen content of the total feces of each rat was determined by the Kjeldahl procedure as was the nitrogen content of the diet.

Fecal fat excretion. The same animals were used for fat absorption studies the week following the protein absorption studies. The total amount of food eaten was determined as described above. All feces from each animal were collected for a period of 6 days. They were air-dried and weighed. Fat was extracted for 24 hours on a Soxhlet extractor, using purified diethyl ether. The fat-extracted feces were weighed again. Fat excretion was calculated by difference. The fat content of the diet was determined by the same procedure.

Fecal excretion of administered I^{131}-labeled triolein. Restricted animals were maintained at approximately 160 g. After the rats has been fed the diet for 3 or 4 weeks, each animal was given, by means of a stomach tube, an amount of I^{131}-labeled triolein[2] containing about 10^6 count/min. All feces were collected for the next 3 days. The radioactivity of the feces was determined using a Packard auto-gamma spectrometer. The portion of the fat excreted and the portion absorbed were calculated from the fecal radioactivity and the radioactivity of the I^{131} triolein.

Fecal energy excretion. The restricted animals were maintained at approximately 160 g. All feces and urine were collected during consecutive 4-day periods for 40 days. Feces were stored in a commercial freezer until the entire experiment was completed. The feces of all restricted animals for each 4-day period were pooled, as were those of all ad libitum-fed animals for each 4-day period. Each sample of feces was air-dried, weighed, and ground to a fine powder with a mortar and pestle. Pellets weighing approximately 1.5 g were prepared, using a hand press. Pellets were weighed to the nearest 0.1 mg and the caloric content of the pellet was determined by combustion in a Gallenkamp Adiabatic bomb calorimeter (model C.B. 140).[3]

Duplicate determinations were carried out. Duplicates differed by less than 0.020 kcal/g. The energy content was calculated as calories per gram and total calories for each 4-day fecal sample.

Another portion of each sample of pulverized feces was incinerated in a muffle furnace at 600°C for 20 hours. The ash content was determined from the difference in weight of the sample before and after incineration.

The creatinine excretion of each animal during each 4-day urine collection was determined by the method of Folin as described by Hawk et al. (6). Results are expressed in milligrams creatinine excreted per rat per day. All statistical analyses were carried out using the *t* test as described in Snedecor (7).

RESULTS

Fecal nitrogen and fat excretion by ad libitum-fed and restricted rats is shown in table 1. The ad libitum-fed rats ingested approximately 40% more nitrogen than did the restricted rats, but the apparent efficiency of absorption[4] was similar in the 2 groups — approximately 95%. Apparent fat absorption by rats fed ad libitum and those calorically restricted, when estimated by the same procedure, differed by less than 2% in the 2 groups and was greater than 95% in both groups.

The fecal excretion of I^{131} following the intragastric administration of I^{131}-labeled triolein is shown in table 2. There is considerable individual variability in I^{131} excretion in each group, as shown by the

[2] Trioleotope, E. R. Squibb and Sons, New York.
[3] A. Gallenkamp and Company, Ltd., London, England.
[4] $\frac{\text{N intake} - \text{N excretion}}{\text{N intake}} \times 100$.

TABLE 1

Fecal nitrogen excretion and fecal fat excretion by rats fed ad libitum and calorically restricted diets[1]

	Fed ad libitum (5)[2]	Calorically restricted (7)[2]
Feces/day,[3]g	0.70 ± 0.31[4]	0.47 ± 0.11
Nitrogen excreted/day, mg	17.1 ± 9.1	11.5 ± 5.0
Nitrogen ingested/day, mg	403	187
Dietary nitrogen excreted, %	4.24	6.15
Dietary nitrogen absorbed, %	95.76	93.85
Fat excreted/day, mg	50.6 ± 31.1	17.1 ± 9.1
Fat ingested/day, mg	1120	520
Dietary fat excreted, %	4.52	3.29
Dietary fat absorbed, %	95.48	96.71

[1] Initial average body weight of both groups was 200 g.
[2] Numbers in parentheses represent numbers of animals used.
[3] Fresh weight.
[4] Mean ± SD.

TABLE 2

Fecal excretion of I^{131} following the ingestion of I^{131}-labeled triolein by rats fed ad libitum and calorically restricted diets[1]

	Fed ad libitum	Calorically restricted
Experiment 1	(5)[2]	(5)[2]
Administered, count/min	10^6	10^6
Recovered, count/min	$4.5 \pm 1.45 \times 10^4$ [3]	$3.6 \pm 1.0 \times 10^4$
Excreted, %	4.5	3.6
Absorbed, %	95.5	96.4
Experiment 2	(4)[2]	(4)[2]
Administered, count/min	7.5×10^5	7.5×10^5
Recovered, count/min	$3.8 \pm 2.3 \times 10^4$	$3.8 \pm 0.7 \times 10^4$
Excreted, %	4.8	5.1
Absorbed, %	95.2	94.9

[1] Initial average body weight of both groups was 160 g.
[2] Numbers in parentheses indicate the number of animals used.
[3] Mean ± SD.

large standard deviations. However, in both experiments the efficiencies of apparent absorption were in agreement with those established by fecal fat analyses and were similar when ad libitum-fed and caloric-restricted rats were compared.

The average energy content of feces collected in 11 consecutive 4-day periods from ad libitum-fed control rats was 4.77 ± .15 kcal/g and from restricted rats was 4.36 ± .18 kcal/g. The difference is significant at a 1% level of confidence ($t = 5.7$). The total weights of feces excreted by animals in the 2 groups were markedly different, (620 ± 173 mg/rat/day for animals fed ad libitum and 371 ± 70 mg/rat/day for restricted animals). Figure 1 shows the total energy excretion per rat per day, during each 4-day period, for the 2 groups of animals. After the 4th collection period, the average energy excretion of ad libitum-fed rats was approximately 3.33 kcal/day and that of restricted rats, was approximately 1.50 kcal/day.

The diet contained 4.59 kcal/g and the restricted rats ate an average of 6.5 g/day, whereas the rats fed ad libitum ate an average of 14.0 g/day. Therefore, the restricted animals ingested approximately 29.85 kcal/day and those fed libitum ingested approximately 64.30 kcal/day. The efficiencies of absorption would therefore, be 95.0% for restricted rats and 94.8% for ad libitum-fed rats. Although the amounts of food ingested by the 2 groups of animals differed by a factor of 2.15, the percentage of ingested energy lost in the feces was virtually identical in the ad libitum-fed and restricted groups.

Figure 2a shows the ash content per gram of feces for each collection period.

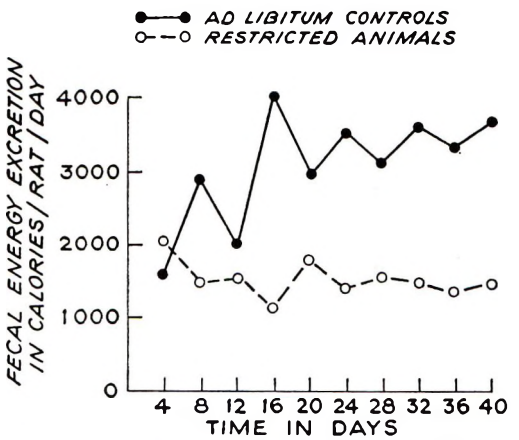

Fig. 1 Total energy excretion of rats fed ad libitum and of rats subjected to caloric restriction. Energy excretion is shown as small calories (1000 × kcal).

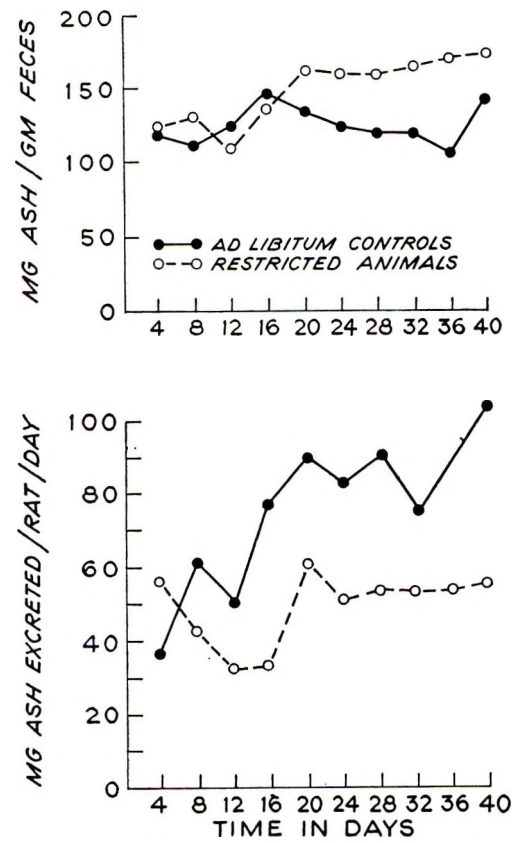

Fig. 2a,b Fecal ash content of rats fed ad libitum and of rats subjected to caloric restriction: 2a, milligrams of ash extract per gram of feces; 2b, total milligrams of ash excreted per 4-day collection period.

For the ad libitum-fed animals the fecal ash represents an average of 12.54% ± 1.06% of the weight of feces and for the restricted animals it is 14.58% ± 2.25%. The difference is significant at a 1% level of confidence ($t = 2.79$). There is a trend toward increasing ash content of feces from restricted rats, the longer the duration of caloric restriction. Figure 2b shows the total ash excreted per day per rat. The average daily ash excreted by restricted rats is 53.7 ± 10.6 mg, whereas that for ad libitum-fed rats is 78.7 ± 2.26 mg. The difference is significant at a 1% level of confidence ($t = 3.4$). In this case, total fecal ash excretion by restricted rats remained relatively constant during the 11 collection periods, whereas that of the ad libitum-fed animals increased markedly. The ash content of the diet is 2.77%. At 6.5 g and 14.0 g food/day for restricted and ad libitum-fed rats, respectively, the total ash ingested per day would be 180 mg for restricted and 388 mg for ad libitum animals. Therefore, only about 20% of the ingested mineral was excreted in the feces of ad libitum-fed animals, whereas 30% of the ingested minerals was excreted in the feces of restricted animals.

Figure 3 shows the excretion of creatinine in the urine of ad libitum and caloric-restricted rats. Creatinine excretion appeared to increase with time in the case of the rats fed ad libitum, while remaining relatively constant in restricted rats.

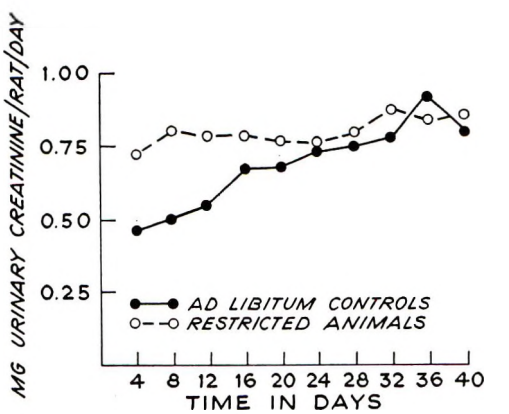

Fig. 3 Urinary creatinine excretion by rats fed ad libitum and by rats subjected to caloric restriction.

DISCUSSION

The data presented in this report indicate that the caloric requirement adaptation is not, to a significant extent, the result of alterations in fecal excretion or in apparent absorption (intake minus fecal excretion) of nutrients.

Since caloric restriction is accompanied by a decrease in caloric requirements of approximately 40%, the differences in the fecal loss of introgen and fat by ad libitum-fed rats as opposed to that by caloric-restricted rats is negligibly small. It has been shown by Carroll (8) and by Carroll and Richards (9) that a portion of the fecal fat, existing as soaps, is not ether extractable. Therefore, actual fecal fat excretion would be somewhat higher than our figures indicate. However, in the case of triolein this represents only a small part of the fecal fat and may be disregarded in this study. The agreement between fecal fat excretion and I^{131} excretion suggest that the data are a valid reflection of fat absorption.

The significantly lower energy content, per gram of feces, in feces from restricted rats, as compared with that from ad libitum-fed controls is also reflected in fecal fat excretion, where the total fat excreted by animals fed ad libitum was 3 times as great as that by restricted animals, but total fecal weight was only about 1.5 times as great. However, these differences in fecal energy per gram of feces can not be a contributing factor in the decreased caloric requirements, since the total energy excreted, as a percentage of the energy intake, is very similar. In fact, the apparent energy retention of 95% in the 2 groups of animals indicates a very high level of absorption, as the diet contains 3% cellulose, which is not metabolically available but which will contribute to fecal energy yields when measured colorimetrically.

The higher mineral excretion by restricted rats, as compared with ad libitum-fed rats may be a reflection of the lack of skeletal growth in the restricted animals. Lee and Lucia (3) showed that in restricted rats there is no significant alteration in gross body composition over a considerable period of time. The relative constancy of creatinine excretion by restricted rats and the gradual increase in creatinine excretion with time by ad libitum-fed rats agrees with the accepted views (10, 11) that creatinine excretion is a reflection of muscle mass.

As pointed out in the introduction, fecal excretion represents only one avenue of energy balance. It is possible that differences in urinary energy excretion exist which are of sufficient magnitude to account for the decreased caloric requirements of restricted animals. It may also be possible that restricted animals expend less energy in total or volitional activity than do ad libitum-fed animals. In this respect, Miller and Payne (12) have reported that they were unable to demonstrate differences in activity of rats or pigs receiving different levels of caloric intake.

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Influence of Dietary Menhaden Oil on Growth Rate and Tissue Fatty Acids of the Chick[1,2]

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ABSTRACT Using purified diets containing 2 sources of protein, an essential fatty acid deficiency was produced in chicks that was characterized by poor growth and a high quantity of eicosatrienoic acid in the liver lipid. The feeding of corn oil caused an increase in the eicosatetraenoic acid and a decrease in the eicosatrienoic acid content of the liver. The feeding of menhaden oil did not change the eicosatetraenoic acid content of the liver lipid, but caused a complete disappearance of eicosatrienoic acid. Eicosapentaenoic acid, docosapentaenoic acid and docosahexaenoic acid were present in the liver only when menhaden oil was fed. The data indicate that the feeding of fish oil with its high content of long-chain polyunsaturated fatty acids inhibits the synthesis of eicosatrienoic acid in the chicken. When menhaden oil was fed with corn oil, the level of arachidonic acid present in the liver and heart fat was decreased from levels resulting when corn oil is fed alone. Some interpretations of these observations are discussed.

Marine oils contain large amounts of long-chain polyunsaturated fatty acids (PUFA). These oils stimulate growth in rats deficient in essential fatty acid (EFA), but do not cure the dermal symptoms resulting from an EFA deficiency (1, review). Purified fractions from tuna oil containing 18, 20 or 22 carbon atoms in length have been shown by Privett et al. (2) to stimulate growth in the EFA-deficient rat but not to relieve the dermal symptoms associated with the deficiency.

The addition of safflower oil or methyl linoleate to diets free from unsaturated fatty acids has been shown by Machlin and Gordon (3) to produce a growth response in chicks. Addition of methyl linolenate failed to cause a growth response under these same conditions. Edwards et al. (4) have shown that menhaden oil is effective in overcoming the growth-depressing effect that results when chickens are fed EFA-deficient diets. Since only small quantities of linoleate are present in menhaden oil, a study was carried out in an attempt to obtain some biochemical explanation for the growth response to this oil.

EXPERIMENTAL

The chicks used were White Plymouth Rock cockerels obtained from a local hatchery. They were housed in electrically heated battery brooders with wire floors and were supplied feed and water ad libitum from one day of age until killed for analytical purposes or until the experiments were terminated. The composition of the basal diets used in this study is shown in table 1. Diet A contains by analysis 0.27% linoleic acid, and diet B contains 0.01% linoleic acid. Saponifiable lipids of the diet were obtained by KOH hydrolysis and analyzed for fatty acids with gas-liquid chromatography.[4] No measurable amounts of linolenic or any 20 or 22 carbon acids were present in either of the basal diets. The lipids to be tested were added to the diets with adjustments being made in the amount of glucose monohydrate, and cellulose to maintain equal metabolizable energy levels.

The fatty acid composition of the corn oil and menhaden oil used in the experiments as determined by gas-liquid chromatography are as follows: (expressed as a percentage of the total fatty acids) Corn

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[4] The gas liquid chromatography methods as well as the method of calculating results are given in: Edwards, H. M., Jr., and J. E. Marion 1963 A simple method of calibrating a GLC column for quantitative fatty acid analysis. J. American Oil Chemists' Society, 55: 299 (Letter to the Editor).

TABLE 1

Composition of basal rations

	Diet	
	A	B
	g	*g*
Glucose monohydrate	65.64	65.30
Isolated soybean protein[1]	26.66	—
Casein	—	18.44
Gelatin	—	9.22
DL-Methionine	0.67	0.34
Glycine	0.33	—
Cellulose[2]	1.00	1.00
Choline Cl concentrate (70.7%)	0.29	0.29
NaCl	0.75	0.75
KCl	0.60	0.60
$MgCO_3$	0.19	0.19
$CaHPO_4$	2.86	2.86
$CaCO_3$	0.54	0.54
1,2 Dihydro-6-ethoxy-2,2,4-trimethyl quinoline	0.03	0.03
Vitamin mixture[3]	0.22	0.22
Mineral mixture[4]	0.22	0.22

[1] Archer-Daniels-Midland Company, Minneapolis.
[2] Solka Floc, Brown Company, Berlin, New Hampshire.
[3] The vitamin mixture supplied the following expressed as milligrams/100 g of diet: vitamin A (384,000 USP units/g), 204; vitamin E (296 IU/g), 22.3; vitamin D_3 (950,000 USP units/g), 0.06; thiamine·HCl, 1.98; riboflavin, 1.98; Ca pantothenate, 3.30; pyridoxine·HCl, 0.99; niacin, 3.96; folic acid, 0.66; biotin, 0.07; vitamin B_{12} (1 mg/g), 3.30; *p*-aminobenzoic acid, 16.5; inositol, 165.0; menadione sodium bisulfite complex, 0.53.
[4] The mineral mixture supplied the following expressed as milligrams/100 g of diet: $FeSO_4 \cdot 7H_2O$, 11.0; $CuSO_4 \cdot 5H_2O$, 1.1; $CoCl_2 \cdot 6H_2O$, 1.1; KI, 1.1; $Na_2MoO_4 \cdot 2H_2O$, 0.11; $ZnSO_4 \cdot 7H_2O$, 16.5; $MnSO_4 \cdot H_2O$, 17.0.

oil, 16:0,[5] 10.7; 18:0, 2.0; 18:1, 29.1; 18:2, 57.2; and 18:3, 1.0. Menhaden oil, 14:0, 11.8; 14:1, 0.4; 16:0, 23.4; 16:1, 15.7; 18:0, 4.8; 18:1, 15.8; 18:2, 1.5; 18:3, 2.4; 18:4, 1.6; 20:3, 0.7; 20:4, 0.8; 20:5, 9.9; 22:5, 1.3; and 22:6, 9.7. Tentative identification of fatty acids in all analyses is based on retention time and hydrogenation to an acid with a retention time similar to the saturated acid of that carbon series. In the menhaden oil analyses some of the material listed as 18:0, 18:1, 18:2 or 18:3 is undoubtedly 16:2, 16:3 and 16:4. This must be true since more 16:0 and less 18:0 was recovered from menhaden oils when they were completely hydrogenated than would be expected from summing the individual analytical values for fatty acids of these carbon series from an unhydrogenated sample. This conclusion is reasonable in view of the report of Stoffel and Ahrens (5) indicating that menhaden body oil contains 2.0% of 16:4, 1.3% of 16:3 and 2.0% of 16:2.

Livers and, in one experiment, hearts from chicks from each dietary treatment were analyzed for their fatty acid composition. They were frozen fresh after being excised from the animal. Approximately 4.0 g of the tissue was heated in a water bath (60°C) with 16 ml of 25% KOH under a stream of nitrogen for 16 hours. This digest was cooled, extracted with diethyl ether 3 times and made acid with HCl. The fatty acids were then extracted 3 times with 30 to 60°C bp petroleum ether. This petroleum ether extract was washed 3 times with cold distilled water and dried over anhydrous sodium sulfate. The fatty acids were then brought to dryness in a 60°C water bath under a stream of nitrogen and refluxed for 3 hours with 10 ml of 5% HCl in superdry methanol. The resulting methyl esters were separated from this solution into 30–60°C bp petroleum ether, cleaned by washing with cold distilled water and dried over sodium sulfate. The petroleum ether was evaporated from the methyl esters in a 60°C water bath under a stream of nitrogen and the methyl esters were analyzed by gas-liquid chromatography.

Growth and fatty acid analysis data were subjected to a statistical analysis, and treatment significance was determined by the multiple range test of Duncan (6) as modified by Kramer (7).

RESULTS

A chick growth response was obtained by adding corn oil and menhaden oil to the soybean-protein and the casein-gelatin diets in experiment 1 (table 2). A significant difference also occurred in the growth rate of the chicks fed the 2 basal rations.

The results of the fatty acid analysis of the liver lipids from the chicks in experiment 1 are presented in table 3. In general, the level of palmitic and palmitoleic acid reflects the fatty acid content of the fat supplements fed. However, the birds fed the casein-gelatin basal diet showed an increase in palmitoleic acid over those receiving the isolated soybean-protein diet. The stearic acid content of the liver fat increased when either corn oil or menhaden oil was fed. When the soybean-

[5] Abbreviations for fatty acids, first number indicates number of carbons and the second the number of double bonds.

TABLE 2

Effect of supplementation of the diet with fats on growth rate (experiment 1)

Supplement to diet	Average wt at 4 weeks					
	Isolated soybean protein			Casein-gelatin		
	g			g		
None	318[1]	(3.48)[2]		385	(4.28)	
4% Corn oil	548	(3.00)		470	(3.09)	
4% Menhaden oil	548	(2.99)		432	(3.53)	
Grouping of treatment results: [3]						
Growth	318	385	432	470	548	548
Liver weight	2.99	3.00	3.09	3.48	3.53	4.28

[1] Average of 3 groups of 10 chicks each.
[2] Liver weight expressed as a percentage of body weight.
[3] Two values not underscored by the same line are significantly different ($P < 0.05$).

TABLE 3

Fatty acid composition of liver lipid (experiment 1)

Fatty acid[1]	Isolated soybean protein diet			Casein-gelatin diet		
	Control	4% Corn oil	4% Menhaden oil	Control	4% Corn oil	4% Menhaden oil
	%	%	%	%	%	%
14:0	0.1	0.1	0.7	0.5	0.2	0.5
16:0	23.9	23.9	28.3	26.4	25.2	27.1
16:1	8.2	5.1	7.9	11.5	7.8	9.5
18:0	17.7	21.0	22.4	13.3	19.2	22.4
18:1	37.8	22.6	21.6	43.5	22.6	27.6
18:2	7.8	18.3	5.6	1.9	15.1	1.8
20:3	2.9	0.7	0.0	2.7	0.7	0.0
20:4	1.6	8.2	1.0	0.2	9.1	1.3
20:5	0.0	0.0	4.6	0.0	0.0	4.0
22:5	0.0	0.0	0.6	0.0	0.0	0.3
22:6	0.0	0.0	6.7	0.0	0.0	5.4
Grouping of treatment results when significance was found: [2]						
16:1	5.1	7.8	7.9	8.2	9.5	11.5
18:0	13.3	17.7	19.2	21.0	22.4	22.5
18:1	21.6	22.6	22.6	27.6	37.6	43.5
18:2	1.8	1.9	5.6	7.8	15.1	18.4
20:3	0.0	0.0	0.7	0.7	2.7	2.9
20:4	0.2	1.0	1.3	1.6	8.2	9.1

[1] See footnote 4 of text.
[2] See footnote 3, table 2.

protein control diet was fed, rather high levels of linoleic acid were present in the liver lipid, whereas the level of arachidonic acid was low. The addition of corn oil increased the linoleic acid content twofold and more than quadrupled the arachidonic acid content. When menhaden oil was fed, the level of both linoleic acid and arachidonic acid was lower than that observed with chicks fed the basal diets. When the casein-gelatin control diet was fed, the liver lipids contained very low

levels of linoleic acid and arachidonic acid, whereas the addition of corn oil to the diet increased the levels of these fatty acids to approximately the same levels observed with the soybean-protein diet when corn oil was fed. The addition of menhaden oil to the casein-gelatin diet resulted in no change in linoleic acid and little change in the arachidonic acid when compared with the chicks receiving the basal diet.

