Synthesis of Serum and Sub-cellular Liver Cholesterol Esters in Fasted and Fed Rats [1]

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ABSTRACT The formation of serum and liver sub-cellular liver cholesterol esters was investigated in fasting and fed rats. Rats were injected with cholesterol-4-^{14}C-labeled serum and the liver and serum cholesterol esters examined at 45, 90, and 180 minutes. There was a marked heterogeneity in the labeling of the cholesterol esters in the liver cell fractions and serum. In both fasting and fed rats the soluble fraction ^{14}C-esters had a higher proportion of ^{14}C-arachidonate than the particulate liver fractions. Fasting rats had a higher percentage of ^{14}C-arachidonate in the liver cell fractions and serum cholesterol esters than fed rats. There were also comparable differences in the cholesterol ester fatty acid composition. The differences in ^{14}C-activity distribution and cholesterol ester fatty acid composition were principally at the expense of monounsaturated esters. The specific activity of the liver cell fraction esters exceeded the specific activity of the corresponding esters in the serum. Cholesterol arachidonate in serum and liver had the highest fractional turnover rate. The data suggest that there is a compartmentalization of cholesterol ester metabolism in the liver and that the liver is the major source of the serum esters. The types of cholesterol esters synthesized in the liver and released into the blood are dependent on the nutritional status of the animal.

Recent studies (1–9) have provided evidence that there is a marked heterogeneity in turnover rates, fatty acid composition and synthesis among the cholesterol esters in rat and human liver and in rat blood. This heterogeneity also extends to the cholesterol esters in the different lipoproteins and sub-cellular liver fractions (5, 7–9). These differences in the metabolism of the cholesterol esters may be due to the existence of different hepatic pools of esters associated with the synthesis of the different lipoprotein fractions or to the enzyme systems involved in the synthesis of the different blood and liver cholesterol esters. Of particular significance is whether the blood cholesterol esters are synthesized predominantly within the liver or by the action of the blood cholesterol transferase enzyme (10, 11). The blood transferase enzyme has the requisite specificity to account for the large proportion of polyunsaturated fatty acids in the blood cholesterol esters. The liver has been shown to contain a particulate cholesterol-esterifying system which favors the formation of monounsaturated esters in the rat and the linoleate ester in man (12–15). In addition, it was recently shown (9, 16) that rat liver has a soluble enzyme system which favors the formation of cholesterol arachidonate. The soluble esterifying system was more active in the fasted than the fed rat (9). This correlates well with the observations that fasted rats have a higher proportion of arachidonic acid in the serum cholesterol esters than fed animals (17). The selective incorporation of polyunsaturated cholesterol esters into the high density lipoproteins and the non-selective incorporation of saturated and monounsaturated cholesterol esters into the low density lipoproteins at different sites in the liver and by different enzyme systems would be consistent with the view that the liver is the chief source of the blood cholesterol esters. This would account for the wide difference in fatty acid composition between the serum and liver esters. In the present report, data are presented which support the concept that the liver is a major source of the serum cholesterol esters, and that the types of cholesterol esters synthesized in the liver and released into the blood are constantly

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changing as a function of the nutritional status of the animal. The data also suggest the existence of several cholesterol ester pools in the liver.

EXPERIMENTAL

Male rats (Wistar strain), weighing 175 to 200 g and fed a stock pellet ration, were used. The terms fed and fasting rats refer to those animals that either had access to food at all times or were fasted 18 hours before they were killed. The solution for injection was prepared by dispersing (by ultrasonic disintegration for 2 minutes at 4°) a tracer amount (10 μCi) of cholesterol-4-^{14}C [2] in 0.5 ml rat serum. The ^{14}C-cholesterol was found to be uniformly dispersed in the serum by this procedure and no chemical changes were noted when the labeled serum was subsequently extracted and analyzed by thin-layer chromatography. The rats were injected in the saphenous vein with the ^{14}C-cholesterol serum, and groups of 5 animals were killed at 45, 90, and 180 minutes after the injection. Blood was obtained from the abdominal aorta and the liver was removed. The blood was immediately chilled at 4°, allowed to clot and centrifuged at 4° to separate the serum. This procedure prevented the formation of cholesterol esters in the blood due to the cholesterol transferase enzyme (10). The livers were homogenized with 0.25 M sucrose (1 part liver to 2.5 parts sucrose solution). The homogenate was centrifuged at 1000 × *g* for 10 minutes to remove nuclei and debris and then fractionated into mitochondria (10,000 × *g* for 30 minutes), microsomes (104,000 × *g* for 60 minutes), and soluble cell fraction. The serum and liver cell fractions were extracted with 20 volumes of 2:1 chloroform-methanol.

The serum and liver cell fraction lipids were separated by silicic acid column chromatography (18). Cholesterol esters were eluted with diethyl ether-petroleum ether (1:99, v/v) and free cholesterol with diethyl ether-petroleum (25:75 v/v). The ^{14}C-activity of the cholesterol fractions was determined by liquid scintillation counting.[3] The free cholesterol concentration was determined by the method of Sperry and Webb (19). The cholesterol ester fraction was treated as follows: The ^{14}C-activity distribution among the various cholesterol esters was determined by separating the cholesterol ester fraction into four major cholesterol ester classes (saturated, monounsaturated, linoleate, and arachidonate) by thin-layer chromatography on silica gel G impregnated with silver nitrate (20). Petroleum ether-diethyl ether (6:1, v/v) was used as the ascending solvent. The esters were visualized by spraying the plates with 2,7-dichlorofluoresceine and examining them under ultraviolet light. The respective ester zones were scraped from the thin-layer plates directly into liquid scintillation vials. Methanol was added to elute the esters and the ^{14}C-activity of the ester fractions determined by liquid scintillation counting. The thin-layer chromatography procedure was checked with known pure cholesterol-4-^{14}C esters (21). The recovery of ^{14}C-activity was determined for each ester when pure standards were analyzed singly and in mixtures. The recovery of each ester in these test mixtures ranged from 93 to 99%. Another portion of the cholesterol ester fraction was transmethylated with BF_3 in methanol to produce methyl esters and free cholesterol (22). The fatty acid composition of the methyl esters was determined by gas-liquid chromatography (6). The free cholesterol liberated from the cholesterol ester fractions was silanized to produce trimethylsilyl ethers and analyzed simultaneously for both mass and radioactivity by gas-liquid radiochromatography (23). The fatty acid composition of the cholesterol esters separated by thin-layer chromatography was determined by gas-liquid chromatography. The saturated ester fraction consisted predominantly of palmitate with a small amount of stearate. The monounsaturated ester fraction contained oleate and palmitoleate; the linoleate and arachidonate fractions contained 93 to 95% of these particular esters. The specific activity of each cholesterol ester was calculated from the specific activity of the total esterified fraction, the ^{14}C-activity distribution among the cholesterol esters, and the fatty acid composition. The validity of this procedure was further checked by comparing

[2] Nuclear-Chicago Corporation, Des Plains, Illinois.
[3] Nuclear-Chicago Corporation liquid spectrometer.

the specific activities obtained by the above procedure with the specific activities of the individual cholesterol ester classes isolated by thin-layer chromatography. The cholesterol ester fractions were isolated by thin-layer chromatography and transmethylated to produce free cholesterol and then analyzed for mass and ^{14}C-activity by gas-liquid radiochromatography (23). The above procedures agreed within ± 7%.

RESULTS

The distribution of ^{14}C-activity among the cholesterol esters of the liver cell fractions and serum 45 minutes after the injection of cholesterol-4-^{14}C is shown in table 1. The cholesterol ester labeling at 45 minutes was at a period considerably before equilibration of the ^{14}C-esters into the different ester pools had occurred. There was marked heterogeneity in the labeling among the cholesterol esters of the liver cell fractions and serum. In both fasted and fed rats the liver soluble fraction ^{14}C-cholesterol esters had a much higher proportion of ^{14}C-arachidonate and lower proportion of ^{14}C-monounsaturated esters than the particulate (microsomes and mitochondria) liver cell fractions ^{14}C-cholesterol esters. The composition of the mitochondrial and microsomal ^{14}C-cholesterol esters was similar. In both fasted and fed rats the liver soluble fraction ^{14}C-cholesterol esters more closely resembled the serum ^{14}C-cholesterol ester composition than the particulate ^{14}C-cholesterol esters. Comparison of the fed and fasted rat indicates that the liver cell fraction ^{14}C-cholesterol esters of the fasted animal contained a higher proportion of ^{14}C-arachidonate than the fed animal. These differences were principally at the expense of ^{14}C-monounsaturated esters. The serum ^{14}C-cholesterol esters of the fasted animal had a higher proportion of ^{14}C-arachidonate and lower percentage of ^{14}C-linoleate than the fasting rat. The above differences in ^{14}C-cholesterol ester composition were found to be significant ($P < 0.01$).

The cholesterol ester fatty acid composition data are shown in table 2. The cholesterol esters of the liver soluble fraction of both fasted and fed animals had a significantly higher proportion ($P < 0.01$) of arachidonic acid than the liver particulate fractions. The liver particulate fractions had nearly the same cholesterol ester fatty acid composition. These differences were principally at the expense of monounsaturated fatty acids. Comparison of the fed and fasted animals showed that the liver particulate and soluble cell fraction cholesterol esters of the fasted animal contained a significantly higher proportion ($P < 0.01$) of arachidonic acid than the fed animal. These differences were principally at the expense of monounsaturated cholesterol esters. The serum cholesterol esters of the fasting animal had a significantly higher proportion ($P < 0.01$) of arachidonic acid and a lower percentage of linoleic acid than the fed animal.

Comparison of the distribution of ^{14}C-activity in the esters and cholesterol ester fatty acid composition (tables 1 and 2) indicate that in both fasted and fed ani-

TABLE 1

Distribution of ^{14}C-activity in cholesterol esters

Tissue [1]	% of total ^{14}C-cholesterol esters			
	Saturated	Monounsaturated	Linoleate	Arachidonate
		Fasted rats		
Liver microsomes	14.1 ± 2.0 [2]	24.5 ± 6.0	25.2 ± 3.8	36.2 ± 8.1
Liver mitochondria	16.9 ± 3.6	25.2 ± 5.1	24.6 ± 3.1	33.3 ± 6.1
Liver soluble fraction	11.9 ± 1.6	16.6 ± 4.0	18.0 ± 4.0	53.5 ± 9.1
Serum	7.7 ± 0.8	4.7 ± 1.0	15.6 ± 3.1	72.0 ± 2.9
		Fed rats		
Liver microsomes	11.0 ± 1.9	37.9 ± 2.1	30.3 ± 2.1	20.8 ± 3.2
Liver mitochondria	14.8 ± 2.1	39.0 ± 3.6	29.7 ± 2.1	16.5 ± 3.7
Liver soluble fraction	7.6 ± 1.1	21.5 ± 2.1	26.4 ± 3.3	44.5 ± 5.7
Serum	10.5 ± 1.1	7.6 ± 1.0	29.0 ± 2.7	52.9 ± 3.0

[1] Rats were injected with 10 μCi cholesterol-4-^{14}C in serum and killed at 45 minutes.
[2] Each value represents the average of 5 to 6 animals ± SD.

TABLE 2

Cholesterol ester fatty acid composition

Tissue	% of total fatty acids [1]			
	Saturated	Monounsaturated	Linoleic	Arachidonic
		Fasted rats		
Liver microsomes	28.9 ± 6.7 [2]	28.0 ± 4.5	23.1 ± 3.8	20.0 ± 4.8
Liver mitochondria	26.4 ± 4.9	32.2 ± 6.0	23.4 ± 3.6	18.0 ± 5.6
Liver soluble fraction	30.4 ± 3.8	21.0 ± 4.7	21.0 ± 5.0	27.6 ± 5.2
Serum	17.0 ± 6.0	9.0 ± 2.3	20.9 ± 3.9	53.1 ± 6.3
		Fed rats		
Liver microsomes	27.5 ± 5.4	34.1 ± 2.1	26.0 ± 3.1	12.4 ± 1.8
Liver mitochondria	22.4 ± 5.6	41.0 ± 2.8	26.3 ± 3.6	10.3 ± 1.8
Liver soluble fraction	27.9 ± 4.1	29.0 ± 3.2	25.5 ± 3.1	17.7 ± 2.0
Serum	15.6 ± 1.6	14.7 ± 1.1	29.7 ± 1.8	40.0 ± 2.1

[1] Values shown are for 45-minute interval after cholesterol-4-^{14}C injection.
[2] Each value represents the average of 5 to 6 animals ± SD.

mals there was a considerably higher percentage of ^{14}C-arachidonate than arachidonic acid. These differences are a reflection of the higher fractional turnover rate of cholesterol arachidonate than the other cholesterol esters.

The specific activities of the individual cholesterol ester classes are shown in figures 1 and 2. In both fasted and fed animals the cholesterol esters of the liver cell fractions had a specific activity equal to and in most cases greater than the specific activities of the corresponding serum cholesterol esters. The specific activity of cholesterol arachidonate exceeded the specific activity of the other cholesterol esters in the serum and liver cell fractions of both fasted and fed rats. The saturated esters had the lowest specific activity. The specific activity of the soluble cell fraction arachidonate at 45 minutes exceeded the specific activity of the other cholesterol

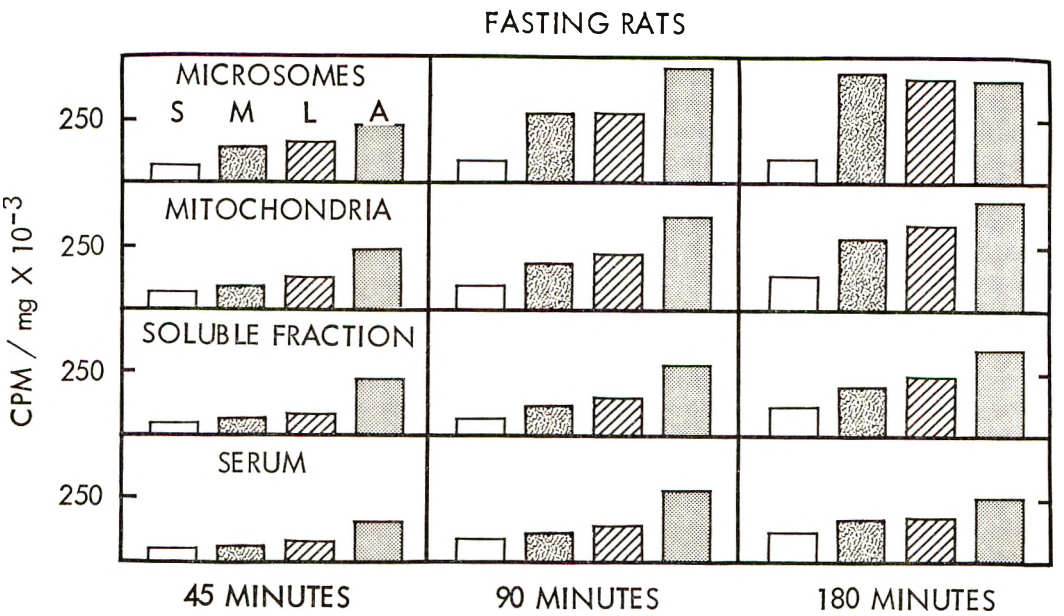

Fig. 1 Specific activities of the liver cell fraction and serum cholesterol esters of fasted rats. S = saturated, M = monounsaturated, L = linoleate, and A = arachidonate. (Values for the counts per minute shown in the figure have been derived by multiplying the actual values by 10^{-3}.)

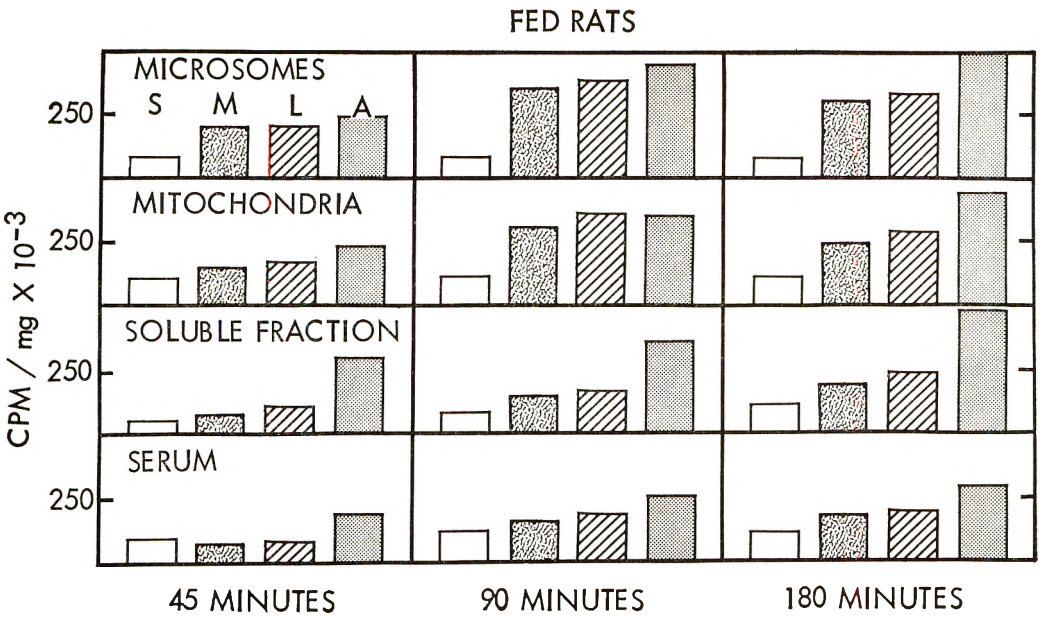

Fig. 2 Specific activities of the liver cell fraction and serum cholesterol esters of fed rats. S = saturated, M = monounsaturated, L = linoleate, and A = arachidonate. (Values for the counts per minutes shown in the figure have been derived by multiplying the actual values by 10^{-3}.)

esters in that cell fraction by a larger proportion than in the particulate fractions. The specific activities of the particulate monounsaturated and linoleate ester tended to be higher than those esters of the soluble cell fraction. The specific activities of the cholesterol esters in serum and liver of the fasted rats approached equilibrium at 180 minutes, whereas in the fed rat there were still marked differences among the different cholesterol esters at that time-period.

The specific activity of the free cholesterol fraction of the liver cell fractions was essentially the same in mitochondria, microsomes, and soluble fraction. There were no differences between fasting and fed animals. The serum free cholesterol fraction was somewhat lower than the free fraction in the liver.

DISCUSSION

The present studies have provided some new parameters with respect to the metabolism of serum and liver cholesterol esters. In an earlier report (9) it was shown that there was marked heterogeneity in the composition of the cholesterol esters synthesized by the liver cell fractions of fasting and fed rats. The results of the present study correlate well with these in vitro findings and suggest that the cholesterol esters synthesized by the liver are markedly influenced by the nutritional status of the animal. This in turn may reflect the types of cholesterol esters being transported from the liver into the blood since the fasted animal had a higher percentage of arachidonate in the serum cholesterol esters than the fed animal. The level of high and low density lipoproteins in the blood could account for the observed differences in fatty acid composition and labeling of the serum cholesterol esters.

The results of the present study also support the view that the serum cholesterol esters arise principally from the liver. The specific activity of the cholesterol esters of the liver exceeded the specific activity of the esters in the serum. The ester with the highest specific activity in both serum and liver was always cholesterol arachidonate. In view of the differences between the fatty acid composition of the serum and liver cholesterol esters it is probable that the serum esters arise from the liver by

highly selective processes. The enzymatic system in the liver soluble cell fraction may be intimately related to the synthesis of the serum cholesterol esters. Comparison of the ^{14}C-cholesterol esters in the soluble cell fraction with that of serum suggests a very close relationship. This has also been reported for human liver (7). Recent studies (9, 16) have indicated that there is present in the soluble cell fraction an enzymatic system which esterifies cholesterol. It has been demonstrated by one group of workers (16) that a cholesterol ester transferase enzyme similar to the one in blood occurs in this liver cell fraction. However, in recent studies [4] it was observed that there is another enzyme system present in rat liver soluble cell fraction which is markedly stimulated by arachidonyl CoA. This enzyme has been partially purified. The particulate rat liver system has been previously (13, 14) characterized and synthesizes predominantly saturated and monounsaturated cholesterol esters. This system may be primarily involved in the synthesis of the saturated and monounsaturated cholesterol esters which are found predominantly in the low density lipoproteins (8). The soluble esterifying system, however, may synthesize the cholesterol esters associated with the high density lipoproteins which are principally polyunsaturated (8).

Thus, it appears that the interplay of a number of factors controls the turnover of the serum and liver cholesterol esters. This includes the enzyme systems involved in the synthesis and hydrolysis of the serum and liver cholesterol esters and the nature of the fatty acid pool available for cholesterol esterification. The latter may be a reflection of the nutritional status of the animal. Finally, it is clear that all these factors must be taken into consideration in evaluating the metabolism of the serum and liver cholesterol esters.

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Urinary Nitrogen Excretion in Fowls Fed Acid or Alkali

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ABSTRACT Experiments were conducted to confirm the assumption that part of the excessive excretion of urinary ammonia of fowls fed a protein-free diet is associated with acid-base balance, and also to examine the influence of dietary administration of acid or alkali on urinary ammonia excretion. The urine of birds fed the protein-free diet showed a lower pH and larger titratable acidity than that of those fed 1 or 2% casein diets. When sodium bicarbonate was administered to fowls fed the protein-free diet, urinary ammonia excretion was markedly depressed. It was concluded from the data that the excessive excretion of urinary ammonia resulting from the protein-free diet is a regulator of body acid-base balance. Significantly increased or decreased excretion of urinary ammonia of birds fed the 20% casein diet was demonstrated by the administration of 10 and 15 mmoles of hydrochloric acid or sodium bicarbonate, respectively. Urinary ammonia excretion reached the minimal level with administration of 15 mmoles of sodium bicarbonate, and this level was considered to be of exogenous origin, being 55 and 37 mg in 20 and 10% casein diets, respectively. It was found that more ammonia appeared in the urine of birds fed the protein-free diet than in that of those fed the casein-containing diets.

According to Tasaki and Okumura (1), urinary excretion of uric acid and ammonia in fowls increased linearly with an increase in nitrogen intake, but when the fowls were fed a protein-free diet the excretion of ammonia was higher than that estimated from the regression equation calculated with the data of varying levels of dietary protein. O'Dell et al. (2) also reported that fowls fed a purified diet excreted more ammonia than those fed a commercial diet, and this difference was due to the acid-base balance of the experimental diets. Since the protein-free diet lowered the pH of urine, Tasaki and Okumura (1) assumed that part of the urinary ammonia thus excessively increased by feeding the protein-free diet was a regulator of the disturbed body acid-base balance. If this assumption were true, the administration of alkali might be expected to decrease the excretion of urinary ammonia.

According to Folin (3), the quantity of urea excretion in mammals is extremely variable as the result of protein ingestion, whereas ammonia, total creatinine and uric acid are excreted at a constant rate. In birds, however, the variable urinary constituents are uric acid and ammonia, and birds excrete much more ammonia than the mammals (1). The origin of urinary ammonia in birds can be considered as follows: (a) most of the ammonia which is deaminated from amino acids is normally converted into uric acid, but a portion escapes as ammonia itself, or (b) the potential metabolic acid loads increase the excretion of urinary ammonia since the end product of nitrogen metabolism in the fowl is uric acid.

The present experiments were carried out to confirm the assumption described, and to determine the effect of an acid or alkali load upon the urinary ammonia excretion of the fowl fed a protein-containing diet.

EXPERIMENTAL

Single Comb White Leghorn cockerels, approximately 8 months old, with an attached artificial anus, were used as the experimental animal. They were kept in individual metabolic cages and received daily 80 g of pelleted experimental diets.

The first experiment was undertaken to determine the major nitrogenous compounds, pH and titratable acidity of the urine when 3 birds in each group were fed zero, 1% or 2% casein diets.

The second experiment was designed to observe the relationship between ammonia

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excretion and alkali administration when a protein-free diet was fed. After the preliminary feeding of the protein-free diet for 5 days, 5 birds received daily 9 mmoles of sodium bicarbonate, in addition to the protein-free diet. The sodium bicarbonate was mixed in the protein-free diet, and the amount used (9 mmoles) was twice the equivalent of the ammonia excreted when the protein-free diet was fed alone.

In the third experiment, the influence of feeding acid or alkali on the excretion of urinary ammonia was examined. After the preliminary feeding of a 20% casein diet for 5 days, the birds were divided into 2 groups of 5 birds each. They were then fed daily for 3 days each at level 5, 10, and 15 mmoles of either sodium bicarbonate or hydrochloric acid mixed into the casein diet.

The fourth experiment was undertaken to find a minimal level of urinary ammonia excretion with the 10 and 20% casein diets. The experimental procedure was the same as in the third experiment and birds were given 5, 10, 15, 20, 25, 30, and 35 mmoles sodium bicarbonate.

In each experiment urine samples were collected daily, and the nitrogenous constituents or pH were analyzed or measured. The experimental diet, experimental procedure and analytical methods for urinary nitrogenous constituents have been described in a previous paper (1). The homogenized urine was neutralized with 0.1 N sodium hydroxide in the presence of a few drops of 0.1% phenolphthalein solution until a faint pink color persisted. The volumes of 0.1 N sodium hydroxide consumed were indicated as the titratable acidity. The pH of the homogenized urine was measured at 37° with a glass rod pH meter.