The quantity of eicosatrienoic acid present in the liver lipids of chicks receiving both basal diets was high. The addition of corn oil to both basal diets lowered the level of eicosatrienoic acid in the liver, whereas the addition of menhaden oil to the diet reduced the liver lipid eicosatrienoic acid content to zero. When menhaden oil was added to the diet, significant amounts of eicosapentaenoic acid, docosapentaenoic acid and docosahexaenoic acid were present in the liver lipids.

Experiment 2 was conducted to determine the age at which significant amounts of eicosatrienoic acid would appear in the liver of chicks fed the casein-gelatin diet, and to determine what level of menhaden oil was necessary to cause a disappearance of eicosatrienoic acid. The results presented in table 4 indicate that appreciable amounts of eicosatrienoic acid begin to appear in the liver lipids at 3 weeks and that even more is present in the liver at 4 weeks. A supplement of one per cent menhaden oil was not sufficient to reduce eicosatrienoic acid content of the liver lipid to zero; however, it definitely caused a decrease in the liver lipids at 4 weeks. Four per cent of menhaden oil caused the eicosatrienoic acid level to be zero at both 3 and 4 weeks. However, a small amount of eicosatrienoic acid was detected in the liver of chicks receiving 4% menhaden oil at 2 weeks of age.

The third experiment was conducted to determine whether feeding menhaden oil would also cause a decrease in the quantity of arachidonic acid in the liver and heart lipids when a source of linoleic acid such as corn oil was present in the casein-gelatin diet. The results of this study are shown in tables 5 and 6.

In this particular experiment a significant growth response was not obtained from menhaden oil and the addition of menhaden oil along with the corn oil caused a decrease in growth rate as compared to corn oil alone.

The addition of corn oil at the 3% and the 6% level, and menhaden oil at the 4% level caused changes in the fatty acid composition of the liver similar to those observed in experiment 1. When 4% of menhaden oil was added to the diet in combination with corn oil, the eicosatrienoic acid content of the liver as well as the arachidonic acid content of the liver was lowered below that obtained when corn oil alone was added, while at the same time almost no change took place in

TABLE 4

Effect of level of supplemental menhaden oil on liver fatty acid composition over a 4-week feeding period (experiment 2)

Fatty acid[1,2]	Diet	Control				Plus 1% menhaden oil				Plus 4% menhaden oil			
	Weeks	1	2	3	4	1	2	3	4	1	2	3	4
		%	%	%	%	%	%	%	%	%	%	%	%
14:0		1.4	0.6	0.5	0.3	1.0	0.2	0.2	0.3	0.8	0.4	0.4	0.6
16:0		22.9	28.4	28.4	20.4	37.1	30.5	27.4	29.9	36.4	32.1	29.8	30.4
16:1		12.0	9.5	11.8	9.2	9.4	14.8	10.8	7.9	11.8	7.5	9.3	5.0
18:0		12.6	12.0	11.7	14.8	13.5	15.1	19.3	18.0	16.7	23.1	20.5	24.4
18:1		49.1	47.2	43.4	44.6	36.5	34.9	32.3	32.3	32.5	26.4	27.8	21.6
18:2		1.9	2.1	2.3	2.2	1.6	3.1	1.6	1.1	1.5	1.9	1.1	1.0
20:1		0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
20:2		0.0	0.0	0.0	0.7	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0
20:3		0.0	0.0	1.8	6.2	0.0	0.0	0.5	1.1	0.0	0.7	0.0	0.0
20:4		0.0	0.0	0.0	0.6	0.3	0.0	0.2	0.9	0.0	0.6	1.9	2.0
20:5		0.0	0.0	0.0	0.1	0.4	1.8	2.2	3.0	0.1	5.8	4.7	5.0
22:5		0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.2	0.0	0.4	0.7	2.3
22:6		0.0	0.0	0.0	0.0	0.3	1.1	4.7	4.2	0.0	1.0	3.6	7.3

[1] See footnote 4 of text.
[2] The first figure represents the number of carbon atoms, the second the number of double bonds.

TABLE 5

Effect of feeding menhaden oil and corn oil, alone and in combination, on growth and fatty acid composition of liver fat (experiment 3)

	Basal	Plus 3% corn oil	Plus 6% corn oil	Plus 3% corn oil + 4% menhaden oil	Plus 6% corn oil + 4% menhaden oil	Plus 4% menhaden oil
	g	g	g	g	g	g
Avg wt, 4 weeks	347[1]	475	452	428	411	371
Fatty acid[2,3]	%	%	%	%	%	%
	Percentage by weight of total fatty acids in liver					
14:0	0.4	0.2	0.1	0.2	0.1	0.8
16:0	23.2	22.8	18.8	24.9	20.6	27.9
16:1	11.4	5.8	5.5	6.0	4.2	6.5
18:0	11.6	20.6	21.6	20.2	24.6	19.5
18:1	45.5	28.1	21.1	22.8	13.7	27.3
18:2	4.9	14.4	19.6	12.4	19.4	2.3
20:3	2.6	0.6	0.4	0.1	0.1	0.0
20:4	0.2	7.7	12.9	1.8	2.4	0.9
20:5	0.0	0.0	0.0	6.5	6.3	8.1
22:5	0.0	0.0	0.0	0.8	1.4	1.9
22:6	0.0	0.0	0.0	4.3	7.9	4.8
Grouping of significant treatment results: [4]						
Growth	347	371	411	428	452	475
16:0	18.8	20.6	22.8	23.2	25.0	27.9
16:1	4.2	5.5	5.8	6.0	6.5	11.4
18:0	11.6	19.5	20.2	20.6	21.6	24.6
18:1	13.7	21.1	22.8	27.3	28.1	45.5
18:2	2.3	4.9	12.4	14.4	19.4	19.6
20:3	0.0	0.1	0.1	0.4	0.6	2.6
20:4	0.2	0.9	1.8	2.4	7.7	12.9

[1] Average of 3 groups of 10 chicks each.
[2] See footnote 4 of text.
[3] The first figure represents the number of carbon atoms, the second the number of double bonds.
[4] Two values not underscored by the same line are significantly different ($P < 0.01$).

the quantity of linoleic acid present in the liver lipid. Similar results were obtained when heart lipids were analyzed. However, the level of eicosatrienoic acid of animals receiving the basal diet was not as great as that present in the liver. The level of several other fatty acids was different from those observed in the liver; however, the effect of the fat supplements was essentially the same.

DISCUSSION

The quantity of eicosatrienoic acid present in the liver lipid of chicks receiving both basal diets was high, indicating essential fatty acid deficiency (8) and abnormal metabolism of oleic acid to eicosatrienoic acid (9). The observations indicate that the presence of the long-chain PUFA of menhaden oil inhibits the synthesis of eicosatrienoic acid from oleic acid. Since the same effect was observed in both liver and heart fat, it appears that although transport of lipid may be effected by feeding oils such as menhaden oil, the decrease in eicosatrienoic acid cannot be explained on this basis. This interpretation is in line with observations by Bozian and Coniglio[6] that oral supplementation of fat-

[6] Bozian, R. C., and J. G. Coniglio 1962 The effect of ethyl arachidonate upon the metabolism of oleic acid in the fat deficient rat. Federation Proc., 21: 286 (abstract). Bozian, R. C., and J. G. Coniglio 1963 The effect of ethyl arachidonate upon eicosatrienaote levels in livers of fat deficient rats. Federation Proc., 22: 489 (abstract).

TABLE 6

Effect of feeding menhaden oil and corn oil, alone and in combination, on fatty acid composition of heart fat

Fatty acid[1,2]	Basal	Plus 3% corn oil	Plus 6% corn oil	Plus 3% corn oil + 4% menhaden oil	Plus 6% corn oil + 4% menhaden oil	Plus 4% menhaden oil
	%	%	%	%	%	%
	Percentage by weight of total fatty acids in heart					
12:0	0.0	0.2	0.2	0.1	0.2	0.6
14:0	0.5	0.3	0.4	1.1	0.7	1.3
14:1	0.0	0.5	0.3	0.8	0.9	0.2
16:0	20.9	22.6	21.7	25.0	20.7	29.2
16:1	10.0	5.1	3.4	4.7	2.7	9.0
18:0	6.4	17.0	16.5	17.2	20.0	16.6
18:1	49.4	26.6	20.8	23.1	19.1	33.9
18:2	11.3	18.0	23.0	19.4	25.3	3.8
20:3	1.3	0.3	0.1	0.0	0.0	0.0
20:4	0.2	9.4	13.6	3.1	5.4	1.5
20:5	0.0	0.0	0.0	4.9	4.4	3.9
22:5	0.0	0.0	0.0	0.5	0.7	0.0
22:6	0.0	0.0	0.0	0.2	0.0	0.0
Grouping of significant treatment results: [3]						
16:0	20.7	20.9	21.7	22.6	25.0	29.2
16:1	2.7	3.4	4.7	5.1	9.0	10.0
18:0	6.4	16.5	16.6	17.0	17.2	20.0
18:1	19.1	20.8	23.1	26.6	33.9	49.4
18:2	3.8	11.3	18.0	19.4	23.0	25.3
20:3	0.0	0.0	0.0	0.1	0.3	1.3
20:4	0.2	1.5	3.1	5.4	9.4	13.6

[1] See footnote 4 of text.
[2] The first figure represents the number of carbon atoms, the second the number of double bonds.
[3] See footnote 4, table 5.

deficient rats with ethyl arachidonate resulted in a decrease of eicosatrienoic acid levels of organ fatty acids. These workers interpreted their results as suggesting that arachidonate may function as a regulator of conversion of oleic acid to eicosatrienoic acid. The data suggest that menhaden oil also inhibits the conversion of linoleic acid to arachidonic acid. This would indicate that the presence of the long-chain PUFA's of menhaden oil may be inhibiting all conversions of intermediate chain length PUFA (such as 18:1, 18:2 and 18:3) to long-chain PUFA's (such as 20:3, 20:4, 20:5 and 22:6). This in turn indicates that the enzymes for the various conversions are not specific for a particular reaction and, therefore, the presence of a large amount of the product will inhibit a reaction common to all conversions. The conversion of a monoene to a diene may be a very slow reaction in the body of the chick since when corn oil is fed, linoleic acid is converted to arachidonic acid, in preference to oleic acid to eicosatrienoic acid or palmitoleic acid to eicosatrienoic acid.

A comparison of the results obtained with chicks to those obtained with rats is pertinent. Bieri et al. (10) reported only a slight decrease in weight gain of chicks reared to 8 weeks with a fat-free ration. However, in a subsequent report Bieri et al. (11) reported that the blood, heart and liver of chicks reared to 8 weeks with the fat-free diet showed a decrease in dienoic

acid content and an increase in trienoic acid content. Machlin and Gordon (3) reported that the addition of safflower oil or methyl linoleate to diets free from unsaturated fatty acids produced a growth response and a decrease in the quantity of 20:3 in the liver. The increase in 20:3 content of tissue of the EFA rat is a criteria of long standing (8), and it has recently been given more stature by Holman (12) by the proposal that triene-to-tetraene ratios of fat from rats be used as a measure of essential fatty acid adequacy of the diet that the rat has been receiving.

The observations reported here indicate that the decreased growth rate and the increased 20:3 content of tissues of chicks fed low fat diets may be comparable with these abnormalities in the EFA-deficient rat, but are not comparable with the dermal symptoms noted in EFA-deficient rats.

The inability of menhaden oil to promote a significant increase in growth rate in experiment 3 of the present study is worthy of comment. Supplementation of either isolated soybean-protein diet or the casein-gelatin diet with menhaden oil has given significant increases in growth response in experiment 1 of the present study, in previous studies from this laboratory (4) and, in recent studies[7] where it was fed alone and in combination with corn oil. The reason for the lack of growth response in experiment 2 from menhaden oil alone and the growth depressing effect when it was fed with corn oil is unknown. A point for consideration is that in this particular experiment (experiment 3) the level of tissue arachidonic acid reached an extremely low level and the menhaden oil fatty acids inhibited further synthesis of arachidonic acid from linoleic acid when corn oil was fed, thereby acting somewhat as an anti-metabolite in this body system. If this should be the case, the level of linoleic acid and arachidonic acid in the one-day-old chick would determine the type of response one could expect from feeding menhaden oil or possibly some other source of long-chain polyunsaturated fatty acids that might inhibit the conversion of linoleic acid to arachidonic acid.

Since only 20:4 and its precursors (18:2, linoleyl alcohol, etc.) cure the dermal symptoms in the rat, it has been concluded by many workers that 20:4 is probably the only essential long-chain polyunsaturated fatty acid. Since a number of long-chain PUFA's may satisfy the requirement of the rat (1, 2) and the chick, experiment 1 and (4), for growth, other interpretations are possible. It is possible that when the diet is devoid of all the PUFA's, the chick may synthesize 20:3 from 16:1 and 18:1, and this fatty acid may be able to substitute for 20:4, 20:5, 22:5, or 22:6 in *some* body processes necessary for rapid growth. That is to say that other than the specific requirement for 18:2 as a precursor of 20:4 for curing dermal lesions in rats, some other fatty acid(s) must be supplied or synthesized by the body in sufficient quantity that can be converted by the body into a long-chain PUFA with normal growth rate resulting.

The difference in growth rate with the 2 basal rations used in experiment 1 is significant and of particular interest since the birds fed the casein-gelatin basal diet containing the least linoleic acid grew the fastest. Undoubtedly, the data presented in this paper, as well as all work on EFA deficiency, is complicated by the fact that the "associative dynamic effect" described by Forbes and Swift (13) is probably operating maximally even though the diets are balanced for metabolizable energy. This means that large amounts of carbohydrate and protein are being converted to fat when the animal is fed the basal EFA-deficient ration. When fat is added, the synthesis of lipid from carbohydrate and protein is greatly inhibited. The carbon-14 studies of metabolism of acetate, glucose and leucine in chicks fed various diets, reported by Marion and Edwards (14), substantiate this concept. Also, other work from this laboratory has shown that the protein level of the diet may have a pronounced influence on fat metabolism in chickens (4, 15–17).

No specific information is available concerning the way in which individual amino acids are catabolized when a fat-free diet is fed. They could be catabolized either proportionately or disproportionately to the amount in the protein. If catabolism of the amino acid is disproportionate to the

[7] Unpublished data, H. M. Edwards, Jr., 1963.

protein, then catabolizing 50% of a particular amino acid, which is already at a critical level in the diet, while most of the other amino acids were being catabolized only to the extent of 30% would lead to a drastic deficiency or inbalance in a diet. Conversely, if the amino acids are catabolized in proportion to the amount present in the protein, then the diet that had an amino acid (or group of amino acids) closest to the critical level for the chick would produce the poorest growth response.

The effect that fat and also specific fatty acids in the diet may have on amino acid metabolism must be given more consideration in future studies on essential fatty acid metabolism. The previous discussion indicates that the amount and kind of fat in the diet may be of great importance in amino acid studies.

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Effects of Several Low Protein Diets on the Serum Iron, Total Iron-binding Capacity, and Serum Cholesterol of Rats[1]

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ABSTRACT Hemoglobin formation and the iron transport system as affected by feeding diets low in protein were investigated. Male rats, 8 weeks of age, were fed for 6 weeks at an elevation of 1.46 km (4800 feet), diets containing 3.5% protein from either 1) beef, 2) ground whole wheat or 3) ground whole wheat with added lysine. Other rats were fed a chow diet. Adequacy of these diets was measured at the end of the feeding period by the following determinations: hematocrit, hemoglobin, serum iron, total iron-binding capacity of the serum, and serum total cholesterol. An impairment in the iron-transport system and hemopoietic processes was indicated when rats were fed the diets with 3.5% protein from whole ground wheat, with or without added lysine, but not when they were fed a similar diet with 3.5% protein from beef. Rats fed the 3.5% beef- or wheat-protein diets had similar serum cholesterol values. Hematocrit and hemoglobin values and total iron-binding capacity of the serum of the chow-fed rats were somewhat higher than those reported in the literature as standard values for rats, suggesting an increase due to altitude.

Although it has been known for sometime that serum contains non-hemoglobin iron, practical methods for its determination were slow in development. During the last few years a number of studies have been reported on the serum iron content and iron-binding capacity of rat, as well as human serum or plasma. Basic studies concerning the iron content and the iron-binding capacity of the plasma of rats with normal nutrition have been reported by Itzhaki (1) and Itzhaki and Belcher (2). Beutler (3) and Beutler and Blaisdell (4, 5) investigated not only the iron content and iron-binding capacity of the plasma but studied the relationship of these factors to the iron enzyme systems. Differences in the iron metabolism system with the age of the subject have been reported by Rechenberger and Hevelke (6). Hamilton et al. (7) studied the problem of diurnal variation in plasma iron in man and reported that iron was highest during the early morning and decreased during the day, reaching its lowest level during the evening. Differences in hemoglobin and serum iron in humans, due to sex, were reported by Verloop et al. (8).

Only a few studies have been reported concerning the effect of dietary variations on the iron transport system. Takeda and Hara (9), based on their work with guinea pigs, stated the opinion that the primary function of ascorbic acid is in the mobilization of ferrous iron. Likewise, Greenberg and Rinehart (10), working with monkeys in a state of chronic ascorbic acid deficiency, observed that combined therapy of ascorbic acid and iron markedly increased the serum iron and hemoglobin levels over that obtained with the administration of only the iron. Vitamin E along with ascorbic acid is also effective for hemoglobin regeneration in milk-fed anemic rats, as reported by Greenberg et al. (11).

The present study was made to determine the effects on the iron transport system and hemoglobin formation of feeding diets in which beef and wheat furnished similar amounts of protein. The protein of wheat is known to be low in lysine; hence, this amino acid was added to one of the wheat-containing diets. Serum cholesterol levels were also determined because of the frequency with which this laboratory has observed lowered

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hemoglobin in conjunction with elevated cholesterol values. Known influencing factors such as age, sex, ascorbic acid, vitamin E, dietary iron, and time of sampling were either controlled or at an optimal level. A low level of 3.5% protein was used to exaggerate possible slight differences between the proteins which might not be evident at a higher level. This low level of protein was similar to a level used in the study on hemopoiesis by Orten and Orten (12) in which the various test proteins were fed at levels of 2.8 and 18% of the diet for a period of 120 days. The 3.5% level of protein was shown to be capable of supporting life and permitting growth in tests made in the authors' laboratory.[2]

METHODS

Male rats of the Holtzman strain,[3] 8 weeks of age, with an average weight of 169 g, were housed individually in screen-bottom cages and maintained in a temperature-controlled room of approximately 24°C at an elevation of 1.46 km (4800 feet) for 6 weeks. Groups 1, 2, and 3, composed of 10 rats each, were fed, ad libitum, diets containing 3.5% protein from beef, 3.5% protein from ground, whole wheat, and 3.5% protein from ground whole wheat with added lysine, respectively. Composition of these 3 experimental diets is shown in table 1. Protein percentages were determined on the dried samples by Kjeldahl analyses for total nitrogen using 6.25 as the conversion factor. The 3 experimental diets contained the same amount of total nitrogen. Group 4, composed of 8 rats, was fed a laboratory chow,[4] ad libitum. Weekly food consumption and rat weights were recorded.

Biochemical measurements. Hemoglobin and hematocrit determinations, using tail blood, were made on the morning preceding the day of killing. Hemoglobin was measured by the cyanmethemoglobin method using a hemoglobin standard.[5] Readings were made using a Beckman B spectrophotometer. Standard, heparanized capillary tubes were used for the microhematocrits. After centrifuging, these were read in a standard microcapillary reader. Prior to withdrawing blood by syringe from the anterior vena cava, rats were anesthetized with an intraperitoneal injection of pentobarbital sodium[6] (0.5 ml of a 4 mg/100 ml solution of pentobarbital sodium in water, per 100 g of rat). These blood withdrawals were made in the morning or early afternoon and serum was prepared and frozen at −23°C until analyzed.

Determinations of serum iron and total iron-binding capacity of the serum were made following Mandel's (13) modification of the method of Peters et al. (14). The percentage of iron saturation was calculated from the amount of serum iron

TABLE 1

Composition of diets

	Diets		
	1 Beef	2 Wheat	3 Wheat with lysine
	%	%	%
Sucrose	10	10	10
Fat[1]	15	15	15
Vitamin mixture[2]	2.2	2.2	2.2
Salt mixture[3]	4	4	4
Dextrin	64.8	47.4	47.14
Beef, dried, ground, fat-extracted[4]	4	—	—
Wheat, ground, whole, hard red winter[5]	—	21.4	21.4
L-Lysine hydrochloride[6]	—	—	0.26

[1] Crisco, Procter and Gamble, Cincinnati.
[2] Vitamin Diet Fortification Mixture, Nutritional Biochemicals Corporation, Cleveland.
[3] Salt mixture, USP XIV, Nutritional Biochemicals Corporation, Cleveland.
[4] Dried beef, 87.6% protein, as determined by Kjeldahl analysis (N × 6.25), to furnish 3.5% protein in the diet.
[5] Ground, whole wheat, 16.3% protein as determined by Kjeldahl analysis (N × 6.25), to furnish 3.5% protein in the diet.
[6] L-Lysine hydrochloride added so that the lysine content of diet 3 equals the 0.30% lysine in the beef-protein diet 1.

[2] Unpublished data; seven out of 9 weanling rats fed a 3.5% beef-protein diet lived for 622 days, at which time the test was terminated. During this time these rats made a slow but steady average gain of 1.85 g/week with a total gain of 165 g. The 2 deaths were due to causes unrelated to the diet.
[3] Obtained from Holtzman Rat Company, Madison, Wisconsin.
[4] Purina Laboratory Chow, Ralston Purina Company, St. Louis; approximate amino acid composition of this diet: (in per cent) lysine, 1.27; tryptophan, 0.22; phenylalanine, 0.82; methionine, 0.36; threonine, 0.78; leucine, 1.40; isoleucine, 1.03; valine, 1.02; histidine, 0.44; arginine, 1.15.
[5] Hemoglobin standard, Acuglobin, Ortho Pharmaceutical Corporation, Raritan, New Jersey.
[6] Nembutal, Abbott Laboratories, North Chicago, Illinois.

found in the serum in relationship to its total iron-binding capacity (TIBC). The transferrin present in the serum was calculated on the basis that the transferrin molecule contains 2 atoms of Fe and its accepted molecular weight of 90,000 according to the method of Mandel (13).

Total cholesterol was measured in the serum samples by the method of Abell et al. (15).

Statistical treatment. Resulting data were subjected to the *t* test to determine any significant differences due to dietary variations.

RESULTS AND DISCUSSION

Information on rat weights, food intake and composition of diets. Average weekly food intake and rat weights for each of the 4 groups of rats are shown in figure 1. Rats in groups 1, 2, and 3, fed diets containing 3.5% protein lost from 13 to 23 g during the first week they were fed the diet, but remained almost constant in weight (groups 2 and 3) or gained slightly (group 1) during the next 5 weeks. Although rats in group 1 consumed more food than those in group 2, and those in group 2 more than those in group 3, the weekly consumption of food within each group varied little. Rats fed the chow diet, group 4, consumed the largest amount of food.

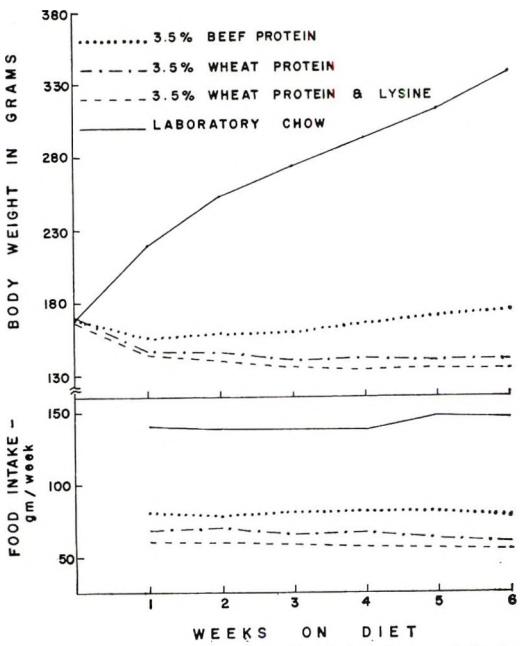

Fig. 1 Average weekly food intake and body weight of rats fed the experimental diets for 6 weeks.

The iron content of the 4 diets was calculated using data from Watt and Merrill (16) and from the known iron content of the diet constituents. Diet 1 contained a total of 104 μg iron/g of diet, 6 μg furnished by the beef and 98 μg from the salt mixture; diets 2 and 3 contained 105 μg iron/g of diet, 7 μg from the ground whole wheat and 98 μg from the salt mixture; diet 4 contained 327 μg iron/g of laboratory chow. These amounts met the requirement of the rat for iron since the smallest weekly intake of food by any group, 58 g for group 3 (fig. 1) contained 6.1 mg of iron or 0.87 mg/day. Farris and Griffith (17) report that 0.25 mg of iron/day is adequate to maintain normal hemoglobin values in the rat.

Based on values presented by Block and Bolling (18), the essential amino acid intake of the rats receiving the diets with protein from beef and whole ground wheat, with or without lysine added, has been estimated. Amino acid intake of the rats in group 4 fed the laboratory chow was estimated from the approximate amino acid composition of that feed furnished by the company.[7] The intakes shown in table 2 are based on the average weekly food consumption of rats in groups 1, 2, 3, and 4, since the graphs in figure 1 indicate that the food consumption for the respective groups was almost constant per week, group 1, 81 g; group 2, 66 g; group 3, 58 g; and group 4, 141 g. Also shown in table 2 are comparisons of these estimated amino acid intakes with the minimal requirements of these amino acids essential for the normal growth of the rat as given by Rama Rao et al. (19). Since these minimal requirements (19) are expressed as percentages of the diet, the calculations and comparisons shown in table 2 are based on the actual food consumption of rats in each of the 3 experimental groups. In explanation of these comparisons shown in table 2, 241 mg of lysine present in 81 g of beef diet represent 33.1% of that present in 81 g of

[7] See footnote 4.

diet had it contained the percentage of lysine given by Rama Rao et al. (19) as the minimal requirement. Likewise, 173 mg of lysine present in 58 g of diet 3, wheat with lysine added, is 33.1% of that present in 58 g of diet had it contained the percentage of lysine given by Rama Rao et al. (19).

Comparative effects of feeding low levels of protein from beef, wheat or wheat with added lysine, on the iron transport system are shown in table 3.

Hematocrit and hemoglobin values. The average hematocrit and hemoglobin measurements for all 4 groups were within the range of standard values for rats given by Albritton (21), hematocrit 39 to 53%, average 46; hemoglobin 12 to 17.5 g/100 ml, average 14.8. Values for some individual rats were below and above these ranges. A comparison of groups 1, 2, and 3 with the control group 4 showed that rats in all 3 low protein groups had significantly lower hematocrits ($P < 0.001$)

TABLE 2

Average weekly intakes of amino acids and their comparison with the minimal requirement of the rat (19)

	Diet 1, beef		Diet 2, wheat		Diet 3, wheat + lysine		Diet 4, laboratory chow	
		% minimal requirement amino acids supplied		% minimal requirement amino acids supplied		% minimal requirement amino acids supplied		% minimal requirement amino acids supplied
		%		%		%		%
Total intake/week:								
Diet, g	81		66		58		141	
Protein, g	2.8		2.3		2.0		33	
Nitrogen, mg	448		368		320		5280	
Amino acids, mg								
Lysine	241	33.1	62	10.4	173	33.1	1269	141.1
Tryptophan	28	31.5	28	38.4	24	37.5	155	200.0
Phenylalanine	109	18.7	117	24.6	102	24.4	1015	113.9
Methionine	76	19.1	58	18.0	50	17.6	691	73.5
Threonine	123	29.8	76	22.6	66	22.3	719	153.0
Leucine	218	39.0	161	35.4	140	35.0	973	202.9
Isoleucine	146	32.7	92	25.3	80	25.1	775	187.3
Valine	143	31.5	99	26.8	86	26.5	832	172.8
Histidine	92	54.1	48	34.5	42	34.4	296	209.4
Arginine	182	112.3[1]	99	75.0[1]	86	74.1[1]	282	574.8[1]

[1] Arginine requirements based on requirements given by Rose et al. (20).

TABLE 3

Changes in blood constituents of rats fed low levels of protein from beef, wheat, and wheat with lysine for 6 weeks

Group no.	1	2	3	4
Protein level,%	3.5	3.5	3.5	26.2
Protein source	Beef	Wheat	Wheat with lysine	Laboratory chow
Hematocrit, %, avg	47.5	45.5	42.5	52.4
Range	45.5–49.9	41.4–49.4	37.6–48.5	50.0–56.8
Hemoglobin, g/100 ml, avg	15.2	15.5	13.4	16.7
Range	13.5–16.2	14.2–16.4	11.8–14.9	15.7–17.8
		Serum constituents		
Iron, μg/100 ml	166 ± 6.1[1]	256 ± 14.9	302 ± 16.5	192 ± 7.9
TIBC, μg/100 ml[2]	627 ± 14.3	638 ± 9.7	660 ± 12.5	666 ± 7.8
Iron saturation, %	26.3 ± 0.9	40.0 ± 2.3	45.7 ± 2.2	28.8 ± 1.1
Transferrin, g/100 ml	0.51 ± 0.04	0.51 ± 0.01	0.53 ± 0.01	0.54 ± 0.04
Total cholesterol, mg/100 ml	86.8 ± 2.4	86.5 ± 2.8	81.8 ± 2.3	65.0 ± 2.7

[1] SE of mean.
[2] TIBC indicates total iron-binding capacity.

and hemoglobin levels ($P < 0.01$) than did those in the control group fed the laboratory chow. Rats fed the 3.5% beef-protein diet did not differ significantly in hematocrit and hemoglobin values from those fed the 3.5% wheat-protein diet. When rats were fed the wheat-protein diet with added lysine, these blood values were significantly lower than when either the unsupplemented wheat-protein diet, or the beef-protein diet which contained a similar amount of lysine was fed ($P < 0.01$).

The blood values of normal male rats used in this study and fed an optimal diet, at an elevation of 1.46 km (4800 feet), group 4 (table 3) are considerably higher than the hemoglobin and hematocrit levels given by Albritton (21), as standard values for the rat. This suggests that the elevation of place of work may be a contributing factor with rats as well as with humans. This suggestion is made on the assumption that a 6-week feeding period at one elevation is sufficient time for the rat's hemopoietic processes to adjust to an increase in altitude.

Serum iron. For the purpose of comparisons made in this paper it is assumed that the values obtained for serum iron, total iron-binding capacity, percentage of iron saturation, and transferrin protein for rats in group 4, represent normal values for 14-week-old rats as used under the experimental conditions of this study. Serum iron values of rats in groups 1, 2, and 3, fed diets with low protein levels, differed significantly from the control group 4, $P < 0.05$, $P < 0.01$, and $P < 0.01$, respectively. However, the serum iron level of rats in group 1, fed the 3.5% beef-protein diet was significantly lower than that of the controls, whereas levels in the other 2 groups were higher. Rats in group 2, fed the 3.5% wheat-protein diet, and group 3, fed the same diet with lysine supplementation, had high serum iron levels and at the same time showed low hematocrit and hemoglobin values. This phenomenon, as discussed by Mandel (13) in his clinical and diagnostic work with humans, suggests an impairment in iron utilization in which the hemopoietic process is disturbed and the iron, although present in the serum in larger amounts than normal, is not used for hemoglobin regeneration.

Total iron-binding capacity and iron saturation. When the 4 groups are examined, the TIBC values of the rats in groups 1 and 2 were lower than those in the control group 4 ($P < 0.05$) whereas group 3 was similar to the controls. When the iron present in the serum is expressed in its relationship to the total iron-binding capacity of that serum (percentage of iron saturation) rats in group 1, fed the 3.5% beef-protein diet, are very similar in their percentage of iron saturation to those in the control group, indicating an almost normal situation for iron utilization in the rats fed the 3.5% beef-protein diet. The percentages of iron saturation in the serum of rats in groups 2 and 3, fed the 3.5% wheat-protein diet, with and without lysine supplementation, are significantly higher ($P < 0.01$) than that of the control group of rats or of the beef fed rats, indicating a disturbance in the utilization of iron or in the iron transport system of the rats fed the wheat-protein diets. Itzhaki and Belcher (2) reported on the total iron-binding capacity in the plasma of normal rats as ranging from 530 to 610 μg/100 ml. This is somewhat lower than that found in the present study for control rats, range 634 to 697, average 666 μg/100 ml serum. The amino acid composition of the chow diet together with the food intake of the rats in group 4 that were fed this diet ad libitum, indicate that the rats in this control group received an excess of all the essential amino acids.

In discussing the adequacy of the 3.5% beef- or wheat-protein diets for normal iron utilization, attention is called to several facts. The 3.5% protein diets fed to groups 1, 2, and 3, contained all minerals and vitamins known to be necessary for the normal nutrition of the rat, including iron as ferric ammonium citrate, copper as cupric sulfate, vitamin E as α-tocopherol and ascorbic acid (table 1). The rats were fed at an elevation of 1.46 km and were not depleted of their body storage of iron prior to the time they were placed on the experimental diets at 8 weeks of age.

Any explanation offered for the difference in performance between the beef- and wheat-containing diets must be one

of postulation since only the unrefined proteins were fed and no supplementations with amino acids, other than the one level of lysine, were used. The 21.4% of whole wheat in the diet could conceivably furnish other compounds which might influence iron utilization. Wheat is one of the cereals known to contain phytic acid which may form insoluble compounds with some of the mineral constituents such as calcium, magnesium, zinc and iron. It may also depress the absorption of other food materials in the intestinal tract (22).

Since the iron present in the 3 experimental diets, 104 μg iron/g of diet, far exceeds the iron requirement of the rat, it appears unlikely that the phytic acid in the wheat was responsible for the poorer utilization of the iron in the wheat diets as compared with the beef diets. Furthermore, Sherman (23) raised many generations of rats with a diet of 5 parts of whole wheat and one part dried whole milk with sodium chloride added in the proportion of 2% of the weight of the wheat. In the Sherman diet all the iron and the other minerals were derived from the wheat and the whole milk, with the bulk of the iron contributed by the wheat. Even with no iron from any other source the diet was adequate for growth and reproduction. In the experimental diets used in the present study, adequacy of iron was insured through the use of 4% salt mixture in each diet.

The essential amino acid intakes of the rats on the beef and wheat diets (table 2) vary considerably. Part of this variation is due to a smaller food consumption of the rats fed the wheat-containing diets 2 and 3, and part to the actual amino acid composition of the beef and wheat proteins. Harper (24) studying the effect of the dietary level of protein on the severity of an amino acid balance reported that dietary additions that caused quite severe imbalances in low protein diets had almost no effect when the protein level was sufficiently high to satisfy the amino acid requirements of the rat. Munaver and Harper (25) showed that the lysine requirement of the rat for maximal growth increased from 0.9 to 1.2% of the diet as the wheat gluten content of their diet was increased from 30 to 60%. The percentage of the lysine requirement for normal growth as shown in table 2 is based on Rama Rao's (19) figure of 0.9%. At the very low level of 3.5% protein used in the present study the requirement for lysine is not known. However, the rats fed the beef diet 1 with 3.5% beef protein, showed nearly normal utilization of iron even though their growth was severely retarded. Although the hematocrit and hemoglobin levels of these rats were lower than the rats receiving the chow diet, they were still within a normal range as given by Albritton (21) and serum iron, total iron-binding capacity of the serum and the percentage of iron saturation were similar to those values for the chow fed rats.

If the phytic acid content of the wheat diets was ruled out as a contributing factor in the poor utilization of the iron by the rats receiving the wheat diets, the more complete utilization of the dietary iron of the rats fed the beef diets indicates that the relationships of the amounts of the amino acids in the beef diets were more conducive to maximal iron utilization than the amino acid balances in the wheat-containing diets. From the poor response to the addition of lysine to the wheat diet it appears that the level of lysine alone is not responsible for this effect, for the iron utilization was not improved, rat growth was retarded and food intake lowered (fig. 1).

A review of the percentages of the minimal requirements of amino acids furnished by the beef and wheat diets (table 2) indicates that the main percentage differences between the beef and wheat diets are in their lysine, histidine and arginine values. O'Dell and Regan (26) and Fisher et al. (27) observed unusually high arginine requirements for guinea pigs and chicks fed casein diets. The results of O'Dell and Regan (26) suggest a lysine-arginine and glycine-arginine antagonism. Retarded growth effects caused by feeding excess lysine could be overcome by feeding additional arginine at the same time. Hemoglobin is known to contain approximately 8% of histidine (18). Nasset and Gatewood (28) noted that the reduction of histidine in their experimental diets re-

sulted in a reduction of hemoglobin in the rat.

Whether any of these amino acid relationships or which of these relationships could be responsible for the poorer utilization of iron with the wheat diets must rest upon further work. However, the authors feel that the aspect of iron utilization and the hemopoietic processes as related to dietary protein and amino acid balances is one that should receive further attention.

Transferrin protein. Since the values for transferrin protein in the serum samples are based on the TIBC values of the serums, the comparisons of transferrin in the various groups are similar to the comparisons for TIBC. These values are presented in table 3 to give quantitative information on this important serum protein.

Serum cholesterol. Even though there were significant differences in the iron transport system between rats fed the beef-containing diet and the wheat diets, with and without added lysine, there were no significant differences in the serum cholesterol of these 3 experimental groups.

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Comparative Protein Evaluation Studies by Carcass Retention and Nitrogen Balance Methods[1]

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ABSTRACT Protein evaluations were carried out with rats and chicks to elucidate certain problems encountered in earlier studies. The following observations were recorded: 1) Linseed and cottonseed meals when supplemented with the limiting amino acids promoted much improved growth in both rats and chickens. Net protein utilization (NPU) in the chicken, in contrast with results in the rat, was only slightly improved. 2) Significant differences were observed in protein utilization when the carbohydrate source was varied. For both the rat and the chicken a combination of corn starch, glucose, and a small amount of dextrin was superior to the single components of the mixture. 3) A cod fillet flour was noted to have an inferior NPU for chicks compared with commercial menhaden fish meal. Subsequent study showed the fillet flour to be deficient in arginine for the chicken. For the rat this flour was noted to be deficient in methionine. 4) Generally good agreement was observed between NPU values obtained by either the nitrogen balance or the carcass retention method in the rat. The former method consistently gave higher values than the latter, and it is suggested that these differences result primarily from a somewhat lower protein intake (and therefore better utilization) due to the equalized feeding practice with the nitrogen balance method.

During the course of a systematic evaluation of protein sources for growing chickens by the carcass nitrogen retention method, certain results were obtained that could not be explained on the strength of the experimental results and therefore merited further investigation (1–3). A marked improvement in growth rate had been noted when either cottonseed or linseed meal was supplemented with its limiting amino acids, whereas net protein utilization (NPU) was improved only slightly. A second problem of concern was the unusually low NPU values obtained in an experiment in which the carbohydrate source of the basal diet had been modified. Finally, in a comparison of fish meals of different origin, better utilization was obtained with a commercial menhaden fish meal than with an edible, supposedly high quality cod fillet flour prepared for human consumption. The studies to be reported are concerned with the exploration of these anomalous findings.

GENERAL PROCEDURES

The experiments were carried out with either chickens or rats. Those with chickens were carried out in accordance with procedures previously described (1, 2). For the rat studies 5 albino Wistar-strain males, each weighing approximately 100 g, were assigned to each treatment group. Rats were housed in individual metabolism cages for quantitative collection of urine and feces. For the nitrogen balance method, the rats were given the test diet for 11 days, with urine and fecal collections carried out during the last 4 days of this period. A protein-free diet was given following the test-protein feeding for a period of 7 days, with urine and fecal collections being again carried out during the last 4 days. For the carcass nitrogen retention studies the rats were given the experimental diets for 10 days, followed by a day without food. The animals were subsequently killed with chloroform and then dried and ground in the same manner as the chickens (1). The diets for chickens were prepared to supply 13% protein and those for the rats 10%. The

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composition of the basal diet for both animal species supplied: (in per cent) mineral mix,[3] 4.9; corn oil, 3.0; cellulose, 3.0; vitamins,[3] 0.25; choline chloride (70% concentrate), 0.3. Unless otherwise indicated in the tables of results, the carbohydrate component of the diets consisted of: cornstarch, 28.0%; dextrin, 5.0%; and glucose to 100% of the diet.

The NPU values were calculated from nitrogen balance or carcass retention as follows:

Nitrogen balance:

$$\text{NPU} = \frac{\text{Biological value} \times \text{true digestibility}}{100}$$

where the biological value is as defined by Mitchell (4) and the true digestibility is that of the test protein.

Carcass retention:

$$\text{NPU} = \frac{B_f - B_k + I_k}{I_f} \times 100$$

where B_f and I_f denote carcass nitrogen and nitrogen intake of animals fed the test diet, respectively, and B_k and I_k equal carcass nitrogen and nitrogen intake with the protein-free control diet, respectively. (Sample calculations are shown in table 5.)

EXPERIMENTAL AND RESULTS

Studies with linseed and cottonseed meal. Table 1 shows typical results obtained in several experiments when linseed or cottonseed meal was fed to growing chickens. Supplementation of linseed and cottonseed meals with amino acids (methionine and lysine) resulted in body weight increases of 30 and 58%, respectively, when compared with the unsupplemented meals. On the other hand, NPU was improved by only 17 and 13%, respectively, as a result of methionine and lysine supplementation. Since generally poor NPU values were noted for both meals, it was believed possible that the diet, which supplied 13% protein, might provide only the minimal maintenance requirement for protein and that the NPU of the amino acid-supplemented meals might be improved at protein intakes greater than 13%.

Table 2 shows the response of the chicken to three levels of dietary linseed meal. Again, however, the improvement due to amino acid supplementation was marked only in terms of body weight change, but was negligible in terms of NPU at all 3 levels of dietary protein. For both protein sources NPU values decreased with increasing level of dietary protein.

These results rule out that the poor NPU response to amino acid supplementation of linseed meal is dependent on a specific level of dietary protein. Next, we considered the possibility that poor digestibility might be a factor. Since amino acid supplementation increased food consumption, the improved growth resulting therefrom is not necessarily in contradiction with unimproved NPU values due to poor protein digestibility. Calculations indicate that a small improvement only, could result from a low digestion coeffi-

[3] For composition, see Summers and Fisher (1).

TABLE 1

Three-week body weights and net protein utilization values for linseed and cottonseed protein fed with or without amino acid supplementation to growing chicks

Protein source	Measurements			
	3-Week wt	Increase[1]	Net protein utilization	Increase[1]
	g	*%*		*%*
Linseed meal	138 ± 7[2]		35.6 ± 0.8	
Linseed meal + 0.2% DL-methionine + 0.46% L-lysine[3]	179 ± 5	30	41.7 ± 1.7	17
Cottonseed meal	107 ± 2		35.0 ± 1.0	
Cottonseed meal + 0.2% DL-methionine + 0.32% L-lysine[3]	169 ± 6	58	39.6 ± 0.9	13

[1] Resulting from amino acid supplementation.
[2] Mean ± SE for quadruplicate lots of 5 chicks.
[3] Added as the monohydrochloride.

cient for these meals. This hypothesis, however, could not be tested without resorting to surgery since the chicken excretes urine and feces jointly. The next experiment, therefore, was carried out with rats to determine whether this species would respond in its utilization of cottonseed and linseed meal in a way similar to that of the chicken.

Table 3 shows that for the rat there is essentially the same rate of improvement due to amino acid supplementation of cottonseed meal in weight gain as in NPU, the latter values having beeen determined by both nitrogen balance and carcass retention methods. For linseed meal, the improvement is larger in weight gain but is still considerable for NPU when com-

TABLE 2

Three week body weights and net protein utilization values of chickens fed increasing levels of linseed meal with or without amino acid supplementation

Protein source	Measurements			
	3-Week wt	Increase[1]	Net protein utilization	Increase[1]
	g	*%*		*%*
10% Protein:				
Linseed meal	125 ± 4[2]		53.5 ± 3.1	
Linseed meal + 0.2% DL-methionine + 0.46% L-lysine[3]	158 ± 4	26	58.2 ± 1.2	9
13% Protein:				
Linseed meal	136 ± 4		42.2 ± 0.1	
Linseed meal + 0.2% DL-methionine + 0.46% L-lysine[3]	196 ± 4	44	45.8 ± 1.4	9
17% Protein:				
Linseed meal	154 ± 4		35.2 ± 0.6	
Linseed meal + 0.2% DL-methionine + 0.46% L-lysine[3]	204 ± 8	32	39.0 ± 2.5	11

[1] Resulting from amino acid supplementation.
[2] Mean ± SE for quadruplicate lots of 5 chicks.
[3] Added as the monohydrochloride.

TABLE 3

Comparison of net protein utilization values of cottonseed and linseed protein for the growing rat by carcass retention and nitrogen balance methods

Protein source	Measurements				
	11-Day wt gain	Nitrogen balance			Carcass retention
		Biological value	True digestibility	Net protein utilization (BV × TD ÷ 100)	Net protein utilization
	g		*%*		
Linseed meal	22 ± 1	57.9 ± 1.7	80.4 ± 1.2	46.5 ± 0.3	44.8 ± 1.5
Linseed meal + 0.2% DL-methionine + 0.46% L-lysine[1]	33 ± 1 (50)[2]	73.2 ± 1.8 (26)	80.3 ± 1.0	58.8 ± 2.1 (25)	58.2 ± 1.2 (29)
Cottonseed meal	25 ± 2	55.6 ± 0.3	76.1 ± 0.8	42.3 ± 0.2	39.3 ± 1.2
Cottonseed meal + 0.2% DL-methionine + 0.32% L-lysine[1]	31 ± 3 (24)	66.0 ± 1.2 (19)	79.3 ± 0.7	52.3 ± 1.1 (24)	48.3 ± 1.4 (23)

[1] Added as the monohydrochloride.
[2] Mean value ± SE for 5 rats; values in parentheses represent % increase resulting from amino acid supplementation.

pared with the results obtained with chickens. Also, the digestion coefficients were not as low as they would have had to be if the discrepancy observed in the chicken was to be explained on this basis. Attention is drawn to the generally good agreement between NPU values determined by the 2 methods, although the nitrogen balance method always gave slightly higher values than the carcass retention method.

Studies with different carbohydrate sources. As mentioned earlier, during a routine assay with chicks, NPU values for a series of proteins were observed to be considerably below expectation based on the poor performance of a standard protein (isolated soy) included in all our protein evaluation studies (1). On investigating possible reasons for these results it was noted that a substitution in one of the carbohydrate components of the diet had been made during mixing as a result of a shortage in this particular ingredient. Subsequently, the following experiments were initiated to determine the extent to which a change in carbohydrate might influence protein utilization.