RESULTS AND DISCUSSION

The major nitrogenous compounds excreted in urine per metabolic body size ($W^{0.705}$) in the first experiment are shown in table 1, where W indicates body weight of the birds in kilograms, and the power, 0.705, is used according to Mitchell (4) for White Leghorn fowls. The total excretion of urinary nitrogen of birds fed the protein-free diet was not significantly different from that of birds fed the 1 or 2% casein diets. Tasaki and Okumura (1) reported previously that there was no significant difference in the total urinary nitrogen excretion between in fowls fed a protein-free diet and a 3% casein diet. Ariyoshi (5) observed that the total urinary nitrogen excretion in birds that received 1 g of whole egg protein daily was not significantly different from that in birds receiving a protein-free diet. Mitchell and Carman (6) also found that the total urinary nitrogen excretion in rats fed a protein-free diet was not significantly different from that in rats fed a diet containing 4% whole egg protein. In this respect, the data obtained in the present experiment agree well with observations of Tasaki and Okumura, Ariyoshi, and Mitchell and Carman, which suggest that there was no significant difference in total urinary nitrogen excretion with feeding a protein-free or a low protein diet.

Table 1 also shows that uric acid excretion was very constant when birds were fed zero, 1, and 2% casein diets, whereas the titratable acidity decreased gradually

TABLE 1

Major nitrogenous compounds,[1] titratable acidity[2] and pH of urine with protein-free or low protein diets

Casein in diet	Total N	Uric acid N	Ammonia N	Urea N	Total creatinine N	Titratable acidity	pH
%							
0	182 ± 16 [3]	97 ± 7	55 ± 8	4 ± 1	10 ± 1	4.99 ± 0.33	6.44
1	152 ± 13	99 ± 8	33 ± 2	4 ± 3	6 ± 2	4.37 ± 0.57	6.68
2	172 ± 11	102 ± 7	46 ± 4	11 ± 2	6 ± 2	3.16 ± 0.32	6.64

[1] Milligrams $N/W_{kg}^{0.705}$ in 24 hours.
[2] Equivalent millimoles of free acids in urine to bring its original pH to the phenolphthalein end point.
[3] Mean ± SE of mean.

with the increase of casein intake. However, more ammonia was excreted when birds were fed the protein-free diet than when fed the 1% casein diet. From these results, it appears that part of the urinary ammonia excreted by the protein-free diet group is accompanied by some acidic substances. The basis for this assumption is the changing pH of the urine. If the markedly increased excretion of ammonia is associated with the acid-base balance of the body fluid, the ammonia excretion should be depressed by the ingestion of alkali- or base-yielding feed.

The effect of ingested alkali on the excretion of ammonia and total nitrogen excretion in the fowl fed the protein-free diet is shown in figure 1. The excretion of ammonia responded markedly to the sodium bicarbonate administration, and the total urinary nitrogen excretion tended to respond, though no significant difference was observed. The result of this experiment provides conclusive evidence that birds fed the protein-free diet do not excrete any essential amount of ammonia, and the amount of ammonia excreted is considered to have resulted from the regulation of acid-base balance in the body.

As shown in table 1, a higher excretion of total creatinine was observed when birds were fed the protein-free diet. This high excretion reflects the excessive breakdown of body protein. This is shown by the loss of body weight in birds fed a protein-free diet (about 13 g for 8 days), whereas birds fed 1 or 2% casein diets maintained their body weight. The excretion of urea nitrogen when the 2% casein diet was fed was about 3 times more than that with the protein-free or 1% casein diets, though in a previous report (1) it was shown that the excretion of urea with the protein-free diet was 2 times more than that with the 3% casein diet. Owen and Robinson (7) concluded that, using the Sperber technique, the chicken could synthesize urea from exogenous or endogenous plasma arginine via a rate-limiting process, and this urea was subsequently excreted into tubular urine by direct release. In this experiment, however, the effect of the casein level on urea excretion of the fowl was not explained.

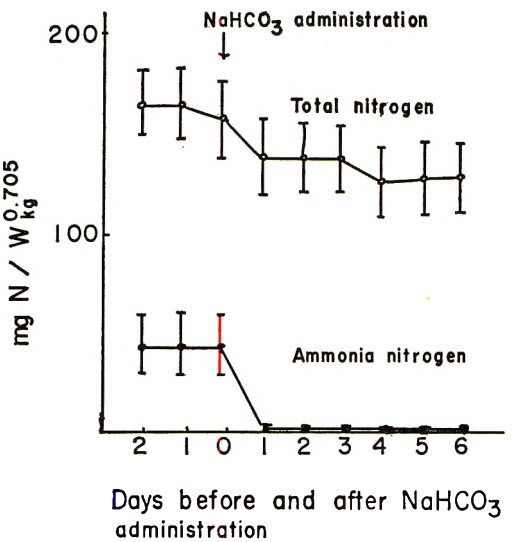

Fig. 1 Effect of sodium bicarbonate administration on urinary excretion of total nitrogen and ammonia in fowls fed a protein-free diet.

Distribution of the major urinary nitrogen compounds of fowls given different loads of acid or alkali when fed the 20% casein diet is shown in table 2.

The administration of sodium bicarbonate or hydrochloric acid tended to decrease or increase the total nitrogen excretion, respectively.

Administration of 10 or 15 mmoles of hydrochloric acid increased the ammonia excretion significantly, whereas no significant effect was observed with administration of 5 mmoles hydrochloric acid. In the case of alkali administration, 5 mmoles of sodium bicarbonate did not have a significant effect on ammonia excretion even though this amount of sodium bicarbonate was equivalent to 140 mg of ammonia nitrogen in alkalinity. A significant depression of urinary ammonia ouput was observed with the administration of more than 10 mmoles of sodium bicarbonate. Specifically, the administration of 15 mmoles of sodium bicarbonate decreased urinary ammonia excretion markedly and subsequently the urinary pH became alkaline.

In the present experiment, the administration of hydrochloric acid or sodium bicarbonate did not significantly change the excretion of uric acid, urea or total creatinine, though Wolbach (8) observed in birds that the administration of hydro-

TABLE 2

Distribution of major nitrogenous compounds [1] of urine of fowls given different loads of acid or alkali when fed 20% casein diet

Load of acid or alkali	Total N	Uric acid N	Ammonia N	Urea N	Total creatinine N	pH
Control	746 ± 31 [2]	641 ± 38	83 ± 4	7 ± 2	5 ± 1	6.15 ± 0.22
HCl, 5 mmoles	755 ± 40	577 ± 47	96 ± 9	8 ± 2	4 ± 1	5.63 ± 0.10
HCl, 10 mmoles	868 ± 40	576 ± 33	157 ± 9	9 ± 4	5 ± 1	5.55 ± 0.08
HCl, 15 mmoles	837 ± 38	593 ± 77	159 ± 8	8 ± 2	5 ± 1	5.58 ± 0.09
Control	795 ± 31	651 ± 31	87 ± 6	5 ± 2	5 ± 1	5.66 ± 0.32
$NaHCO_3$, 5 mmoles	744 ± 38	637 ± 33	77 ± 5	7 ± 3	5 ± 1	6.52 ± 0.23
$NaHCO_3$, 10 mmoles	701 ± 24	608 ± 27	63 ± 5	5 ± 1	5 ± 1	6.88 ± 0.21
$NaHCO_3$, 15 mmoles	687 ± 31	611 ± 57	39 ± 3	5 ± 1	5 ± 1	8.31 ± 0.56

[1] Milligrams $N/W_{kg}^{0.705}$ in 24 hours.

[2] Mean ± SE of mean.

chloric acid increased the renal excretion of ammonia, and simultaneously decreased the excretion of uric acid.

The question remains whether the administration of more than 15 mmoles of sodium bicarbonate would further lower ammonia excretion, and whether the lowering effect of sodium bicarbonate would also be shown in birds fed other levels of protein. Figure 2 shows the effect of the administration of graded levels of sodium bicarbonate on the excretion of urinary ammonia in fowls fed 10 and 20% casein diets. Birds fed the 20% casein diet excreted more ammonia than those fed the 10% casein diet, but the administration of sodium bicarbonate lowered ammonia excretion at the same rate in both groups. In birds fed both protein levels, urinary ammonia excretion reached the minimum with administration of 15 mmoles of sodium bicarbonate, and no greater ammonia excretion was observed with administration of more than 15 mmoles of sodium bicarbonate. The minimal levels of the urinary ammonia excretion with the 10 and 20% casein diets were 37 and 55 mg nitrogen per $W^{0.705}$ kg, respectively. These levels of ammonia excretion are considered to be of exogenous origin.

Comparison of figures 1 and 2 shows that the rate of depression of ammonia ex-

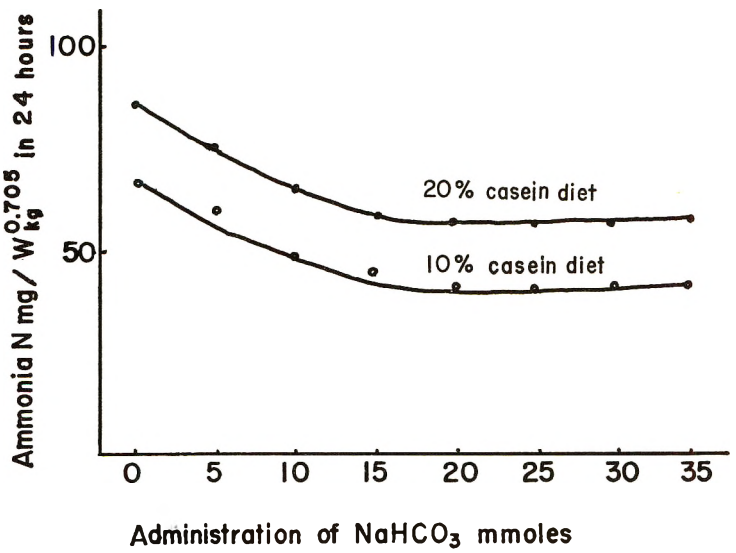

Fig. 2 Effect of administration of graded levels of sodium bicarbonate on the excretion of urinary ammonia in fowls fed 10 and 20% casein diets.

cretion caused by sodium bicarbonate administration was much greater with the protein-free diet than with the casein diet. This fact suggests that more ammonia is associated with the acid-base balance in birds fed the protein-free diet than in birds fed the protein-containing diet.

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Effects of Pyridoxine Deficiency on the Lymphatic Organs and Certain Blood Components of the Neonatal Chicken [1]

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ABSTRACT The effects of pyridoxide deficiency on the lymphatic organs and selected blood components were studied in the neonatal chicken. One hundred and fifty newly hatched broiler-type chicks were alloted to 3 treatments in three replicate experiments of 2-week duration. Treatments were 1) purified vitamin B_6-deficient diet, 2) purified vitamin B_6-adequate diet, and 3) practical vitamin B_6-adequate diet, ad libitum. Typical clinical signs of vitamin B_6 deficiency were observed with the first treatment. There was a significant atrophy of the spleen, thymus and bursa in the deficient chicks as well as a microcytic polychromatic hypochromic anemia. Total serum protein concentration was signifiantly lower than normal, as was the serum albumin which declined to one-third to one-fifth of the control values. The deficiency caused a significant increase in a β_2-macroglobulin and β_1-lipoprotein. Immune γ_2-globulin (IgG) was not affected. The data indicate that vitamin B_6 deficiency in the young chick depresses erythrocytopoiesis and hemoglobin synthesis, disturbs metabolic functions of the liver cell and hinders development of the lymphatic organs.

The need of the chicken for vitamin B_6 at all ages is well-recognized; however, the effects of the deficiency of this nutrient in the fowl on body tissues and fluids have received little attention. Changes observed in laying hens subjected to pyridoxine deprivation have been reported previously (1). In the present study, attention was focused on the effects of a vitamin B_6-deficient ration fed for 2 weeks to newly hatched chicks, that is, during a period of fast growth when tissues and fluids are rapidly evolving from embryonic to more mature forms. Evidence of the deficiency was based on the usual criteria of body weight gain, feed efficiency, serum glutamic oxaloacetic transaminase activity as well as the occurrence of clinical signs and mortality. Determinations consisted of hematocrit and hemoglobin values, total serum proteins, the electrophoretic and immunoelectrophoretic patterns thereof, weights of the thymus, spleen, bursa of Fabricius, and liver. Also included were histologic sections of the same organs plus the bone marrow. Preliminary results of this study were previously reported.[4]

EXPERIMENTAL PROCEDURES

A total of 150 commercial one-day-old male Cornish × White Plymouth Rock chicks were used in three consecutive experiments of 2 weeks' duration. In each experiment, 3 groups of birds were raised in 5-deck battery brooders with wire floors and thermostatically controlled electric heating elements of the back-warming type. One group of 20 chicks (experimental) was raised with a purified diet supplemented with all known nutrients except pyridoxine; the second or diet control group of 15 chicks received the same feed as above but with 6 mg of pyridoxine·HCl/kg of feed; and a normal control group of 15 chicks received a practical diet known to support normal growth and performance in this type of chicken. The composition of the diets is presented in tables 1 and 2. Water and feed were provided ad libitum and care was taken to scrub and wash the waterers twice daily to minimize bacterial

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[4] Asmar, J. A., N. J. Daghir and H. A. Azar 1967 Serum proteins in pyridoxine-deficient chicks. Federation Proc., 26: 413 (abstract).

TABLE 1

Composition of purified diet

	%
Dextrose	64.75
Isolated soybean protein	22.00
Corn oil	3.00
Nonnutritive fiber [1]	3.00
Mineral mixture [2]	5.30
Vitamin mixture [3]	1.00
Choline chloride (70% aqueous)	0.25
DL-Methionine	0.50
Glycine	0.20

[1] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

[2] Fox, M. R. S., and G. M. Briggs, J. Nutr., 72: 243, 1960 (obtained from Nutritional Biochemicals Corporation).

[3] Vitamin mixture supplied the following per kg of diet: vitamin A palmitate, 9000 IU; vitamin D_3, 1000 IU; and (in milligrams) α-tocopherol, 50; ascorbic acid, 450; inositol, 50; choline chloride, 750; riboflavin, 10; menadione, 2.25; *p*-aminobenzoic acid, 50; niacin, 45; thiamine·HCl, 10; Ca pantothenate, 30; biotin, 0.2; folic acid, 0.9; vitamin B_{12}, 0.01. Pyridoxine hydrochloride (Merck) was added to this mixture at the rate of 6 mg/kg when fed to the diet control group.

TABLE 2

Composition of practical diet

	%
Ground yellow corn	67.0
Soybean oil meal (44% protein)	21.0
Alfalfa meal (17% protein)	1.0
Steamed bone meal	1.0
Chick concentrate [1]	10.0

[1] Chick concentrate furnished the following nutrients per kilogram of diet according to manufacturer's specifications: protein, 58 g; calcium, 6.4 g; phosphorus, 3.7 g; sodium chloride, 4.0 g; vitamin A palmitate, 6000 IU; vitamin D_3, 1500 IU; riboflavin, 4.4 mg; nicotinic acid, 24.2 mg; pantothenic acid, 5.5 mg; choline, 539 mg; vitamin B_{12}, 11 μg; iron, 20 mg; copper, 2 mg; manganese, 50 mg; iodine, 2 mg; cobalt, 1 mg; and zinc, 50 mg.

growth and the ensuing import of microbial vitamin B_6. At 1 and 2 weeks of age the birds were individually weighed and their serum glutamic oxaloacetic transaminase activity was determined by the method of Karmen.[5] At the end of the 2-week period the chicks were killed individually by chloroform inhalation and exsanguinated by open-chest heart puncture. Individual blood samples were immediately drawn into heparinized capillary tubes that were centrifuged for 6 minutes in an IEC model B microcapillary centrifuge, and the hematocrit values were read in an IEC reader.[6] The remainder of the individual blood samples was divided between 2 sets of tubes, one set containing Na citrate for hemoglobin determination, with the other tubes being held for serum. The spleen, bursa, thymus and liver were carefully excised, and weighed to 0.1 mg in closed petri dishes with a Mettler precision balance.[7] Pieces of the same organs and the proximal one-third of the femur were fixed in 10% formalin for sectioning and staining with hematoxylin and eosin. Hemoglobin concentrations were determined by the cyanmethemoglobin method using a Lumetron colorimeter [8] fitted with a 540 mμ filter, whereas the biuret method was used in determining total serum protein. Paper electrophoresis, included to determine the effect of B_6 deficiency on individual protein fractions, was carried out according to the technique described by Aronsson and Gronwall (2) in an LKB electrophoresis chamber [9] and the amido black-stained paper strips were scanned in a Spinco Analytrol.[10] The agar gel for electrophoresis and immunoelectrophoresis was made with Ionagar no. 2 [11] in a barbital·HCl buffer (3) and the serum protein migration completed in 2 hours at 2.5 ma/row of 3 slides. Anti-chicken serum antibodies raised in rabbits and concentrated by precipitation with $(NH_4)_2SO_4$ was used for the immunodiffusion reaction. In agar gel, protein fractions or their immunoprecipitin bands were stained with thiazine red, and oil red O was used for staining lipoproteins.

RESULTS AND DISCUSSION

The clinical signs of pyridoxine deficiency in the young chicken conformed to previously published observations, including a significant reduction in serum glutamic oxaloacetic transaminase activity, stunted growth, feather follicle hemorrhage and up to 60% mortality (4,5). Typical results from one of the experiments are summarized in table 3. The controls raised with the vitamin B_6-adequate diet consistently failed to gain as much weight as those fed the practical ration. The etiology of this reduced growth rate was not

[5] A simplified method for colorimetric determination of glutamic-oxalacetic and glutamic-pyruvic transaminases. Sigma Technical Bull. No. 505, Sigma Chemical Company, St. Louis.

[6] International Equipment Company, 300 Second Avenue, Needham Heights, Massachusetts.

[7] Mettler Instrument Corporation, Princeton, New Jersey.

[8] Photovolt Corporation, New York.

[9] LKB-Produkter AB, Stockholm.

[10] Beckman Scientific Instruments, Fullerton, California.

[11] Oxoid Division of Oxo Ltd. London, E.C. 4.

identified but it did not, however, affect the experimental results. At termination of the experiments, except for marked flaccidity and distension of the crop, the viscera of the deficient birds were apparently normal. Data on weights of lymphatic organs are presented in table 4. The liver weights, included for comparison, were unaffected by the deficiency whereas the spleen, bursa of Fabricius and thymus were significantly reduced in relation to body weight. The most striking gross changes were observed in the thymus whose lobes in the deficient birds looked like empty shells of faintly vascularized fibrous tissue in contrast with the plump succulent thymus lobes of the controls. In the latter, the bone marrow was consistently red as opposed to its marked paleness in deficiency. Histologic sections revealed in the vitamin B_6-deficient birds a state of cellular depletion of the lymphatic organs of which a detailed description will be published elsewhere with the hematologic findings. The liver, however, appeared normal both anatomically and histologically.

The depressing effect of vitamin B_6 deficiency on erythrocytopoiesis was described in a number of animals (6) including avian species in which a microcytic hypochromic anemia was reported in vitamin B_6 deficiency (5, 7, 8). In the present study, vitamin B_6 deficiency caused a significant reduction of hematocrit and hemoglobin values (table 5), hemoglobin being more depressed (66% of controls) than the packed cell volume (79% of controls), indicating a hypochromic type of anemia. In blood smears of deficient chicks there was polychromasia, anisocytosis and microcytosis together with a number of immature erythrocytic cells. Pyridoxine deficiency was demonstrated [12] to interfere with heme synthesis (9,10). The present data suggest that hypochromic vitamin B_6 deficiency anemia in the young chick is due to unequally depressed erythrocytogenesis and hemoglobin synthesis, the latter being more affected.

In a previous study in laying hens, vitamin B_6 deficiency had no detectable effect on serum proteins and their paper electrophoresis patterns (1), which was later con-

[12] Schulman, M. P., and D. A. Richert 1956 Utilization of glycine succinate and aminolevulinic acid for heme synthesis. Federation Proc., *15*: 399 (abstract).

TABLE 3

Effects of vitamin B_6 deficiency on growth, feed conversion and viability of the neonatal chick

	Purified vitamin B_6-deficient diet	Purified vitamin B_6-adequate diet	Practical diet
Body wt gain, g/bird	23.8 ±1.93 ** [1]	101.7 ±4.66 *	158.2 ±3.91
Feed consumed, g/bird/day	7.5 ±0.08 **	13.3 ±0.05 *	20.0 ±0.50
Feed/gain	4.46±0.33 **	1.85±0.03	1.71±0.01
Viability [2]	6/20	10/15	13/15

[1] Means ± SE based on individual body weights and group feed consumption, respectively.
[2] Chicks alive at 2 weeks/chicks started.
* $P < 0.05$.
** $P < 0.01$.

TABLE 4

Effect of vitamin B_6 deficiency on lymphatic organs (liver included for comparison) [1]

	Purified vitamin B_6-deficient diet	Purified vitamin B_6-adequate diet	Practical diet
Spleen	0.0542±0.0011 ** [2]	0.1196±0.0437	0.1272±0.0020
Bursa	0.0997±0.0142 **	0.3406±0.0347	0.3288±0.0300
Thymus	0.3006±0.0142 **	0.4987±0.0224	0.4875±0.1270
Liver	3.5999±0.1620	3.8275±0.2160	4.3878±0.1450

[1] Organ weight in g/100 g body weight.
[2] Means ± SE.
** $P < 0.01$.

TABLE 5

Blood component values in 2-week-old chicks raised with a vitamin B_6-deficient diet

	Purified vitamin B_6-deficient diet	Purified vitamin B_6-adequate diet	Practical diet
Hematocrit, PCV%	22.5 ±2.22 * [1]	28.5 ±1.51	28.9 ±0.50
Hemoglobin, g/100 ml	5.6 ±0.70 **	8.3 ±0.53	8.5 ±0.24
Serum protein, g/100 ml	1.9 ±0.09 **	2.6 ±0.07	2.5 ±0.08
Serum albumin, g/100 ml	0.5 ±0.08 **	1.6 ±0.04	1.5 ±0.06
β-Globulin, g/100 ml	0.5 ±0.06 **	0.3 ±0.03	0.3 ±0.03
γ-Globulin, g/100 ml	0.17 ±0.04	0.19 ±0.02	0.17 ±0.03

[1] Means ± SE.
* $P = 0.01$.
** $P < 0.01$.

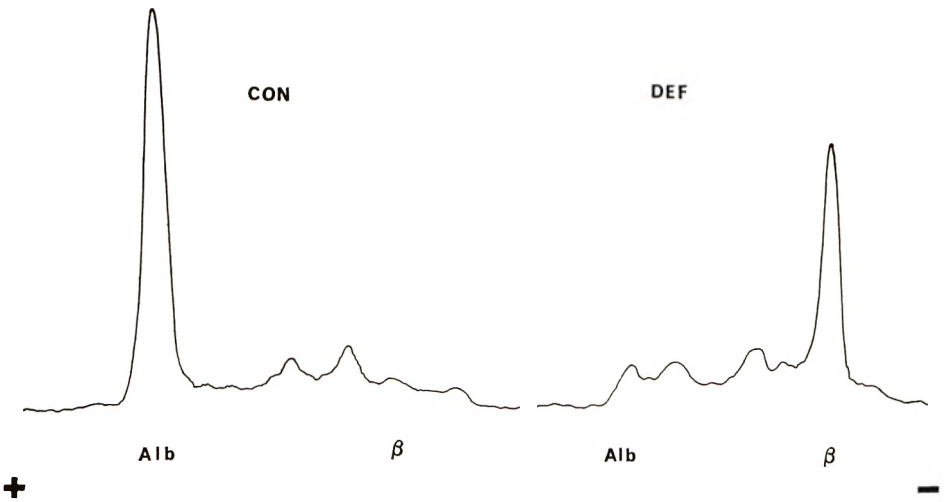

Fig. 1 Paper electrophoresis of deficient (DEF) and control (CON) chick serum. The Analytrol tracing is markedly altered in deficiency because of the marked albumin decrease (Alb) and the increase of the β-globulin fraction.

firmed by immunoelectrophoresis.[13] In contradistinction to this, vitamin B_6 deficiency caused marked alterations in the serum protein patterns in the neonatal chick (table 5). Not only did the deficiency cause an overall reduction of the serum protein content, but it had a particularly depressing effect on serum albumin which decreased to one-third to one-fifth the amounts observed in the controls. Furthermore, a significant increase was observed in the β-globulin group (fig. 1). Agar gel electrophoresis and immunoelectrophoresis confirmed the above results and showed the β-globulin involved to be a β_2-macroglobulin forming a thick precipitin band near the antigen well, with its larger portion located on the cathodic side of the well. This β_2-macroglobulin was also found in the controls but in lesser amounts (figs. 2 and 3). In contrast with the foregoing changes, the γ-globulin was observed in equal amounts in deficient as well as in control birds. In the immunoelectrophoretic (IE) patterns, the γ_2-globulin (IgG) formed a visible precipitin band which generally had comparable shape and size for the deficient and control chicks alike (fig. 3). The agar gel electrophoresis patterns stained with oil red O showed an increase in the β_1-lipoprotein associating consistently with pyridoxine deficiency (fig. 4).

In the 2-week-old chick, γ-globulin is considered as being of maternal origin, absorbed from the yolk into which it is transferred from maternal plasma during the

[13] Unpublished data, J. A. Asmar.

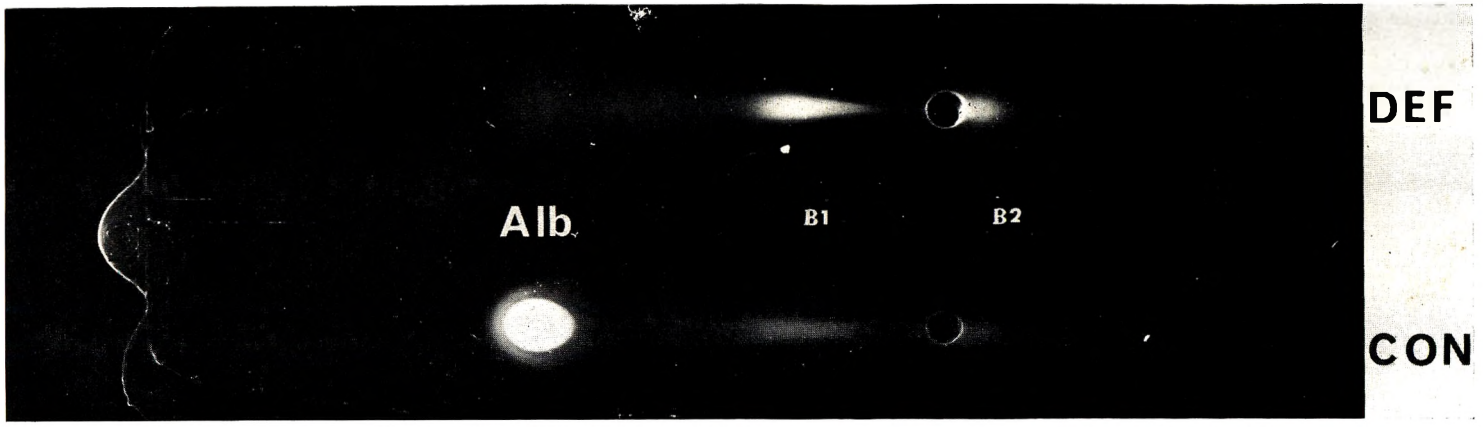

Fig. 2 Electrophoresis of deficient (DEF) and control (CON) chick serum in agar gel. Proteins stained with thiazine red. Albumin (Alb) reduction and β_2-globulin (B_2) increase, clearly visible in DEF, were consistently observed in deficiency. The size of the β_1-population (B_1) varied among individuals but not between treatments. The B_2 patch here corresponds to the B_2 arc in figure 3.

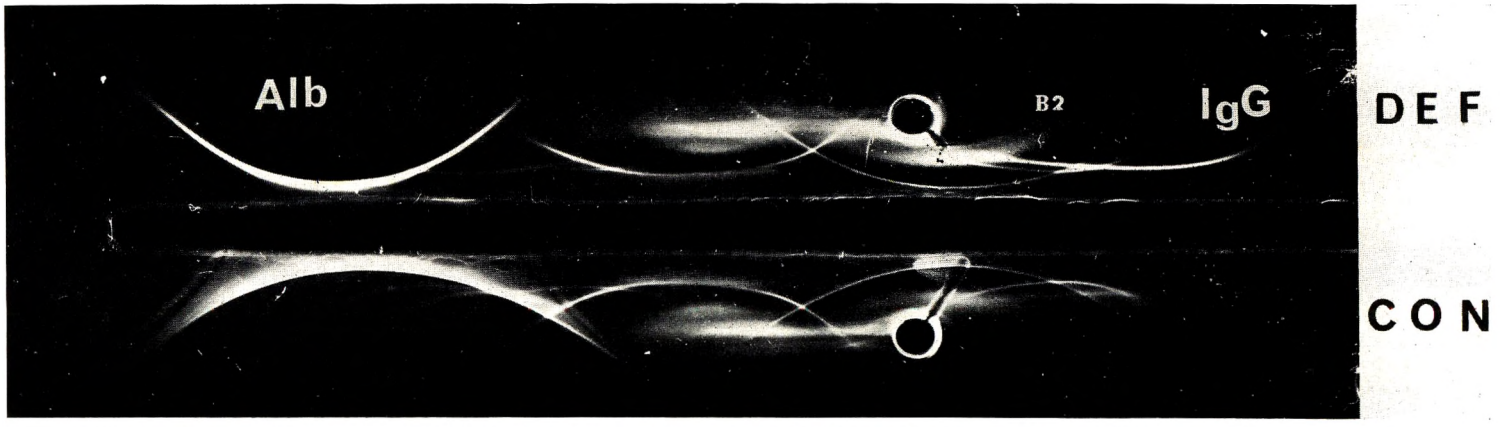

Fig. 3 Immunoelectrophoresis of deficient (DEF) and control (CON) chick serum. In the DEF pattern albumin (Alb) is reduced to a slim arc, whereas the β_2-macroglobulin (B_2) arc is noticeably increased. The fainter immune γ_2-globulin (IgG) arc in the control pattern corresponds to hypogammaglobulinemia observed in a few individuals in all treatments.

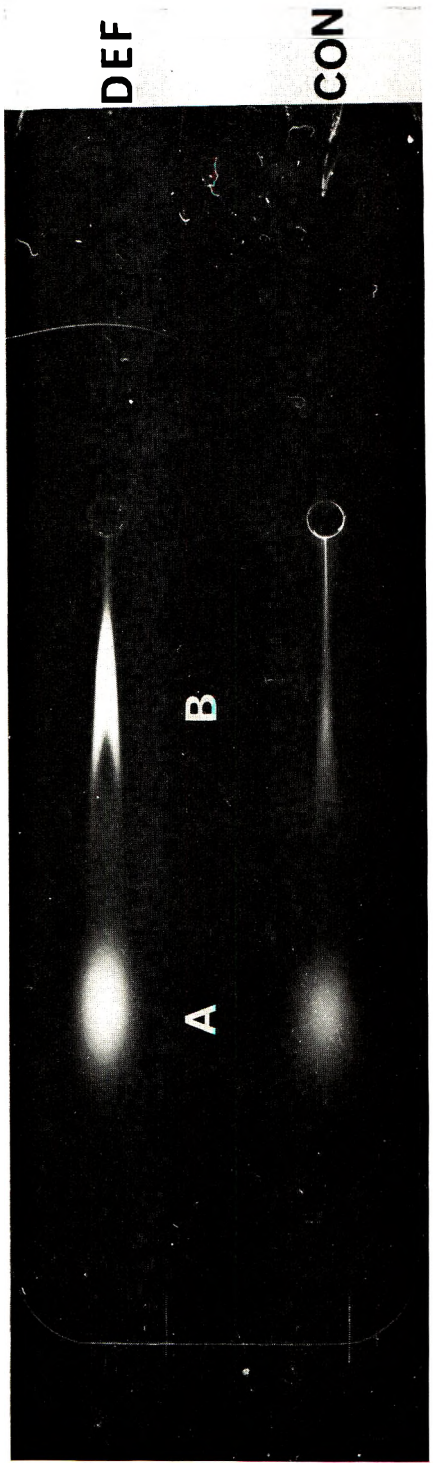

Fig. 4 Serum of deficient (DEF) and control chicks (CON) electrophoresed in agar gel and stained for lipoproteins with oil red O. The β-lipoprotein (B) was consistently increased in deficiency, whereas the α-lipoprotein (A) varied among individuals but not between treatments.

process of yolk formation. Thus stored, the γ-globulin passes into the tissues of the developing chick from the eleventh day of incubation through a few days after hatching, the larger portion being absorbed after eclosion (11). The finding in the vitamin B_6-deficient chicks of normal concentrations of circulating γ-globulin indicates the absorption process had taken place; moreover, the transfer of this protein from the yolk to the tissues of the chicks must have been complete since no residual yolk existed in the abdominal cavities of the birds at postmortem. There remains to explain, however, the reason the deficient chicks did not have twice as much γ-globulin in their blood since they had not grown to more than 40% the size of the controls, and they must have received a similar amount of the same protein. It is not clear whether in the deficient chicks the extra amounts of γ-globulin were excreted, degraded at a faster rate or merely shifted to the extravascular compartment, unless, contrary to conventional thinking, the biosynthesis of γ-globulin in the chick begins at 2 weeks of age which would then account for increased concentrations of this protein in the controls.

Hypoalbuminemia is often observed in nephrotic syndromes or other pathological conditions similarly characterized by abnormal losses of plasma proteins through the kidney or gut. These conditions are usually accompanied by edema in various parts of the body. In the present study the deficient birds showed no anatomical or pathological evidence of nephrosis nor did they display any tendency toward edema. There is apparently no indication of abnormal protein losses to account for the observed hypoalbuminemia.

Reduced concentrations of albumin may also be observed in advanced inanition, and since there was consistently lower feed intake in the deficient birds, inanition effects must be considered. Pair-feeding is the usual device to determine what complications are due to partial starvation; it was not used, however, in this study because strong competition for food within the restricted control groups would leave the less competitive chicks close to starvation while the more aggressive individuals

eat an almost normal share. There is indirect evidence that reduced feed consumption is not the cause of hypoalbuminemia. Pyridoxine deficiency had no effect on serum albumin in the mature hen (1) nor in the young chick when the deficiency was initiated at 2 weeks instead of at one day of age,[14] even though in both instances there was marked loss of appetite. The lack of pyridoxine in the ration did not interfere with albumin formation past the second week of age, which may indicate that the primary result of the deficiency in this respect is interference with the formation of the biosynthetic apparatus that manufactures albumin. Vitamin B_6 was previously reported to be necessary for the formation of messenger RNA and the tissues of vitamin B_6-deficient rats contained fewer polysomes than normal rats (12). The chicken embryo begins the synthesis of albumin at 11 days of incubation in amounts increasing gradually with time (13). The site of albumin synthesis in birds is the liver cell as it is in other species studied (14, 15). The liver-to-body weight ratio was practically normal in the deficient chicks as was the histology of this organ. Depressed albumin synthesis did not then correlate with reduced liver growth or pathological changes and it remains to be seen whether alterations of the subcellular structures of the liver cell would account for the observed lower albumin concentrations.

The increased β_2-macroglobulin in the vitamin B_6-deficient chicks is of unknown nature, particularly, as it is not clear whether it holds any relationship to the immunoglobulins. Apparently it is not an abnormal protein since lesser amounts of it are also observed in the controls, nor is it a neonatal globulin persisting in its original form since a similar increase is observed when the deficiency is initiated at 2 weeks of age.[14]

The altered lipoprotein pattern is an additional indication that vitamin B_6 deficiency in the very young chick interferes with liver function.

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Metabolic Changes in Liver Associated with Spontaneous Ketosis and Starvation in Cows [1,2]

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ABSTRACT Although some information is available concerning the physiological alterations in vivo associated with spontaneous bovine ketosis, a broad study of pathways and possible changes of enzyme activities has not been carried out. To this aim, liver samples were taken from spontaneously ketotic cows, from the same cows when normal, and when starved for 96 hours. Measurements were made to ascertain the degree of ketosis and to compare changes in lipid synthesis, substrate oxidation, enzyme activities and the level of several metabolic intermediates. This research was particularly directed to a comparison of differences and similarities between the normal and spontaneously ketotic animal and between spontaneous ketosis and starvation ketosis. The liver of the spontaneously ketotic cow had a lower activity of mitochondrial NAD-malate dehydrogenase than the normal, higher levels of lactate, pyruvate, acetoacetate and β-hydroxybutyrate, and a substantially lower concentration of citrate. Livers from the spontaneously ketotic cows, when compared to with those of the starved animal, had a lower activity of mitochondrial pyruvate carboxylase, lower levels of NADH and higher levels of citrate, β-hydroxybutyrate and acetoacetate. Many other parameters of carbohydrate and lipid metabolism were measured and found to be unchanged from the normal in the two ketotic conditions. Notable among these was the lack of any alterations in the activity of phosphoenolpyruvate carboxykinase or levels of oxaloacetate. These findings conflict with the oxaloacetate shortage theory of bovine ketosis as generally stated.

Spontaneous ketosis occurs most commonly during the first 2 months of lactation, when the milk production of dairy cows often exceeds their feeding capacity. Such cows utilize considerable body fat and protein (1, 2). Most cows adapt harmoniously and present a paradox: They are feeding at maximal capacity yet undergoing some adaptations that are more characteristic of starvation. Spontaneous ketosis may be thought to arise as a failure to adapt to a form of relative starvation.

Although rumen mucosa (3) and mammary gland (4) produce ketone bodies, most theories of bovine ketosis assume an increase in both hepatic ketogenesis and release of ketone bodies from the liver. Central to these theories is the concept that acetyl-CoA production from fatty acids exceeds its removal by the citric acid cycle or lipogenesis, and hence acetyl-CoA tends to accumulate and contributes to enhanced ketone body synthesis (5–7). The most common theme is a postulated deficiency of oxaloacetate in ruminants due to an inadequate oxaloacetate production from propionate, amino acids and lactate relative to its utilization for gluconeogenesis and eventually milk lactose synthesis (8, 9). More recently, Krebs (10) has suggested that a high rate of gluconeogenesis and specifically an increase in phosphoenolpyruvate carboxykinase activity relative to pyruvate carboxylase would lower the oxaloacetate concentration during spontaneous ketosis and he has supported this suggestion by comparison with enzyme data from alloxan-diabetic rats. Another suggestion proposed for ketotic rat liver by Wieland and Löffler (11) is that the oxaloacetate-to-malate ratio is influenced by the redox state of the mitochondria so that under certain circumstances a lower mitochondrial NAD^+-to-NADH ratio results in a

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fall in the mitochondrial oxaloacetate concentration. A second major theory is that impaired lipogenesis would enhance ketogenesis, again due to an increase in the acetyl-CoA concentration (12, 6).

There is, however, insufficient information available concerning the levels of intermediates and enzymes in the liver of spontaneously ketotic cows to permit an evaluation of the proposed theories. In the present investigation we have attempted to provide this information by the study of relevant parameters in the liver of spontaneously ketotic, normal and fasted cows.

MATERIALS AND METHODS

Cows. Six cows diagnosed by practicing veterinarians to have primary uncomplicated ketosis were used for these studies. The clinical condition of these cows at the time of the experiments is summarized in table 1 along with the plasma concentration of ketone bodies, glucose and free fatty acids. A liver biopsy sample was taken, and the cow was then treated once with dexamethasone (10 mg), cyanocobalamin (1 mg) and nicotinic acid (1 g) by intravenous injection, and protamine zinc insulin (200 units) by intramuscular injection. A second liver biopsy was taken when the cow appeared normal, i.e., was exhibiting no clinical signs and when the packed cell volume and plasma concentrations of ketone bodies, free fatty acids and glucose, and the milk production (with one exception) indicated the cow was completely recovered (see table 1). A third liver sample was taken after the cow had been deprived of food (but not water) for 96 hours. Milk production decreased in all the starved cows, but otherwise they showed few or no clinical signs. Except during starvation, the cows were offered a good quality lucerne hay ad libitum together with a grain concentrate mixture at a rate of 1 kg/3 kg milk produced.

Liver biopsy involved laparotomy. The right flank was prepared for aseptic surgery. Local anesthesia was achieved with a T-line infiltration of the skin muscles and peritoneum with about 100 ml of 2% procaine solution. A vertical incision about 17 cm long was made parallel to and 5 to 10 cm from the last rib. Scissors were used to start an excision line in the lower edge of either the caudate or diaphragmatic lobe of the liver. The excision of a piece of liver about 10 cm × 3 cm × 3 cm was achieved partly with the scissors and partly by compression of the soft tissue and stripping it out of the tough liver capsule with the fingers. After separation of the sample, a piece was cut off within seconds and immersed in liquid dichlorodifluoromethane for the determination of metabolic intermediates. Other sections were immediately placed in appropriate ice-cold solutions for the assay of nicotinamide coenzymes, oxaloacetate and β-hydroxybutyrate and homogenized or used for the preparation of liver slices and enzyme extracts. Sutures were placed in the peritoneum, in the combined muscle layers, and in the skin. Subsequent flank incisions were performed 2 to 3 cm in front or behind the previous incision(s), and liver samples were taken from different portions of the liver. One cow (K32) died soon after an operation in which a major blood vessel was accidentally severed, another (K33) died of peritonitis. A third (K35) did not respond well to the ketosis therapy. It ate poorly for several weeks and milk production decreased severely. When its food intake improved, the milk production remained low (table 1). Otherwise the cows appeared to recover well from surgery and respond well to the ketosis therapy.

Additional liver samples were obtained from five non-lactating normal cows and were used for the measurement of certain enzyme activities.

Blood. Samples were taken from the mammary vein using heparin as anticoagulant. The packed cell volume was determined with a microhematocrit (13,000 × *g*, 10 minutes). Plasma concentrations were determined for acetoacetate (13), β-hydroxybutyrate (14), glucose (15), modified by Campbell and Kronfeld (16), and free fatty acids (17).

Incubation studies. Liver was sliced as described previously (18) and incubated at 37° in 5.0 ml Krebs-Ringer bicarbonate buffer, pH 7.4 (19) containing 50 mM (2.5 μCi) glucose, acetate or potassium pyruvate as indicated in table 2. This procedure has been described in detail previously (20).

TABLE 1

Clinical description of experimental animals [1]

Cow	Lactation	Milk produced /day	Clinical conditions	Plasma concentrations			
				Acetoacetate	β-hydroxybutyrate	Glucose	Free fatty acids
	weeks	*kg*		*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mEq/liter*
K31	2	10	SK, (1 day) nervous myoclonics, feeding undiminished, milk production down 25%	5.0	28	41	0.50
	4	15	N, no clinical signs	0.1	4	41	0.22
	12	5	St, slightly depressed, unresponsive	14.8	41	28	1.87
K32	4	18	SK, (2 days) depressed, nervous episodes eating hay only, milk production down 50%	10.6	39	35	1.05
K36	4	12	SK, (2 days) depressed very weak, eating slowly, salivating, milk production down 30%	18.7	58	25	1.18
	8	15	N, no clinical signs	3.2	32	43	0.46
	13	5	St, appeared weak when undisturbed but responsive to handling	6.2	24	41	1.12
K34	3	17	SK, (3 days) depressed, unresponsive, not eating, milk production down 50%	5.9	22	44	0.87
	6	19	N, no clinical signs	0.8	7	50	0.39
	10	7	St, no clinical signs	3.1	10	40	1.56
K33	1	15	SK, (5 days) eating hay, not grain, milk production down 40%, no depression or excitement, almost normal	1.4	6	59	0.24
	4	14	N, no clinical signs	0.8	9	49	0.44
K35	5	32	SK, (7 days) depressed nervous episodes for 2 days, milk production down 15%, refused grain	4.5	15	21	1.41
	11	11	N, apparently normal except for milk production	1.1	5	43	0.34
	16	4	St, no clinical signs	1.2	14	56	0.97

[1] Liver biopsies were taken from the cows when spontaneously ketotic (SK), later when apparently normal (N), and again later when starved for 96 hours (St). Under clinical conditions, the time in parentheses represents the interval between the first observed clinical sign of ketosis and time of the biopsy. Milk production is reported for the day before the biopsy. The blood sample for the plasma analysis was taken about 2 hours before the liver biopsy.

TABLE 2

Metabolism of ^{14}C-labeled acetate, glucose and pyruvate by cow liver slices [1]

Labeled substrate	Labeled product	Ketotic	Normal	Starved
		μmoles incorporated/3 hr/g tissue at 37°		
Acetate	CO_2	5.64 ± 2.67 [2]	8.04 ± 2.09	6.67 ± 1.88
	fatty acid	0.04 ± 0.02	0.09 ± 0.04	0.04 ± 0.02
	non-saponifiable lipid	0.06 ± 0.04	0.16 ± 0.07	* 0.01 ± 0.001
Glucose	CO_2	0.82 ± 0.22	1.03 ± 0.15	1.24 ± 0.30
	fatty acid	0.005 ± 0.001	0.009 ± 0.004	0.006 ± 0.003
	non-saponifiable lipid	0.006 ± 0.002	0.026 ± 0.008	* 0.003 ± 0.001
Pyruvate	CO_2	3.38 ± 1.35	5.15 ± 1.45	2.25 ± 0.98

[1] Values significantly different (5% probability level) from the normal are indicated by an asterisk.
[2] Mean ± SE.

The ^{14}C-labeled CO_2, fatty acid and non-saponifiable lipid were isolated and the the radioactivity was measured (21). All radioactivity determinations were performed on a Nuclear-Chicago liquid scintillation spectrometer. The efficiency of the system as determined by channels ratio was 60 to 70% and all values were corrected to 100% efficiency on this basis.

Preparation of liver extracts for enzyme measurements. Liver samples were homogenized with a coaxial homogenizer in 10 volumes of a buffered isotonic sucrose solution (22). Homogenates were centrifuged at 100,000 × *g* for 30 minutes in a Spinco model L ultracentrifuge at 2°. The intracellular distribution of the particulate enzymes measured in this study showed their activity to be associated with the mitochondrial fraction. Therefore, to reduce the time involved in preparing the enzymes for assay, their activity was routinely measured in the total particulate fraction. Supernatants were used for the assay of glucose 6-phosphate dehydrogenase, NAD-malate dehydrogenase, citrate synthase, ATP-citrate lyase, acetyl-CoA synthetase, pyruvate carboxylase and phosphoenolpyruvate carboxykinase. The particulate fraction was suspended in buffered sucrose, freeze-dried, resuspended in water and used for the assay of citrate synthase, NAD-malate dehydrogenase, pyruvate carboxylase and phosphoenolpyruvate carboxykinase.

Enzyme assays. Standard assay procedures were used for the measurement of the activity of glucose 6-phosphate dehydrogenase (23), 6-phosphogluconate dehydrogenase (24), NAD-malate dehydrogenase and NADP-malate dehydrogenase (25) and ATP-citrate lyase (26). Modifications of assays for pyruvate carboxylase (27) and phosphoenolpyruvate carboxykinase (28) have been reported previously (29) as have changes in the assay of citrate synthase (30) and acetyl-CoA synthetase (31) by Hanson and Ballard (20). All assays were carried out at 37° with appropriate blanks.

Preparation of liver extracts for the measurement of intermediates. Samples of frozen liver were used within 2 hours after freezing. The tissue was weighed, homogenized in 5 volumes of 6% perchloric acid and centrifuged. The pH of the supernatant was adjusted to approximately 2 with 4 N KOH and to pH 6 with 2 M $KHCO_3$. The suspension was centrifuged again and the clear supernatant used for the determination of malate, lactate, pyruvate and citrate. All steps were carried out at zero to 4°. Other pieces of liver were homogenized in various ice-cold solutions, 3.75% trichloroacetic acid for oxaloacetate and acetoacetate, 0.15 M KCl buffered with 0.02 M $KHCO_3$ to pH 7.6 for β-hydroxybutyrate, acid and alkaline solutions for the nicotinamide coenzymes (32).

Determination of intermediates. Lactate (33) and malate (34) were measured spectrophotometrically. As the spectrophotometric measurements of pyruvate and citrate were not sufficiently sensitive, these methods were modified to detect fluorometrically concentrations of intermediates in the range 0.2 to 10 mμmoles. All fluorescence measurements were performed with a Turner fluorometer model 110 or 111 using as a primary filter a Corning

no. 7–60 with a maximum transmission at 360 mμ and as a secondary filter a Wratten 2A and 47B combination with a maximum transmission at 435 mμ. Since the native fluorescence of the NADH is strongly affected by variations in temperature, the fluorometer was equipped with a temperature-stabilized door and all measurements were performed at 37°.

For the assay of pyruvate and citrate, 0.1 or 0.2 ml of the liver extract was added to 300 μmoles of Tris (hydroxymethyl) aminomethane (pH 7.5) in a final volume of 2.5 ml. At this point 5 mμmoles of NADH [3] was added and the increase in fluorescence was noted. The decrease in fluorescence upon sequential addition of lactate dehydrogenase, malate dehydrogenase and citrate lyase was due to pyruvate, oxaloacetate and citrate, respectively. Usually there was not sufficient oxaloacetate present to measure in the concentration of extract used. Occasionally there was more citrate plus pyruvate present than the 5 mμmoles of NADH added. In these instances, additional, known amounts of NADH were added and the subsequent oxidation of NADH measured.

Oxaloacetate and acetoacetate were measured according to Kalnitsky and Tapley (35). Both substances form a diazo derivative which is measured spectrophotometrically at 450 mμ. The oxaloacetate is destroyed by acid, only the acetoacetate then being measured, and the oxaloacetate is taken to represent the difference. The reliability of this difference diminishes at high concentrations of acetoacetate relative to the concentration of oxaloacetate. The range of 5 to 20 mμmoles oxaloacetate/g wet weight which we have found in sheep and cow liver agrees with previous results obtained by this method applied to rat liver (35, 36). Assays of oxaloacetate in rat liver by enzymatic methods have shown concentrations of about 5 mμmoles/g wet weight (7, 37).

The β-hydroxybutyrate was assayed as described by Williamson et al. (14).

NAD^+, NADH, $NADP^+$ and NADPH were measured by the method of Bassham et al. (38). The exact procedure for extraction of the nucleotides from liver and minor modifications in the procedure have been described previously (39).

RESULTS

The 6 cows were at different stages in the course of the disease at the time of the first biopsy. The most severe clinical signs were being displayed by cows K32, K36 and K35, those with the lowest plasma glucose concentrations (table 1). Cows K32 and K36 also had the highest ketone body levels and these cows were in the early course of the disease (table 1). Cow K33 exhibited severe nervous signs, 2 to 4 days before the experiment, but on the day of the experiment it appeared normal, apart from its rejection of grain. This cow was eating hay well, and its plasma constituents suggested that it had recovered (table 1). Consequently, values obtained from cow K-33 when "ketotic" have not been included in the statistical comparisons. As no obvious trends were discerned between the days of duration of spontaneous ketosis and hepatic function we have presented the means ± the standard error within groups of cows, and significance of differences between normal and ketotic states calculated by paired *t* tests. In all parameters animals K31, K32, K34, K35, K36 were used for spontaneous ketosis; animals K31, K33, K34, K35, K36 for normals and animals K31, K34, K35, K36 for starvation.

Significantly lower rates of incorporation of both ^{14}C-acetate and ^{14}C-glucose into nonsaponifiable lipids were found in liver slices from starved cows compared with those obtained from the same animals when normal (table 2). No other significant differences were noted between incorporation rates under the various conditions.