Table 4 shows the results of a study with chicks. Significant differences ($P < 0.01$) were observed between cornstarch, dextrin, glucose, and sucrose, and a mixture

TABLE 4

Three-week body weights and net protein utilization values of chicks fed isolated soybean protein diets[1] in which the carbohydrate source was varied

Carbohydrate source	Measurements			
	3-Week wt	Feed consumption	Carcass nitrogen	Net protein utilization
% of diet	*g*	*g/bird*	*% dry wt*	
Cornstarch,28 + dextrin, 5 + glucose, 38	221 ± 5[2]	325	7.35	62.9 ± 1.3
Dextrin, 71	184 ± 6	401	7.59	38.6 ± 1.9
Cornstarch, 71	182 ± 5	269	7.70	53.5 ± 0.7
Glucose, 71	203 ± 5	304	7.63	55.2 ± 0.9
Sucrose, 71	211 ± 5	296	7.14	59.3 ± 1.3

[1] ADM C-1 Assay Protein, Archer-Daniels-Midland Company, Minneapolis, supplying 13% protein (N × 6.25) and supplemented with 0.2% DL-methionine.
[2] Mean ± SE for quadruplicate groups of 5 chicks.

TABLE 5

Net protein utilization values by carcass retention and nitrogen balance methods for rats given isolated soy protein diets[1] varying in the source of carbohydrate

Carbohydrate source	Measurements				
	11-Day wt gain[2]	Nitrogen balance			Carcass retention
		Biological value	True digestibility	Net protein utilization[3] (BV × TD ÷ 100)	Net protein utilization[3]
% of diet	*g*		*%*		
Glucose, 76	29 ± 4[4]	66.7 ± 1.1	93.8 ± 0.2	62.6 ± 1.1	64.6 ± 7.0
Cornstarch, 76	30 ± 2	69.5 ± 0.2	94.5 ± 0.1	65.7 ± 0.1	59.1 ± 2.7
Dextrin, 76	24 ± 4	81.1 ± 1.6	86.7 ± 0.2	70.7 ± 1.8	52.0 ± 15.4
Sucrose, 76	37 ± 2	73.2 ± 0.2	92.6 ± 0.3	67.8 ± 0.2	57.8 ± 2.0
Cornstarch, 28 + dextrin, 5 + glucose, 43	33 ± 2	79.0 ± 1.0	93.4 ± 0.1	73.8 ± 1.0	56.6 ± 2.5

[1] ADM C-1 Assay Protein, Archer-Daniels-Midland Company, Minneapolis, supplying 10% protein (N × 6.25) supplemented with 0.2% DL-methionine.
[2] Fed ad libitum during carcass retention study.
[3] Sample calculation for glucose diet: Nitrogen balance: NPU = 66.7 × 93.8 ÷ 100 = 62.6; carcass retention: $\text{NPU} = \frac{4.106 - 2.716 + 0}{2.153} \times 100 = 64.6$.
[4] Mean ± SE for 5 rats.

containing different proportions of starch, glucose, and dextrin. The superiority of the carbohydrate mixture is relevant because its individual components (cornstarch, dextrin or glucose) were markedly inferior either to the mixture or to sucrose. The mixture was originally designed to permit pelleting of the diet in a laboratory pelleting machine, no prior attempt having been made to evaluate this particular mixture nutritionally. Attention is also drawn to the unusually poor NPU value obtained with dextrin. Although this low value is not reflected in particularly poor growth, dextrin appears to be a very poor source of calories for chicks since they consumed approximately 25% more of the dextrin-containing feed compared with that containing the mixed carbohydrates.

The same carbohydrate sources were next compared by nitrogen balance and carcass retention methods in the rat (table 5). There were significant differences among groups ($P < 0.01$) in NPU values determined by the nitrogen balance method; with the carcass retention procedure, individual variations were exceptionally large, particularly in the groups given glucose and dextrin (the 2 extreme values). In comparing the values obtained by the nitrogen balance method, the carbohydrate mixture was again superior to any of the individual components of the mixture. None of the differences were as marked as those observed with the chicks.

TABLE 6

Three week body weights and net protein utilization values for chicks given soy and fish protein with or without amino acid supplementation

Protein source	Measurements	
	3-Week wt	Net protein utilization
	g	
Isolated soy protein[1]	126 ± 7[2]	46.5
+0.2% DL-methionine	210 ± 9	62.3
Cod fillet flour[3]	172 ± 5	59.9
(menhaden fish meal[4]	234 ± 10	63.2)
Cod fillet flour	170 ± 5	57.6
+0.2% DL-methionine	158 ± 7	58.8
+0.32% L-lysine[5]	154 ± 7	58.5
+0.42% L-arginine[5]	194 ± 7	67.8
+0.2% DL-methionine + 0.32% L-lysine + 0.42% L-arginine	196 ± 9	61.6

[1] ADM C-1 Assay Protein, Archer-Daniels-Midland Company, Minneapolis.
[2] Mean ± SE for triplicate groups of 5 chicks.
[3] An ethylene dichloride extracted high protein concentrate (93%, N × 6.25), Viobin Corporation, Monticello, Illinois.
[4] Included for comparison purposes from previous studies (2).
[5] Added as the hydrochloride.

TABLE 7

Net protein utilization values by carcass retention and nitrogen balance methods for rats given soy and fish protein with or without amino acid supplementation

Protein source	Measurements			
	Nitrogen balance			Carcass retention
	Biological value	True digestibility	Net protein utilization (BV × TD ÷ 100)	Net protein utilization
	%			
Isolated soy protein[1]	66.4 ± 1.2[2]	95.5 ± 0.6	63.3 ± 0.8	52.7
+0.2% DL-methionine	79.1 ± 1.2	96.8 ± 0.8	76.8 ± 0.5	68.2
Cod fillet flour[3]	71.8 ± 2.4	94.8 ± 0.8	68.2 ± 2.9	66.8
+0.2% DL-methionine	79.2 ± 5.3	97.2 ± 1.4	76.7 ± 4.5	74.7

[1] ADM C-1 Assay Protein, Archer-Daniels-Midland Company, Minneapolis.
[2] Mean ± SE for 5 rats.
[3] An ethylene dichloride extracted high protein concentrate (93%, N × 6.25), Viobin Corporation, Monticello, Illinois.

Studies with fish protein. A comparison (table 6) of a supposedly high quality, edible cod fillet flour with feed-grade menhaden fish meal showed the latter protein source to be slightly better for growth and NPU than the former. Several amino acids were tried as supplements for the cod fillet flour, as shown in the same table; for the chick the fillet flour was found to be deficient in arginine. Table 7 shows that, for the rat, the fillet flour was markedly improved by methionine supplementation (other amino acids were not tried).

Attention is also drawn to results obtained with rats and chicks fed soy protein with and without methionine supplementation (tables 6 and 7). This comparison (with rats) was included because Henry and Toothill (5) had suggested that large discrepancies occurred in NPU values between nitrogen balance and carcass retention methods for proteins that were severely deficient in sulfur amino acids. Table 7 shows excellent agreement in NPU value between the 2 methods for the cod fillet flour, but a 20% difference for the unsupplemented soy flour.

DISCUSSION

Studies with linseed and cottonseed meal. Results obtained with the rat and chick for linseed and cottonseed meal and supplementation with their limiting amino acids (methionine and lysine) indicate that a species difference exists, the rat utilizing more efficiently than the chicken the 2 meals supplemented with amino acids. These studies, therefore, fail to resolve for the chick the slight improvement in NPU upon amino acid supplementation, despite a marked improvement in growth rate. Since the chicken has a much shorter digestive tract per unit body size than the rat, the possibility still exists that the discrepancy results from differences in digestibility.

The chick studies point up one of the weaknesses of the NPU method as an indicator of protein quality. Whereas for most proteins there is good agreement between growth rate and efficiency of utilization (NPU), exceptions exist where the protein is readily consumed, even to excess, to promote reasonably good growth, without a concomitant reflection of this growth rate on efficiency of utilization.

The values obtained for the rat for linseed meal are similar to the value given by Henry and Toothill (5); the cottonseed meal values obtained in our studies were, however, generally lower than those obtained by these workers. As in most of our rat studies, there was relatively good agreement between the nitrogen balance and carcass retention NPU values, although the former were always higher than the latter.

Studies with different carbohydrate sources. With the suboptimal level of protein supplied by the test diets in these evaluation studies for both the chick and the rat, the source of carbohydrate influenced the utilization of dietary protein. Differences in growth rate with low protein rations as a result of varying the carbohydrate source have been noted for both chicken and rat (6–8). In our studies, the mixture of starch, glucose, and a small amount of dextrin was superior in both rat and chick to any of the single carbohydrates. In the chick, dextrin resulted in unusually poor protein utilization, despite relatively good growth. This discrepancy can be explained on the basis of the increased food intake on the dextrin diets, leading to an increased protein intake and its consequent poorer utilization. These results also emphasize the potential sparing effect of dietary energy on nitrogen retention.

Attention is called to the inconsistencies in NPU values for the rat, obtained by nitrogen balance versus the carcass retention method. The values obtained by the latter method varied greatly apparently due to large individual variations in the food intake pattern. Food intake had been equalized among rats for the nitrogen balance method.

These studies with different carbohydrates emphasize the importance of standardizing not only the protein level in protein evaluation studies, but also other components of the diet, such as the carbohydrate and, as recently suggested by the work of Morrison et al. (9), the vitamins and minerals. The report by Howe and Gilfillan (10) concerning the role which fat may play in the utilization of dietary

protein will also need careful consideration in the context of the present experimentation.

Studies with fish meal. Morrison et al. (11) have recently shown differences in the nutritive value of fish protein that had been extracted with different solvents. Our observations of an arginine deficiency in cod fillet flour for the chick might not, however, be related to the ethylene dichloride extraction but might simply reflect a deficiency of this amino acid due to the absence of gelatin protein from bone, skin and scales, which are high in arginine. Thus, for avian species, a whole fish flour might be a superior product in terms of its amino acid balance than a fillet meal. Again, species differences between rat and chicken are manifest in that the rat responded well to methionine supplementation, in agreement with Morrison's observations (11) that a similarly extracted fillet flour was deficient in methionine for the rat. Methionine was not even the second most limiting amino acid for the chicken, as was shown by the NPU results obtained with the combined supplementation of lysine, methionine and arginine; it was not even as effective as arginine supplementation alone.

Nitrogen balance versus carcass retention method. Although we compared the 2 methods in the rat for only a few proteins, we believe that under carefully controlled conditions the 2 methods give not only highly correlated results among the proteins tested, but also agree well in absolute terms. Henry and Toothill (5) observed very large differences in NPU for a large number of proteins, and suggested that well-balanced as well as sulfur amino acid-deficient proteins gave divergent results by the 2 methods. Our values for cod fillet meal (table 7) and values for other proteins (12) indicate that good agreement can be obtained for the type of protein for which Henry and Toothill claim large discrepancies.

Without exception, the nitrogen balance method gives somewhat higher values in our hands than the carcass retention method. Although this could result from cumulative errors, particularly nitrogen losses, in the nitrogen balance method, we feel that it is more likely to be due to a lower protein intake under the equalized feeding procedure followed for the nitrogen balance method. Forbes et al. (13) and Summers and Fisher (1) have, among others, shown an inverse relationship between protein intake and its utilization (see also results in table 2). The one-day fast before analysis of the carcass might also explain, in part, the lower values by the retention method. It remains to be determined whether this procedure incurs a larger error than would obtain if undigested food were to be considered as part of the retained nitrogen.

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Distribution and Phosphorylation of Oxythiamine in Rat Tissues[1]

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ABSTRACT The phosphorylation of oxythiamine was studied in vivo by determinations of the content of oxythiamine plus oxythiamine monophosphate and oxythiamine diphosphate in the liver, brain and heart of rats under different experimental conditions. The liver and, to a lesser extent, the heart were able to phosphorylate oxythiamine to its diphosphate. The degree of phosphorylation was inversely proportional to the thiamine (thiamine diphosphate) content of the organs. Oxythiamine does not penetrate the brain and therefore oxythiamine diphosphate was not observed there. The antithiamine activity of oxythiamine was essentially dependent on the thiamine level of the tissues.

The only effect of oxythiamine (OT) so far demonstrated in vitro is inhibition of carboxylase (1–3) and transketolase (4) by oxythiamine diphosphate (ODP), especially when the latter is added to the apoenzyme before thiamine diphosphate (TDP) (cocarboxylase). This observation led to the hypothesis (3, 5, 6) that in vivo the effect of OT is also through specific inhibition of thiamine diphosphate. In this case it must be assumed that OT is phosphorylated to the diphosphate, since only this substance can act as specific inhibitor. However, lack of a suitable analytical method for separation and determination of the phosphoric esters of thiamine and OT in animal tissues has until now prevented experimental proof of this hypothesis. We have now developed such a method (7) and have used it to study phosphorylation of OT in vivo and its distribution in rat tissues. We report here the results of some of our experiments.

EXPERIMENTAL

Male albino rats (Wistar strain), of 90 to 110 g starting weight, were kept in individual cages at 22°C and fed a thiamine-deficient diet. The percentage composition of the diet was : casein, washed and defatted, 18; corn starch, 69; olive oil, 10; cod liver oil, 2 and Osborne and Mendel's salt mixture,[2] 5. This was supplemented by suitable doses of the vitamin B complex, minus thiamine, given by mouth on alternate days. In addition, 1 mg α-tocopherol was given weekly.

Oxythiamine and oxythiamine-S^{35} were prepared by the method of Rydon (8) from thiamine and thiamine-S^{35}, respectively.[3] The phosphoric esters of oxythiamine and thiamine were determined by the chromatographic method of de Giuseppe and Rindi (7). The method does not permit separation of either thiamine or oxythiamine from their monophosphates. Using this procedure, the values of OT plus oxythiamine monophosphate (OMP) < 0.49 μg/g and of ODP < 1.03 μg/g were not statistically significant ($P = 0.05$).

Experiment 1. Rats fed either a complete or thiamine-deficient diet for 15 days were given 1 mg OT by intraperitoneal injection. Then all the animals were fed the thiamine-deficient diet and were decapitated at various times after injection (table 1). The content of OT, thiamine and their phosphates were determined in liver and brain.

Experiment 2. Rats fed as in the experiment 1 were given OT-S^{35} also by intraperitoneal injection. The total OT was determined after 1 and 24 hours in liver, heart and brain. The OT content was calculated as the ratio of activity, in counts per minute, of a trichloroacetic (TCA) extract of the organs of treated rats to the extract of the corresponding organs of

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[2] Osborne, T. B., and L. B. Mendel, J. Biol. Chem., *12*: 81, 1912.

[3] Kindly supplied by Hoffmann — La Roche, Basle, Switzerland.

TABLE 1

Content of thiamine, oxythiamine and their phosphoric esters[1] *(μg/g fresh tissue) of rat tissues after intraperitoneal injection of 1 mg of oxythiamine*

Hours after injection	Liver				Brain			
	T + TMP[2]	TDP	OT + OMP[3]	ODP	T + TMP	TDP	OT + OMP	ODP
				Normal rats				
0	1.92 ± 0.20	6.00 ± 0.53	—	—	0.97 ± 0.03	1.90 ± 0.05	—	—
1	1.83 ± 0.14	5.95 ± 0.23	2.56 ± 0.23	0.46 ± 0.06	1.08 ± 0.01	1.76 ± 0.07	0.28 ± 0.06	0.33 ± 0.05
12	1.82 ± 0.05	3.23 ± 0.10	0.57 ± 0.13	0.29 ± 0.15	0.96 ± 0.06	1.94 ± 0.06	0.44 ± 0.03	0.40 ± 0.1
24	1.58 ± 0.31	3.98 ± 0.33	0.34 ± 0.07	0.17 ± 0.02	0.93 ± 0.03	1.68 ± 0.07	0.18 ± 0.02	0.18 ± 0.04
				Thiamine-deficient rats (15 days)				
0	0.14 ± 0.03	0.26 ± 0.03	—	—	0.57 ± 0.05	1.48 ± 0.10	—	—
1	0.13 ± 0.02	0.22 ± 0.02	5.92 ± 0.42	9.48 ± 0.19	—	—	—	—
12	0.18 ± 0.03	0.32 ± 0.07	1.17 ± 0.13	2.69 ± 0.14	0.70 ± 0.04	1.60 ± 0.07	0.33 ± 0.07	0.26 ± 0.05
24	0.21 ± 0.06	0.24 ± 0.01	1.16 ± 0.08	2.47 ± 0.25	0.68 ± 0.07	1.57 ± 0.09	0.13 ± 0.01	0.38 ± 0.02

[1] Mean ± SE of 2 to 3 determinations, each being made on tissues pooled from 3 (liver) or 5 (brain) rats.
[2] T, TMP, TDP = thiamine, thiamine mono-, diphosphate.
[3] OT, OMP, ODP = oxythiamine, oxythiamine mono-, diphosphate.

untreated rats with addition of a known amount of OT-S^{35}, and subjected to the same analytical procedure; namely, tissue extraction with TCA, purification of the extract on charcoal, dephosphorylation with phosphatase (9); chromatography on amberlite[4] IRC 50 (H^+); washing with water and elution 0.2 N HCl. The radioactivity was measured on the total eluate or aliquots of it using a Geiger-Müller counter with a 2-mg/cm^2 mica window.

Experiment 3. Rats fed for 15 days a thiamine-deficient diet were injected intraperitoneally with 1 mg of OT plus amounts of thiamine from 0.01 to 1 mg. The liver content of thiamine and OT and their phosphates was determined after one hour.

Experiment 4. Suitable proportions of OT and thiamine were injected subcutaneously for a maximum of 20 days. The design of the experiment was practically identical with that of Gubler (10). Four groups of rats were fed a thiamine-deficient diet for 4 days and then each day were injected subcutaneously: first group, with 10 μg thiamine; second, 10 μg thiamine + 2 mg OT; third, 40 μg thiamine; and fourth, 40 μg thiamine + 8 mg OT. The ratio of thiamine-to-oxythiamine was therefore constant at 1:200, but the absolute amounts of thiamine were varied to obtain different tissue levels. The body weight of the animals was checked every other day during treatment.

The rats, maintained with the thiamine-deficient diet, were decapitated after treatment for 5, 10 and 15 and in some cases 20 days. The liver and brain content of thiamine, OT and their phosphates was assayed.

Experiment 5. The content of OT, thiamine and their phosphoric esters was determined in the liver and brain 1 and 24 hours after intraperitoneal injection of 1 mg OT in rats with severe thiamine deficiency (27 days deprivation).

RESULTS

The results of the experiments are shown in figures 1, 2 and 3, and in tables 1, 2 and 3.

[4] Amberlite, Rohm and Haas Company, Philadelphia 5, Pennsylvania.

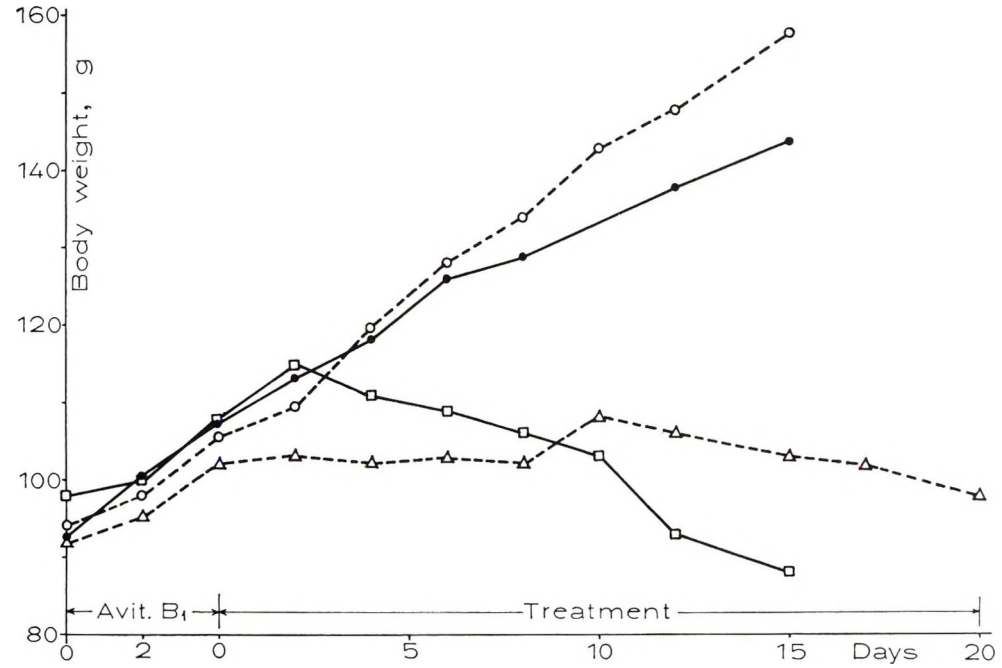

Fig. 1 Body weight of rats receiving a daily subcutaneous injection of: ——●—— 10 μg of thiamine; ——□—— 10 μg of thiamine + 2 mg of oxythiamine; ——○—— 40 μg of thiamine, ——△—— 40 μg of thiamine + 8 mg of oxythiamine. Each point is the average of 10 rats.

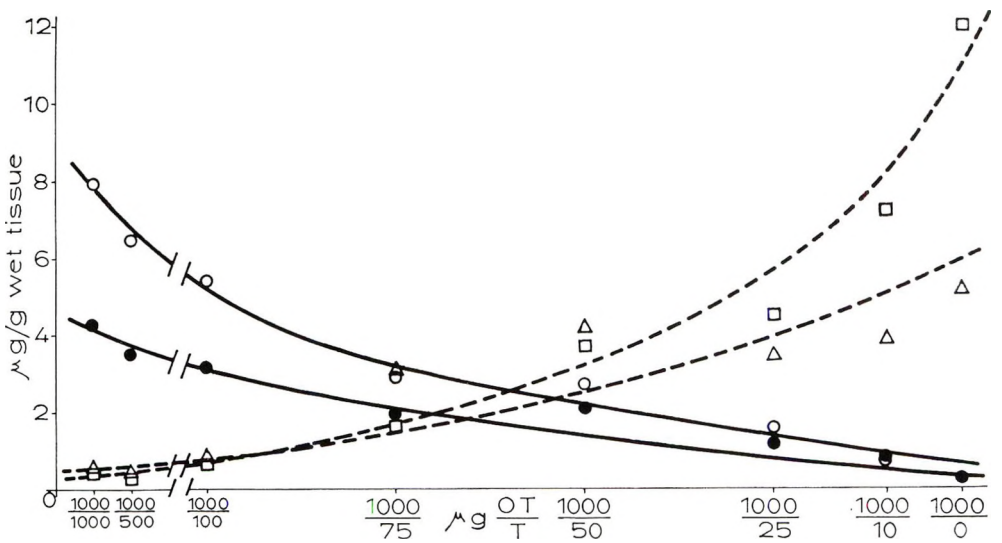

Fig. 2 Content of: ——●—— thiamine + thiamine monophosphate; ——○—— thiamine diphosphate; ——△—— oxythiamine + oxythiamine monophosphate; ——□—— oxythiamine diphosphate in liver of thiamine-deficient rats, 1 hour after a single intraperitoneal injection of 1 mg of thiamine (T) together with different amounts of oxythiamine (OT). The points are the average of 3 different determinations, each made on tissues pooled from 2 animals.

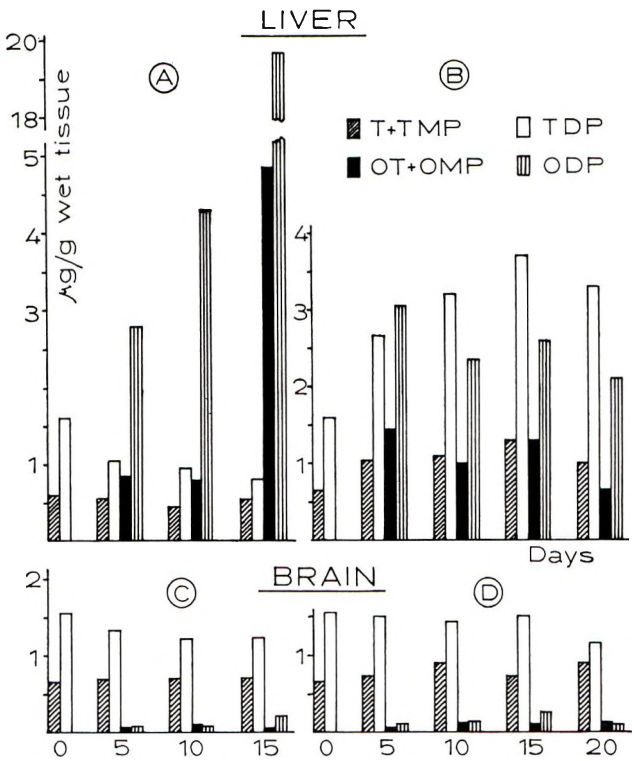

Fig. 3 Content of thiamine, oxythiamine and their phosphates of tissues of rats receiving a daily subcutaneous injection of: A, C 10 μg of thiamine + 2 mg of oxythiamine; B, D 40 μg of thiamine + 8 mg of oxythiamine. Each value was obtained on tissues pooled from 10 rats (for abbreviations see table 1).

TABLE 2

Oxythiamine-S^{35} content[1] (μg/g wet tissue) of rat tissues after intraperitoneal injection of 1 mg of oxythiamine-S^{35}

Hours after injection	Normal			Thiamine-deficient (17 days)		
	Liver	Brain	Heart	Liver	Brain	Heart
1	1.15	0.013	0.180	8.80	0.070	0.520
24	0.039	0.010	0.041	0.76	0.030	0.031

[1] Each value was obtained on tissues pooled from 10 rats.

TABLE 3

Content of thiamine, oxythiamine and their phosphoric esters[1] (μg/g wet tissue) of organs of rats with severe thiamine-deficiency (27 days) after an intraperitoneal injection of 1 mg of oxythiamine

Hours after injection	Liver				Brain			
	T + TMP[2]	TDP	OT + OMP	ODP	T + TMP	TDP	OT + OMP	ODP
1	0.13	0.16	7.33	16.85	0.19	0.39	0.05	0.13
24	0.09	0.13	1.18	2.81	0.34	0.46	0.06	0.25

[1] Each value is the mean of 2 determinations, each obtained on tissues pooled from 7 rats.
[2] For the abbreviations see table 1.

Experiment 1 (table 1). Practically no phosphorylation of OT was noted in the liver and brain of normal rats with a high tissue level of total thiamine. The ODP content was in fact always far below the statistically significant minimum for the method used, both 1 and 24 hours after injection. The only significant increase was observed in the hepatic levels of OT and OMP in the first hour. In the thiamine-deficient rats there was a low content of total thiamine in the liver and other organs, with the exception of the brain. At this time a conspicuous accumulation of OT was noted in the liver, mainly as diphosphate. Its presence was particularly high after 1 hour, but was still evident after 24 hours, the diphosphate content being always higher than the combined OT + OMP content. In the brain, on the other hand, where thiamine - deficiency over 15 days only slightly altered the total thiamine level, there was, as in normal brain, practically no phosphorylation of OT.

Experiment 2 (table 2). The content of OT-S^{35} was practically negligible in the organs of normal animals 1 and 24 hours after injection, with the exception of the liver and (although not to the same extent) the heart, but only in the first hour. A high level was observed in the liver and heart of thiamine-deficient rats 1 hour after injection, particularly in the liver where OT-S^{35} could still be observed at 24 hours. There was only an extremely minor amount of OT-S^{35} in the brain of both the normal and thiamine-deficient rats.

Experiment 3 (figure 2). When both compounds were simultaneously injected in suitable amounts, thiamine had a marked influence on phosphorylation of OT in the thiamine-deficient liver (fig. 2). The ODP content of the liver 1 hour after injection was down from 12.0 µg/g with injection of OT only (1 mg), to 0.40 µg/g with simultaneous injection of 1 mg each of thiamine and OT. Values lying between these 2 extremes were observed for intermediate amounts of thiamine. The pattern of the OT and OMP fraction in the liver was similar, although not so regular.

Experiment 4 (figure 3). There was a constant, although different, increase in body weight of the rats fed 10 and 40 µg thiamine (fig. 1). Simultaneous administration of OT greatly modified the body weights which, in relation to dose, were at the completion of the experiment either slightly lower (− 7%) or higher (7.7%) than the initial weight.

As compared with normal (11), a daily dose of 40 µg thiamine stabilized the thiamine compounds at a low level in the liver and brain (fig. 3, B, D), whereas 10 µg gave a slow and continuous decrease in the liver, but not the brain, where the level remained practically unchanged (fig. 3, A, C).

Conversely, the tissues of rats given 10 µg thiamine and 2 mg OT showed a steady increase in the level of OT compounds (fig. 3, A) and leveling off at constant, but lower, values for those receiving 40 µg thiamine plus 8 mg OT (fig. 3, B). There was no fixation of OT in the brain in either experimental variant (fig. 3, C, D).

Experiment 5 (table 3). Derivatives of oxythiamine were not observed in the brain after injection even in rats with very low levels of thiamine compounds in the brain as well as the liver. On the contrary, their level in the liver, which was fairly high at the first hour, was still evident at 24 hours.

DISCUSSION

Our results point to the ability of animal tissues to phosphorylate OT to the diphosphate. Moreover, the degree of phosphorylation is strictly related to the thiamine content of the organ or to the amount of thiamine introduced with the OT: the lower the amount of thiamine, the higher the amount of ODP. We may explain this by the fact (1, 3, 12, 13) that OT cannot inhibit thiaminokinase of animal tissues and hence cannot prevent formation of TDP. The OT is only likely to act as the substrate for thiaminokinase and thus be phosphorylated, when the content of thiamine is very low and that of OT high.

By interpolation of the graph in figure 2, we find that in the liver of thiamine-deficient rats 0.26 µmoles thiamine are needed to block phosphorylation of 3.31 µmoles

OT. Thus, it is conceivable that no antivitamin activity is displayed for molar ratios of OT:T = 13:1. Indeed, the ratios used by different authors in pigeons (14), mice (15) and rats (10, 16) were always greater than indicated above. Our results show, therefore, that if the antivitamin action of OT rests on its conversion to ODP, as suggested by in vitro investigations (1–3, 5), it will be manifest only in animals with low tissue levels of thiamine, since only in them is phosphorylation of quantitative importance.

However, given equal thiamine content, there is some difference between the organs in relation to fixation and phosphorylation of OT. For example, while the liver and, to a lesser extent, the heart [5] are able to phosphorylate OT in thiamine-deficient rats, the brain is never able to do this, even with a low thiamine content. Apparently, OT does not pass the blood-brain barrier, a prerequisite for its phosphorylation there. This may explain the well-known fact, which we too have always observed, that animals treated with OT never show the neuromuscular symptoms of dietary thiamine-deficiency. A characteristic effect of OT (10, 17 — 22) is the conspicuous increase in blood pyruvate, which, however, disappears in about 2 to 3 days upon stopping administration of OT (16, 20).

It is generally accepted, and our results on ODP formation in the tissues support this, that the increase in pyruvate in the blood comes, at least in part, from blocking of TDP by ODP. Such a blockage, and hence the high blood levels of pyruvate, are maintained for as long as ODP is present in the body. Since, as our experiments showed, ODP tends to disappear rapidly from rat tissues when its administration is stopped, this may explain why the pyruvate level returns to normal. The antivitamin effect of OT depends on its conversion to ODP and persists as long as the latter remains in the tissues.

This property of OT is quite different from that of pyrithiamine, which acts as inhibitor of thiaminokinase (1, 3, 12) voiding the organs of their thiamine content (16, 20, 23 — 27). This is particularly true of the brain, in which accumulation of pyrithiamine produces the neuromuscular symptoms of beri-beri (27, 28). Its effect thus is derived mainly from reduction in the tissue level of thiamine as a consequence of increased urinary excretion (16, 19, 29, 30) and persists even after administration ceases, until thiamine is introduced.

The results of experiment 4 are particularly instructive (figs. 1 and 3) as they correspond to the type of treatment commonly used in the study of the antivitamin activity of OT. A daily dose both of 10 and 40 μg thiamine caused a similar weight gain in the rats, but the thiamine content of the rat tissues showed quite different patterns for both doses. For a dose of 10 μg there was slight aneurin-hypovitaminosis, with steady decrease in liver thiamine; this did not occur in the brain, an organ which only very slowly loses its thiamine even in avitaminosis. For the dose of 40 μg the thiamine level of the tissues was stabilized at constant values, below normal ones. In other words, the body weight, which showed a continuous increase in both cases, is not a reliable indication of the thiamine content of the tissues. Naturally, the administration of an amount of OT 200 times that of thiamine had different effects on body weight: on 10 μg thiamine, the body weight decreased continuously after an initial increase, and 40% of the rats died in the 15-day period; on 40 μg, the body weight remained practically stationary throughout the experiment and only a few (9%) of the rats died within the 15 days. Therefore, the effect (antivitamin) on growth did not depend so much on the thiamine-to-oxythiamine ratio as on the absolute amount of thiamine introduced, since the latter regulates the vitamin level of the tissue (fig. 3) and thus the antivitamin effect of OT. It is interesting to compare our results on the organ content of ODP with those of Gubler (10) who estimated some thiamine-dependent enzymatic activities of rat organs in the same experimental conditions used in our experiment 4 (10 μg thiamine and 2 mg OT). He observed a notable decrease in the in vitro oxidation of pyruvate both in the mitochondria of liver and heart homogenates, whereas there was no change

[5] Unpublished results.

in the oxidation of pyruvate and α-ketoglutarate of brain homogenates. The inhibitory effect of the antivitamin was therefore observed only in those organs in which we detected more or less marked accumulation of ODP. Recently, Brin (31) reported decreased tranketolase activity in all, except brain, tissues of rats treated with OT.

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Metabolism of Sodium Acetate-1-C^{14} and Palmitic Acid-1-C^{14} in the Pantothenic Acid-deficient Rat

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ABSTRACT Fat utilization in the intact pantothenic acid-deficient rat was studied by the use of sodium acetate-1-C^{14} and palmitic acid-1-C^{14}. The rate of CO_2 production was normal, as was the absorption of fatty acid from the gastrointestinal tract of the deficient animals. Contrary to expectation, however, the rate of oxidation of both acetate-1-C^{14} and palmitate-1-C^{14} to $C^{14}O_2$ was increased in the deficient rats. Nearly 3 times as much of the C^{14} from acetate was incorporated into the liver cholesterol of the deficient animals as into that of the pair-fed controls, and more than twice as much of the C^{14} from palmitic acid was incorporated into the carcass cholesterol of the deficient animals as into that of the pair-weighed controls. No impairment of lipogenesis was observed. Cholesterol synthesis appeared to be more rapid in the deficient animals. The failure of liver fat deposition and the lowering of liver and serum cholesterol levels observed in the pantothenic acid-deficient rat may be tentatively ascribed to excessive utilization of fat, but decreased biosynthesis of fat and cholesterol cannot be ruled out on the basis of these results.

Pantothenic acid as a constituent of coenzyme A is essential for both synthesis and oxidation of fatty acids, and for the synthesis of cholesterol, as well as for the oxidation of pyruvate. Nearly all the evidence for this has come from the use of liver slices from deficient animals (1,2), from microorganisms (3,4) or from purified enzyme systems (5). One might expect these processes to be depressed in pantothenic acid-deficient animals, but so far, this has not been demonstrated conclusively in the intact animal.

That lipid metabolism is not normal in the pantothenic acid-deficient animals is clear from the work of Guehring et al. (6). These investigators observed that not only did fatty livers not occur in pantothenic acid-deficient rats but that these rats were resistant to the liver fat deposition ordinarily caused by a cholesterol-rich diet. That the deficient animals did not develop fatty livers could not be ascribed to inadequate food intake. Furthermore, rats made deficient in either riboflavin or pyridoxine responded normally to cholesterol feeding by developing fatty livers. A disturbed metabolism of cholesterol and fat in the pantothenic acid-deficient rat was thus indicated. In addition, Morgan and Lewis (7) reported that pantothenic acid deficiency prevented production of fatty livers characteristic of choline deficiency.

Additional evidence that fatty acid synthesis is decreased in pantothenic acid-deficient rats has been obtained by Boyd (8) and by Swell et al. (9). Boyd noted that pantothenic acid-deficient rats fed fat-free diets had reduced levels of plasma and liver cholesterol esters and reduced liver coenzyme A, but those fed fat-containing diets had normal levels of cholesterol esters. Swell et al. reported that feeding cholesterol and bile salts did not increase liver or serum cholesterol in pantothenic acid-deficient rats unless fat was added to the diet. These results (8,9) suggested that pantothenic acid-deficient rats were unable to form sufficient fatty acid to make serum and liver cholesterol esters or for the absorption of dietary cholesterol from the intestine.

The results of isotope studies of lipid metabolism in pantothenic acid-deficient rats have been contradictory. Guggenheim and Olson (10) observed no differences in the incorporation of C^{14} into serum and tissue fatty acids and cholesterol in deficient and control groups fed sodium acetate-1-C^{14}. Neither were there differences in the levels of fatty acids and cholesterol

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in livers, heart, and sera from the normal and deficient rats, but the adrenals of the latter group were low in cholesterol. Tolbert et al. (11) gave sodium acetate-2-C^{14} or sodium heptanoate-7-C^{14} by injection to normal and deficient rats and observed more and faster excretion of $C^{14}O_2$ by the latter.

Although Klein and Lipmann (1) reported a decreased incorporation of acetate-1-C^{14} into liver fatty acids and cholesterol in liver slices from pantothenic acid-deficient rats, other studies with liver slices have shown the opposite effect. Wolff and Thiaville-Dubost (12) noted incorporation of more acetate-1-C^{14} into $C^{14}O_2$, cholesterol, and fatty acids in liver slices from young pantothenic acid-deficient rats. Lata and Anderson (13) also observed an increased incorporation of acetate-1-C^{14} into cholesterol in liver slices from pantothenic acid-deficient rats which were mature at the start of the depletion period.

Thus, the in vivo non-isotopic experiments carried on in this laboratory (6, 7) have indicated significant lowering of liver fat and adrenal and serum cholesterol levels in deficient rats. This is in contrast with the inconclusive results of isotopic experiments (1, 10–13). The state of deficiency of the rats may have varied in these studies. The present study was undertaken 1) to compare the metabolism of acetate-1-C^{14} in normal and pantothenic acid-deficient rats prepared as were the intact animals previously reported upon; and 2) to compare the metabolism of C^{14} injected as acetate-1-C^{14} with that derived from a long-chain fatty acid, palmitic acid-1-C^{14}, given orally.

EXPERIMENTAL

Female rats of the Long-Evans-Wistar strain were given the purified pantothenic acid-deficient lactation diet when their young were 15 days of age. At 21 days of age the young were weaned, placed in individual cages and groups according to litter and weight. Male rats were used throughout the experiment. The normal control animals were pair-fed in the sodium-1-C^{14} experiment and pair-weighed, that is, fed only enough diet to maintain body weight equal to that of the deficient animals. in the palmitic acid-1-C^{14} experiment. In the latter experiment 3 normal animals fed ad libitum were also included as control.

The diet given the mothers of the litters when the young were 15 days old contained: 36% vitamin-free casein, 44.8% sucrose, 8% hydrogenated vegetable fat, 6% salts,[1] plus vitamins in sucrose to 100%. The basal diet given the weanlings contained: 22% vitamin-free casein, 65% sucrose, 9% hydrogenated vegetable. fat and 4% salts.[2] In addition vitamin supplements fed 3 times a week provided/rat/day: (in μg) thiamine·HCl, 20; pyridoxine·HCl, 20; and folic acid, 20; riboflavin, 40; Ca pantothenate, 100; nicotinamide, 66; *p*-aminobenzoic acid, 100; biotin, 2; menadione, 50; choline, 10 mg; inositol, 2.5 mg; vitamin A, 100 IU, vitamin D, 10 IU, and 1 mg mixed tocopherols. The dosage of all vitamins was doubled for the lactating females.[3]

The young rats were maintained with these diets for 5 weeks when the usual signs of deficiency appeared in the deprived group. Growth ceased in all but a few animals, which were discarded. It had been observed previously in this laboratory that mortality increases rapidly after the fifth week of depletion. After the rats had been fed the diet for the fourth week, they gained no more than 2 or 3 g. The usual greying of the fur and porphyrin-stained whiskers were observed.

Sodium acetate-1-C^{14} with a specific activity of 14 μc/mg was dissolved in isotonic saline. The rats were injected intraperitoneally with 0.5 ml of this solution containing 5.6 to 7.5 μc of radioactivity.

The injected animals were placed immediately in individual closed metabolism chambers. Carbon dioxide-free air was drawn past the animal and into carbon dioxide collectors containing 2 N NaOH. The carbon dioxide collectors were changed twice during the first hour and then at hourly intervals. After 3 hours in the metabolism chamber, during which time they had access to food and water, the rats were killed by exsanguination and their livers removed and frozen for analysis at a later date.

[1] Hubbell, R. B., L. B. Mendel and A. J. Wakeman 1937 J. Nutrition, 14: 273.

[2] See footnote 1.

[3] Vitamin B_{12} was not included in the vitamin mixture because of the relatively high content of casein in the diets.

Palmitic acid-1-C^{14} with a specific activity of 1 mc/mmole was dissolved in fresh cottonseed oil.[4] One-half milliliter of the oil solution, containing 20 μc of radioactivity was administered to each rat via stomach tube. Immediately after the palmitic acid-1-C^{14} was given, the rats were placed in individual closed metabolism chambers as described for the acetate experiment. The rats had free access to water and were fed 3 to 4 g of diet 8 hours after receiving the labeled acid. After 24 hours during which 8 or 9 samples of CO_2 were collected the animals were killed.

The $C^{14}O_2$ was precipitated as $BaC^{14}O_2$ and counted in an end window counter. Necessary corrections for background, coincidence, and self absorption were made. A known aliquot was weighed to determine total CO_2.

The livers were digested with alcoholic KOH, 30% KOH in 48% ethanol, and extracted with petroleum ether. Cholesterol was determined by a modification of the Sperry-Webb method (14) and fatty acids by Bloor's chromic acid oxidation technique (15). The C^{14} in the saponifiable fraction was measured by plating 1-ml aliquots directly on aluminum cups and counting them with the end window counter. Mass was assumed to be negligible.

The cholesterol was precipitated as the digitonide, washed free of digitonin, plated on aluminum plates and counted. The results were corrected for coincidence when necessary and for self-absorption. The specific activity of cholesterol (count/min/mg) was calculated by taking the cholesterol content of cholesterol digitonide as the theoretical value 23.9%. A self-absorption corrections curve was prepared for cholesterol digitonide as described by Calvin et al. (16).

Unabsorbed palmitic acid-1-C^{14} was measured as follows: the entire gastrointestinal tract was dissected out and slit open. The contents were washed into a beaker with approximately 200 ml of 95% ethanol. The feces collected on a sheet of perforated aluminum foil lining the floor of the metabolism chamber were also added. The ethanol slurry was heated in an air oven at 60°C for 2 hours with occasional stirring. The mixture was then filtered and solid material extracted 24 hours with redistilled ethyl ether in a Soxhlet extractor. The ethyl ether extract was combined with the ethanol fraction and made to volume. Aliquots of the alcohol-ether extract were then plated on aluminum planchets and counted in an end window counter. All of the activity was considered to be due to unabsorbed palmitic acid-1-C^{14}. The amount of fatty acid absorbed was calculated by subtracting the amount found in the gastrointestinal tract and feces from the administered dose.

The carcasses were digested with alcoholic KOH, 30% KOH in 48% ethanol, the digest filtered through glass wool after evaporation of the alcohol and made to volume. An aliquot was then extracted with petroleum ether and analyzed for cholesterol radioactivity as for the livers. Saponifiable fat was determined gravimetrically.

The amount of radioactivity in the urine was determined by plating 1-ml aliquots of a 50-fold dilution of 24-hour urine collection onto aluminum plates and counting with the mass in the aliquot assumed to be negligible.

RESULTS

The incorporation of acetate-1-C^{14} during a 3-hour period into $C^{14}O_2$ and liver lipids by the deficient rats and their pair-fed controls is presented in table 1 and figures 1 and 2. As shown in figure 1 the deficient group in 3 hours incorporated 79.7% of the injected dose of acetate-1-C^{14} into $C^{14}O_2$ as compared with 67.2% by the pair-fed control group. This difference was found to be highly significant ($P < 0.01$). The deficient rats incorporated more acetate-1-C^{14} into $C^{14}O_2$ in spite of their smaller total CO_2 production, 5290 mg $BaCO_3$ compared with 6385 mg by the controls. The difference would therefore be greater when expressed in terms of the amount of CO_2 produced per gram of body weight, 12572 count/min/g body weight compared with 9972 by the pair-fed controls.

All of the pair-fed control rats and 4 of the 6 deficient rats achieved maximal specific activity in respiratory CO_2 during the

[4] Wesson Oil, The Wesson Oil Company, New Orleans, Louisiana.

TABLE 1

Incorporation of acetate-1-C^{14} into cholesterol and fatty acids of livers of deficient and pair-fed control rats after diet was fed for 5 weeks

	Pantothenic acid-deficient	Pair-fed controls
No. of rats	6	6
Body weight, g	83 ± 4[1]	96 ± 3
Liver weight, g	3.64 ± 0.17	4.50 ± 0.21
Acetate-1-C^{14} dose total count/min × 10^{-3}	1283	1422
Total CO_2 production, mg $BaCO_3$	5290 ± 556	6383 ± 542
Liver cholesterol, %	0.183 ± 0.008	0.176 ± 0.005
Incorporation of acetate-1-C^{14} into liver cholesterol, %	0.118 ± 0.033	0.042 ± 0.009
Liver fatty acids, %	2.94 ± 0.19	2.96 ± 0.30
Incorporation of acetate-1-C^{14} into liver fatty acids, %	2.81 ± 1.00	3.37 ± 0.38

[1] Mean ± SE.

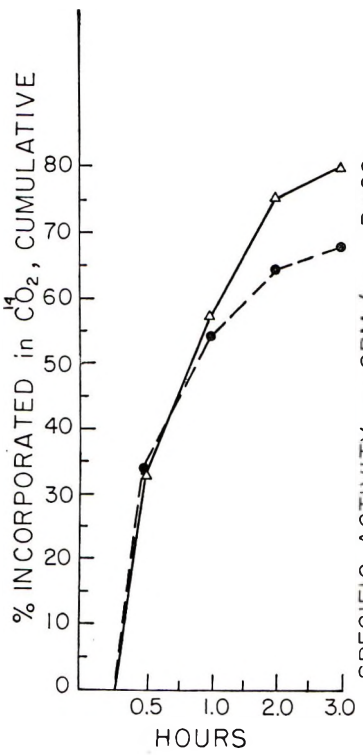

Figure 1

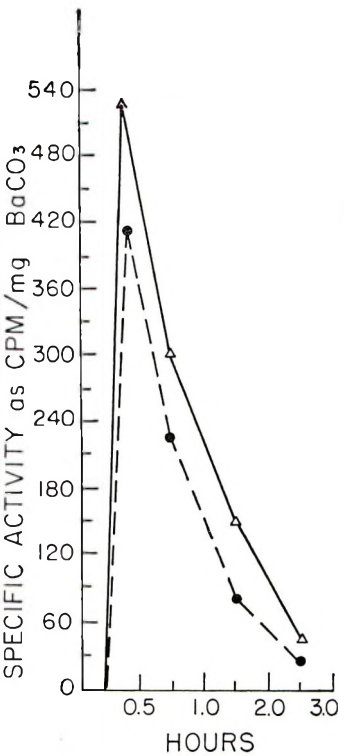

Figure 2

Fig. 1 Rate of incorporation of acetate-1-C^{14} into expired $C^{14}O_2$ in 3 hours by deficient, △ — △, and pair-fed rats, ● — ●.