The activities of several enzymes in cow liver during spontaneous ketosis, when normal and during fasting, are presented in tables 3 and 4 together with additional assays of gluconeogenic enzymes in nonlactating animals. NAD-malate dehydrogenase from the particulate fraction decreased in activity in both the spontaneously ketotic and starved animals. The activity of neither cytoplasmic nor particulate phosphoenolpyruvate carboxykinase changes significantly during spontaneous ketosis or starvation, and furthermore the activity of this enzyme was the same in livers from

[3] P-L Biochemicals, Milwaukee, Wisconsin.

both lactating and nonlactating cows. Cytoplasmic pyruvate carboxylase, however, was more active in the liver from lactating than from nonlactating cows. Starvation during lactation increased the activity of this enzyme, whereas no change in activity was noted during spontaneous ketosis. Similar results were found with the mitochondrial enzyme.

The concentrations of selected metabolic intermediates in cow liver are given in table 5. During spontaneous ketosis the hepatic concentrations of β-hydroxybutyrate and acetoacetate were greater than were noted in the liver of the same cows when normal or starved. Significantly higher lactate and pyruvate concentrations were found in the liver of spontaneously

TABLE 3

Enzyme activities in liver from spontaneously ketotic, normal and starved cows [1]

Enzyme	Spontaneously ketotic	Normal	Starved
		μmoles/min/g liver	
Glucose 6-phosphate dehydrogenase	1.61 ± 0.25 [2]	1.31 ± 0.15	1.88 ± 0.18
6-Phosphogluconate dehydrogenase	2.48 ± 0.30	2.37 ± 0.28	2.36 ± 0.20
Citrate synthase, particulate	1.20 ± 0.04	0.99 ± 0.21	1.30 ± 0.29
Citrate synthase, soluble	0.43 ± 0.09	0.49 ± 0.11	0.40 ± 0.07
NAD-malate dehydrogenase, particulate	* 85.5 ± 30.5	243 ± 74	* 69.3 ± 21.1
NAD-malate dehydrogenase, soluble	324 ± 175	137 ± 33	122 ± 57
NADP-malate dehydrogenase	0.08 ± 0.02	0.03 ± 0.03	0.10 ± 0.04
ATP-citrate lyase	0.02 ± 0.01	0.04 ± 0.01	0.03 ± 0.01
Acetyl-CoA synthetase	0.20 ± 0.05	0.16 ± 0.06	0.11 ± 0.07

[1] All assays were carried out at 37°. Values significantly different (5% probability level) from the normal are indicated by an asterisk and those significantly different from the spontaneously ketotic are indicated by a plus sign.
[2] Mean ± SE.

TABLE 4

Phosphoenolpyruvate carboxykinase and pyruvate carboxylase activities in cow liver [1]

Enzyme	Non-lactating cows [2]	Lactating, spontaneously ketotic cows	Lactating, normal cows	Lactating, starved cows
		μmoles/min/g liver		
Phosphoenolpyruvate carboxykinase:				
Particulate	7.21 ± 0.43 [3]	4.24 ± 0.82	5.46 ± 0.83	4.97 ± 1.02
Soluble	5.07 ± 1.10	4.36 ± 0.96	4.73 ± 0.69	4.25 ± 0.33
Pyruvate carboxylase:				
Particulate	* 4.53 ± 0.13	6.96 ± 1.47	8.28 ± 2.29	+ * 14.9 ± 2.15
Soluble	* 1.09 ± 0.29	2.45 ± 0.73	2.62 ± 0.45	3.92 ± 0.54

[1] All assays were carried out at 37°. Values significantly different (5% probability level) from the lactating normal are indicated by an asterisk, and those significantly different from the spontaneously ketotic are indicated by a plus sign.
[2] Five cows used.
[3] Mean ± SE.

TABLE 5

Levels of metabolic intermediates in liver from spontaneously ketotic, normal and starved cows [1]

Intermediates	Spontaneously ketotic	Normal	Fasted
		mμmoles/g liver	
Lactate	* 1774 ± 183 [2]	1178 ± 140	1983 ± 454
Pyruvate	* 51.8 ± 5.7	33.7 ± 5.1	35.5 ± 5.3
Malate	463 ± 62	319 ± 55	* 484 ± 54
Citrate	* 71.6 ± 10.3	209 ± 21	+ * 33.1 ± 2.5
Oxaloacetate	14 ± 5	12 ± 2	17 ± 3
β-Hydroxybutyrate	* 1831 ± 315	409 ± 71	+ 675 ± 236
Acetoacetate	* 280 ± 18	68.2 ± 16.1	+ 130 ± 44

[1] Values significantly different (5% probability level) from the normal are indicated by an asterisk and those significantly different from the ketotic by a plus sign.
[2] Mean ± SE.

ketotic animals as compared with the normals. Citrate was present at 35% of the normal in spontaneously ketotic animals and 16% of the normal in livers from starved animals. The difference in citrate concentration between all groups of animals was significant at the 1% probability level.

The concentrations of NAD^+, NADH, $NADP^+$ and NADPH in liver are shown in table 6. The only significant change noted was an increased level of NADH during starvation, which resulted in a lowered NAD^+-to-NADH ratio (table 7). Using the assumptions outlined by Williamson et al. (40) the NAD^+-to-NADH ratio in both the cytoplasmic and mitochondrial fractions may be calculated from the lactate-to-pyruvate and β-hydroxybutyrate-to-acetoacetate systems. These ratios, as shown in table 7, are similar in the spontaneously ketotic and normal conditions and show a lower cytoplasmic and slightly higher mitochondrial ratio during starvation.

Although only these few statistically significant differences were found in the present investigations, several other trends were noticeable but were not statistically significant, mainly due to variation between animals. For example, the high soluble NAD-malate dehydrogenase in liver from spontaneously ketotic cows (236% of normal) was not significant because one value was within the normal range. Other examples are the high soluble pyruvate carboxylase activity in livers from starved animals (150% of normal, 160% of ketotic), higher rates of $^{14}CO_2$ and fatty acid formation from all labeled substrates in liver slices from normal cows compared with starved cows (with the exception of CO_2 formation from glucose) and high lactate levels in livers from starved cows (168% of normal).

DISCUSSION

One major metabolic difference between monogastrics and ruminants is the almost complete fermentation of dietary sugar in the latter, resulting in an absolute requirement for glucose synthesis from propionate and amino acids. Lactation at the levels

TABLE 6

Nicotinamide coenzymes in the liver of spontaneously ketotic, normal and starved cows [1]

Nucleotide	Ketotic	Normal	Starved
		mμmoles/g liver	
NAD^+	230 ± 37 [2]	267 ± 43	212 ± 38
NADH	188 ± 38	237 ± 55	+ 333 ± 24
$NADP^+$	53 ± 6	71 ± 9	78 ± 29
NADPH	165 ± 59	240 ± 60	276 ± 89
Total	637 ± 113	799 ± 126	904 ± 127

[1] Values significantly different (5% probability level) from the ketotic are indicated by a plus sign. No values were significantly different from the normal at this level.
[2] Mean ± SE.

TABLE 7

Observed and calculated NAD^+ to NADH ratios in liver from cows when spontaneously ketotic, normal and when starved for 4 days [1]

Condition	$\frac{\text{Lactate}}{\text{pyruvate}}$	$\frac{\beta\text{-Hydroxybutyrate}}{\text{acetoacetate}}$	Ratios of NAD^+ to NADH calculated from		
			Lactate-pyruvate couplet (cytosol)	β-Hydroxybutyrate acetoacetate couplet (mitochondria)	Observed ratio: $\frac{NAD^+}{NADH}$
Spontaneously ketotic	34.3	6.5	269	3.1	1.4
Normal	35.0	6.0	261	3.4	1.2
Starved	55.9	5.2	164	3.9	0.6

[1] Lactate and pyruvate values and β-hydroxybutyrate and acetoacetate values are obtained from table 5, and the NAD^+ and NADH concentrations are from table 6. The ratios of NAD^+ to NADH determined from the lactate-pyruvate or β-hydroxybutyrate-acetoacetate couplets are calculated using the respective equilibrium constants reported by Williamson et al. (40).

attained by the highly productive dairy cow so taxes the gluconeogenic capacity of the liver that dietary sources of propionate are insufficient and the animal must use considerable amounts of body protein for this purpose. Furthermore, the lactating cow is in negative energy balance, and utilizes large amounts of stored fats for oxidation in the citric acid cycle. It is this dependence on propionate, gluconeogenic amino acids and fat oxidation that links gluconeogenesis and ketosis in this animal, and serves as a starting point in any study of the role of the liver in spontaneous bovine ketosis.

Propionate is produced by ruminal fermentation at the rate of about 1 kg/day (41). One- to two-thirds of this is converted to glucose (42, 43) by a series of reactions which initially involve the intramitochondrial conversion of propionate to malate (fig. 1). Malate would also be derived from many amino acids via α-ketoglutarate and succinate (10). Cow liver contains a mitochondrial phosphoenolpyruvate carboxykinase in addition to a cytoplasmic enzyme, and hence oxaloacetate formed within the mitochondria is probably converted to phosphoenolpyruvate which in turn leaves the mitochondria for the subsequent steps in gluconeogenesis. It has been suggested, however, that during gluconeogenesis in rat liver, malate is the principal product that leaves the mitochondria for the formation of phosphoenolpyruvate (44, 45). The cow, however, has 2 pathways available for phosphoenolpyruvate synthesis from propionate. First, malate formed from propionate via succinate could be converted to oxaloacetate and then to phosphoenolpyruvate which passes out of the mitochondria (pathway A, fig. 1); or second, the malate could leave the mitochondria and be converted to oxaloacetate and then to phosphoenolpyruvate by enzymes in the cytoplasm

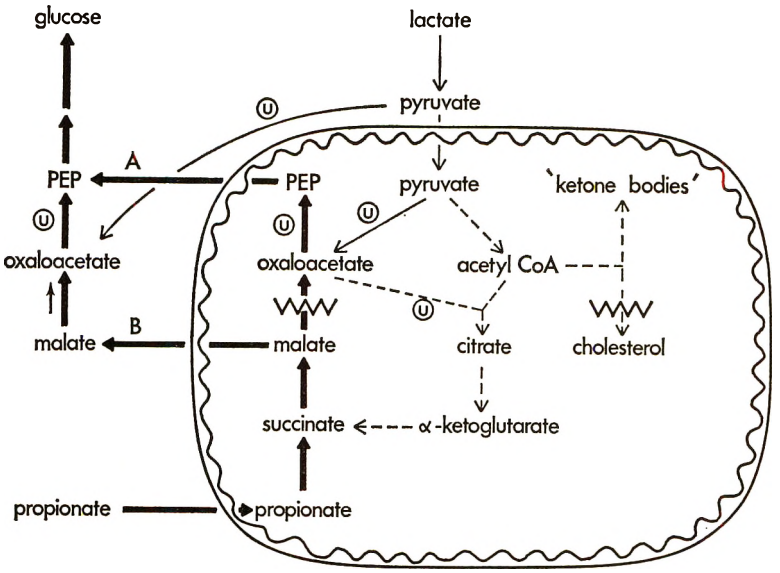

Fig. 1 Postulated pathways of gluconeogenesis in cow liver. Two alternate pathways of propionate conversion to phosphoenolpyruvate (wide arrows) differ as in pathway A, phosphoenolpyruvate leaves the mitochondrion, while in pathway B, malate is the gluconeogenic precursor that leaves the mitochondrion. The relatively minor pathways of oxaloacetate formation from pyruvate or lactate are indicated by narrow arrows and other mitochondrial reactions are shown by dashed lines. For simplicity, the positions at which the various glucogenic amino acids enter the scheme are not shown. Enzyme activities that were measured in spontaneously ketotic cows in the present study are indicated as unchanged from the normal (u); increased (↑) or decreased (ww). In addition, lactate, pyruvate, and perhaps malate levels are increased in spontaneous ketosis and citrate is depressed. These findings are not shown in the figure as the intracellular distribution of these intermediates is not known. PEP indicates phosphoenolpyruvate.

(pathway B, fig. 1). In addition, oxaloacetate may be derived from pyruvate carboxylation, but this is probably minor in ruminant liver.

The possible shortage of oxaloacetate. What clearly emerges from a consideration of these pathways is the central role of oxaloacetate in the synthesis of glucose from propionate and many amino acids. Oxaloacetate is also important in the regulation of the synthesis of ketone bodies in liver. Several investigations have shown that unless the amount of available oxaloacetate within the mitochondria is sufficient to condense with the acetyl-CoA formed from fatty acid breakdown, the excess acetyl-CoA might be shunted into the synthesis of ketone bodies (5–7). It is during periods of rapid gluconeogenesis, such as lactation in the cow, that the great demand for oxaloacetate for glucose synthesis might reduce the concentration of mitochondrial oxaloacetate with ketosis a result. The oxaloacetate concentration in whole liver (table 5) was not altered during spontaneous ketosis or starvation, but this does not preclude a change in the mitochondrial oxaloacetate concentration. This has not been measured directly, and it is likely to be extremely low (40). Krebs (10) has proposed a general concept that the functionally important level of oxaloacetate may be inferred from the relative activities of the enzymes that generate and utilize it; he has also suggested that in severe bovine ketosis as in diabetic ketosis the activity of phosphoenolpyruvate carboxykinase would increase, whereas that of pyruvate carboxylase would not. Substantial increases in hepatic phosphoenolpyruvate carboxykinase have, in fact, been found in diabetic and starved rats (46, 47), but we report no such changes in starved or spontaneously ketotic cows as compared with the lactating cow or even the nonlactating animal. Although Krebs (10) reported no changes in hepatic pyruvate carboxylase activity in diabetic rats, increases in activity were found by Freedman and Kohn (48) and also by Prinz and Seubert (49). We noted no change in pyruvate carboxylase activity in the ketotic cow liver as compared with the normal, but there was an almost twofold increase in starved cows. Furthermore, when compared with the nonlactating cows, particulate and cytoplasmic pyruvate carboxylase activities are significantly higher in all groups of lactating animals.

The formation of oxaloacetate from pyruvate would be favored by an increase in acetyl-CoA concentration as this is an obligatory cofactor for pyruvate carboxylase (50). Black,[4] by measuring the distribution of label in glutamate from 2-^{14}C-pyruvate, has assessed the relative importance of the two main pathways that pyruvate can enter the citric acid cycle, i.e., decarboxylation to acetyl-CoA or carboxylation to oxaloacetate. His work shows that a greater proportion of pyruvate is metabolized via oxaloacetate as compared with acetyl-CoA upon the onset of spontaneous ketosis. Our results show an accumulation of pyruvate in the liver of the spontaneously ketotic cow, and this may be interpreted as impaired utilization. However, the lower citrate levels in both types of ketosis reported in the present investigation may imply a shortage of oxaloacetate in the mitochondria, especially as no changes in citrate synthase activity were found and because it is unlikely that acetyl-CoA would be limiting citrate formation in ketotic conditions where fatty acid breakdown is active. Complementary changes in blood concentrations of pyruvate and citrate have been reported by Bach and Hibbitt (51).

If propionate is converted to oxaloacetate in cow liver mitochondria, as has been suggested in figure 1 (pathway A), changes in mitochondrial NAD-malate dehydrogenase activity are important. This enzyme activity was reduced to about one-third of normal during both spontaneous ketosis and starvation. Within this general framework of the theory proposed by Krebs (10), relating bovine ketosis to gluconeogenesis at the enzyme level, this was the only change noted that is likely to lead to a decrease in mitochondrial oxaloacetate, and during starvation this change is opposed by an increased activity of mitochondrial pyruvate carboxylase. Thus mitochondrial oxaloacetate may be depleted in spontane-

[4] Black, A. L. 1966 Metabolic changes in the utilization of glucose carbon in the tricarboxylic acid (TCA) cycle in bovine ketosis. Federation Proc. 22, 611 (abstract).

ous ketosis but not during starvation, and this difference may be responsible for the difference in the degree of ketosis between these two conditions. In this sense our results support the general concept of Krebs (10), although the exact enzyme changes observed by us differ from those he predicted.

In addition to changes in the activity of malate dehydrogenase, alterations in the redox state of the mitochondria and in the cell cytoplasm would also affect the levels of oxaloacetate. It may be inferred from the calculations based on the concentration of substrates and products of lactate dehydrogenase and β-hydroxybutyrate dehydrogenase (table 7) that these are representative of the NAD^+ to NADH ratios in the cytoplasm and mitochondria, respectively. The assumptions involved in these calculations are numerous and have been discussed recently (40). The differences between the calculated and observed ratios are undoubtedly due to the selective binding of NADH (40). Nonetheless, the lactate-to-pyruvate ratio, the β-hydroxybutyrate-to-acetoacetate ratio and the total NAD^+-to-NADH ratio are similar in livers from spontaneously ketotic and normal cows, whereas deviations from these values occur during starvation (table 7). If these ratios are used to calculate mitochondrial oxaloacetate levels as done by Williamson et al. (40), we would find, as these authors did, both a decrease in cytoplasmic oxaloacetate and an *increase* in mitochondrial oxaloacetate during starvation, but no differences between the normal and the spontaneously ketotic.

Ketogenesis and lipogenesis. From the high levels of hepatic β-hydroxybutyrate and acetoacetate in spontaneously ketotic and starved cows, we may infer that liver ketogenesis is increased under these conditions. We also might expect higher hepatic production of acetyl-CoA due to higher plasma concentrations of fatty acids. These are probably being oxidized in the liver (52, 53). Higher acyl-CoA concentrations could lead to an inhibition of citrate synthase with a resultant increase in ketone body formation (7). In some preliminary measurements of hepatic acetyl-CoA levels by fluorometric methods adapted from Ochoa (30), we found an average concentration of 9.1 mμmoles/g liver in normal cows, and only a slight increase to 11.1 mμmoles/g in livers from the same animals after starvation. No measurements were carried out on the spontaneously ketotic animals.

In confirmation of an earlier study (20), the rates of lipogenesis in cow liver were found to be extremely low. The slightly lower rates of lipid synthesis in ketosis might be explained by the lower hepatic citrate concentrations since citrate is required for activation of acetyl-CoA carboxylase (54), a limiting step in fatty acid synthesis (55). Elema (56) has also reported lower rates of fatty acid synthesis in spontaneously ketotic cows.

Gluconeogenesis. During spontaneous ketosis the activity of NAD-malate dehydrogenase was decreased in the mitochondria (table 3) and greatly increased in the soluble fraction (except in one cow). These findings suggest that pathway A (fig. 1) may be depressed, whereas pathway B is enhanced, and hence overall gluconeogenesis might not be depressed by these enzyme changes. In contrast, during starvation, no increase in soluble NAD-malate dehydrogenase activity was detected, although the mitochondrial activity was depressed. Consequently, depression of pathway A would not be compensated for by enhancement of pathway B, and the overall gluconeogenic ability of the liver might be depressed.

These results complement estimates of glucose production obtained from the rate of dilution of intravenously injected ^{14}C-glucose. Glucose entry rate was about one gram per minute in normal and spontaneously ketotic cows, but only 0.5 g/minute in starved cows (32, 57). Glucose production would reflect the function of gluconeogenic pathways and the availability of precursors. Ruminal propionate concentration is depressed by diminished food intake, though it is not depressed in the early stages of spontaneous ketosis (58). Thus, gluconeogenesis appears to be *depressed* by starvation in cows through decreased availability of propionate and decreased activity of NAD-malate dehydrogenase. This contrasts with the situation in the rat, in which starvation

causes an *increase* in the rate of gluconeogenesis (59, 60).

Comparative aspects. Differences in hepatic gluconeogenesis in response to starvation and ketosis are but one type of evidence that indicates a basic difference in the metabolism of monogastric and ruminant animals. The activities of glucose 6-phosphate dehydrogenase (61), NADP-malate dehydrogenase (10) and ATP-citrate lyase (62) are markedly depressed in diabetic rat liver but not in livers from ketotic cows. Increased activity of phosphoenolypyruvate carboxykinase (10) and decreased oxaloacetate levels (7) have been observed in the diabetic rat but not in the spontaneously ketotic cow. Reasons for these differences may be the extremely low rate of fatty acid synthesis in bovine liver (20), the absence of the citrate cleavage pathway in the cow (20) and the already high activity of phosphoenolpyruvate carboxykinase as compared with rat liver. Using the same enzymatic assay we have found a combined activity (soluble + particulate) of 2.7 ± 0.3 units of phosphoenolpyruvate carboxykinase in rat liver as compared with 10.2 units in cow liver (table 4). The high activity of this gluconeogenic enzyme probably reflects the nutritional status of an animal that is secreting large amounts of sugar-containing milk but obtains essentially no glucose from the diet. In view of these differences we should like to stress the need to discriminate between the ketotic states in various animals for the purpose of explaining the biochemical basis of ketosis.

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Effect of Protein Deficiency on the Spleen and Antibody Formation in Rats [1,2]

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ABSTRACT As protein malnutrition frequently has been associated with lowered resistance and poor immune response, a study was undertaken to determine which aspects of the primary immune response may be impaired in protein deficiency and to describe such effects in terms of tissue components related to synthesis of plasma proteins. Protein-deficient and normal adult male rats were immunized with sheep erythrocytes and killed 6 days later. Spleens of protein-deficient rats were smaller and contained fewer cells, less nitrogen, less RNA, and more DNA per cell than those of controls. These changes were compared with those observed in liver as a result of protein depletion. In depleted animals, numbers of specific antibody-forming cells (AFC) and amounts of circulating antibody were about one-third of normal, while the amount of γ-globulin was two-thirds of normal. Since antibody production per AFC did not decrease in deficient animals, the depression of antibody titers in protein deficiency could be attributed largely to the reduction in numbers of AFC's in the spleen.

Resistance to infection is often low in undernourished children (1), and nutritional stresses have caused poor immune response and high mortality in infected animals (2–5). This susceptibility may result, in part, from the reduction in spleen size characteristic of malnutrition (6, 7). The spleen is important in phagocytosis (8) and in synthesis of antibodies (9, 10) and other immunoglobulins (11); splenectomy has lowered resistance to certain diseases (12). Effects of such factors as age on the antibody-forming potential of the spleen have been described (13). However, little work has been carried out in various nutritional states to characterize quantitatively the relationship of size and composition of the spleen to its production of immunoglobulins. In the present study, the spleen and serum immunoglobulins were examined in protein-depleted and normal adult rats. For comparison with a commonly studied organ, data are also presented for composition of liver and for concentrations of one of its biosynthetic products, serum albumin.

PROCEDURE

Adult male rats of the Wistar strain were depleted of protein by feeding ad libitum a diet containing 10% fat, 4% salts, 2% fiber, and 84% cornstarch, plus a daily supplement of vitamins (5). In experiment 1, nine rats were fed this low protein diet; they lost 24% of their initial body weight (443 $\pm$ 3 g) in 5 weeks. Twelve older and heavier animals in experiment 2 lost 22% of their initial body weight (553 $\pm$ 9 g) after only 4 weeks of depletion. Control rats (10 and 12 in experiments 1 and 2, respectively) were fed the laboratory stock diet (5) which provided about 25% protein. Six days before the end of the experiment, all rats were immunized by intravenous injection of 1.0 ml 2% washed sheep erythrocytes.

In the first experiment, serum was obtained from blood taken from the tail 6 days after immunization. Rats were then stunned by a blow on the head. The spleen was excised and placed immediately in a tared container of balanced salt solution (14) and weighed. After the spleen cells had been teased apart and washed, they were diluted to a known volume and counted. The percentage of nucleated cells was determined from examination

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of stained smears. To identify cells producing antibody to sheep red blood cells (AFC), spleen cells and antigen were plated in agar in chemically defined tissue culture medium (15) and overlaid with complement, according to the method of Jerne et al. (16). After incubation, a plaque of lysis surrounded each cell that produced specific antibody. For each animal, plaques on each of 3 plates were counted under low power magnification; total counts from 2 series of 5 fields each were averaged.

In experiment 2, blood was drawn from the abdominal aorta or heart after anesthetizing animals with sodium pentobarbital. Blood was centrifuged in the cold; all serum was frozen until it was analyzed. A portion of the spleen was weighed, minced, and prepared for cell counts. The remainder of the spleen and the liver were weighed separately and frozen in liquid nitrogen before storage at $-20°$ for subsequent analysis.

Nucleic acids were precipitated from cold homogenates of spleen or liver by adding perchloric acid. After dissolving the precipitate, RNA was hydrolyzed with alkali. DNA and protein were again precipitated, and RNA in the supernatant was measured directly by ultraviolet absorption (17). DNA was reacted with indole, extracted with chloroform, and measured as the colored reaction product (18). To measure nitrogen, spleen homogenates were digested with sulfuric acid and hydrogen peroxide; the resulting ammonia was reacted with modified Nessler's reagent and measured colorimetrically (19). Homogenates of liver were analyzed for nitrogen by the Kjeldahl procedure and for total fat by ethanol-hexane-ether extraction (20).

Antibody was determined by microadaptation of standard procedures (21). All washings and dilutions were made with Veronal buffer, pH 7.4, containing 1.5 mEq Ca^{++} and 0.5 mEq Mg^{++} per liter. For hemolysin, double dilutions of serum were incubated for 45 minutes at 37° with equal volumes of 2% sheep erythrocytes and diluted guinea pig serum (containing 4 units of complement). To measure agglutinin, dilutions of serum were incubated at room temperature for 1 hour with an equal volume of 1.5% red blood cells. End points were taken as lysis or agglutination of half the cells in the reaction mixture. Reciprocals of end-point dilutions were transformed to logarithms for statistical evaluation of titers.

γ-Globulin in 0.5 ml serum was precipitated 3 times in one-third saturated ammonium sulfate. Precipitates were dissolved each time in 0.9% sodium chloride. After treatment with biuret reagent, solutions of γ-globulin were compared colorimetrically with standard solutions of bovine serum albumin. Electrophoresis in acrylamide gel demonstrated that protein separated in this manner contained 2 γ- components. Serum albumin was obtained by diluting an aliquot of serum with 23% sodium sulfate and adding diethyl ether. Biuret reagent was added to a portion of the aqueous phase.

In experiment 2, a known amount of Evans blue dye was injected intravenously 15 minutes before the animals were killed. Plasma volume was calculated from the concentration of the dye in serum.

RESULTS

Spleens of protein-deficient rats were reduced in actual weight, to about 60% of normal, and in proportion to body weight (table 1). Loss of splenic tissue resulted from reduction in cell numbers rather than decreased mean cell volume. The percentage of nucleated cells in the spleen was approximately the same in all groups. In depleted rats, cells actively forming antibody to sheep erythrocytes administered during the experiment (AFC) were only about one-third as numerous as in control animals. Liver weight averaged 12.51 and 12.71 g in controls and 8.28 and 11.65 g in depleted groups in experiments 1 and 2. The larger rats in experiment 2 had been depleted only 4 weeks; livers of these rats were heavier on a fat-free basis and contained more fat than livers of animals in experiment 1 which had been depleted for 5 weeks.

Loss of nitrogen from the spleen in protein deficiency paralleled the loss in weight and in numbers of cells in that organ (table 1). Although both components were lost at about the same rate, the ratio of N/RNA was slightly increased in deficient animals. The DNA content of depleted

TABLE 1

Composition of spleens and livers of protein-depleted and control rats

Measurement	Exp. no.	Spleen			Liver		
		Control	Depleted	Depleted / Control × 100	Control	Depleted	Depleted / Control × 100
Organ wt, g	1	0.89 ± 0.04 [1]	0.48 ± 0.03	54 ** [2]	12.5 ± 0.4	8.3 ± 0.2	66 **
	2	0.92 ± 0.03	0.59 ± 0.02	64 **	12.7 ± 0.3	11.6 ± 0.5	91
fat, g/100 g	1	—	—	—	5.0 ± 0.2	9.2 ± 0.8	184 **
	2	—	—	—	5.4 ± 0.2	14.4 ± 1.1	267 **
fat-free wt, g	1	—	—	—	11.9 ± 0.4	7.5 ± 0.2	63 **
	2	—	—	—	12.0 ± 0.3	9.9 ± 0.4	82 **
mg/g body wt	1	1.90 ± 0.09	1.43 ± 0.07	75 **	25.2 ± 0.9 [3]	22.4 ± 0.5	89 *
	2	1.81 ± 0.07	1.39 ± 0.05	77 **	23.5 ± 0.6	23.3 ± 0.9	99
Cell counts × 10^{-6}	1	1190 ± 180	640 ± 80	54 **	—	—	—
	2	950 ± 105	590 ± 84	62 *	—	—	—
nucleated × 10^{-6}	1	750 ± 140	310 ± 70	41 **	—	—	—
	2	480 ± 40	260 ± 45	54 **	—	—	—
log AFC [4] plaques	1	2.07 ± 0.10	1.62 ± 0.18	36 * [5]	—	—	—
Nitrogen, mg	2	15.8 ± 0.6	9.8 ± 0.3	62 **	460 ± 10	291 ± 7	63 **
mg/g tissue	2	16.3 ± 0.3	16.5 ± 0.3	101	38.4 ± 0.5 [3]	29.5 ± 0.2	77 **
RNA, mg	2	5.50 ± 0.29	3.16 ± 0.12	57 **	80.1 ± 1.7	71.2 ± 1.7	89 **
mg/g tissue	2	5.92 ± 0.16	5.33 ± 0.12	90 **	6.71 ± 0.22 [3]	7.22 ± 0.17	108
N/RNA, mg/mg	2	2.65 ± 0.07	3.03 ± 0.09	114 **	5.8 ± 0.1	4.1 ± 0.1	71 **
DNA, mg	2	9.4 ± 0.4	7.4 ± 0.3	79 **	21.5 ± 0.7	26.6 ± 0.9	124 **
mg/g tissue	2	10.2 ± 0.2	12.5 ± 0.5	123 **	1.80 ± 0.08 [3]	2.70 ± 0.11	750 **
RNA/DNA, mg/mg	2	0.58 ± 0.01	0.43 ± 0.02	74 **	3.8 ± 0.2	2.7 ± 0.1	71 **

[1] Mean ± SE.

[2] * $P < 0.05$; ** $P < 0.01$ that depleted and control values do not differ, based on t test; $P > 0.05$ for all other comparisons.

[3] All calculations based on fat-free weight of liver.

[4] Antibody-forming cells.

[5] Based on ratio of antilogarithms.

spleens was lowered, but not so much as was the weight or the number of nucleated cells. The resulting concentration of DNA in spleens of depleted rats was so high that the amount of DNA per nucleus appeared to have increased by about half. Since DNA was lost less rapidly than other components, the RNA/DNA ratio of the spleen was lowered from 0.58 in controls to 0.43 in depleted animals.

The nitrogen content of the depleted livers was 63% of normal (table 1). In contrast with the spleen, the concentration of nitrogen in fat-free liver was reduced significantly in depletion, as was the ratio of N/RNA. Proportionately less RNA was lost from the liver than from the spleen. The total amount of hepatic DNA in deficient animals exceeded that in controls. It may be concluded that total nucleic acids of the liver were less adversely affected by protein deficiency than those of the spleen. However, the ratios of RNA/DNA in both tissues were decreased to about the same degree by protein deprivation.

Total circulating γ-globulin was lowered by protein deficiency (table 2) to a greater extent than has been reported by some workers (22). The amount of γ-globulin bore a constant relationship to the RNA content and weight of the spleen in both normal and depleted animals. The amount of serum albumin was also reduced by protein depletion, but less albumin was present per milligram of hepatic RNA in depleted rats than in controls. Similarly, nitrogen content of the liver, relative to hepatic RNA, was lowered by dietary deficiency, whereas splenic nitrogen, relative to splenic RNA, was not (table 1).

Because the mean plasma volumes per unit body weight differed significantly between normal and depleted rats (table 2), those values measured in experiment 2 were used to estimate plasma volumes of animals in experiment 1. The total amount of antibody thus estimated to be in the serum in protein-deficient rats was only

TABLE 2

Serum γ-globulin, albumin and hemolysin in relation to components of liver and spleen in normal and protein-depleted rats

Measurement	Exp.	Control	Depleted	$\frac{\text{Depleted}}{\text{Control}} \times 100$
Plasma volume, ml/100 g body wt	2	3.62 ± 0.09 [1]	4.10 ± 0.12	113 ** [2]
γ-Globulin, g/100 ml	2	0.51 ± 0.4	0.38 ± 0.11	74 *
mg	2	93 ± 6	66 ± 7	71 **
mg/mg spleen RNA	2	17.3 ± 1.3	20.6 ± 2.0	119
mg/g spleen	2	101 ± 7	111 ± 11	110
Albumin, g/100 ml	2	4.92 ± 0.14	2.89 ± 0.23	59 **
g	2	0.90 ± 0.02	0.57 ± 0.03	63 **
mg/mg liver RNA	2	11.0 ± 0.4	8.2 ± 0.7	75 *
mg/g fat-free liver	2	74 ± 2	62 ± 5	84 *
Hemolysin, log units/ml	1	3.07 ± 0.21	2.77 ± 0.23	50 [3] ┐
	2	3.64 ± 0.06	3.15 ± 0.09	32 ┘ *
log units	1	4.30 ± 0.21	3.91 ± 0.23	40 [3] ┐
	2	4.90 ± 0.06	4.32 ± 0.10	26 ┘ *
units /AFC [4] plaque × 10^{-3}	1	0.36 ± 0.12	0.36 ± 0.10	100
units/μg spleen RNA	2	15.6 ± 2.6	8.4 ± 2.1	54
units/mg spleen	2	96 ± 17	45 ± 11	47 *
units/μg γ-globulin	2	1.05 ± 0.27	0.46 ± 0.07	44
units/unit agglutinin	1	76 ± 18	49 ± 15	64
	2	149 ± 11	148 ± 36	99

[1] Mean ± SE.
[2] * $P < 0.05$; ** $P < 0.01$ that depleted and control values do not differ, based on t test; $P > 0.05$ for all other comparisons.
[3] Ratio of antilogarithms.
[4] Antibody-forming cell.

about 30% of that observed in normal animals (table 2). Protein deficiency reduced antibody output to the same extent as it inhibited formation of antibody-synthesizing cells, but total γ-globulin was not affected to that degree. The average amount of circulating antibody per AFC was not altered in protein depletion. Ratios of antibody to splenic RNA, spleen weight, and γ-globulin tended to be lowered in depleted rats ($P < 0.10$), although variation was in some instances too large to permit detection of significant differences with the numbers of animals examined. Hemolysin and hemagglutinin 6 days after immunization were affected similarly by protein deficiency.

DISCUSSION

Although the concentration of circulating antibody has been related to the number of specific antibody-forming cells (AFC) (16, 23), correlations between size or composition of the whole spleen and total immunoglobulins in serum are more difficult to demonstrate. Immunoglobulins are formed by several tissues, all of which may not respond to antigenic stimulus as does the spleen. Though convenient for study, the spleen may contain varying numbers of erythrocytes and other cells that do not form antibodies. Further, only a small fraction of the nucleated cells of the spleen are thought potentially capable of antibody synthesis (13). Despite the inherent variability of procedures, such as antibody titrations and AFC counts, some relationships have become evident.

Compared with maintenance of plasma γ-globulins, formation of AFC's and synthesis of a new antibody were particularly sensitive to lack of dietary protein. Thus, protein may not have been preferentially used to enable the animals to produce clones of AFC's in response to the new antigenic stimulus, but rather to maintain globulin synthesis by those fully differentiated cells already committed to forming specific immunoglobulins. The lack of dietary effect on the amount of γ-globulin per spleen cell or per milligram of RNA in the spleen and on the titer of hemolysin in relation to numbers of AFC's support this concept.

γ-Globulin was correlated significantly with antibody titers only in protein-deficient rats. Thus, γ-globulin concentrations may not serve as a satisfactory basis for predicting capacity to respond to new antigens, except perhaps when malnutrition is severe. Even then, small body size, chronic infections and poor sanitation, frequently associated with malnutrition in the human, tend to elevate the concentration of γ-globulin and may mask any decrease in globulin output resulting from malnutrition. Effects of a diet devoid of protein fed to adult rats under controlled laboratory conditions, as reported here, may differ from those of diets containing limited amounts of poor quality protein, such as those consumed by young children in areas where episodes of malnutrition and infectious disease are frequent.

Comparison of changes induced in the 2 tissues by protein deprivation showed that the spleen was in some respects more sensitive to lack of protein than the liver. Even though percentage losses of nitrogen from spleen and liver were comparable, losses of nucleic acids from spleen exceeded any losses from liver. Although the data of Thomson et al. (24) supported the concept that the DNA per cell in livers of adult rats remained constant despite dietary modification, total DNA per liver (25), as well as DNA per nucleus and DNA per gram of dry tissue (26), has increased in livers of adult rats fed diets containing little or no protein. Controls in experiment 1 were 7% heavier (472 ± 9 g) and those in experiment 2 were 7% lighter (512 ± 4 g) at killing than depleted rats had been initially. However, this difference in maximal weight was not sufficient to account entirely for the high content of hepatic DNA or fat-free weight of the livers of deficient animals in experiment 2, when compared with controls. Lowered concentrations of γ-globulin accompanied decreases in weight, cellularity, nitrogen, and RNA of spleen, but the average amount of γ-globulin produced by surviving spleen cells was at least normal. In contrast, serum albumin was reduced in depleted rats in relation to weight, nitrogen, and nucleic acids of liver. Spleen and liver might be expected to respond differently to nutritional deficiency. Although the

spleen is subject to only relatively small alterations in its environment (the plasma), the liver may also be influenced considerably by cessation of the normal influx via the portal circulation of dietary amino acids. Further, inherent differences in sensitivities of key enzyme systems in the 2 tissues (27) may lead to diverse responses to malnutrition. Whatever the cause, detrimental effects of protein deficiency on the spleen and on its function in synthesizing immunoglobulins were clearly evident from the data presented.

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Effects of Age and Impending Parturition upon Serum Copper of Thoroughbred Mares [1]

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ABSTRACT During an investigation of the possible relationship between serum copper and the incidence of parturient uterine artery rupture of mares, sera from 475 Thoroughbred mares less than 30 days prepartum were assayed for copper and protein. Serum copper decreased significantly from a predicted mean of 1.70 μg Cu/ml at age 4 years to 1.52 μg/ml at age 20 years. There was a significant increase in serum copper between 30 and zero days prepartum. Mares fatally hemorrhaging from ruptured uterine artery at parturition had significantly lower serum copper levels than the predicted levels for non-hemorrhaging mares of comparable ages, whereas nonfatal cases of apparent uterine artery rupture at parturition had serum copper levels not significantly different from predicted levels for mares of comparable ages.

Factors affecting the copper content of serum include diet (1–3), neonatal age changes (4, 5), pregnancy (6), nephrosis (7), Wilson's disease (8), Hodgkin's disease (9), molybdenosis (10) and parasitism (11). Cardiovascular lesions of poultry (12) and swine (13) have been associated with a deficiency of copper, an important element in the formation of aortic elastin (14).

Data for the present report were obtained from 475 preparturient Thoroughbred mares as part of an investigation of the possible relationship between serum copper and the incidence of parturient uterine artery rupture which causes fatal internal hemorrhage of aged mares (15).

EXPERIMENTAL

During the 1966 foaling season, blood samples from 120 mares on one farm were obtained within one month of the anticipated foaling dates. Glassware used for blood-collecting, serum storage and copper analyses was washed with a biodegradable detergent and rinsed in standard distilled water followed by double glass-distilled water. The serum was assayed for copper by the oxalyldihydrazide acetaldehyde procedure of Stark and Dawson (16) and total protein was determined by the biuret method (17).

During the 1967 foaling season, serum samples were obtained from 355 mares one month or less prepartum and representing 4 farms. These blood samples were obtained with commercial disposable equipment [2] without special preparation. Glassware for the serum was processed as previously described. The copper and protein analyses on these serums were made by automated procedures.[3] The automated copper procedure was an adaptation of the pressure dialysis method of Summers (18), which also uses oxalyldihydrazide acetaldehyde color reagent. The total protein procedure used was the automated biuret procedure adapted by Failing et al. (19). Regression analyses using the coded class value system and correlation analyses of the serum copper data, expressed in terms of micrograms of copper per milliliter of serum and per gram of serum protein, were made according to Goulden (20). The 1966 and 1967 data were statistically analyzed separately because different assay procedures were used each year. Copper data from fatal and nonfatal cases of parturient hemmorrhage occurring among the mares sampled were compared with appropriate mean serum copper values for respective groups of non-hemorrhaging mares.

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[2] Vacutainer, Becton-Dickinson Company, Rutherford, New Jersey.

[3] AutoAnalyzer Equipment, Technicon Instruments Corporation, Research Park, Chauncey, New York.

RESULTS AND DISCUSSION

The serum protein and copper values arranged according to age of pre-parturient mares are presented in table 1 and the data are graphically represented in figure 1. Regression analyses demonstrate an inverse relationship between serum copper and mare age. The regression is significant for the 1967 data with $P < 0.01$ for Cu/ml serum and $P < 0.05$ for Cu/g serum protein. The regression coefficients are -0.1045 and -0.1123, respectively, for copper per milliliter serum and grams of serum protein. There was a significant correlation $P < 0.05$ between mare age and copper per gram of protein ($r = -0.108$) for the 1967 data.

The extent of displacement of the 1967 data from the 1966 data on the ordinate is attributed to the reduced opportunity for ion contamination in the automated copper analyses and a change from the Kjeldahl nitrogen assayed reference standard used in the 1966 (manual) protein assays to the bovine albumen reference standard used in the 1967 (automated) protein analyses.

The 1967 serum protein and copper values according to days prepartum are shown in table 2. There was a significant elevation in copper per milliter of serum ($P < 0.05$) and copper per gram of serum protein ($P < 0.01$) during the last 30 days of gestation with regression coefficients of -0.0381 and -0.0638 for copper per milliliter of serum and copper per gram of serum protein, respectively. As parturition approached (10–0 days prepartum) there appeared to be a marked tendency toward elevation of total protein, copper per milliliter of serum and copper per gram of protein; however, this period was also marked by a large variation in copper levels and a limited number of samples analyzed. Neither regression nor correlation coefficients indicated significant relationships between days prepartum and serum copper or protein for the 30- to 10-day and 10- to zero-day prepartum periods.

Using the prediction equation for the regression line for the 1967 data in figure 1 and adjusting the 10- to zero-day prepartum copper data to a common mare age (10 years) the regression coefficients for copper per milliliter of serum and per gram of serum protein were -0.175 and -0.076, respectively, each insignificant.

Data for 4 fatal and 3 nonfatal, clinically diagnosed cases of parturient rupture of the uterine artery are presented in table 3. These mares ranged in age from 15 to 25 years which, according to Rooney (15) is

TABLE 1

Serum protein and copper levels of preparturient Thoroughbred mares [1]

Age	No. assayed		Total protein		Serum copper			
	1966 [2]	1967 [3]	1966	1967	1966	1967	1966	1967
years			*g/100 ml*		*μg/ml*		*μg/g protein*	
4	1	6	6.38	7.90 ± 0.11 [4]	1.55	1.84 ± 0.13	24.30	23.23 ± 1.39
5	7	11	6.71 ± 0.23	7.78 ± 0.10	2.24 ± 0.17	1.71 ± 0.01	33.70 ± 2.99	21.90 ± 0.95
6	7	33	7.08 ± 0.16	7.87 ± 0.05	2.04 ± 0.15	1.69 ± 0.06	28.25 ± 2.24	21.37 ± 0.62
7	9	38	6.89 ± 0.16	7.90 ± 0.08	1.96 ± 0.08	1.71 ± 0.06	28.88 ± 1.12	21.28 ± 0.75
8	12	34	6.70 ± 0.17	7.79 ± 0.07	1.93 ± 0.10	1.57 ± 0.05	29.13 ± 1.74	20.26 ± 1.17
9	10	36	7.05 ± 0.21	7.82 ± 0.06	1.93 ± 0.08	1.62 ± 0.05	27.62 ± 1.29	20.64 ± 0.61
10	11	40	6.92 ± 0.15	7.80 ± 0.07	1.85 ± 0.05	1.54 ± 0.04	26.67 ± 0.70	20.10 ± 0.70
11	10	34	6.67 ± 0.22	7.94 ± 0.03	2.08 ± 0.10	1.66 ± 0.06	31.18 ± 0.99	21.05 ± 0.69
12	6	21	6.88 ± 0.15	7.92 ± 0.04	1.97 ± 0.22	1.67 ± 0.01	28.81 ± 3.47	20.97 ± 0.79
13	12	21	7.04 ± 0.19	7.75 ± 0.08	1.91 ± 0.09	1.42 ± 0.04	27.57 ± 1.36	18.41 ± 0.55
14	8	13	7.04 ± 0.12	7.81 ± 0.15	2.02 ± 0.13	1.57 ± 0.01	28.83 ± 2.15	19.81 ± 0.64
15	3	16	6.93 ± 0.10	7.84 ± 0.09	2.00 ± 0.13	1.64 ± 0.01	28.89 ± 1.71	20.62 ± 0.94
16	5	9	6.83 ± 0.19	7.82 ± 0.17	1.77 ± 0.12	1.70 ± 0.01	26.02 ± 1.64	21.69 ± 0.95
17	5	8	6.87 ± 0.29	7.82 ± 0.08	1.62 ± 0.05	1.55 ± 0.01	23.85 ± 1.23	19.61 ± 1.04
18	3	8	6.87 ± 0.41	7.97 ± 0.09	1.76 ± 0.06	1.48 ± 0.01	25.94 ± 2.00	19.17 ± 1.07
19	3	3	6.79 ± 0.15	7.90 ± 0.24	1.95 ± 0.07	1.65 ± 0.02	28.80 ± 1.81	20.98 ± 0.77
20	2	9	7.72 ± 0.29	7.60 ± 0.19	1.95 ± 0.11	1.50 ± 0.01	25.43 ± 2.31	19.88 ± 0.64

[1] Less than 30 days prepartum.
[2] Manual assay procedure.
[3] Automated assay procedure.
[4] Averages ± SE of mean.

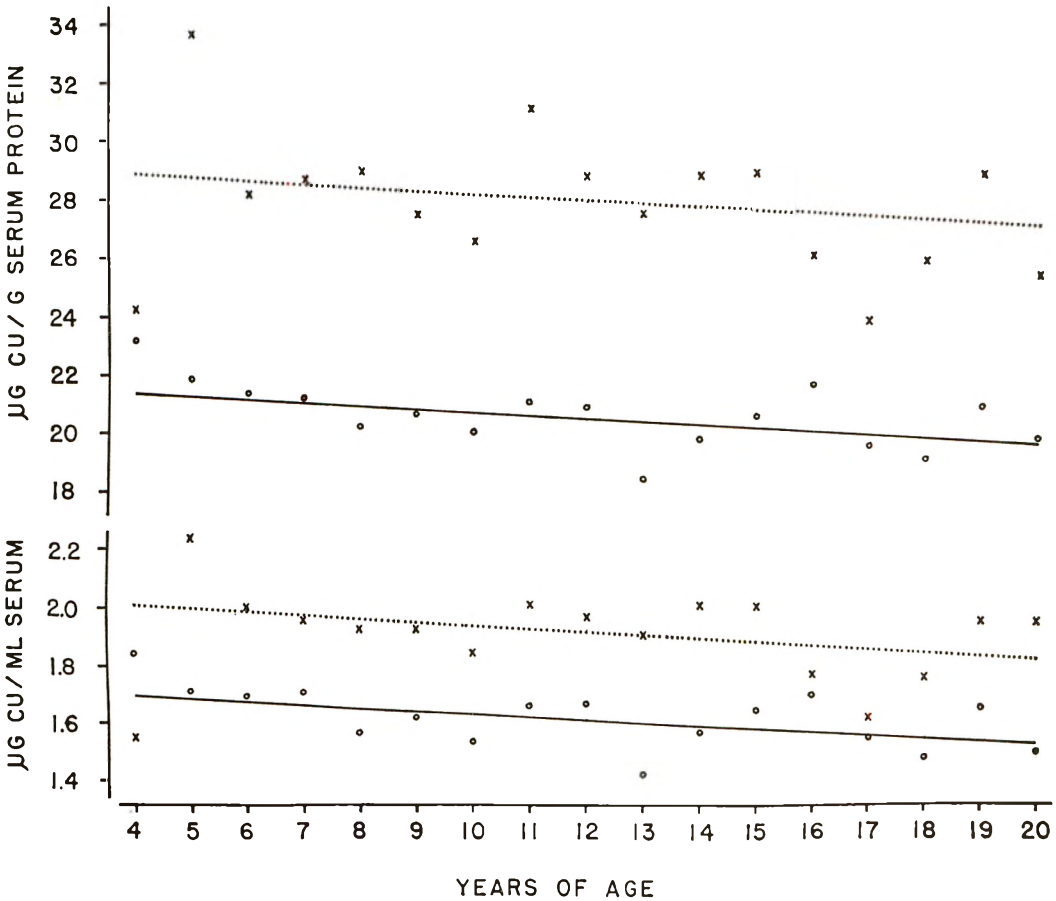

Fig. 1 Serum copper levels of Thoroughbred mares less than 30 days prepartum; ×, means of 120 serums assayed manually (1966); ○, means of 355 serums assayed with automatic techniques (1967); ..... insignificant regression; and ——— significant regression.

TABLE 2

Serum protein and copper levels of Thoroughbred mares 1 to 30 days preparatum

Days prepartum	No. assayed [1]	Total protein	Serum copper	
		g/100 ml	*µg/ml*	*µg/g protein*
1	6	8.15 ± 0.17 [2]	1.62 ± 0.09	20.15 ± 1.02
2	3	8.05 ± 0.09	2.17 ± 0.22	26.95 ± 3.72
3	2	8.35 ± 0.11	1.58 ± 0.04	18.95 ± 0.74
4	2	7.62 ± 0.08	1.81 ± 0.04	23.78 ± 0.14
5	3	7.78 ± 0.32	2.02 ± 0.20	25.79 ± 1.71
6	6	7.92 ± 0.08	1.58 ± 0.10	19.91 ± 1.17
7	9	7.82 ± 0.14	1.69 ± 0.14	21.46 ± 1.43
8	3	7.54 ± 0.51	1.84 ± 0.24	24.30 ± 2.79
9	6	7.90 ± 0.21	1.87 ± 0.23	23.33 ± 2.33
10	8	7.73 ± 0.18	1.60 ± 0.11	20.89 ± 1.52
11–12	19	7.83 ± 0.13	1.63 ± 0.07	20.82 ± 0.82
13–14	19	7.89 ± 0.09	1.61 ± 0.10	20.40 ± 1.14
15–16	16	7.76 ± 0.06	1.63 ± 0.12	20.61 ± 1.10
17–18	27	7.95 ± 0.17	1.57 ± 0.07	19.94 ± 0.92
19–20	15	7.92 ± 0.17	1.51 ± 0.11	18.92 ± 1.33
21–22	18	7.87 ± 0.13	1.67 ± 0.11	21.28 ± 1.51
23–24	25	7.71 ± 0.08	1.56 ± 0.06	20.58 ± 0.65
25–26	22	7.67 ± 0.17	1.59 ± 0.08	20.77 ± 0.95
27–28	30	7.84 ± 0.12	1.58 ± 0.08	20.25 ± 0.93
29–30	19	7.90 ± 0.10	1.57 ± 0.08	19.71 ± 0.85

[1] Automated procedure.
[2] Averages ± SE of mean.