Fig. 2 Rate of incorporation of acetate-1-C^{14} expressed as count/min/mg $BaCO_3$ in expired $C^{14}O_2$ in 3 hours by deficient, △ — △, and pair-fed rats, ● — ●.

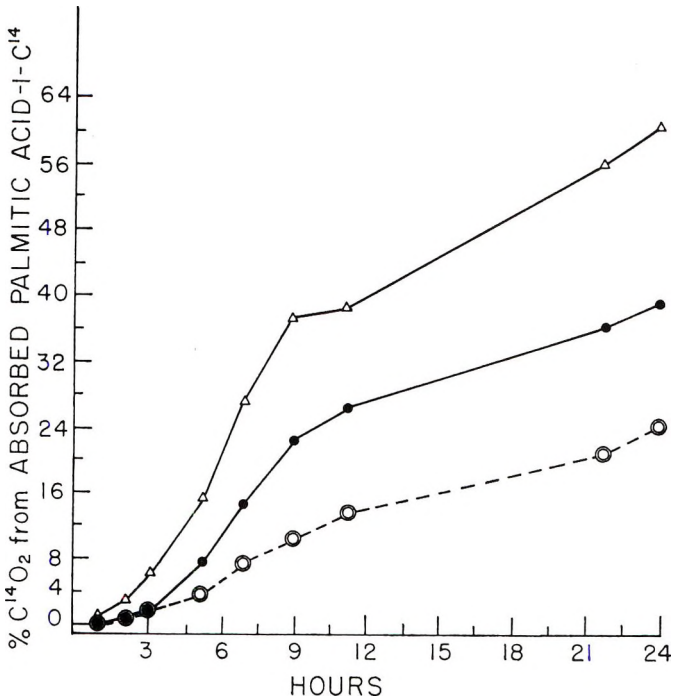

Fig. 3 Percentage of $C^{14}O_2$ produced from absorbed palmitic acid-1-C^{14} in 24 hours by deficient, △ — △, pair-weighed, ◎ — ◎, and ad libitum-fed normal control rats, ● — ●.

first half hour after injection of the acetate. Two of the deficient animals achieved maximal specific activity in their respiratory CO_2 by the end of the second half hour. Utilization of the acetate was thus initially slower in these 2 rats but despite this they incorporated more acetate-1-C^{14} into $C^{14}O_2$ by the end of the third hour than did their pair-fed controls (fig. 2).

The deficient rats incorporated 3 times as much acetate-1-C^{14} into liver cholesterol as did the pair-fed controls (table 1) although the cholesterol concentration in the liver did not differ significantly between the 2 groups. Due to large variations in the percentage incorporation of acetate-1-C^{14} into liver fatty acids, the difference between the means for the 2 groups of animals was not significant. However, incorporation of acetate-1-C^{14} into liver fatty acids was not decreased in the pantothenic acid-deficient rat.

The adrenal glands of the deficient rats contained 1.18% cholesterol and those of the pair-fed controls 2.35%. Except for one animal that had 1345 count/min in the glands, moderate amounts of activity, average 22 count/min, were present in the adrenal glands of the deficient rats. The glands of 3 of the pair-fed control group had small amounts of activity, the other 3 had none. The mean total adrenal cholesterol of the deficient rats was 0.225 mg, of the pair-fed rats 0.490. The deficient animals appeared to have incorporated more acetate-1-C^{14} into the adrenal cholesterol in 3 hours than did the pair-fed controls.

In a second experiment the absorption, oxidation, and incorporation of palmitic acid-1-C^{14} into liver and body cholesterol by pantothenic acid-deficient rats and their pair-weighed controls was studied. In figure 3 are presented the data for the oxidation of palmitic acid-1-C^{14} by these rats.

The mean body weights of the animals in this series were, 9 deficient, 86 g; 9 pair-weighed, 87 g; and 3 ad libitum controls, 178 g. The percentages of absorption of palmitic acid-1-C^{14} were, deficient, 89.4 ± 3.1; pair-weighed, 82.9 ± 9.3; and ad libitum, 93.0. The CO_2 production in milligrams $BaCO_3$ per gram body weight

TABLE 2

Incorporation of palmitic acid-1-C^{14} into liver and carcass cholesterol and fat in 24 hours

	Liver			Carcass		
	Deficient	Pair-weighed	Ad libitum	Deficient	Pair-weighed	Ad libitum
No. of rats	9	9	3	9	9	3
Weight, g	3.03 ± 0.005[2]	2.94 ± 0.09	6.66	74.9 ± 1.5	75.1 ± 2.3	149.3
Cholesterol, %	0.223 ± 0.005	0.237 ± 0.008	0.251	0.082 ± 0.006	0.069 ± 0.006	0.035
Absorbed activity in cholesterol, %	0.043 ± 0.004	0.026 ± 0.003	0.016	0.067 ± 0.002[1]	0.028 ± 0.002	0.016
Fatty acids, %	2.94 ± 0.023	2.86 ± 0.35	2.59			
Absorbed activity in fatty acids, %	3.26 ± 0.34	2.77 ± 0.40	2.15			
Saponifiable fat, %				2.78 ± 0.26[1]	4.05 ± 0.36	7.16
Absorbed activity in saponifiable fat, %				21.5 ± 1.75[1]	35.3 ± 3.07	25.6

[1] Significant difference ($P < 0.01$).
[2] Mean ± SE.

per hour was, by the deficient group 14.2 ± 3.4, by the pair-weighed group 14.1 ± 1.7 and by the ad libitum group 12.5.

No impairment of absorption of fatty acid by pantothenic acid-deprivation was indicated. The deficient group oxidized almost 3 times as much of the labeled material during 24 hours as did the pair-weighed control group. The difference was not due to increased general metabolism, the CO_2 production being essentially the same for both groups of rats. Oxidation of palmitic acid-1-C^{14} by the deficient group was considerably greater than that of the control groups at all times during the experimental period.

Table 2 presents the data for the incorporation of palmitic acid-1-C^{14} into liver and carcass cholesterol. The deficient group incorporated 0.043% of the absorbed C^{14} dose into liver cholesterol compared with 0.026% by the pair-weighed control group. The 3 ad libitum-fed rats averaged 0.016% of the absorbed C^{14} dose in their liver cholesterol. The differences were even more pronounced in the carcass cholesterol-C^{14} values. These were 0.067, 0.028, and 0.016%, respectively, for the deficient, pair-weighed, and ad libitum-fed groups. There was no significant difference in cholesterol concentration in the liver or carcass of deficient and pair-weighed control groups.

The amount of palmitic acid-1-C^{14} remaining as fatty acid is of interest. In the liver there was no significant difference in the amount of radioactivity present as fatty acid-C^{14} among the 3 groups of rats. In the carcass the deficient group had 21.5% of the absorbed activity remaining as fatty acid-C^{14} after 24 hours compared with 35.3% for the pair-weighed control group. The percentage fat in the carcass was not significantly different in the deficient and pair-weighed control groups.

The amount of C^{14} excreted in the urine during the 24-hour period was found to be 1.31% of the absorbed dose for the deficient group, 0.74% for the pair-weighed, and 0.75% in the ad libitum control group. These differences were not significant.

DISCUSSION

The results indicate that fat utilization is at least not decreased in the pantothenic

acid-deficient rat. The normal rate of CO_2 production and the increased rate of oxidation of both acetate-1-C^{14} and palmitic acid-1-C^{14} suggest that as the deficient state progresses the rat may metabolize fat in preference to carbohydrate. After a 24-hour period the deficient group retained 21.5% of the radioactivity from palmitic acid-1-C^{14} in the saponifiable lipid fraction of the carcass as compared with 35.3% for the pair-weighed group. A block in carbohydrate utilization at the pyruvate level in pantothenic acid deficiency has been well demonstrated (2,3,17,18).

The incorporation of C^{14} from both acetate and palmitic acid into carcass cholesterol was significantly higher in the deficient group. Since the concentration of cholesterol in the liver and carcass of the deficient rats did not differ from that of the pair-weighed control group, this may be an indication of increased turnover of cholesterol in the deficient rats.

These results, however, can be interpreted by the explanation suggested by Tolbert et al. (11). These authors postulated that the pool of acetyl coenzyme A may be reduced in the pantothenic acid-deficient rats so that there will be less dilution of the labeled acetate and the specific activity of the acetate being oxidized or incorporated into fatty acids and cholesterol would be greater.

Also, since only tracer amounts of acetate-1-C^{14} or palmitate-1-C^{14} were given, the size of the endogenous pool is more important in this study. In the in vitro work of Klein and Lipmann (1), the possible effect of the endogenous pool of acetate was virtually eliminated by a large amount of nonlabeled acetate (30 μmoles/5 ml) together with the labeled acetate. This procedure may have permitted the effect of the decreased coenzyme A concentration to be demonstrated. On the other hand, Lata and Anderson (13) observed that liver slices from rats fed a pantothenic acid-deficient diet for 4 to 5 months incorporated more acetate-1-C^{14} into cholesterol even when the total amount of nonlabeled acetate was 125 μmoles in 5 ml. Their rats however were older than those of Klein and Lipmann, and the deficient rats had lower levels of liver cholesterol.

The greater oxidation of palmitic acid-1-C^{14} might also reflect that acetate-C^{14} from palmitic acid-C^{14} may not have mixed with the acetate from other sources since acetyl coenzyme A derived from palmitate stays bound to coenzyme A and is not mixed with the acetate pool of the liver (5).

If fat oxidation were increased, one might expect ketosis in the pantothenic acid-deficient rat. However, ketosis is not a known symptom of pantothenic acid deficiency. This is probably due to the fact that pyruvic acid metabolism, because of the block in the oxidation pathway, proceeds via CO_2 fixation to L-malic acid, which in turn is a precursor of oxaloacetic acid. An adequate supply of oxaloacetic acid facilitates the oxidation of acetyl coenzyme A via the citric acid cycle.

One would expect the oxidation of both acetate and long-chain fatty acids to be slower in the deficient animals since both processes require coenzyme A. Under these conditions, accumulation of fat in the livers might be expected. But the failure of such fat accumulation may be due to the excessive utilization of fat along oxidative pathways as suggested in these experiments. That body and skin fat are significantly lower in animals deprived of pantothenic acid was found by Morgan and Lewis (7). This may be secondary to excessive utilization rather than to impaired lipogenesis as these experiments failed to demonstrate a significant difference in the rate of incorporation of acetate-1-C^{14} into liver fatty acids.

These experiments also demonstrated no impairment of absorption of fatty acid in the deficient rat. Bloom et al. (19) reported no difference in the rate of absorption of palmitic acid and tripalmitin. Thus, unless there is a specific block in triglyceride hydrolysis in the deficient rat, the absorption of fatty acids from dietary fat would not be expected to be impaired.

That survival of pantothenic acid-deficient rats is dependent upon adequate dietary fat has been demonstrated by Granados and Verzar (20). These investigators observed that elimination of fat from the diet reduced survival of the deficient rats by 50% compared with only 7% of the controls. A similar importance of dietary fat was noted for adrenalectomized ani-

mals. Since pantothenic acid-deficient rats have been shown to demonstrate symptoms of adrenal insufficiency one might speculate that the increased utilization of fat by the pantothenic acid-deficient rats is influenced by 11-oxysteroid insufficiency.

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Human Metabolism of L-Ascorbic Acid and Erythorbic Acid[1,2]

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ABSTRACT Human subjects were partially depleted of ascorbic acid and then were given supplements of either L-ascorbic acid or erythorbic acid. At the end of supplementation, subjects who had received erythorbic acid were given a 300-mg load dose of this isomer. White cell and plasma concentrations and urinary excretion of total ascorbic acid were followed. The plasma was first to deplete in progressive ascorbic acid deficiency and with L-ascorbic acid supplementation plasma concentrations remained low until white cells were repleted. Plasma concentrations near 0.2 mg/100 ml were associated with white cell concentrations ranging from 9 to 23 mg/100 g. Results with erythorbic acid supplementation show that the uptake or "tissue fixation" of L-ascorbic acid is structurally specific for the L-configuration about carbon 5. The rejection of erythorbic acid by the white cells explains the high urinary excretion of this isomer and its apparent lack of anti-scorbutic activity. Maximal total body ascorbic acid, based on the assumption that white cell concentrations are proportional to body tissues in general, was calculated to be 4 g.

Erythorbic acid, differing from L-ascorbic acid only in the spacial configuration about the fifth carbon atom, has little or no anti-scorbutic activity (1–3), yet it is fully as active as the vitamin in several cell-free enzyme reactions (4–6). Wang (7) and Kadin and Osadca (8) have reported that erythorbic acid and L-ascorbic acid, when given in load doses to human subjects, produce comparable elevations in plasma ascorbic acid concentration for periods of 3 to 4 hours, but the investigation in this laboratory (7) showed that erythorbic acid supplementation to low ascorbic acid diets would not maintain fasting plasma ascorbic acid concentrations in human subjects. It has also been shown in the human that from 50 to 70% of a dose of erythorbic acid is excreted within 24 hours if given orally (7) and within 6 hours if given intravenously (9).

The studies cited suggest that the demonstrated lack of anti-scorbutic activity of erythorbic acid is due primarily to its molecular structure which may limit its uptake by the tissues. The present investigation is a comparison of the physiological response of healthy human male subjects, partially depleted of ascorbic acid, to oral intakes of the 2 isomers. The criteria were white cells and plasma total ascorbic acid concentrations and urinary excretion of total ascorbic acid. A balance study and a load test were conducted.

EXPERIMENTAL PROCEDURE

Four healthy men, 20 to 21 years old, were fed diets low in ascorbic acid for the 12 weeks of the study. The analyzed total ascorbic acid content of the daily diet for the first 5 weeks was 13.0 ± 2.6 mg; for the remaining 7 weeks, 6.8 ± 2.1 mg. The ascorbic acid-containing foods of the diets were analyzed for total ascorbic acid by the method of Roe and Oesterling (10). The calculated intake of all nutrients, other than ascorbic acid, exceeded the recommended dietary allowances of the National Research Council (11). Details of the dietary regimen, sampling, recording and calculation are described by Rivers.[5]

The subjects were fed the ascorbic acid depletion diet for 8 weeks. Following this

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[5] Rivers, J. M. 1962 Human metabolism of L-ascorbic acid and erythorbic acid. Ph.D. Thesis, The Pennsylvania State University.

the diets of 2 subjects were supplemented with L-ascorbic acid and the diets of the other two with erythorbic acid. The daily oral dose was given with the noon meal and for the first 2 weeks of supplementations it was 50 mg; for the last 2 weeks, 100 mg.

At the end of the supplementation period the 2 subjects who had received erythorbic acid were given a 300-mg oral load dose of this compound, one hour after the noon meal. The meal contained 4.2 mg of total ascorbic acid by analysis.

White cell and plasma total ascorbic acid determinations were made weekly for each subject during the depletion period; at 3- and 4-day intervals throughout the supplementation period; and at predetermined intervals for a period of 19 hours following the ingestion of the load dose. Venous blood was used. Except for the load test, all blood samples were collected with the subjects in the post-absorptive state. Plasma was analyzed for total ascorbic acid by the method of Bessey (12). White cell total ascorbic acid was determined by the method of Bessey et al. (13). Triplicate determinations were made on plasma and white cells obtained from one blood sample.

Duplicate determinations of urinary total ascorbic acid were made for each subject on one 24-hour collection each week during depletion and at 3- and 4-day intervals during supplementation. Each voiding of urine, for a 19-hour period following the ingestion of the load dose, was collected and analyzed for total ascorbic acid. The samples were preserved during collection with toluene and oxalic acid and the total ascorbic acid content was determined by the method of Roe and Kuether (14).

Because of the difference in K values of the isomers (7), calculation of any increase over pre-dose levels in plasma ascorbic acid concentrations following the ingestion of erythorbic acid was made using the K value for erythorbic acid. Calculations of urinary total ascorbic acid following the ingestion of erythorbic acid were based on a calibration curve of this isomer.

Levels of probability were determined by an analysis of variance of the data.

RESULTS

Depletion period. White cell total ascorbic acid concentration decreased from an average of 27.6 mg to 15.3 mg/100 g (table 1); plasma ascorbic acid concentration decreased from an average of 0.54 mg to 0.17 mg/100 ml (table 1). These decreases over the 8-week period were highly significant ($P < 0.001$). The rate of white cell ascorbic acid depletion was not constant for all subjects, but at the end of the depletion period there was no significant difference ($P > 0.05$) between the 2 pairs which were to receive different treatments during supplementation, in either white cell or plasma ascorbic acid concentrations.

The average excretion of total ascorbic acid for all subjects during depletion was 9.4 mg/24 hours (table 1). The decrease in urinary excretion of ascorbic acid during the depletion period was not significant ($P > 0.05$). Although there was a highly significant variation among the 4 subjects in urinary total ascorbic acid ($P < 0.001$), the difference between the 2 pairs which were to receive the 2 forms of supplementation was not significant ($P > 0.05$).

Supplementation period. The 50-mg daily supplement of L-ascorbic acid for 14 days caused an average increase in white cell ascorbic acid concentration of 5.9 mg/100 g; with the 100-mg supplement, average white cell ascorbic acid concentrations increased an additional 8.5 mg/100 g (table 1). This increase with time was highly significant ($P < 0.001$). White cell ascorbic acid concentrations reached saturation levels in both subjects since no further increase was observed for the last 7 days of supplementation. Plasma ascorbic acid concentrations increased only slightly (0.04 mg/100 ml) with the 50-mg daily L-ascorbic acid supplement (table 1). The 100-mg daily supplement caused plasma ascorbic acid concentrations to increase to 0.51 mg/100 ml which was approximately equal to that noted at the start of the experiment. This increase in plasma ascorbic acid concentrations was highly significant ($P < 0.001$).

The L-ascorbic acid supplementations caused a slight but inconsistent increase in urinary excretion of ascorbic acid in the partially depleted subjects (table 1).

TABLE 1

Average total ascorbic acid values for white cells, plasma and urine of subjects[1] during depletion and supplementation with either L-ascorbic acid or erythorbic acid

Time	Form of supplement									
	Amount of supplement	White cell total ascorbic acid			Plasma total ascorbic acid			Urinary total ascorbic acid		
		L-Ascorbic acid	None	Erythorbic acid	L-Ascorbic acid	None	Erythorbic acid	L-Ascorbic acid	None	Erythorbic acid
days	*mg/day*	*mg/100 g*			*mg/100 g*			*mg/100 g*		
0	0		27.6			0.54			11.5	
7	0		25.5			0.38			13.6	
14	0		25.7			0.29			12.0	
21	0		22.6			0.25			8.7	
28	0		21.4			0.19			8.8	
35	0		21.0			0.17			7.1	
42	0		20.4			0.16			6.8	
49	0		16.9			0.20			9.0	
64	0	15.1	15.3	15.5	0.17	0.17	0.16	7.3	7.1	6.9
64	50									
67	50	16.3		11.5	0.17		0.15	8.1		37.9
71	50	17.9		11.6	0.22		0.15	10.6		37.3
74	50	22.3		14.4	0.17		0.12	8.3		35.5
78	50	21.0		11.3	0.21		0.18	9.3		42.8
78	100									
81	100	27.0		12.4	0.34		0.21	10.2		53.4
85	100	27.7		11.7	0.35		0.20	11.5		57.4
88	100	32.1		10.8	0.46		0.18	13.2		58.8
91	100	30.8		11.3	0.46		0.16	—		—
95	100	29.5		9.7	0.51		0.17	—		—

[1] Average of 4 subjects during depletion and of 2 subjects receiving each type of supplementation.

This increase was not significant ($P > 0.05$).

Results obtained with equal quantities of erythorbic acid supplementation are in sharp contrast with those obtained with L-ascorbic acid. White cell ascorbic acid concentrations declined 4.2 mg/100 g during 50-mg supplementation and an additional 1.6 mg/100 g during the 100-mg supplementation (table 1). This decrease was highly significant ($P < 0.001$). The change in fasting plasma concentration of ascorbic acid was not significant ($P > 0.05$). With the 50-mg daily supplement, the average change in plasma ascorbic acid concentration was from 0.16 to 0.18 mg/100 ml; with 100-mg daily supplement from 0.18 to 0.17 mg/100 ml (table 1).

The average 24-hour excretion of ascorbic acid during supplementation with erythorbic acid is presented in table 1. With the 50- and 100-mg supplement the average daily excretion was 38.4 and 56.6 mg, respectively.

Load test. Results obtained following the ingestion of a 300-mg load dose of erythorbic acid are presented in table 2.

TABLE 2

Average white cell, plasma and urine levels of total ascorbic acid following ingestion of 300 mg of erythorbic acid by two subjects partially depleted of ascorbic acid.

Time	Ascorbic acid		
	White cell	Plasma	Urine
hours	*mg/100 g*	*mg/100 ml*	*mg*
0	11.28	0.16	
0.5	9.84	0.21	
1.25	10.18	0.41	
3	11.53	0.50	39.3
6	11.81	0.33	
7			39.4
12			24.8
19	9.61	0.17	15.7

The elevation in plasma ascorbic acid concentration after the load dose was highly significant ($P < 0.001$). The highest average concentration measured was 0.5 mg/100 ml and this was reached at the 3-hour sampling. Fasting plasma ascorbic acid concentrations, 19 hours after ingestion of the dose, were down to pre-dose levels.

The urinary return of total ascorbic acid in 19 hours accounted for 37.2% of the ingested load dose. Twenty-five per cent

was excreted within the first 7 hours after ingestion of the load dose.

DISCUSSION

Depletion period. Plasma ascorbic acid concentrations averaged 0.19 mg/100 ml at the fourth week and varied little from this value for the remainder of the depletion period. White cell ascorbic acid concentrations which ranged from 9 to 23 mg/100 g were associated with plasma concentrations near 0.2 mg/100 ml. Plasma concentrations near this level are therefore indicative of a poor level of ascorbic acid nutriture, but are not an accurate basis for assessing the extent of deficiency. A comparison of white cell and plasma ascorbic acid concentrations at other time intervals shows that plasma concentrations greater than 0.5 mg/100 ml were accompanied by white cell concentrations at or near saturation levels; when plasma concentrations were greater than 0.3 mg/100 ml, white cell concentrations were greater than 25 mg/100 g. These results are in close agreement with those reported by Lowry et al. (15), Steele et al. (16), and Davey et al. (17) under similar conditions of ascorbic acid deprivation.

Urinary excretion of total ascorbic acid on the 6.8 ± 2.1-mg ascorbic acid diet without supplementation (last 3 weeks of depletion) averaged 7.7 mg daily for the two subjects who were to receive supplements of L-ascorbic acid and 7.6 mg daily for the two who were to receive supplements of erythorbic acid. These values are assumed to represent basal excretion at this level of dietary intake and would therefore exist during the supplementation period. In the discussion which follows, these basal values have been subtracted from total excretion to obtain an estimate of the excretion due to supplementation.