TABLE 3

Comparison of serum protein and copper values of parturient hemorrhaging and non-hemorrhaging mares

Hemorrhaging mare no.	Age	Days prepartum sample obtained	Total protein	Serum copper		Deviation from means for comparable age groups of non-hemorrhaging mares		Disposition
						Total protein [1]	Serum copper [2]	
			g/100 ml	*µg/ml*	*µg/g protein*	*g/100 ml*	*µg/ml* *µg/g protein*	
29	17	13	7.25	1.82	25.10	+0.38	−0.03 − 2.30	died
36	15	10	6.90	1.21	17.54	+0.07	−0.67 − 10.06	died
74	20	10	7.80	1.32	16.90	−0.20	−0.20 − 2.70	died
795	25	0 [3]	7.50	1.36	23.40	−0.30	−0.45 + 0.20	died
	Mean	19.2	7.42	1.43	20.7	−0.01	−0.34 [4] − 3.66	
74	20	27	7.31	2.11	28.9	−0.41	+0.29 + 1.80	recovered
77	19	30	6.60	1.85	28.0	−0.19	+0.02 + 0.80	recovered
923	20	10	7.80	1.32	16.9	+0.10	−0.01 0.00	recovered
	Mean	19.6	7.20	1.76	24.6	−0.16	+0.10 + 0.93	

[1] Actual.
[2] Predicted.
[3] Postmortem serum.
[4] Significantly ($P < 0.05$) less than predicted serum copper levels of non-hemorrhaging mares.

the age range of mares seen with this condition at necropsy. There appeared to be no relationship between total protein and the incidence of uterine artery rupture. The fatally hemorrhaging mares averaged 0.33 µg Cu/ml serum and 3.9 µg Cu/g serum protein less than the nonfatally hemorrhaging mares. The copper per milliliter of serum of the fatally hemorrhaging mares was significantly ($P < 0.05$) lower than (actually 0.34 µg Cu/ml serum below) predicted serum copper levels of non-hemorrhaging prepartum mares of comparable age groups. Copper per gram of serum protein of the fatally hemorrhaging mares was also below (3.66 µg Cu/g serum protein below) predicted levels for non-hemorrhaging mares. The nonfatally hemorrhaging mares averaged 0.10 µg Cu/ml and 0.93 µg Cu/g serum protein above the predicted levels for non-hemorrhaging mares of comparable ages.

The decrease in serum copper with advancing age of mares, the indication that there is a prepartum elevation of serum copper in the mare as Nielsen (21) has demonstrated in the human and the observed low copper levels in fatally hemorrhaging, aged, parturient mares make feasible a relationship between serum copper and the incidence of uterine artery rupture at foaling. Were the prepartum serum copper elevation a physiological process to assure optimal amine oxidase activity (22) for the maintenance of vascular elasticity during the rigors of parturition, the aged, hypocupremic mare would possess inadequate endogenous copper and be more susceptible to vascular rupture than a younger mare.

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Some Effects of Amino Acid Deficiencies on Antibody Formation in the Rat [1]

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ABSTRACT The effects of a variety of nutritional deficiencies on antibody formation in rats have been studied. Deficiencies of vitamin B_6, tryptophan and phenylalanine in rats decreased the antibody response to the synthetic antigen, poly $Glu^{52}Lys^{33}Tyr^{15}$ (no. 3) or to the natural antigen, sheep red blood cells. Methionine deficiency, methionine excess, or caloric restriction did not alter the antibody response. The metabolic antagonist deoxypyridoxine further decreased the antibody response in vitamin B_6-deficient rats. The effect was reversed partially by glycine or serine, serine being more effective on a molar basis. Ethionine, a methionine antagonist decreased the antibody response, and its effect was not reversed by adenine or by methionine. The antibody response in germfree rats was higher than in normal animals.

Since a relationship between resistance to infectious disease and nutrition has been generally accepted, there have been many studies of the effects of nutritional deficiency on antibody formation. These studies have been restricted generally to vitamin deficiencies, particularly vitamin B_6, and to a lesser extent, to protein deprivation. In the present work, the effects on antibody formation of feeding rats diets containing varying amounts of methionine, phenylalanine and tryptophan have been studied. The effects of the methionine antagonist ethionine and the vitamin B_6 antagonist, deoxypyridoxine, which limits the availability of glycine and serine, were also investigated. Finally, the effects of restricted feeding and lack of gastrointestinal flora on antibody formation were studied.

METHODS

Male rats of the Charles River CD strain were used, except in the germfree studies. The animals were housed in group cages, and were fed water and food ad libitum unless otherwise specified. In the germfree studies, male rats of the Charles River CDF strain were used. The germfree rats were housed in sterile isolation units at the Charles River laboratories. One control group with normal gastrointestinal flora was maintained under the same housing conditions as the germfree rats, and another control group was maintained in a conventional rat cage in our animal room.

The control diets are shown in table 1. In the phenylalanine experiment the diet consisted of a commercial formula diet for patients with phenylketonuria.[2] It contained approximately 15% protein and 0.06 to 0.1% phenylalanine; 1.2 g of DL-phenylalanine were added to each 100 g of the control diet.

The rats were immunized either with the synthetic polypeptide poly $Glu^{52}Lys^{33}Tyr^{15}$ (no. 3),[3] or with sheep red blood cells. The immunization schedule for the polypeptide consisted of 0.75 mg of antigen in 0.5 ml of complete Freund's adjuvant injected into both hind foot pads and subcutaneously into the back of the neck (primary

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[2] Lofenalac, Mead Johnson Company, Evansville, Indiana.

[3] The nomenclature of the synthetic polypeptides is systematically defined in Biopolymers, 2: 283, 1964. The superscripts are the molar percentages of each amino acid residue and the number following the polypeptide formula denotes the preparation. The synthetic polypeptide used in this study has a molecular weight of 86,000.

TABLE 1

Composition of control diet used in vitamin B_6, methionine and tryptophan experiments [1]

	Diet 1	Diet 2	Diet 3
	Vitamin B_6 study	Methionine study	Tryptophan study
	%	%	%
Casein	15	—	—
Salt-free acid hydrolyzed casein	—	—	20
Peanut meal	—	20	—
Gelatin	—	10	—
DL-Methionine	—	0.6	0.6
L-Tryptophan	—	0.15	0.24
L-Lysine	—	0.3	—
L-Phenylalanine	—	0.3	1.0
DL-Threonine	—	0.6	—
Glycine	3	—	—
Sucrose	67.7	53.75	63.86
Salt mixture IV (1)	4	4	4
Corn oil	9	9	9
Cod liver oil	1	1	1
Choline	0.3	0.3	0.3

[1] Each diet contained per kg of diet: (in milligrams) thiamine·HCl, 4; riboflavin, 8; pyridoxine·HCl, 4; niacin, 40; Ca pantothenate, 20; folic acid, 1; biotin, 0.2; vitamin B_{12}, 0.05; menadione, 1; and *dl*-α-tocopheryl, 100. Changes in the amino acid composition of the experimental diets were accompanied by appropriate alterations in sucrose levels.

immunization) followed by an intraperitoneal injection of 0.75 mg of antigen in 0.5 ml of water (secondary immunization) in approximately 3 weeks. With the sheep red blood cells, a single immunization was used, except in the studies with the germfree rats: 0.8 ml of 10% suspension of erythrocytes was injected subcutaneously in the back of the neck and 0.1 ml was injected into each of the hind foot pads.

Antibody titers elicited with the synthetic antigen were measured by the passive hemagglutination technique (2). Using this technique, antibody formation was not detected before the second injection of the synthetic antigen. Antibody titers elicited with the sheep red cells were measured by the direct hemagglutination method, in which untanned uncoated sheep red cells were substituted for the tanned, antigen-coated sheep red cells. For the hemagglutination titrations the sera were diluted tenfold and subsequently in twofold dilutions. The results are expressed as the geometric mean of the logarithm of the dilution to the base 2 ± the standard error of the mean. Differences in the amount of antibody formed had to be significant at $P < 0.01$ in order to be considered meaningful (3).

RESULTS

Vitamin B_6 deoxypyridoxine, glycine, and serine studies. Table 2 presents data from 2 experiments on the ability of glycine or serine to reverse the inhibition of antibody formation caused by vitamin B_6 deficiency and deoxypyridoxine. In the first study a group of rats weighing approximately 74 g were fed the experimental diets 3 days before the primary immunization with poly $Glu^{52}Lys^{33}Tyr^{15}$ (no. 3). The secondary immunization followed 17 days after the primary, and 5 days later the rats were bled. Vitamin B_6 deficiency inhibited the formation of antibody, and the deficiency plus the feeding of deoxypyridoxine completely prevented antibody formation in all but one of 13 rats. The addition of glycine to this diet partially overcame the inhibition of growth and antibody formation caused by the deoxypyridoxine.

Similar results were obtained in the second experiment in which sheep red blood cells were used as the antigen. In this experiment, weanling rats weighing about 45 g were fed the experimental diets 10 days before being immunized. Ten days later, their hemagglutination titers were determined. Glycine partially prevented

the inhibitory effects of deoxypyridoxine, as in the first experiment, and serine at half the molar concentration of the glycine was considerably more effective in protecting the rats against the deoxypyridoxine effects.

Methionine and ethionine studies. Table 3 shows the results of feeding ethionine and different levels of methionine on antibody formation in rats immunized with poly $Glu^{52}Lys^{33}Tyr^{15}$ (no. 3). Groups of rats weighing approximately 164 g were fed the experimental diets 6 days before the primary immunization. The second dose of antigen was given 17 days after the first, and the rats were bled 5 days later. The diets deficient in methionine or containing a large enough amount of DL-methionine (5%) to inhibit growth did not significantly effect antibody formation. The addition of ethionine to the low methionine diet significantly reduced the amount of antibody formed. This effect of ethionine was not altered by adding methionine to the diet, although the inhibitory effect of ethionine on growth was reversed. Since one mechanism of ethionine action is to trap adenine due to the formation of S-adenosyl-L-ethionine (4–6) adenine was fed to one group of rats in an attempt to reverse the effects of ethionine. The administration of adenine, however, did not have any demonstrable effect on either the inhibition of growth or antibody formation produced by ethionine.

TABLE 2

Effects of glycine and serine on antibody formation in vitamin B_6-deficient rats treated with deoxypyridoxine

	Wt gain	No. rats responding	Geometric mean hemagglutination titer
	g		
Antigen: poly $Glu^{52}Lys^{33}Tyr^{15}$ (no. 3)			
Diet 1			
with vitamin B_6	89 [1]	10/10	7.8 ± 0.6 [2]
without vitamin B_6	49	10/10	5.5 ± 0.6
without vitamin B_6 + 3% glycine	28	8/10	4.4 ± 0.8
without vitamin B_6 + 10 mg DOP [3]/kg diet	3	1/13	0.3
without vitamin B_6 + 10 mg DOP/kg diet + 3% glycine	15	5/10	2.4 ± 0.9
Antigen: sheep red blood cells			
Diet 1			
with vitamin B_6	55 [4]	13/13	8.7 ± 0.1
without vitamin B_6	21	13/13	7.2 ± 0.4
without vitamin B_6 + 5 mg DOP/kg diet	3	0/11	0
without vitamin B_6 + 5 mg DOP/kg diet + 3% glycine	8	3/12	0.9
without vitamin B_6 + 5 mg DOP/kg diet + 2.1% L-serine	13	13/13	4.2 ± 0.8

[1] Grams/18 days.
[2] ± SE.
[3] DOP: deoxypyridoxine.
[4] Grams/20 days.

TABLE 3

Effects of methionine and ethionine on antibody formation in rats [1]

Diet	Wt gain /28 days	No. rats responding	Geometric mean hemagglutination titer
	g		
Low methionine basal (diet 2)	100	12/12	10.3 ± 0.3 [2]
Basal + 0.6% DL-methionine	124	14/14	10.1 ± 0.3
Basal + 0.1% DL-ethionine	7	15/15	7.5 ± 0.4
Basal + 0.1% DL-ethionine + 0.2% adenine	6	12/13	6.0 ± 0.7
Basal + 0.1% DL-ethionine + 0.6% DL-methionine	131	14/14	8.1 ± 0.4
Basal + 5% DL-methionine	58	15/15	9.6 ± 0.4

[1] Rats immunized with poly $Glu^{52}Lys^{33}Tyr^{15}$ (no. 3).
[2] ± SE.

Tryptophan and phenylalanine studies. Table 4 shows the effects of feeding diets deficient in tryptophan or phenylalanine on antibody formation in rats immunized against sheep erythrocytes. The groups of weanling rats used in both of these experiments were fed the experimental diets 5 days before immunization, and they were bled 9 days after immunization. The tryptophan-deficient diet contained 0.04% added L-tryptophan compared with the 0.24% added in the control diet. The phenylalanine-deficient diet consisted of the control diet without any additional phenylalanine. Both the tryptophan and the phenylalanine-deficient animals showed an impaired ability to form antibodies to sheep red blood cells.

Restricted feeding studies. Table 5 shows the result of restricting caloric intake on antibody formation in rats fed laboratory ration. Initially the rats weighed approximately 155 g. They were fed the restricted diets 7 days before immunization with poly $Glu^{52}Lys^{33}Tyr^{15}$ (no. 3). The second dose of antigen was given 21 days after the first, and the animals were bled 9 days later. The data show that restricted feeding did not have a significant effect on antibody formation.

Studies in germfree rats. Studies on germfree rats were undertaken to determine whether the lack of a gastrointestinal flora, with its ill-defined nutritional significance, or the lack of previous antigenic stimulus provided by the bacteria would influence antibody formation in rats. The rats were immunized with sheep red blood cells by injecting 0.5 ml of a 2% suspension of cells intravenously through the tail vein. Ten days later their antibody titers were measured, and they were given a second intravenous injection of 0.5 ml of a 2% suspension of red cells. The secondary response was measured 5 and 11 days after the second injection of antigen. The results of this study (table 6) show that germfree rats consistently had higher antibody titers than the animals in either control group. The type of housing and handling which the germfree animals received did not have any effect on the results obtained, since no statistical differences were observed between the amounts of antibody in the two control groups, one of which was handled in the same way as the germfree animals.

TABLE 4

Effects of diets deficient in tryptophan or phenylalanine on antibody formation in rats [1]

Diet	Wt change /14 days	No. rats responding	Geometric mean hemagglutination titer
	g		
Control (diet 3)	52	12/12	6.6 ± 0.4 [2]
Tryptophan-deficient	−2	12/14	4.7 ± 0.6
Control (Lofenalac [3] + 1.2% DL-phenylalanine	42	12/12	7.3 ± 0.4
Phenylalanine-deficient	−4	13/13	5.3 ± 0.5

[1] Rats immunized with sheep red blood cells.
[2] ± SE.
[3] Mead Johnson Company, Evansville, Indiana.

TABLE 5

Effect of restricted feeding on antibody formation in rats [1]

Amount of ration /rat/day	Wt change /37 days	No. rats responding	Geometric mean hemagglutination titer
g	*g*		
Ad libitum	184	15/15	7.5 ± 0.4 [2]
15–16	116	16/16	8.1 ± 0.4
12–13	66	15/15	8.3 ± 0.4
8–10	12	11/11	8.4 ± 0.9

[1] Rats immunized with poly $Glu^{52}Lys^{33}Tyr^{15}$ (no. 3).
[2] ± SE.

TABLE 6

Antibody formation in germfree rats [1]

Group	Time after immunization	No. of rats [2]	Geometric mean hemagglutination titer
Germfree	10 days after primary	15	7.8 ± 0.1 [3]
	5 days after secondary	13	8.3 ± 0.1
	11 days after secondary	9	9.6 ± 0.4
Controls in isolation	10 days after primary	8	5.6 ± 0.5
	5 days after secondary	8	5.8 ± 0.4
	11 days after secondary	8	6.5 ± 0.5
Non-isolated controls	10 days after primary	7	5.4 ± 0.7
	5 days after secondary	5	6.5 ± 0.9
	11 days after secondary	5	7.1 ± 0.7

[1] Rats immunized with sheep red blood cells and fed sterilized laboratory ration.
[2] Every rat produced antibody.
[3] ± SE.

DISCUSSION

Cannon (7) suggested that globulin production is dependent upon the intake of amino acids and that antibody production must similarly depend on protein intake. Since then there have been a number of studies in man and animals of the effects of protein deficiency on the antibody response and of the relationships between protein malnutrition and infection (8, 9). There have been few studies, however, of the effects of deficiencies of individual amino acids on the immune response.

In the present study, deficiencies of phenylalanine and tryptophan, but not of methionine, resulted in impaired antibody formation. The inability of the methionine deficiency to alter the antibody response may be related to the relatively less intense deficiency state produced by the low methionine diet as shown by the growth response compared with that produced by the tryptophan and phenylalanine-deficient diets.

Nutrition studies often use metabolic inhibitors to enhance the deficiency effects of the nutrients they antagonize. For example, Wissler et al. (10) inhibited the antibody response in rats immunized with sheep erythrocytes by feeding them β_3-thienylalanine, and they reversed the effect by adding more phenylalanine to the diet. However, the data obtained with metabolic antagonists must be interpreted with caution, since the effects of the inhibitors can be quite complex and go beyond the simple intensification of the deficiency of their nutrient analogues. It has been established through the years, particularly by the work of Axelrod and his associates that vitamin B_6 deficiency inhibits the immune response, and the degree of inhibition can be increased by the use of deoxypyridoxine (11). Gershoff and Faragalla (12) have suggested that the availability of serine and glycine is blocked by deoxypyridoxine. The results of the present study support this hypothesis, since they show that the effect of deoxypyridoxine in inhibiting antibody synthesis can be partially reversed by glycine or serine supplementation, serine being much more effective than glycine on a molar basis.

Ethionine, which is a methionine antagonist, can inhibit protein synthesis by inducing an adenosine triphosphate (ATP) deficiency due to trapping adenine as S-adenosyl-L-ethionine. This effect can be reversed by ATP or adenine (4–6). When ethionine was added to a low methionine diet, both antibody formation and growth were markedly depressed. Adenine did not alter the effect of ethionine. The addition of methionine to the ethionine-containing diet resulted in normal growth, but did not significantly affect antibody production. In a recent study, Gill and Gershoff (13) reported that ethionine added to a diet low in methionine inhibited antibody formation in Cebus albifron monkeys immunized with poly $Glu^{52}Lys^{33}Tyr^{15}$ (no. 3) and that feeding excessive quantities of methionine also depressed the immune response. In the present study, however, the feeding of

growth-inhibiting quantities of methionine to rats did not inhibit their ability to form antibodies to the same antigen.

It is often difficult to distinguish between the effects produced by a specific vitamin or amino acid deficiency and the more general effects of inanition produced by the nutrient deficiency. In the present study, however, food restriction did not alter the antibody response (tables 3 and 5). Thus the effects due to the various deficiencies appear specific. Axelrod and Pruzansky (14) have provided similar data in studies of the effects of vitamin B_6 deficiency on circulating antibody production to diphtheria toxoid in rats.

The enhanced antibody response in the germfree rats has been observed by others also, and they have attributed it to alterations in the avidity of the macrophage system (15). However, two alternative explanations may be offered. The first is suggested by considering the parallel phenomenon of radiation enhancement of the antibody response. An appropriate dose of radiation administered a short time after an antigenic stimulus enhances the antibody response (16).

It has been postulated that depletion of lymphoid cells by radiation allows a disproportionate repopulation of the lymphoid tissue by surviving antigen-stimulated cells, which multiply more rapidly than surviving non-stimulated cells. In the germfree animals, the grossly underdeveloped lymphoid tissue might provide a similar situation in which the antigen-stimulated cells would proliferate more rapidly than the unstimulated cells, thus producing higher than normal amounts of circulating antibody. The second alternative would be a decreased catabolism of γ-globulin in the germfree rats, thus allowing a higher concentration of antibody to be attained in the serum.

The effects of various nutritional deficiencies have been demonstrated with both sheep red blood cells and a synthetic polypeptide as antigens. The antibody response to the synthetic antigen is affected by the same types of metabolic alterations that affect the response to a natural antigen. Thus, the synthetic antigens can be used in nutritional and metabolic experiments, and they offer the additional advantages of detailed chemical control of structure and a relatively limited variety of different antigenic sites. The latter property can be of great advantage in studying the mechanism of action of nutritional deficiencies and of the genetic control of the antibody response.

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Copper-Molybdenum Interaction in Sheep and Chicks [1,2]

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ABSTRACT A series of experiments was conducted in an attempt to gain further insight into the biological interaction between copper and molybdenum. In sheep fed a purified, low copper diet containing varying levels of molybdenum and inorganic sulfate, anemia developed only in those animals receiving molybdenum. All these sheep showed a diminution of plasma copper as the trial progressed. High levels of dietary molybdenum did not significantly depress the induction of ceruloplasmin activity in chicks stressed with fowl typhoid. High levels of molybdenum in the incubation solution reduced the in vitro uptake of copper by rat liver and kidney slices. It was observed that $CuSO_4$ and Na_2MoO_4 form a complex which precipitates in a near neutral solution. The ratio of copper to molybdenum in this complex was 4:3. The results of these studies were used to develop a hypothesis that molybdenum complexes with copper and that copper bound in this state is biologically inactive.

A number of reports in the literature have indicated that molybdenum and inorganic sulfate alter the normal metabolism of copper, but the mechanism or site of this interaction remains elusive.

The first indication of an interaction between copper and molybdenum arose from the work of Ferguson et al. (1). These workers observed scouring in cattle that grazed forages containing a high level of molybdenum and found that it could be prevented or cured by feeding or drenching with copper sulfate even though the copper content of the forage was normal. Thus, it appeared that molybdenum precipitated a physiological copper deficiency.

Dick (2) observed that dietary inorganic sulfate intensified the harmful effect of molybdenum upon copper metabolism in sheep. Other workers (3, 4) have demonstrated a beneficial effect of sulfate on molybdenum-induced copper deficiency in the rat. Gray and Daniel (5) have attempted to reconcile these apparently anomalous effects of inorganic sulfate by showing that, in the rat, prior copper status affects the response. If copper stores were low, inorganic sulfate intensified the molybdenum effect; if copper stores were adequate, sulfate prevented the harmful effects of molybdenum. An interaction between copper and molybdenum has also been observed in rabbits (6) and in the guinea pig (7).

A striking difference between ruminants and nonruminants, noted from these reports, is that the adverse effects of molybdenum in the ruminant occur at much lower dietary levels.

In the present investigation the interacting effects of copper and molybdenum were studied under three experimental conditions. In the first, lambs were fed a purified (8), low copper diet containing varying levels of molybdenum and sulfate. The purpose of this experiment was to study the effect of molybdenum and inorganic sulfate on hemoglobin and plasma copper concentrations of the lambs under a defined dietary regimen. The second experiment, using chicks, was performed to study the effect of molybdenum and sulfate on ceruloplasmin induction. Normally, chicks have very low levels of ceruloplasmin activity, but it can be increased by stressing

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the birds if copper is present in the diet (9). The third experiment consisted of incubating rat liver and kidney slices in varying concentrations of molybdenum and copper to study the effect of molybdenum on tissue uptake of copper in vitro. In addition to these experiments, a fourth study is also presented characterizing a copper-molybdenum complex.

EXPERIMENTAL

General procedures. Hemoglobin concentrations were determined by the method of Shenk et al. (10). Copper determinations were made according to the method of Parks et al. (11), as modified by Matrone et al. (12). Ceruloplasmin activity was determined by the procedure of Houchin (13), as modified by Rice (14), with the following alterations: when the activity was very low, 0.2 ml of serum was used instead of 0.1 ml, and incubation time was increased from 15 minutes to 30 minutes or 1 hour. Radioactivity of ^{64}Cu was determined by a gamma scintillation counter.[5] The analysis of variance method was used for statistical analyses of the data (15).

Sheep experiment. Ten young lambs weighing approximately 23 kg each were divided into 5 groups of 2 lambs each. Each group was housed in an individual wooden pen with a diamond-mesh wire floor. Water was provided ad libitum. The lambs were fed twice daily. Each group was assigned to 1 of the 5 experimental diets (table 1). The variables in the experimental diets are summarized in table 2.

Blood samples were collected from the jugular vein every 2 weeks for the first 10 weeks and again at weeks 36 and 43. Sodium heparin was used to prevent coagulation. Hemoglobin and plasma copper determinations were made on all blood samples.

Chick experiment. Thirty-six young chicks (White Plymouth Rock) weighing about 300 g each were divided into 4 groups of 9 birds each. Each group was housed individually in battery brooders and assigned at random to one of the experimental diets. The experimental diets and drinking water were provided ad libitum. The composition of the basal diet is shown in table 3. Four experimental diets were prepared from this basal mixture. Diet 1 was the basal and served as the control. Diets 2 and 4 were supplemented with $Na_2MoO_4 \cdot 2H_2O$ to provide 500 ppm molybdenum. Diets 3 and 4 were supplemented with $MgSO_4$ (in lieu of $MgCO_3$) to provide 1000 ppm inorganic sulfate.

After the birds had been consuming their respective diets for 2 weeks, blood samples were collected (zero-day sample) from each bird by cardiac puncture. Immediately after this sample was taken, each bird was given an intraperitoneal injection of a saline suspension containing approximately 10^6 *Salmonella gallinarum* organisms. Further blood samples were collected

TABLE 1

Composition of sheep diets

	%
Casein [1]	20.0
Glucose [2]	29.2
Cornstarch [2]	23.0
Hydrogenated vegetable oil [3]	4.0
Cellulose [4]	3.0
$KHCO_3$	4.0
$NaHCO_3$	6.0
$CaCO_3$	1.0
$CaHPO_4$	1.8
Vitamin mixture [5]	5.0 [6]
Mineral mixture [7]	3.0

[1] Nutritional Biochemicals Corporation, Cleveland.
[2] Corn Products Sales Company, Norfolk, Virginia.
[3] Primex B and C (pure vegetable shortening), Procter and Gamble Company, Cincinnati.
[4] Alphacel, Nutritional Biochemicals Corporation.
[5] Contained per kg diet: (in milligrams) thiamine·HCl, 8.82; riboflavin, 18.74; nicotinic acid, 24.91; Ca pantothenate, 31.31; pyridoxine·HCl. 12.57; folic acid, 1.26; p-aminobenzoic acid, 24.91; inositol, 250.22; biotin, 0.25; choline chloride, 2500; menadione, 2.54; 0.1% vitamin B_{12} (with mannitol), 102.73; α-tocopheryl acetate, 12.57; and glucose, 47 g.
[6] Administered 88.2 IU of vitamin A and 9.0 IU of vitamin D/day/kg body weight by capsule.
[7] Contained per kg diet: (in milligrams) KCl, 6020; NaCl, 5270; $MgCO_3$, 3110; Na_2SO_4, 109; $FeSO_4 \cdot 2H_2O$, 169; $MnSO_4 \cdot H_2O$, 30.8; ZnO, 49.9; $CoCO_3$, 0.20; KI, 0.13; and glucose, 15.34 g. This was for the basal diet (17D). The other diets were supplemented (with concomitant reduction in glucose) as follows: (mg/kg diet) 17E, $Na_2MoO_4 \cdot 2H_2O$, 4.23; 17F, $Na_2MoO_4 \cdot 2H_2O$, 8.47; 17G, $Na_2MoO_4 \cdot 2H_2O$, 4.23 and Na_2SO_4, 600; 17H, $Na_2MoO_4 \cdot 2H_2O$, 8.47 and Na_2SO_4, 1200.

TABLE 2

Experimental variables in sheep diets

Diet	Copper	Molybdenum	Sulfur as sulfate
	ppm	*ppm*	%
17D	1	0	0.03
17E	1	2	0.03
17F	1	4	0.03
17G	1	2	0.06
17H	1	4	0.12

[5] Gamma Scintillation Counter, Nuclear-Chicago Corporation, Des Plaines, Illinois.

TABLE 3

Composition of basal chick diet

	%
Glucose [1]	32.5
Dried skim milk	60.0
Vegetable oil [2]	5.0
DL-Methionine	0.3
L-Arginine·HCl	0.5
Glycine	0.5
NaCl	0.5
	mg/kg
Vitamin mixture [3]	1980
$MgCO_3$	1855
Choline chloride	1540
$FeSO_4 \cdot 7H_2O$	528
$MnSO_4 \cdot H_2O$ (reagent grade)	220
$CuSO_4 \cdot 5H_2O$	40

[1] Cerelose, Corn Products Refining Company, New York.
[2] Wesson Oil, The Wesson Oil Company, New Orleans, Louisiana.
[3] Supplied per kg of diet: (in milligrams) thiamine, 3.52; riboflavin, 5.27; folic acid, 1.10; α-tocopheryl acetate, 22.00; menadione sodium bisulfite, 0.792; biotin, 0.176; pantothenic acid, 18.3; niacin, 52.3; pyridoxine, 5.7; and vitamin B_{12}, 8.8 μg; vitamin A, 11,000 USP units; and vitamin D, 1980 ICU.

by the same procedure at 24-hour intervals for the subsequent 5 days. Each blood sample was assayed for serum ceruloplasmin activity.

Tissue uptake experiments. Tissues used in this study were obtained from adult rats that had been consuming a commercial laboratory diet.[6] The rats were killed by stunning and decapitation. The liver or kidneys were removed immediately and placed in chilled Krebs-Ringer solution. Tissue slices ranging in weight from 100 mg to 200 mg were prepared using a Stadie-Riggs microtome. Following slicing, the tissue slices were washed twice in chilled Krebs-Ringer solution. Tissue aliquots ranging in weight from either 0.3 g to 0.5 g for liver or 0.2 g to 0.4 g for kidney were used in the incubation. The incubations were performed in Krebs-Ringer solution in 25-ml Erlenmeyer flasks at 37° in a constant-shaking water bath. Liver slices were incubated for 60 minutes and kidney slices for 30 minutes. Three levels of molybdenum (0, 500 and 1000 μg) and 4 levels of copper (100, 250, 500 and 1000 μg) were used in a factorial design. Molybdenum as $Na_2MoO_4 \cdot 2H_2O$ and copper as $CuSO_4 \cdot 5H_2O$ were dissolved in Krebs-Ringer solution. The total incubation volume, excluding the tissue slices, in each flask was 5.0 ml.

Immediately before the start of the incubation period, approximately 20,000 to 25,000 cpm of ^{64}Cu were added to each flask. After incubation the tissue slices were washed in cold Krebs-Ringer solution, blotted and placed in counting tubes for radioactivity determinations.

Characterization of Cu-Mo complex. During the course of this investigation it was observed that copper sulfate and sodium molybdate formed a complex at near neutral pH. The method of continuous variation, as outlined by Job (16), was used to determine the ratio of copper to molybdenum in the Cu-Mo complex. Copper and molybdate solutions (0.1 M) were made in water from copper (II) sulfate and sodium molybdate, respectively. They were then mixed according to the continuous variation procedure. Both the absorbance at 340 mμ of the supernatant solution and the weight of the precipitate formed were measured. The absorbance at 340 mμ was used as a measure of the concentration of the Cu-Mo complex in solution since the complex, but neither copper sulfate nor sodium molybdate, shows an absorption band of visible light in this region.

RESULTS AND DISCUSSION

Lamb experiment. As shown in figure 1, all animals exhibited decreasing plasma copper levels as the trial progressed, regardless of the diet fed. These results indicate that the basal diet, which contained 1 ppm Cu, was marginal or inadequate in respect to copper. Further examination of the data in figure 1 shows that the rate of decline in plasma copper was similar for

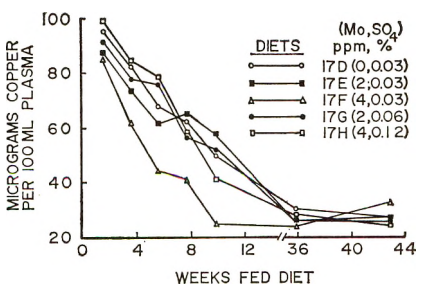

Fig. 1 Effect of diet on plasma copper levels of sheep. Mo added in the form of $MoO_4 \cdot 2H_2O$, sulfur in the form of Na_2SO_4.

[6] Purina Laboratory Chow, Ralston Purina Company, St. Louis.

the animals fed diets 17D (basal), 17E, 17G and 17H but lower for those fed 17F which contained 4 ppm of molybdenum and 0.03% sulfate. A comparison of the results obtained with plasma copper concentrations in figure 1 with hemoglobin concentrations in figure 2 shows a low order of correlation between these 2 parameters. In contrast with the results obtained with plasma copper, hemoglobin levels of the lambs fed diet 17D (basal) and those fed diet 17E, with the low level of molybdenum (2 ppm) and sulfate (0.03%), remained rather constant and at a normal level; all other experimental groups developed anemia. The effect of dietary sulfate on hemoglobin level is shown strikingly in the comparison between lambs fed diet 17E and 17G. Both diets contained the same level of molybdenum (2 ppm), but diet 17G contained twice as much sulfate; animals fed the former diet maintained a normal level of hemoglobin, whereas those fed the higher level of sulfate developed the severest anemia of any group in the experiment. However, the degree of anemia was less, and the effect of sulfate was ambiguous in the groups fed the high level of molybdenum (4 ppm). Thus at the low level of molybdenum the effect of increased dietary sulfate was synergistic with molybdenum, whereas at high levels the predominant depressing factor on hemoglobin appeared to be associated with the level of molybdenum.

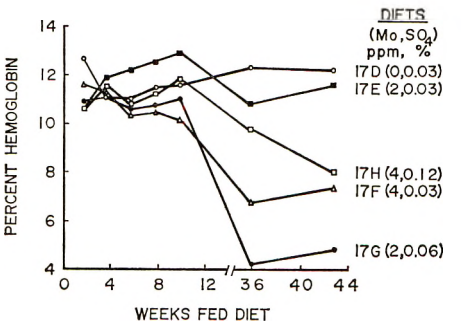

Fig. 2 Effect of diet on hemoglobin concentration of sheep. Mo added in the form of $MoO_4 \cdot 2H_2O$, sulfur in the form of Na_2SO_4.

Chick experiment. The effect of the diet on chick ceruloplasmin activity is presented in table 4. Recently it was reported (9) that induction of ceruloplasmin synthesis in chicks, which normally have low ceruloplasmin activity, could be brought about by certain stressor agents. As shown in table 4, ceruloplasmin activity of chicks in all groups responded similarly, reaching a peak on the third day after inoculation with fowl typhoid organism, followed by a small decline. Although the average values of the chicks fed high molybdenum, diet 2, are slightly lower than those for other groups, a statistical analysis of the data indicates that the differences were not significant ($P > 0.05$). Thus, neither molybdenum, nor sulfate, nor the combination of the two affected the induction of ceruloplasmin appreciably. Liver copper values of these chicks (table 5) also show no significant differences between treatments.

Tissue uptake experiments. The effect of molybdenum on the in vitro uptake of copper by rat liver and kidney tissues is shown in table 6. In every instance, the presence of molybdenum in the incuba-

TABLE 4

Effect of diet on chick serum ceruloplasmin activity [1]

Days after inoculation of fowl typhoid organism	Ceruloplasmin activity [2]			
	Diet			
	1 Control	2 High Mo	3 High sulfate	4 High Mo + high sulfate
0	0.91	0.82	0.97	1.01
1	3.95	4.18	4.15	3.77
2	5.84	5.65	6.33	5.85
3	7.27	6.40	7.00	7.14
4	6.85	5.98	6.51	6.43
5	7.08	5.84	6.45	6.26

[1] Each value represents the mean of 9 chicks.
[2] Ceruloplasmin activity expressed as international units.

TABLE 5

Effect of diet on liver copper of chicks [1]

Diet	Liver copper
	ppm (dry liver basis)
1 Control	13.89 ± 0.56 [2]
2 Migh Mo	13.19 ± 0.53
3 High sulfate	14.18 ± 1.12
4 High Mo + high sulfate	13.17 ± 0.59

[1] Each value represents the mean of 9 chicks.
[2] Mean ± SE.

TABLE 6

Effect of molybdenum on incorporation of copper into rat liver and kidney slices

Amount in flask		Copper incorporated	
Copper	Molybdenum	Liver	Kidney
μg	μg	μg/g tissue [1]	
100	—	164	140
100	500	151	109
100	1000	140	112
250	—	324	237
250	500	277	174
250	1000	264	182
500	—	499	334
500	500	432	294
500	1000	411	299
1000	—	571	450
1000	500	499	431
1000	1000	490	432

[1] Values calculated by the following equation:

$$\mu g \text{ incorporated/g tissue} = \frac{\text{observed cpm}}{\text{g tissue}} \div \frac{\text{cpm in incubation flask}}{\mu g \text{ Cu in incubation flask}}$$

where observed counts per minute have been corrected for background and radioactive decay.

tion flask reduced the uptake of copper by the tissue slices. Statistical analysis of these data shows that while the two high levels of molybdenum significantly reduced ($P < 0.005$) the uptake of copper when compared with the zero-molybdenum level, there were no significant differences in copper uptake between tissue slices exposed to either 500 or 1000 μg of molybdenum.

Presumably the reduced uptake of copper by the rat liver slices exposed to the molybdate could have been effected either through interference with the absorption process of copper in some unknown manner or by molybdate complexing the copper before absorption. Observations made accidentally showed that under certain instances a mixture of sodium molybdate and copper sulfate resulted in a precipitate, suggesting that the latter alternative might have a rational basis. Accordingly a study was conducted to investigate the characteristics of the precipitate.

Characterization of Cu-Mo complex. The results of the continuous variation method of Job (16) are presented in figures 3 and 4. Both the absorbance at 340 mμ and the weight of the precipitate formed indicate that the molar ratio of copper to molybdenum is approximately 4:3. Others (17, 18) have reported the in vitro formation of a copper molybdate complex in which the ratio of copper to molybdenum was 1:1. Tests for sulfate in the precipitate were negative. Another characteristic of the precipitate was that it formed optimally at around neutral pH.

The results obtained with sheep and chicks again emphasized the high susceptibility of ruminants to molybdenum tox-

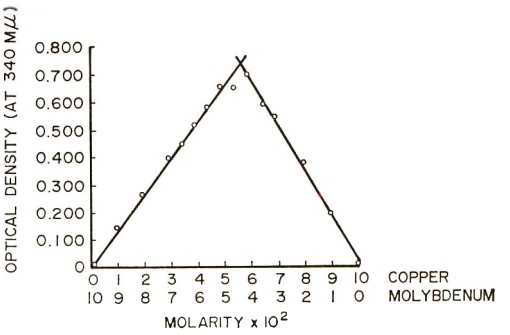

Fig. 3 Effect of molar variation of $CuSO_4 \cdot 5H_2O$ and Na_2MoO_4 upon amount of Cu-Mo complex in solution. (Values for molarity shown in the figure have been derived by multiplying the actual values by 10^2.)

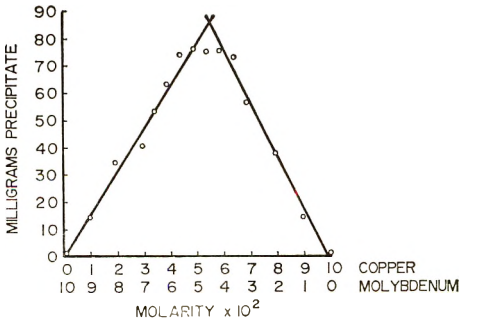

Fig. 4 Effect of molar variation of $CuSO_4 \cdot 5H_2O$ and Na_2MoO_4 on formation of Cu-Mo complex precipitate. (Values for molarity shown in the figure have been derived by multiplying the actual values by 10^2.)

icity as contrasted with the nonruminant. The study of copper uptake by liver tissue slices and the study demonstrating the formation of a Cu-Mo complex suggested the hypothesis that molybdenum was complexing copper, rendering it biologically inactive.

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A Copper-Molybdenum Complex: Its effects and movement in the piglet and sheep [1,2]

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ABSTRACT Experiments were conducted to study the in vivo availability and movement of copper of a copper-molybdenum (Cu-Mo) complex. In baby pigs fed the Cu-Mo complex as the only copper supplement, serum copper levels were similar to those in pigs fed copper sulfate. However, the pigs receiving the Cu-Mo complex showed an increase in ceruloplasmin activity which was no greater than that in pigs fed a diet without supplemental copper. Tissue molybdenum concentrations were higher in pigs fed the Cu-Mo complex than in pigs fed an equal amount of molybdenum as the sodium salt. Conversely, tissue copper levels in the pigs fed the Cu-Mo complex were generally lower than those in pigs fed either copper sulfate or copper citrate plus sodium molybdate in equivalent amounts. In sheep given an intravenous injection of the Cu-Mo complex made from ^{64}Cu and ^{99}Mo, the rates of removal from the blood of the copper and molybdenum were equal, and this rate was more rapid than the removal of molybdenum when ^{99}Mo was injected alone. Conversely, the rate of urinary excretion of molybdenum from the ^{64}Cu-^{99}Mo-injected sheep was slower than from the ^{99}Mo-injected animal. These results support the hypothesis that copper bound in a Cu-Mo complex is biologically unavailable and indicate that such a complex can exist in vivo.

The observations that molybdenum can initiate copper deficiency and that copper can alleviate molybdenum toxicity have been well-documented (1–4). The nature of this interaction is not known. From the results in a previous study (5), it was suggested that the formation of a "Cu-Mo complex" renders the copper biologically inactive.

The purpose of the present study was to test the hypothesis that the formation of a Cu-Mo complex may be the cause of the interaction between the 2 metals. Two experiments were conducted. In the first experiment, baby pigs were fed various copper supplements, including the Cu-Mo complex, to determine whether the complexed copper was metabolically available. Serum copper, ceruloplasmin activity, and tissue copper and molybdenum determinations were made. Two ewe lambs were used in the second experiment to determine the rates of removal from the blood of copper and molybdenum from an intravenously injected dose of the Cu-Mo complex. These lambs were also used to study the rate of urinary molybdenum excretion, both from the injected Cu-Mo complex and from molybdenum injected as the sodium salt.

EXPERIMENTAL

General procedures. Copper determinations were made by the method of Parks et al. (6), as modified by Matrone et al. (7). Molybdenum was determined by the procedure of Evans et al. (8) except that a chloroform-butanol (70:30) solution was used as the extracting agent rather than isopropyl ether. Ceruloplasmin activity was assayed according to the procedure of Houchin (9), as modified by Rice (10). Radioactivity of ^{64}Cu and ^{99}Mo was determined by a gamma scintillation counter.[5] The analysis of variance method was used for statistical analysis of the data (11).

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[3] This report constitutes a portion of a thesis submitted by R. P. Dowdy in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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[5] Gamma Scintillation Counter, Nuclear-Chicago Corporation, Des Plaines, Illinois.

Baby pig experiment. A litter of 12 baby pigs was farrowed, divided into 4 groups of 3 pigs each and raised artificially for 3 weeks according to the regimen of Gomez-Garcia and Matrone (12). The 4 experimental diets consisted of whole raw cow's milk plus the supplements shown in table 1. The Cu-Mo complex used for diet 3 was made in vitro by mixing aqueous solutions of copper (II) sulfate and sodium molybdate. The precipitate which formed was washed 5 times with distilled water and dissolved in dilute hydrochloric acid. When the complex was diluted with milk to feeding concentration, it contained 20 ppm copper and 24 ppm molybdenum.

Blood samples were collected from the vena cava of each pig at 36 hours of age and again at 7, 14 and 21 days of age. The blood was allowed to coagulate, and, subsequently, ceruloplasmin activity and copper determinations were made on the serum. At the end of the experiment (21 days) the pigs were killed and the following tissues removed and frozen: brain, heart, liver, kidney and spleen. All tissues were analyzed for their content of copper and molybdenum.

Copper and molybdenum uptake study in sheep. Two ewe lambs, equipped with urinary catheters, were placed in metabolism crates. No feed was given during this short-term experiment, but water was provided. One animal was given an intravenous injection of ^{99}Mo via the jugular vein. The other animal was similarly injected with the Cu-Mo complex solution made from ^{64}Cu and ^{99}Mo. The dose given to each animal had equivalent amounts of molybdenum. Blood and urine samples were taken at various time-intervals following injection of the isotope(s). Blood was collected from the jugular vein and urine from the catheters. Radioactivity determinations were made on blood and urine. The blood samples from the animal that received the Cu-Mo complex injection were counted twice; once at a zero time and again 72 hours later. From the half-life of the 2 isotopes used and the total number of counts, equations were constructed by which the total counts were partitioned into those resulting from copper and those resulting from molybdenum.

TABLE 1

Whole raw milk supplements for pig diets

Supplement	Diets			
	1	2	3	4
	ppm			
Iron [1]	80	80	80	80
Copper	—	20 [2]	20 [3]	20 [4]
Molybdenum	—	—	24 [3]	24 [5]

[1] Iron as ferrous sulfate.
[2] Copper as copper sulfate.
[3] Copper and molybdenum as the copper-molybdenum complex.
[4] Copper as copper citrate.
[5] Molybdenum as sodium molybdate.

RESULTS AND DISCUSSION

Baby pig experiment. The effect of the various treatments on serum copper is presented in table 2. The pigs receiving no supplemental copper (diet 1) maintained subnormal levels of serum copper throughout the experiment. As will be shown later, the initial increase in serum copper in these pigs probably resulted from a depletion of tissue copper. The increase in serum copper in pigs fed the other 3 diets progressed

TABLE 2

Effect of diet on serum copper of piglets

Diet [1]	Age			
	36 hours	7 days	14 days	21 days
	μg/100 ml serum			
1 [2]	33.9 ± 0.6	82.2 ± 6.4	92.2 ± 8.7	91.2 ± 11.8
2 [3]	32.4 ± 2.7	132.7 ± 5.0	246.0 ± 28.0	248.0 ± 6.0
3 [2]	33.9 ± 0.6	172.2 ± 8.7	212.3 ± 19.2	266.8 ± 24.6
4 [2]	31.6 ± 1.0	119.8 ± 6.8	223.0 ± 4.7	232.5 ± 5.5

[1] Diet 1, no copper; diet 2, 20 ppm copper as copper sulfate; diet 3, 20 ppm copper and 24 ppm molybdenum as Cu-Mo complex; diet 4, 20 ppm copper as copper citrate and 24 ppm molybdenum as sodium molybdate.
[2] Each value represents the mean of 3 observations ± SE.
[3] Each value represents the mean of 2 observations ± SE.

at a rate normal for young pigs (12). This suggests that the intestinal absorption of copper from the Cu-Mo complex was not impeded nor did molybdenum in diet 4 interfere with the absorption of copper from copper citrate.

There was a marked effect of diet on serum ceruloplasmin activity (fig. 1). As expected, the initial ceruloplasmin activity was low (12). The ceruloplasmin activity from pigs fed copper sulfate (diet 2) increased at a rate which is assumed to be normal for the 3-week experimental period. The presence of molybdenum in the diet

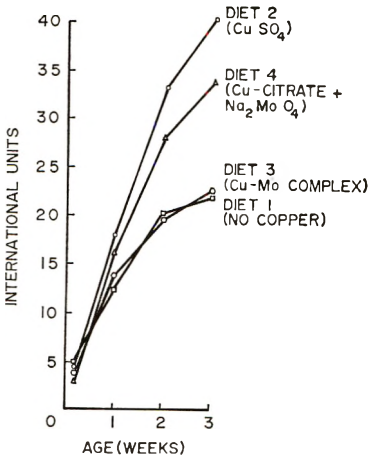

Fig. 1 Effect of diet on ceruloplasmin activity of piglets.

containing copper citrate had no significant effect on the normal increase of ceruloplasmin activity. Conversely, the increase in ceruloplasmin activity in the serum of pigs receiving the Cu-Mo complex (diet 3) very nearly paralleled that of pigs receiving no copper (diet 1); these were both significantly reduced ($P < 0.05$) compared with copper sulfate-fed controls. These results indicate that the copper from the Cu-Mo complex was unavailable for ceruloplasmin synthesis.

The effect of diet on tissue copper and molybdenum concentrations is shown in table 3. The pattern for molybdenum was the same for all tissues examined. Diet 4 (copper citrate, sodium molybdate) resulted in significantly higher ($P < 0.01$) molybdenum concentrations than either of the non-molybdenum diets (diets 1 and 2), but significantly lower ($P < 0.01$) molybdenum concentrations than the Cu-Mo complex-supplemented diet (diet 3).

The copper distribution pattern is not as well defined. In the liver, all copper-containing diets (diets 2, 3 and 4) resulted in significantly higher ($P < 0.05$) copper concentrations than the copper-deficient diet (diet 1). The Cu-Mo complex-supplemented diet (diet 3) resulted in significantly lower ($P < 0.01$) copper concentrations in the kidney than the diet supplemented with copper citrate and sodium molybdate (diet 4). Further, diet

TABLE 3

Effect of diet on tissue copper and molybdenum concentrations of piglets

Tissue	Diet			
	1 [1] No copper	2 [2] $CuSO_4$	3 [1] Cu-Mo complex	4 [1] Cu-citrate + Na_2MoO_4
	ppm (dry matter basis)			
	Molybdenum			
Liver	1.8 ± 0.1	2.3 ± 0.2	25.9 ± 2.4	16.0 ± 2.7
Kidney	4.5 ± 0.2	5.9 ± 0.9	79.8 ± 2.5	36.5 ± 2.4
Heart	2.4 ± 0.5	3.0 ± 0.8	14.6 ± 0.2	10.5 ± 0.1
Brain	0.9 ± 0.1	0.9 ± 0.4	7.2 ± 0.3	5.1 ± 0.2
Spleen	3.1 ± 0.2	2.5 ± 0.2	25.4 ± 1.5	15.4 ± 0.7
	Copper			
Liver	45.0 ± 3.5	210.0 ± 5.0	158.0 ± 10.2	198.0 ± 42.0
Kidney	24.0 ± 0.4	211.0 ± 4.0	150.0 ± 2.4	194.0 ± 5.9
Heart	22.3 ± 2.0	33.4 ± 3.0	18.0 ± 2.5	31.4 ± 4.2
Brain	21.0 ± 0.5	33.3 ± 0.3	29.8 ± 0.4	31.4 ± 0.7
Spleen	11.8 ± 1.2	15.5 ± 0.5	22.1 ± 1.4	18.1 ± 0.5

[1] Each value represents the mean of 3 observations.
[2] Each value represents the mean of 2 observations.

4 resulted in significantly ($P < 0.05$) less kidney copper than diet 2 (copper sulfate, no molybdenum). The heart copper concentration resulting from diet 3 (Cu-Mo complex) was not significantly different from that resulting from no copper supplement (diet 1). In the brain, the copper concentrations resulting from the 2 diets containing molybdenum (diets 3 and 4) were significantly lower ($P < 0.05$) than the concentration resulting from the copper sulfate control (diet 2), but significantly higher ($P < 0.01$) than that with the copper-deficient diet (diet 1). The dietary effect on spleen copper differed markedly from the other 4 tissues studied. In the spleen, the Cu-Mo complex supplement resulted in the highest ($P < 0.05$) copper concentration, whereas diets 2 and 4 (copper sulfate and copper citrate plus sodium molybdate, respectively) resulted in copper levels which were not significantly different from each other.