Supplementation period. During the initial 14 days of supplementation with L-ascorbic acid, white cell ascorbic acid levels appeared to reach a plateau, suggesting that a daily 50-mg supplement to a 6.8 ± 2.1-mg ascorbic acid diet will not produce white cell saturation levels in young men. Morse et al. (18) reported that a daily intake of 57 mg of ascorbic acid resulted in saturation of white cells in women. Haines et al. (19) in a study of tissue reserves of ascorbic acid, observed that a 70-mg daily intake would not maintain tissue saturation but a plateau was reached below the saturation level.

A comparison of plasma and white cell ascorbic acid concentrations during repletion with L-ascorbic acid shows the relationship to agree very closely with that obtained during depletion. White cell ascorbic acid concentrations increased to 21 mg/100 g before any significant increase occurred in plasma concentrations. When plasma concentrations reached 0.34 mg/100 ml, white cell concentrations were 27 mg/100 g, or approaching saturation levels. Thus, the plasma was the first to be depleted in progressive ascorbic acid deficiency and with supplementation plasma concentrations remained very low until the tissues had taken up their required amounts.

The L-ascorbic acid supplementation did not increase urinary ascorbic acid significantly. Deduction of basal excretion from total ascorbic acid excretion during supplementation shows that 97.6 and 96.1% of the 50-mg and 100-mg supplements, respectively, were retained.

The decline in white cell ascorbic acid concentration with erythorbic acid supplementation shows that the uptake or tissue fixation of L-ascorbic acid by white cells is structurally specific for the L- configuration about carbon 5. The fact that white cells reject erythorbic acid explains the high urinary excretion of this isomer, which was noted in this study, and as reported by Wang (7) and Ikeuchi (9).

That erythorbic acid will prolong the survival of guinea pigs fed a scorbutigenic diet is well documented (1–3). Reiff and Free (20) have suggested that this effect of erythorbic acid is due to its protection of residual stores of L-ascorbic acid in the body. The rejection of erythorbic acid by white cells and the decrease in the rate of white cell depletion with the increase in the size of erythorbic acid supplement shown in this study also indicate that the action of erythorbic acid is due to its protection of L-ascorbic acid rather than vitamin C activity, per se. The apparent lack of anti-scorbutic activity of this isomer in

the intact animal must be due primarily to limitations on its uptake by the tissues.

Erythorbic acid supplementation caused no change in fasting plasma ascorbic acid concentrations. That further decrease did not occur concurrently with the decrease in white cell ascorbic acid concentration was expected. Lowry et al. (15) observed plasma concentrations to remain close to 0.2 mg/100 ml, while white cell concentrations decreased to approximately 12 mg/100 g.

Urinary excretion data during erythorbic acid supplementation substantiate the conclusion that white cells reject this isomer. The high returns in partially depleted subjects, 61.8 and 49.1% of the 50-mg and 100-mg supplements, respectively, agree with the results of Wang (7) who noted a 50 to 60% return in subjects who apparently had no tissue deficit. A logical explanation for that part of the ingested dose of erythorbic acid which was not accounted for in the urine is that oxidation proceeded beyond the stage of 2,3-diketo-D-gluconic acid and therefore was not detected with the phenylhydrazine method.

Load test. Results obtained with the load test substantiate the conclusions drawn from the balance study. Plasma ascorbic acid concentrations increased for a period of hours after ingestion of the dose but after 19 hours plasma concentrations had decreased to pre-dose levels. A slight elevation in white cell ascorbic acid concentrations was noted at the 3- and 6-hour sampling. The significance of this increase is questionable; it might represent erythorbic acid diffusion with a lack of cellular fixation since fasting concentrations were down to pre-dose levels. The rapidity of excretion of the ingested erythorbic acid agrees with results reported by Wang (7) and by Ikeuchi (9).

Estimate of total body ascorbic acid. Calculations based on these data have been made for the maximal amount of L-ascorbic acid required to increase white cell ascorbic acid concentrations by 1 mg/100 g. The calculations have been extended to maximal total body ascorbic acid concentrations. The calculated values are presented in table 3. Unless the formula for calculation is specified, the values are averages of the analyzed results.

The basic assumptions are that 1) white cell ascorbic acid concentrations are proportional to the mass of metabolizing body tissue and that the amount of ascorbic acid required to saturate the white cells is a gauge of the total amount required to saturate the body, and 2) all L-ascorbic acid retained during supplementation was utilized for tissue repletion.

The maximal L-ascorbic acid required to increase white cell ascorbic acid concentrations 1 mg/100 g was 115 and 137 mg at the 50- and 100-mg level of supplementations, respectively. The greater amount required at the 100-mg level of supplementation indicates that more L-ascorbic acid was destroyed at the higher intake.

From zero supplementation time through 28 days, the change in white cell ascorbic acid concentrations for these 2 subjects

TABLE 3

Calculation of L-*ascorbic acid retention*

Ascorbic acid	Duration of supplementation		
	0–14 days	14–28 days	0–28 days
Daily supplement, L-ascorbic acid, mg	50	100	
Daily total excretion, total ascorbic acid, mg	9.1	11.6	
Daily basal excretion, total ascorbic acid, mg	7.7	7.7	
Increase in white cell total ascorbic acid, mg/100 g	5.9	9.8	15.7
Maximum retention, total ascorbic acid, mg/14 days	680	1345	2025
Maximum retention per mg/100 g increase in total white cell ascorbic acid, mg	115	137	129

[1] Maximum retention, mg/14 days = [Amount of supplement – (total excretion – basal excretion)] × 14.

was from 15.1 to 30.8 mg/100 g, an increase of 15.7 mg/100 g. The maximal L-ascorbic acid retention during this time was 2025 mg. Extrapolation of this retained ascorbic acid needed to increase white cell concentration by 15.7 mg/100 g gives a value of 1948 mg of ascorbic acid to obtain the initial 15.1 mg/100 g above zero level. The calculated total ascorbic acid in the body at the final saturation level of 30.8 mg/100 g is the sum of these or 3975 mg. Lowry et al. (15), on the basis of results obtained by giving massive load doses to partially depleted subjects, estimated maximal total body ascorbic content at about 4 g.

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Effect of Lysine Deficiency on Chagas' Disease in Laboratory Rats[1,2]

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ABSTRACT Laboratory rats fed a purified diet containing a low quality protein, gluten, were more susceptible to infection with *Trypanosoma cruzi* than rats fed a diet containing a high quality protein, casein. Furthermore, supplementation of the gluten diet with lysine markedly reduced the susceptibility of rats to near control values. Chagas' disease, caused by an infection with *T. cruzi*, manifests itself by high blood parasite counts, extensive cardiac damage, and an increased mortality in the rats fed the unsupplemented gluten diet. Although supplementation of the gluten diet with the most limiting amino acid, lysine, reduced the signs and symptoms of Chagas' disease in rats, the average parasitemia and degree of cardiac damage were not reduced to the values shown for the control animals.

Nutritional problems are commonly encountered in areas where infectious and parasitic disease are most prevalent. The relationship between nutritional deficiency and susceptibility to disease has been studied by many investigators for several decades but much additional work is needed in this field. Scrimshaw et al. (1) published a comprehensive review on the interactions of nutrition and infection.

Trypanosoma cruzi, a flagellated protozoon, is the etiologic agent of Chagas' disease. A World Health Organization report (2) estimates that several million of the inhabitants of South and Central America are infected with this species of parasite. The disease is most often chronic in nature, and myocarditis is one of the more common symptoms. Although the course of the disease can be modified in many instances by chemotherapy, the infection is not, as yet, curable. Consequently, preventive measures are most important in control of Chagas' disease.

T. cruzi differs from the other species of trypanosomes which infect man in that multiplication neither occurs extracellularly nor in the trypanosome stage. Instead, the trypanosome penetrates a tissue cell, transforms into an oval nonflagellated leishmania stage, multiplies by binary fission, transforms into the trypanosome stage, and then escapes from the ruptured cell into the body fluids. This cycle results in the destruction of many cells and eventual organ dysfunction in acute infections as well as many chronic cases.

Earlier we reported (3–6) on the effect of deficiencies of thiamine, pantothenic acid, pyridoxine and riboflavin on the course of *T. cruzi* infection in albino rats; only in studies on riboflavin were we unable to demonstrate a detrimental effect. More recently we found that acute deficiencies of vitamin A (7) or biotin[3] produced a significant reduction in the resistance of rats to infection with *T. cruzi*. The following is a report of similar studies on the effect of lysine deficiency, one of a series of investigations on protein malnutrition.

MATERIALS AND METHODS

Sixty weanling male rats, Carworth CFN strain,[4] were divided into 3 groups of 20 each. Each group was fed ad libitum one of the isonitrogenous diets shown in table 1. Control diet A contained 18% casein which provided sufficient quantities of the amino acids essential for excellent growth of young rats. Diet B contained

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[3] Yaeger, R. G., and O. N. Miller 1962 Effect of biotin deficiency on parasitemia by *T. cruzi*. Federation Proc., 21(2): 390 (abstract).

[4] Carworth Farms, New City, New York.

TABLE 1

Percentage composition of experimental diets

	Diet		
	A	B	C
Casein	18.0	—	—
Gluten (wheat)	—	19.6	19.0
Glycine	—	0.5	—
L-Lysine·HCl	—	—	1.1
Salt mixture[1]	4.0	4.0	4.0
Vitamin mixture, fat-soluble	2.0	2.0	2.0
Vitamin mixture, water-soluble	1.0	1.0	1.0
Corn oil	3.0	3.0	3.0
Choline chloride	0.2	0.2	0.2
Dextrose	71.8	69.7	69.7

[1] Hegsted et al. 1941 J. Biol. Chem., *138*: 459.

19.6% gluten which provided sufficient amounts of all essential amino acids except lysine which was present in approximately one-third of the required amount. Diet C was the same as gluten diet B except that lysine was added in sufficient quantity to bring the lysine content up to that of the control diet A. The vitamin mixtures and the salt mixture were the same as described previously (3). The rats were housed in individual metal cages with wire mesh floors to reduce coprophagy. Food consumption was determined daily; paper was placed under the cages to collect and correct for spilled food. Allowances were also made for food soilage by urine and feces. Water was given ad libitum in 250-ml bottles. All animals were weighed 3 times each week.

Twenty-three days after the start of the experiment, 10 rats from each group were inoculated intraperitoneally with a suspension of blood forms of the Tulahuen strain of *T. cruzi* obtained from stock-infected mice. Rats in groups A and C were each given 400,000 trypanosomes; rats in Group B were given 200,000 trypanosomes each because of their significantly smaller size. Parasitemia determinations were made by examining cover glass preparations of fresh tail blood. Counts were made on uniformly thin fields and were expressed as the number of trypanosomes per 200 fields, using 430 × magnification and wide field eyepieces. In instances where high parasite counts were encountered, the numbers of fields counted were reduced accordingly. The experiment was terminated on the 19th day post-infection when 6 of the infected group B rats had already died. Tissues of all rats that died or were killed were fixed in formalin; sections were later prepared by routine histologic methods.

A second experiment was carried out in the same manner as the first. In the later experiment, mortality was much lower in group B, but all rats were killed as before in order to have comparable studies of the tissues.

RESULTS

No significant differences in food intake or growth were observed between the 2 experiments. Hence only these data from the first experiment are presented.

Noninfected rats that received the casein control diet consumed this in normal amounts and grew at a normal rate (figs. 1, 2). Noninfected group B rats which had received the unsupplemented gluten-diet ate significantly less; their growth rate was even more depressed than food intake, suggesting inefficient utilization of the diet. Food consumption of the uninfected rats of group C, which had received the lysine-supplemented diet, fluctuated between those of groups A and C; growth more closely paralleled that of the control group that received the casein diet. As observed in previous experiments, food intake decreased significantly in all groups after infection. Growth rates for the infected series were only slightly less than observed for the noninfected series. This also had been noted in other studies and is attributed to the greatly reduced activity of the parasitized rats during the acute phase of infection. In studies in which the rats were observed for longer periods, food intake returned to normal in those animals that had recovered from the acute phase.

Figures 3 and 4 show the results of trypanosome counts on blood samples from inoculated rats in both experiments. Trypanosomes appeared in the blood earlier, increased more rapidly and reached significantly higher densities in rats fed the lysine-deficient diet. In the second experiment parasitemia was somewhat higher in all groups when the study was terminated. The 2 studies were made at different times of the year on different lots of rats and

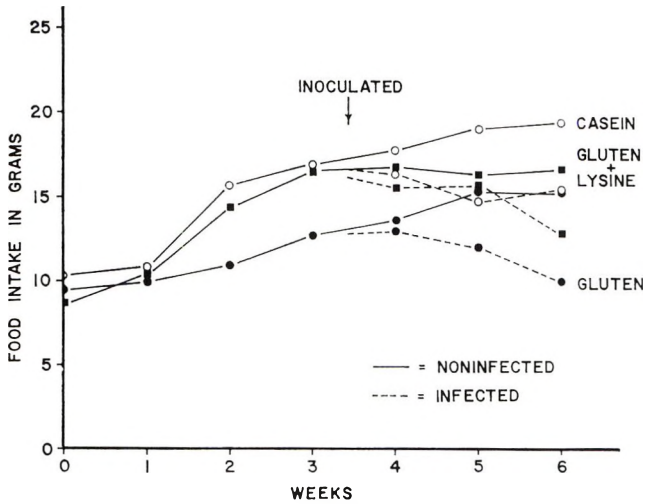

Fig. 1 The effect of protein quality and infection with *Trypanosoma cruzi* on daily food intake of rats in experiment 1; ○——○ represents rats fed a diet containing casein, ■——■ represents rats fed a diet containing gluten and supplemented with lysine, and ●——● represents rats fed a diet containing gluten.

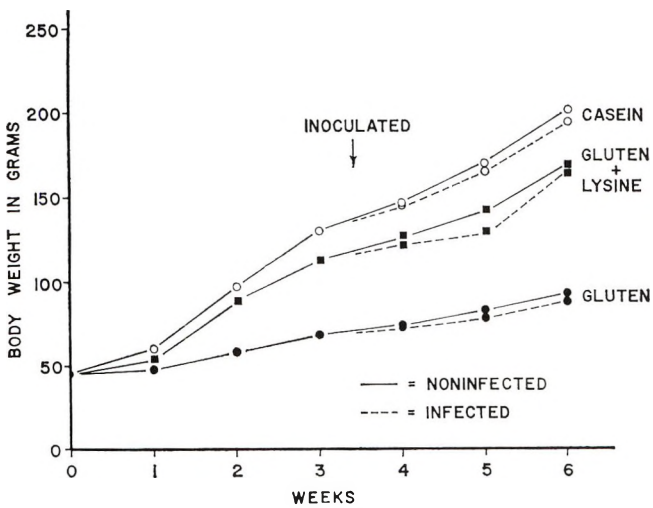

Fig. 2 The effect of protein quality and infection with *Trypanosoma cruzi* on the rate of growth of rats in experiment 1. See legend of figure 1 for code of symbols used.

there may have been some slight differences in susceptibility of the 2 series of rats. In both experiments the addition of lysine to the gluten-diet resulted in a significantly lower parasitemia.

As in previous studies, examination of tissue sections showed that the heart contained many parasites whereas, except for skeletal muscle, parasites were very few in other tissues. Tissue damage and parasites were significantly less in skeletal muscle than in heart muscle and because of the much greater importance of cardiac pathology, only data on this organ are presented. Cardiac damage was classified into 4 degrees. First degree [1] consisted of occasional small areas of infiltrated mononuclear cells; leishmania parasites were invariably absent in such instances. When these infiltrated areas were somewhat

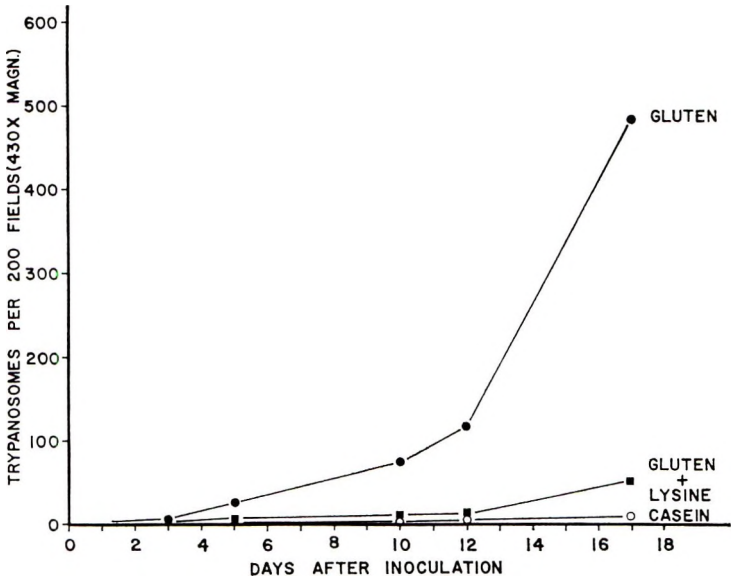

Fig. 3 The parasite count in the blood of rats in experiment 1 is plotted against time (days) after inoculation with *Trypanosoma cruzi*. See text for other details and figure 1 for code of symbols.

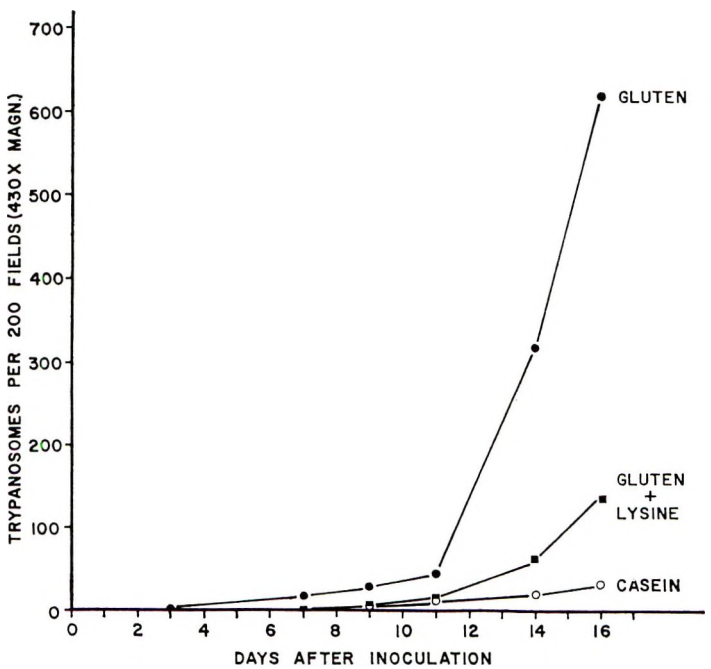

Fig. 4 The parasite count in the blood of rats in experiment 2 is plotted against time (days) after inoculation with *Trypanosoma cruzi*. See text for other details and figure 1 for code of symbols.

TABLE 2

Summary of observations on Trypanosoma cruzi infections in rats fed diets with casein, gluten or lysine-supplemented gluten as source of protein

Group	Experiment	Parasitemia[1]			Degree of cardiac damage[2]		
		Range of maximum counts	Range of averages for each rat	Group average	Range of individual rats	Group average	Mortality rate at time of killing
A Casein diet ad libitum	1	6–92	0.6–27.8	6.7	1–4	1.7	1/10
	2	2–202	3.0–37.8	14.9	1–4	2.2	2/10
B Gluten diet ad libitum	1	14–750	8.6–239.0	91.0	1–4	3.2	6/10
	2	16–2580	4.2–585.5	152.4	2–4	3.6	2/10
C Gluten diet plus lysine, ad libitum	1	4–278	0.2–60.2	17.5	1–4	2.2	1/10
	2	9–700	6.7–186.8	41.4	2–4	2.7	2/10

[1] Trypanosome counts are expressed as the number of parasites in 200 microscope fields at 430 × magnification with wide field eye pieces.
[2] Classification of cardiac damage is given in the text.

larger and more numerous and some areas of hypertrophy were observed, it was classed as second degree [2]; parasites were rarely found. Third degree [3] was used for hearts where more extensive infiltration, necrosis, edema and hypertrophy were observed; leishmania forms were usually present in small numbers. When extensive areas of necrosis, edema, marked infiltration with macrophages, polymorphonuclear leucocytes, lymphocytes and plasma cells were observed, it was classed as fourth degree [4] damage; in these instances leishmania parasites were usually numerous.

Studies of cardiac damage are presented in table 2 together with a summary of parasitemia and mortality observations. These data as well as the graphs on parasitemia are based on 10 rats/group for each experiment. Rats started to die on the afternoon of the 17th day and 16th day of experiments 1 and 2, respectively, but most deaths occurred on the following day in each instance. As observed in our previous studies, rats with the higher trypanosome counts usually showed more extensive cardiac pathology. Occasionally a rat with only a moderate parasitemia died. In such instances heart muscle contained many leishmania forms, and had the rat lived a day or two longer these would have appeared in the blood as trypanosomes. This has been observed more often in the few control rats that died and may be a reflection of the larger number of trypanosomes inoculated.

Mortality in rats fed the lysine-deficient diet was higher than in the other 2 groups of the first experiment. In addition, 3 rats of the group B in the second experiment were moribund at the time they were killed and probably would have died within a day or two. In studies in which the infections were permitted to run their full course, occasional rats have been observed to linger moribund for several days with a very high parasitemia; however, in most instances rats with a high parasitemia die sooner because of extensive tissue damage.

DISCUSSION

The weanling laboratory rat has proved to be an excellent host for studies on nutritional deficiency and susceptibility to *T. cruzi* infection. If these rats are infected after having been fed an adequate diet for 3 or 4 weeks, the majority of animals will develop a mildly acute infection which reaches a peak during the third or fourth week then subsides into a chronic or latent phase. After 3 or 4 months it is difficult and frequently impossible to detect infection by isolation of the parasite from blood, although antibody studies suggest that some parasites do persist, probably in the tissues. Parasites have been recovered by blood cultures but the percentage of positive cultures is indirectly proportional to the age of infection. Chagas' disease in man is similar in that most infections become chronic and persist, it is believed, for the life of the individual.

The data obtained in the present study showed higher average, as well as maximal parasitemias in the lysine-deficient rats. This is a reflection of the number of parasites that multiplied intracellularly and hence the degree of tissue damage; confirmation was obtained by histologic studies. Actor (8) studied infections with *Leishmania donovani* in mice that were fed protein-deficient diets; this species of parasite occurs intracellularly as the leishmania form, primarily in liver and spleen. Higher parasite densities were noted in the livers and spleens of mice that had received a protein-free diet prior to infection or when given such a diet 10 days post-infection; the increase in parasites was largely reversed by feeding the animals an adequate diet. The need for protein in sufficient quantity and adequate with respect to quality for maintenance of resistance to some bacterial infections was shown by Dubos and Schaedler (9). Among the experiments they reported were those in which mice receiving diets with gluten as the source of protein were found to be more susceptible than mice receiving diets in which an equal amount of casein was used. In contrast, Carrera et al. (10) reported no significant differences in susceptibility to infection with *Entamoeba histolytica* in guinea pigs given diets containing 30% casein, 5% casein, or 30% zein. *E. histolytica*, however, live in the lumen of the large intestine and may or may not invade the intestinal wall, depending upon factors not yet understood or known.

Observations made in the present study together with those of previous studies suggest that morbidity and mortality rates would be lower in individuals with Chagas' disease if they received an adequate diet for a sufficient period prior to infection. Getz et al. (11) studied a group of 1100 men and observed clearly active tuberculosis in individuals with markedly substandard blood serum values for vitamins A and C. Similar studies in regions where Chagas' disease is highly endemic may reveal a higher incidence as well as more severe manifestations of this infection in individuals with nutritional deficiencies. Of particular interest would be the lower income groups in the more rural areas where both malnutrition and the disease-transmitting triatomid bugs are found.

Thus far, the majority of studies of parasitic infections in animals have shown that nutritionally deficient animals are more susceptible than those that are adequately fed. Furthermore, such infections are more common in areas where malnutrition is most prevalent. Inasmuch as we cannot yet eradicate the majority of parasites, as well as other types of infections which are more severe when the individual is malnourished, adequate nutrition appears to be the best hope for controlling many kinds of infection in the populations of the world.

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Relationship of Excess Calcium and Phosphorus to Magnesium Requirement and Toxicity in Guinea Pigs[1,2]

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ABSTRACT Approximately 300 weanling male guinea pigs were used to determine the order of magnitude of the effect of excess dietary calcium and phosphorus upon the magnesium requirement. Growth rate and hemoglobin level were used as criteria of magnesium adequacy. Blood inorganic phosphorus was inversely correlated with the dietary magnesium level but was not a useful criterion. The nature of the toxicity of excess magnesium was also studied. In the presence of 0.8% phosphorus and 0.9% calcium the average magnesium requirement as measured by growth rate and hemoglobin level was 0.12%. Increasing calcium to 3.2% gave an average requirement of 0.20%. When calcium was 2.5% and phosphorus 1.7% the requirement was 0.40%. It was concluded that excess calcium and phosphorus independently increase the minimal magnesium requirement and that their effects are additive. The addition of 1.2% magnesium to a basal diet containing 0.9% calcium and 0.6% phosphorus had no detrimental effects. When the diet contained 1.7% phosphorus, excess magnesium caused high mortality and an extremely poor rate of growth. The damaging effect was alleviated completely by increasing calcium to 2.5%. Thus, excess magnesium was deleterious only when calcium was limiting.

Several investigators (1–3) have shown that high levels of dietary calcium increase the severity of magnesium-deficiency symptoms in the rat, but in these studies the effect on the minimal magnesium requirement was not determined. Hegsted et al. (3) concluded that excess calcium was detrimental at low levels of magnesium intake but, when the magnesium consumption was near the minimal requirement, the effect was too small to be detected. McAleese and Forbes (4) made a quantitative study of the effect of dietary calcium level on the magnesium requirement of the rat, and noted that the minimal requirement, as measured by several criteria, increased as the level of dietary calcium was increased. O'Dell et al. (5) observed that excess calcium increased the severity of magnesium deficiency in the guinea pig, but the quantitative aspects of the relationship were not investigated.

An excess of dietary phosphorus has also been shown to increase the magnesium requirement of the guinea pig (5). Even when adequate magnesium was provided, the maximal growth rate with the high phosphorus diet was about 2 g/day less than that attained with the more normal phosphorus levels. Additional calcium alleviated the growth-depressing effect of the high phosphorus diets, but the effect of the more favorable calcium-to-phosphorus ratio on the magnesium requirement was not determined. Bunce et al. (6) observed that an increase in the dietary phosphorus level caused more severe symptoms of magnesium deficiency in the weanling dog. The high calcium diets used by Tufts and Greenberg (1) contained more phosphorus than the lower calcium diets; thus some of the effects attributed to high calcium may have been due in part to the added phosphorus.

Elmslie and Steenbock (7) found 4 g of $MgCl_2$/100 g of diet to be toxic for the rat. Watchorn (8) observed that piebald rats fed a diet containing 0.4% calcium and 1.6% of magnesium grew poorly and developed urinary calculi, whereas those

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receiving 2.0% of calcium and 1.6% magnesium grew at a normal rate and remained in good health. Chickens fed a diet that contained 1.7% calcium, 1.2% of phosphorus and 1.4% of magnesium became nervous and weak and by 6 weeks were unable to stand erect and moved only when disturbed (9). Birds that received about the same percentages of calcium and phosphorus but only 0.14% of magnesium in the diet performed normally. Guinea pigs reared with a diet that contained 0.9% of calcium, 1.7% of phosphorus and 0.6% of magnesium grew at a slower rate than those fed 0.3% of magnesium (10).

The objectives of this investigation were to determine quantitatively the effect of excess calcium and of excess calcium and phosphorus on the magnesium requirement; to study the effect of excess dietary magnesium; and to further explore the pathology of magnesium deficiency in the guinea pig.

EXPERIMENTAL

Experimental conditions were the same as those described by O'Dell et al. (5). The major components of the magnesium-low basal diets were: Acid-washed casein, 30.0; sucrose, 43.3; cellulose,[4] 15.0; potassium acetate, 2.7; soybean oil, 4.0; and salts,[5] 5.0%. The vitamin supplement,[6] which was similar to that previously described (12), supplied the following quantities/100 g of diet: Thiamine·HCl, 1; riboflavin, 1; pyridoxine·HCl, 1; Ca pantothenate, 3; niacin, 5; folacin, 0.6, biotin, 0.02, cyanocobalamin, 0.003; α-tocopheryl acetate, 2; menadione, 1; inositol, 100; and ascorbic acid, 200 mg. Vitamin A, 2000 IU, and vitamin D, 285 IU, were added in a soybean oil premix which contained 12.5 mg of an antioxidant, ethoxyquin[7] (1, 2-dihydro-6-ethoxy-2, 2, 4-trimethylquinoline). Chlortetracycline·HCl was added at a level of 2.5 mg/100 g of diet. By analysis this diet contained 50 ppm of magnesium. Supplementary magnesium was added as the oxide, calcium as the carbonate and phosphorus as monosodium phosphate. Additions were made at the expense of sucrose.

The basal diets used in the requirement studies contained 3 combinations of calcium and phosphorus levels. These levels and the designation of the diets were as follows: Normal calcium and phosphorus (N-Ca), 0.9% calcium and 0.8% phosphorus; high calcium and normal phosphorus (H-Ca), 3.2% calcium and 0.8% phosphorus; high calcium and high phosphorus (H-Ca-P) 2.5% calcium and 1.7% phosphorus. In the magnesium toxicity study calcium was varied from 0.9 to 2.5% and phosphorus from 0.6 to 1.7%.

Blood inorganic phosphorus was determined by a modification of the method of King (13). Hemoglobin concentration was determined by the cyanmethemoglobin method of Drabkin (14) using a commer-

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

[5] The salts mixture supplied the following minerals, expressed as percentage of diet: $CaHPO_4$, 1.64; KH_2PO_4, 1.24; $CaCO_3$, 1.05; NaCl, 0.72; $FePO_4$ (soluble), 0.158; $MnSO_4 \cdot H_2O$, 0.098; KCl, 0.083; $CuSO_4 \cdot 5H_2O$, 0.0062; NaF, 0.0039; KI, 0.0024; $ZnSO_4 \cdot 7H_2O$, 0.0020; $AlK(SO_4)_2 \cdot 12H_2O$, 0.00094; $CoCl_2 \cdot 6H_2O$, 0.00024.

[6] The authors gratefully acknowledge gifts from the following donors: Ascorbic acid and biotin from Hoffmann-LaRoche, Inc., Nutley, New Jersey; folacin and Aureomycin from American Cyanamid Company, Pearl River, N. Y.; choline from Hoffmann-Taft, Springfield, Missouri; vitamin A from Distillation Products Industries, Rochester, New York and other vitamins from Merck Sharp and Dohme, Rahway, New Jersey.

[7] Santoquin, Monsanto Chemical Company, St. Louis.

TABLE 1

Performance of guinea pigs fed low magnesium diets and fed a control diet

Diet[1]	Dietary Ca	Dietary P	Growth rate[2]	Mortality rate[3]	Visible calcification[4]	Blood P	Hemoglobin
	%	%	*g/day*	%	%	*mg/100 ml*	*g/100 ml*
N-Ca	0.9	0.8	2.4 ± 0.3[5]	31(16)	80	7.2 ± 0.2(10)	10.6 ± 0.7(10)
H-Ca	3.2	0.8	0.6 ± 0.6	92(11)	64	5.8 ± 0.3(7)	10.8 ± 0.4(7)
H-Ca-P[6]	2.5	1.7	0.5 ± 0.3	58(12)	73	6.1 ± 0.9(4)	10.6 ± 0.3(4)
Stock diet	1.1	0.6	7.4 ± 0.6	0(12)	0	4.9 ± 0.2(12)	14.8 ± 0.2(12)

[1] See Experimental section in text for description of diets.
[2] Growth rate for a 4-week period. No mortality.
[3] Percentage of animals that died within 8 weeks. Number of animals started shown in parentheses.
[4] Percentage of animals that showed visible calcification at time of death or after 8 weeks.
[5] SE of mean.
[6] Contained 0.03% of supplemental magnesium.

cially prepared diluent and standard.[8] Both determinations were made on survivors of an 8-week trial.

RESULTS

Requirement studies. The performance of guinea pigs fed the lowest level of magnesium in each dietary series and of those fed a stock diet is summarized in table 1. In the H-Ca-P series the lowest level was the basal supplemented with 0.03% of magnesium and in the others it was the respective basal diets. The growth rates of the magnesium-deficient animals were grossly subnormal and the mortality rates and incidence of visible calcification were high. The blood inorganic phosphorus of the deficient animals was 0.9 to 2.3 mg/100 ml greater than of those fed the stock diet. Blood phosphorus, however, averaged 1.4 mg/100 ml less in animals fed the H-Ca basal than in those supplied with the N-Ca basal. All of the animals reared with the magnesium-deficient diets became anemic. Other symptoms typical of magnesium deficiency in the guinea pig (5, 15) were observed in animals fed these low-magnesium diets.

Growth rate was used as one criterion of the minimal magnesium requirement. Shown in figure 1 is a plot of the average daily gain for an 8-week period versus the logarithm of the dietary magnesium level.[9] Each point represents the average of 8 to 12 animals. The ascending limb of each curve was fitted to the data by the method of least squares, whereas the horizontal limb represents the average of the groups judged to have attained the maximal rate for the respective series. The point of intersection is considered to be the minimal requirement since the amount of magnesium in the basal diet was negligible. For animals fed the N-Ca diets (0.9% Ca and 0.8% P) the requirement for growth was about 100 mg/100 g of diet. The requirement for growth with the H-Ca diet (3.2% Ca and 0.8% P) was approximately 240 mg/100 g of diet, slightly greater than twice that for the N-Ca series. The maximal growth rate with the H-Ca-P diets (2.5% Ca and 1.7%

[8] Aculute diluent and Acuglobin standard from Ortho Pharmaceutical Corporation, Raritan, New Jersey.

[9] Snedecor, G. W. 1946 Statistical Methods, ed. 4. The Iowa State College Press, Ames, p. 108.

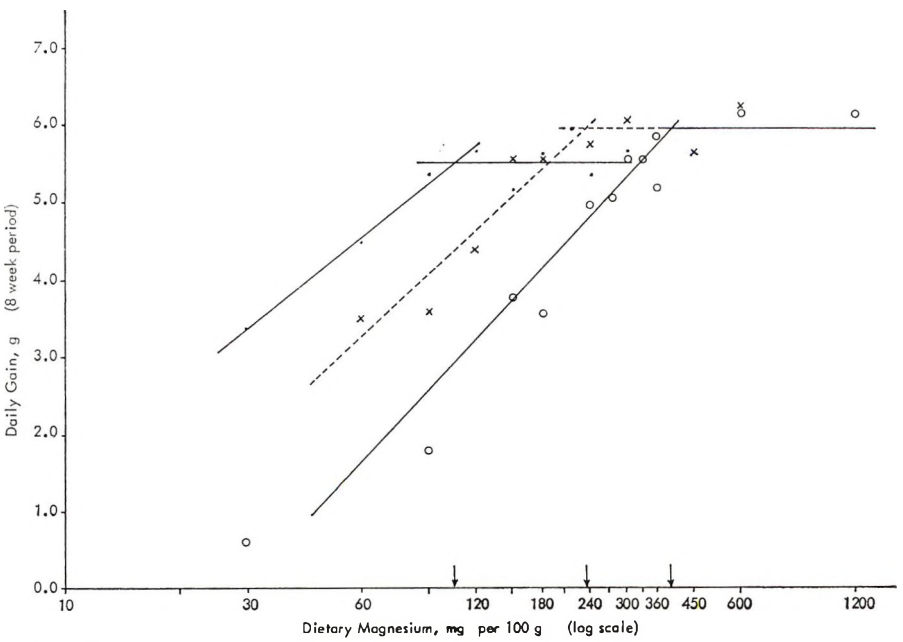

Fig. 1 Effect of calcium and phosphorus content of diet upon the magnesium requirement of the guinea pig. Growth rate used as criterion of adequacy. ●——● 0.9% Ca, 0.8% P; ×– – –× 3.2% Ca, 0.8% P; ○——○ 2.5% Ca, 1.7% P.

P) was attained only after the magnesium level was increased to 400 mg/100 g of diet. Thus, high levels of calcium and phosphorus, even when fed in an acceptable ratio, are additive in their effect on the magnesium requirement.

Since guinea pigs fed the low-magnesium diets developed anemia, hemoglobin concentration was also used as a criterion of adequacy. Determinations were made on blood from animals whose growth rates are shown in figure 1 and the results are shown in figure 2. Hemoglobin concentration in grams per 100 milliliters was plotted versus the logarithm of dietary magnesium and curves drawn as described for figure 1. Using maintenance of a maximal hemoglobin level as the criterion of adequacy, the dietary magnesium requirement was found to be 140, 170 and 400 mg/100 g of diet, respectively, for the N-Ca, H-Ca and H-Ca-P diets. These values are in reasonably good agreement with those determined using growth rate as the criterion of adequacy.

An elevated blood phosphorous concentration has been observed in guinea pigs fed diets deficient in potassium and magnesium (12) and also in the same species with an uncomplicated magnesium deficiency (15). In this investigation an attempt was made to correlate blood inorganic phosphorus with dietary magnesium levels. As shown by the results summar-

TABLE 2

Effect of dietary magnesium on blood inorganic phosphorus in guinea pigs fed various levels of calcium and phosphorus[1]

Added magnesium	Dietary calcium and phosphorus, %		
	0.8 P 0.9 Ca	0.8 P 3.2 Ca	1.7 P 2.5 Ca
%	Blood inorganic phosphorus, mg/100 ml		
None	7.2 ± 0.2[2]	5.8 ± 0.3	—
0.03	7.2 ± 0.5	4.8 ± 0.2	—
0.06	6.2 ± 0.5	5.0 ± 0.3	—
0.09	6.4 ± 0.2	4.6 ± 0.1	8.9 ± 0.7
0.12	6.5 ± 0.3	4.7 ± 0.2	—
0.15	6.1 ± 0.1	5.0 ± 0.3	7.2 ± 0.9
0.18	5.9 ± 0.3	4.8 ± 0.2	7.1 ± 0.3
0.24	6.0 ± 0.2	4.9 ± 0.3	8.6 ± 1.2
0.27	—	—	6.3 ± 0.2
0.30	5.5 ± 0.2	4.8 ± 0.2	7.1 ± 0.2
0.33	—	—	5.2 ± 0.2
0.36	—	—	6.2 ± 0.2

[1] The average values presented represent analysis of blood from 8 to 12 animals.
[2] SE of mean.

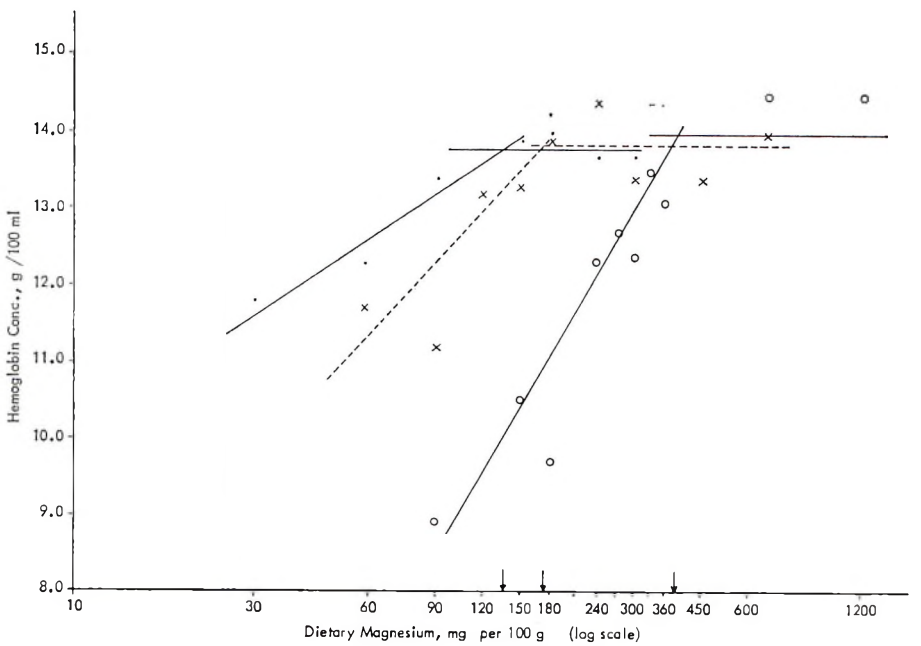

Fig. 2 Effect of calcium and phosphorus content of diet upon the magnesium requirement of the guinea pig. Hemoglobin concentration used as criterion of adequacy. —— 0.9% Ca, 0.8% P; ×– – – –× 3.2% Ca, 0.8% P; ○——○ 2.5% Ca, 1.7% P.

ized in table 2, magnesium decreased the blood inorganic phosphorus concentration when added to the deficient diets regardless of the calcium and phosphorus content. However, blood phosphorus did not prove to be a useful criterion for quantitative evaluation of the magnesium requirement. Excess dietary calcium decreased the blood phosphorus level at all levels of dietary magnesium when dietary phosphorus was maintained at 0.8%. However, those animals fed the H-Ca-P diets had elevated blood phosphorus levels even though the calcium-to-phosphorus ratio was 1.5 to 1.

TABLE 3

Effect of dietary calcium and phosphorus on the magnesium requirement of the guinea pig

Criterion of adequacy	Dietary calcium and phosphorus, %				
	0.4 P 0.9 Ca	0.8 P 0.9 Ca	1.7 P 0.9 Ca	0.8 P 3.2 Ca	1.7 P 2.5 Ca
	Magnesium requirement, % of diet				
Growth rate	0.08[1]	0.10	0.24[1]	0.24	0.40
Hemoglobin	—	0.14	—	0.17	0.39

[1] O'Dell et al. (5).

Table 3 summarizes the magnesium requirement of the guinea pig as determined in this and a previous study (5), using growth and maintenance of normal hemoglobin level as criteria of adequacy. It is clear that excess levels of both calcium and phosphorus increase the magnesium requirement and that their effects are roughly additive.

Excess dietary magnesium. Guinea pigs fed the H-Ca-P diets with 0.6 or 1.2% of magnesium grew at the maximal rate and had normal hemoglobin values (figs. 1, 2). However, in an earlier trial, in which the basal diet contained 0.9% of calcium and 1.7% of phosphorus, increasing magnesium from 0.3 to 0.6% was found to be highly detrimental to growth (10). Thus, guinea pigs were fed diets that contained various levels of calcium and phosphorus with 0.3 and 1.2% of magnesium. The results are summarized in table 4. The high level of magnesium, 1.2%, had no effect on the performance of guinea pigs when the diet contained 0.9 and 0.6% respectively, of calcium and phosphorus. However, when the diet contained 0.9% of calcium and 1.7% of phosphorus, the inclusion of 1.2% of magnesium caused a high mortality rate and depressed the growth rate markedly. Although the hemoglobin level was normal, these animals were lethargic, diarrheic, and exhibited a poor general appearance. When the calcium level was increased to 2.5% and the phosphorus level held at 1.7% the toxic effects of high magnesium were alleviated and the animals grew at a maximal rate.

Discussion. Previous work (5) showed that the magnesium requirement for maximal growth rate of guinea pigs was increased from 0.08 to 0.24% when the phosphorus level was changed from 0.4 to 1.7%. That the magnesium requirement is directly correlated with dietary phosphorus was confirmed in the present study. A portion of the effect of phosphorus may be attributed to decreased absorption of magnesium (16). If the low apparent absorption is due to the formation of slightly soluble magnesium phosphate, the effect would be expected to be most pronounced when the phosphorus is considerably in excess of the calcium. The data presented in table 3 appear to support such an hypothesis.

TABLE 4

Effect of dietary calcium and phosphorus concentrations on the toxicity of excess dietary magnesium (Eight-week period)

	Calculated diet composition, %					
Calcium	0.9	0.9	0.9	0.9	2.5	2.5
Phosphorus	0.6	0.6	1.7	1.7	1.7	1.7
Magnesium	0.3	1.2	0.3	1.2	0.3	1.2
Avg daily gain, g	6.1 ± 0.3[1]	5.8 ± 0.4	4.2 ± 0.3[2]	0.3 ± 0.2[2]	5.6 ± 0.3	6.2 ± 0.8
Mortality, %	8(23)[3]	0(8)	0(15)	42(12)	0(12)	0(7)
Hemoglobin, g/100 ml	13.6 ± 0.2	14.4 ± 0.1	12.8 ± 0.8	14.0 ± 0.1	12.4 ± 0.3	14.5 ± 1.8

[1] SE of mean.
[2] Difference between values is statistically significant, $P < 0.01$.
[3] Number of animals started on experiment.

The calcium-to-phosphorus ratio of the H-Ca diets used in the present study was greater than that used by McAleese and Forbes (4) and the effect on the magnesium requirement was also greater than in their studies. Although the mechanism by which calcium accentuates magnesium deficiency is not clear, there is evidence to suggest a direct physiological antagonism. Mendel and Benedict (17) injected calcium chloride intravenously into dogs and observed an increase in the urinary output of magnesium. Heller and Haddad (18) provided rats with calcium chloride or calcium hydroxide solutions instead of drinking water and reported that the magnesium balance was greatly depressed and the magnesium excretion by way of urine was elevated. Calcium-magnesium antagonism may be particularly significant for the guinea pig since this species exhibits a high apparent absorption of calcium and thus excretes a high proportion of any excess by way of the kidneys (16).

The physical and chemical properties of various compounds in the intestinal tract, particularly at the site of absorption, may materially affect the mineral metabolism of an animal. The high concentrations of calcium and phosphorus fed in the present study probably produced large quantities of insoluble calcium phosphate in the intestine. The large surface area thus formed by the calcium phosphate precipitate may adsorb ions and thus compete with the absorption process. Magnesium may be adsorbed onto the insoluble aggregates as has been postulated for manganese (19). In the case of the chick, an excess of both calcium and phosphorus increases the manganese requirement, whereas calcium alone has little effect.

Anemia due to magnesium deficiency has not been clearly demonstrated previously. Kruse et al. (20) reported low hematocrits in 2 magnesium-deficient dogs, but hemoglobin level was not mentioned. Wiancko (21) could find no effect of magnesium deficiency on the hemoglobin concentration, hematocrit, or red cell count of the rat. The minimal magnesium requirement of the guinea pig as assessed by maintenance of normal hemoglobin level agreed well with that determined by growth rate.

Another interrelationship among the various mineral constituents of the diet is shown by the effect of excess magnesium on the physiological performance of guinea pigs. In agreement with observations made on other species (8, 9) the present study shows that rather high levels of magnesium can be tolerated so long as calcium is not a limiting factor. Haag and Palmer (20) observed that excess magnesium depressed growth with high phosphorus diets only when the calcium level was low, but they also reported that high magnesium depressed the growth rate of rats fed a diet low in phosphorus regardless of the level of calcium. In the guinea pig 1.2% magnesium was highly detrimental when the diet contained 0.9% calcium and 1.7% phosphorus, but was completely innocuous when calcium was increased to 2.5%. Presumably excess phosphorus decreases the calcium availability to such a degree that 0.9% is suboptimal. Magnesium, acting as a physiological antagonist of calcium, then depresses the growth rate by precipitating a calcium deficiency.

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