From the data obtained for the liver, kidney, heart and brain, it appeared that the Cu-Mo complex was taken up and distributed as a unit in the body and that this copper was metabolically unavailable. In such a case, previously stored copper would be utilized for normal metabolism, and the tissue copper concentrations would be lower in pigs receiving the Cu-Mo complex than in pigs receiving an equal amount of copper in another form. It further appeared that the Cu-Mo complex bound molybdenum was turned over less rapidly by the tissues since tissue molybdenum levels resulting from the Cu-Mo complex were higher than those resulting from an equal amount of molybdenum as sodium molybdate.

Copper and molybdenum uptake study. Data concerning the disappearance of the isotope(s) from the blood of sheep are presented in figure 2. These data indicate that the copper and molybdenum from the Cu-Mo complex were removed from the blood at the same rates. Further, it is apparent that the disappearance pattern of molybdenum from the Cu-Mo complex was not the same as for molybdenum alone. From the disappearance pattern of molybdenum alone, it appears that, under normal conditions, molybdenum is initially removed from the blood into some pool and then re-enters the bloodstream before its permanent removal.

The rate of urinary excretion of molybdenum, both alone and from the Cu-Mo complex, is shown in table 4. These data indicate that molybdenum from the injected dose of the Cu-Mo complex was excreted less rapidly than molybdenum injected alone.

In the baby pig experiment, the normal increase in serum ceruloplasmin activity during early postnatal development was depressed in piglets receiving their copper in the form of the Cu-Mo complex. This indicates that the copper bound in the Cu-Mo complex was not available for ceruloplasmin synthesis.

The data concerning tissue levels of molybdenum in the baby pig experiment indicate that the turnover rate for molybdenum was reduced when it was in the Cu-Mo complex. This suggestion was sup-

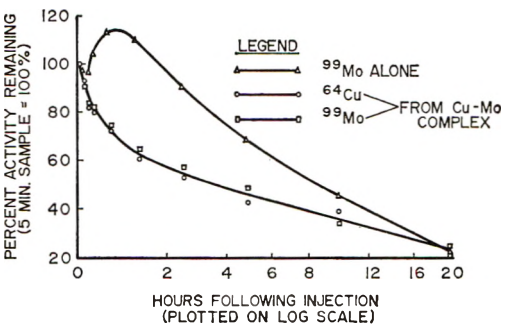

Fig. 2 Rate of isotope uptake from the blood of sheep. △, received injection of ^{99}Mo alone; ○, received ^{64}Cu from Cu-Mo complex; □, received ^{99}Mo from Cu-Mo complex.

TABLE 4

Rate of ^{99}Mo excretion in the urine

Time following injection	^{99}Mo from Cu-Mo complex	^{99}Mo alone
hours	*cpm* [1]	*cpm* [1]
0 to 0.5	1097	1191
0.5 to 1.0	1307	3209
1.0 to 1.5	1533	3809
1.5 to 2.0	1518	4081
2 to 3	1345	2736
3 to 4	1257	2722
4 to 5	1176	2200
5 to 8	936	1270

[1] Values are expressed as counts per minute which were excreted per minute (as an average) between the time intervals indicated.

ported by the results of the sheep experiment in which the molybdenum from the Cu-Mo complex was excreted more slowly in the urine than when molybdenum alone was injected.

Since tissue copper was generally lower in pigs receiving the Cu-Mo complex than in pigs receiving an equal amount of copper in another form, it appears that some of the tissue stores of copper had been used for normal copper metabolism.

The suggestion that the Cu-Mo complex can exist in vivo is supported by the results of the sheep study showing that the rates of removal from the blood of copper and molybdenum arising from the injected Cu-Mo complex were equal. Further, this rate of removal for molybdenum was more rapid than the removal rate of molybdenum alone. One further implication of the existence of the Cu-Mo complex in vivo is that the urinary excretion of copper should be increased when animals are fed high molybdenum diets; this has been reported by various workers (13).[6]

The present data support the hypothesis that the formation of a Cu-Mo complex may be the site of the nutritional interaction between the two metal ions. The chemical nature of such a complex is not known at the present time. Two possibilities are: 1) that copper is bound in a 4-member chelate ring (14) by the divalent molybdate anion; or, 2) that a heteropoly ion (15, 16) is formed between copper and the oxyanion of molybdenum.

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Parathyroid Influences upon Phosphorus Balance and Homeostasis in Cows [1,2]

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ABSTRACT Phosphorus balance studies were conducted on 4 cows during periods of experimentally altered parathyroid status. Parathyroid extract administration to intact and parathyroidectomized cows was accompanied by an increase of urinary phosphorus and a decrease in fecal phosphorus. Parathyroidectomy was followed by a decline in urinary phosphorus output and a rise in fecal phosphorus. The reciprocal changes between urinary and fecal phosphorus excretion during periods of altered parathyroid status resulted in minimal changes in plasma inorganic phosphorus concentration and phosphorus balance. Phosphorus homeostasis during parathyroid extract administration appeared to be preserved principally through a reduction of fecal phosphorus output rather than by liberation of phosphorus from bone. Although the changes in fecal phosphorus excretion are compatible with a parathyroid hormone-induced enhancement of phosphorus absorption, the data do not permit a definitive conclusion regarding such an effect.

Parathyroid extract promotes renal excretion of phosphorus in several species (1) including the bovine (2). Parathyroid extract-induced urinary loss of phosphorus, without replenishment of blood phosphorus from other sources, could be sufficient to deplete body fluids of phosphorus. Previous reports have emphasized the release of phosphorus from bone during parathyroid extract administration (3, 4). The present paper draws attention to changes in fecal phosphorus output of cows, which might partly compensate for changes of urinary phosphorus excretion during parathyroid extract administration and following parathyroidectomy.

METHODS

Six experiments were conducted on 4 six-year-old nonpregnant, nonlactating cows in various parathyroid states (table 1). The cows were kept in metabolic stalls and fed 6.36 kg/day of a ground mixture of timothy hay (4 parts), corn and cob meal (9 parts), alfalfa and bromgrass hay (2 parts), and NaCl (0.14 parts). Before a balance trial, 2 weeks were allowed for equilibration with the ration. The content of the Ca and P in the diet met the recommendation of Morrison (5), and the molar Ca:P ratio ranged from 0.86 to 1.4 in the lots of feed mixed for the various experiments. Water, provided ad libitum, did not contain detectable phosphorus. Parathyroid extract [3] was injected either subcutaneously or intramuscularly once daily at 8 AM or 3 times a day at 8 AM, 4 PM, and midnight (table 1). Parathyroidectomy was accomplished with approximately 25% ablation of the thyroid gland (6).

Urine was collected by means of an indwelling self-retaining catheter (Bardex-Foley with 75 ml inflatable fluted ovoid balloon, size 26 French) into a 24-liter polyethylene carboy. Feces were collected in a pan lined with 6-mil polyethylene sheeting. Urine and fecal collections were made daily at 8 AM. The feces were mixed with a power stirrer, weighed, and a 250-g aliquot was saved in an air-tight plastic container for analysis. The urine volume was measured, and a 100-ml aliquot was frozen for analysis.

Duplicate samples of the fecal aliquot were weighed into tared dishes and dried in an oven at 105° for 48 hours to determine dry matter content. The resultant dry feces were ground and stored. Dupli-

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[2] Concurrent studies on calcium metabolism are reported in a separate publication (J. Nutr., *92:* 253, 1967).

[3] Parathyroid injection, USP, was donated by Eli Lilly & Company, Greenfield, Indiana.

TABLE 1

Types of experiments

Cow no.	Breed	Body wt	Condition	Duration	Beginning date	PTE [1]
		kg		*days*		*USP units/day*
J-1	Jersey	400	intact	14	6-23-63	
			intact+PTE [1]	7	7- 7-63	3000
J-2	Jersey	427	intact	8	1- 7-64	
			intact+PTE	7	1-15-64	3000
J-3	Jersey	369	intact	8	9-15-64	
			intact+PTE	7	9-23-64	3000–5000
		398	PTX [2]	16	8- 4-65	
			PTX+PTE	10	8-20-65	5000 [3]
G-4	Guernsey	427	intact	7	5- 7-64	
			intact+PTE	7	5-14-64	3000
		522	PTX [2]	11	2- 6-65	
			PTX+PTE	8	2-17-65	5000 [3]

[1] PTE, parathyroid injection, USP.
[2] PTX, parathyroidectomized; surgery was performed on cow no. J-3 on 7-30-65, and on cow no. G-4 on 8-25-64. Although complete parathyroidectomy was not confirmed in cow no. G-4, an estimated 92% of its parathyroid tissue was removed (6).
[3] Divided into 3 equal doses at 8-hour intervals.

cate samples (approx. 1 g) of the dry feces were redried to a constant weight in tared crucibles, and ashed in a muffle furnace overnight at 550° to 600°. The ash was dissolved in HCl (1+4), evaporated to dryness to dehydrate the silica, redissolved, and brought to a constant volume before filtering (7). The same procedure was used to ash aliquots of the feed except that weight was determined on air-dried samples.

Heparinized blood samples were collected through indwelling jugular catheters of Teflon tubing (1.2 mm I.D.) before feeding at 8 AM and 4 PM and again at midnight. The plasma was separated by centrifugation within 1 hour and frozen.

Inorganic phosphorus was determined in urine, feed and fecal ash solution, and protein-free filtrates of plasma were assayed by the method of Fiske and Subbarow (8).

RESULTS

Although parathyroid extract administration to intact and parathyroidectomized cows was accompanied by pronounced phosphaturia, phosphorus balance was affected to a much lesser extent (fig. 1). Increases in urine phosphorus ranged from 64 to 300 mmoles/day, whereas net phosphorus elimination from the body was increased by only 6 to 60 mmoles/day, and in one experiment (parathyroidectomized cow, G-4) phosphorus balance was improved by 9 mmoles/day during parathyroid extract administration despite a large increase in urinary loss. The decrease in fecal phosphorus appeared to be the major factor offsetting the increased loss of phosphorus in the urine (fig. 1) since the change in fecal phosphorus ranged from 50 to 107% of the increase in urine phosphorus.

The decrease in fecal phosphorus (expressed as a percentage of the value before extract administration) during parathyroid extract administration to intact cows, J-1, J-2, J-3, and G-4 was 20, 18, 33, and 14%, respectively, and the decrease in parathyroidectomized cows, J-3 and G-4, in consecutive order was 57 and 40%. Larger doses of parathyroid extract and more frequent administration (table 1) may have contributed to the greater changes observed in the urinary and fecal phosphorus output of the parathyroidectomized cows.

Following parathyroidectomy, urinary phosphorus decreased to less than 1 mmole/day in both cows, and fecal phosphorus increased despite a slightly lower dietary phosphorus intake (fig. 1). These were the only experiments in which the fecal phosphorus output exceeded the dietary phosphorus intake. In cow G-4, the fecal phosphorus increased by an amount equal to 51% of the fecal excretion while intact. This cow was in an apparent state of parathyroid hyperactivity before parathyroidectomy, e.g., renal clearance studies

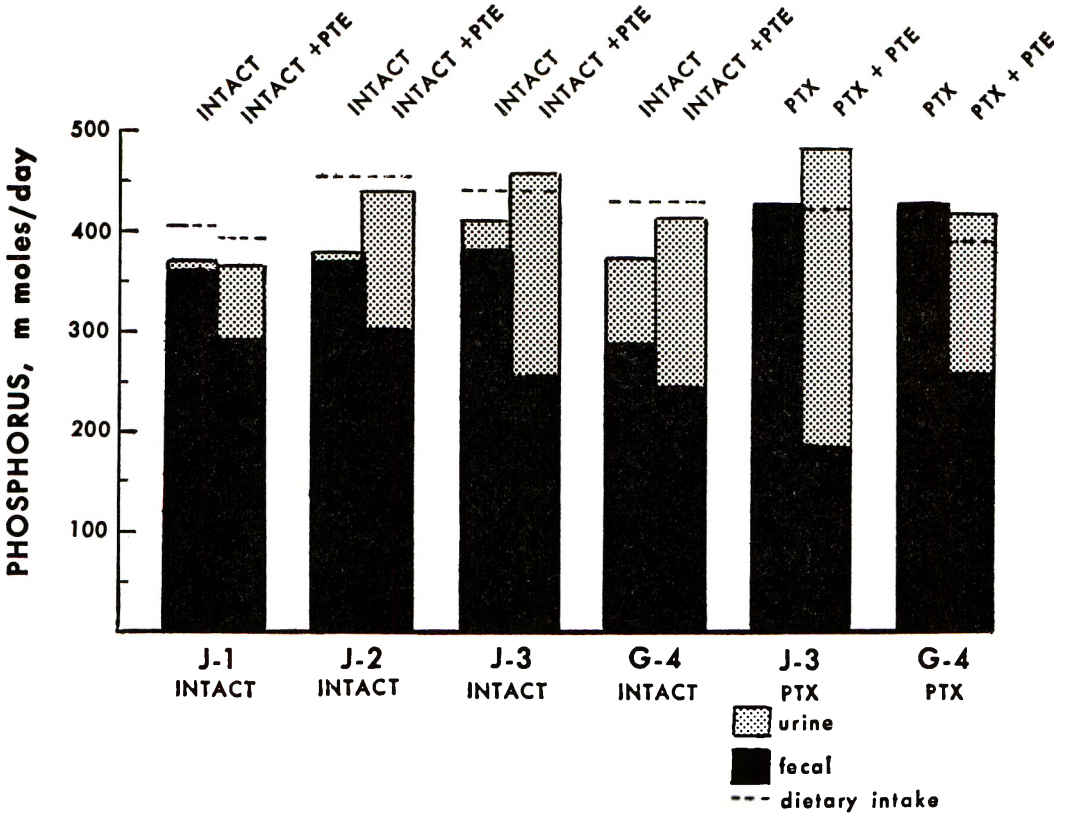

Fig. 1 Changes in fecal and urinary phosphorus excretion of cows associated with parathyroid extract (PTE) administration and parathyroidectomy (PTX). Each value represents the mean of at least 7 days of study. See table 1 for exact duration of each period. The dashed line represents the level of dietary phosphorus intake. Note that parathyroid extract administration was always accompanied by an increase of urinary phosphorus with an offsetting decrease in fecal phosphorus so that changes in total phosphorus excretion were small. Parathyroidectomy was followed by a decline in urinary phosphorus to less than 1 mmole/day and an increase in fecal phosphorus despite a slightly lower dietary intake. Also note that the largest changes in fecal phosphorus output occurred in cows showing the greatest changes in urinary phosphorus.

showed that it reabsorbed a lesser fraction of the glomerular filtered phosphorus than two other intact cows (2). The greater urinary phosphorus output of this cow relative to the other intact cows before extract treatment (fig. 1) probably resulted from depressed renal tubular phosphorus reabsorption.

The changes in fecal and urinary phosphorus output associated with altered parathyroid status appeared to be related in magnitude (fig. 1). The largest changes in fecal phosphorus occurred in association with the greatest changes in urinary phosphorus. The regression (fig. 2) of fecal phosphorus output (Y, mmoles/day) upon urinary phosphorus (X, mmoles/day) was $Y = 388 - 0.734X$. The correlation of these 2 variables ($r = -0.90$) was highly significant ($t = 6.33$, $P < 0.001$). No correction was made for the slight variation in dietary phosphorus intake between cows (fig. 1).

Only minor changes in plasma inorganic phosphorus concentration were observed despite wide shifts in urinary and fecal phosphorus excretion (table 2). Although plasma inorganic phosphorus concentration was slightly increased during parathyroid extract administration in 5 of the 6 experiments, all values remained within the normal range reported for cows.

DISCUSSION

Despite the induction of a pronounced phosphaturia during parathyroid extract administration to cows, phosphorus balance and plasma inorganic phosphorus concentration were only slightly altered (fig. 1 and table 2). Previous reports have emphasized the removal of phosphorus from bone during parathyroid extract administration (3, 4); however, phosphorus homeostasis during parathyroid extract administration to cows appears to be preserved principally by a reduction in fecal phosphorus output rather than by liberation of phosphorus from bone. Although our findings serve to emphasize the role of the gastrointestinal tract in phosphorus metabolism of the cow, they do not preclude an effect of parathyroid extract on bone. In fact, a slight decrease in phosphorus balance in 4 of our 6 experiments suggests some mobilization of phosphorus from body reservoirs, presumably bone. Also, it is conceivable that accelerated turnover of bone phosphorus during parathyroid extract administration, as indicated by investigations with radioactive phosphorus (3, 4), could occur without increasing the net loss of phosphorus from bone.

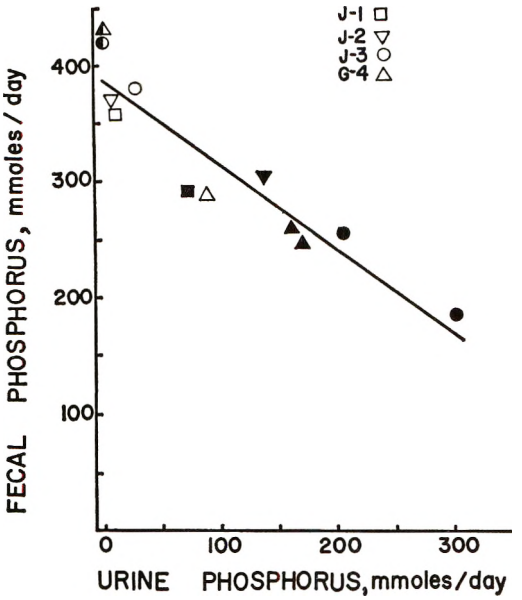

Fig. 2 Relationship between fecal and urinary phosphorus output. Open symbols, solid symbols, and half-closed symbols refer to intact, parathyroid extract treated, and parthyroidectomized cows, respectively. The regression line ($Y = 388 - 0.73X$) calculated by least squares is shown by the solid line. The correlation coefficient ($r = -0.90$) was highly significant ($t = 6.33$, $P < 0.001$).

Although the phosphaturic effect of parathyroid hormone is well-established in several species (1), including the cow (2), support for an effect of the hormone on gastrointestinal transport of phosphorus is less prevalent. Parathyroid extract has been shown to increase the mucosa to serosa flux of phosphorus by everted rat duodenal loops perfused in vitro (9), an effect which suggests enhancement of phosphorus absorption by parathyroid hormone. In our experiments, the decrease in fecal phosphorus associated with parathyroid extract administration and the increase following parathyroidectomy are compatible with the thought that parathyroid hormone may enhance phosphorus absorption from the gastrointestinal tract. However, we are cautious in drawing such an interpretation from our experiments for 2 reasons. First, the changes in fecal phosphorus do not necessarily indicate changes in rate of absorption since large quantities of phos-

TABLE 2

Plasma inorganic phosphorus in intact and parathyroidectomized cows given parathyroid extract

Condition	Plasma inorganic phosphorus			
	Cow no. J-1	Cow no. J-2	Cow no. J-3	Cow no. G-4
	mmoles/liter			
Intact	1.63 ± 0.19 [1]	1.60 ± 0.13	1.42 ± 0.19	1.52 ± 0.24
Intact + PTE [2]	1.57 ± 0.20	1.71 ± 0.27	1.46 ± 0.19	1.62 ± 0.20
PTX [3]			1.53 ± 0.14	1.44 ± 0.25
PTX + PTE			1.87 ± 0.25	1.69 ± 0.31

[1] Mean ± SD.
[2] PTE, parathyroid injection, USP.
[3] PTX, parathyroidectomized.

phorus are secreted into the gastrointestinal tract of cows (10), i.e., alterations in rate of endogenous secretion could influence fecal phosphorus output. Second, the effect on fecal phosphorus in our experiments may not represent a direct hormonal action on the gut. Reciprocal changes, related in magnitude, between urinary and fecal phosphorus in response to alterations in parathyroid status (figs. 1 and 2) suggest that one of these variables may have influenced the other. A direct effect of parathyroid hormone on the renal tubule is more firmly established (1, 11, 12) than a possible effect on the gut, suggesting that the changes in fecal phosphorus may be secondary to the renal effect of the hormone. However, another possible explanation is compatible with direct hormonal effects on both kidney and gut, and with the close reciprocal relationship between urinary and fecal phosphorus output. Enhancement of phosphorus absorption by parathyroid hormone would tend to elevate plasma inorganic phosphorus concentration, as observed during parathyroid extract administration in 5 of our 6 experiments (table 1). This would tend to increase the amount of phosphorus filtered through the glomerulus and thus augment the phosphaturia induced by the direct hormonal effect on the renal tubule. Under such circumstances the magnitude of the increase in urinary phosphorus would be somewhat dependent upon the extent of the increase in phosphorus absorption.

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Systematic Oscillations in Tyrosine Transaminase and Other Metabolic Functions in Liver of Normal and Adrenalectomized Rats on Controlled Feeding Schedules [1]

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ABSTRACT In an attempt to clarify the regulatory mechanism of synthesis and degradation of enzymes in mammalian cells in vivo, rats were fed 12, 30 and 60% protein diets and were adapted to 8 hours' feeding in either a 24-hour cycle or a 48-hour cycle under controlled lighting conditions. The rats were killed at various times in the cycle to determine any oscillatory changes in the activity of tyrosine transaminase, serine dehydratase, glucose 6-phosphate dehydrogenase and citrate cleavage enzyme in liver, compared with changes of glycogen deposition in liver and corticosterone level in plasma. Tyrosine transaminase shows increased activity 6 hours after the onset of food intake, and the increased activities are in proportion to the protein content of the diets. Direct proportionality of activity to protein level in the diet was also found for serine dehydratase and inverse proportionality for glucose 6-phosphate dehydrogenase and citrate cleavage enzyme. A secondary rise in tyrosine transaminase activity observed 16 hours into the fasting periods in rats adapted to 8 hours' feeding in 48-hour cycle is clearly dependent upon the protein content of the diet previously ingested, and is also dependent upon the availability of adrenal hormone. Glucose 6-phosphate dehydrogenase shows higher activity in rats adapted to 8 hours' feeding in a 48-hour cycle than in rats in a 24-hour cycle, and, on the contrary, serine dehydratase presents higher activity in the latter group of rats.

The activity of tyrosine transaminase was shown by Lin and Knox (1) to increase rapidly in liver following the injection of hydrocortisone to intact or adrenalectomized rats or of tyrosine to intact rats. Knox (2) later confirmed the failure of injected tyrosine to induce the enzyme in 4 to 8 hours in adrenalectomized animals but noted striking increases at 24 hours or after 10 days of feeding 5% tyrosine in the diet. The increases produced by feeding tyrosine to adrenalectomized rats were obtained at age 7 weeks but not at age 15 weeks. Kenney (3) showed that the increased activity after cortisone injection was associated with an increased amount of enzyme and with incorporation of labeled amino acid. Berlin and Schimke (4) reported that cortisone stimulated the synthesis of several enzymes about fourfold, but the various enzymes showed marked differences in rate of increase in activity due to inherent differences in decay rates. Tyrosine transaminase, with a half-life of 1 to 2 hours, increased rapidly for several hours and then rapidly declined, whereas arginase with a half-life of 4 days continued to increase for at least 4 days. Kenney (5) recently studied the effect of the well-known inhibitors of protein synthesis, puromycin and cycloheximide, on tyrosine transaminase activity following its induction and showed that, whereas in untreated animals the half-life was 1.5 hours, the enzyme was completely stable for 5 hours in treated animals. It was suggested that the degradation of tyrosine transaminase might require the synthesis of a specific degradation system, and Schimke et al. (6) have confirmed earlier indications that enzyme decay is an energy-requiring process. The above studies make it clear that the amount of any given enzyme in a mammalian tissue is the resultant of opposing rates of specific synthesis and specific degradation. Relevant work by Rechcigl and

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Heston (7), using liver catalase as a model, showed what was believed to be the first demonstration of a genetically controlled rate of degradation for a specific enzyme.

The regulated synthesis and degradation of an enzyme with a physiological half-life of only 1.5 hours raises serious questions about enzyme assays on tissue from animals whose daily cycles of activity are unspecified and uncontrolled and whose feeding habits are defined as ad libitum, as in most previous studies.

We have established in 3 reports that the tyrosine transaminase activity in rat liver is subject to wide systematic oscillations. In rats fed only in the dark from 6 PM (1800) to 6 AM (0600) liver transaminase activity was much higher at midnight than at any other time studied. The midnight activity was directly proportional to the protein content of the diet, while at other times there was almost no effect of diet (8). Potter et al. (9) reported data on male rats that were fed a 60% protein diet for 12 hours in each 48 and killed at 6-hour intervals. These "36-hour fasting-adapted" rats were placed on the regimen at approximately 42 days of age and adapted to it for 30 days. During this period their weight increased from about 150 g to about 225 g in comparison with ad libitum controls that weighed about 325 g. It was subsequently found (10) that 40-hour fasting-adapted rats could be produced and that the "midnight peak" in tyrosine transaminase activity could be obtained at 1500 by inverting the day-night schedule to give a dark period from 0900 to 2100 with food available for only 8 hours every other day from 0900 to 1700. Thus the peak in tyrosine transaminase activity was clearly associated with food intake and followed a pattern that was determined by the time of feeding, yielding an eightfold increase at approximately 6 hours after the onset of ingestion of a 60% protein diet, followed by a rapid decay in activity. Ad libitum controls also showed a significant increase in tyrosine transaminase activity when fed the same diet during the same interval of darkness.

Oscillations in tyrosine transaminase activity in liver of rats fed ad libitum have been independently confirmed by Wurtman and Axelrod (11), who were unaware of our earlier studies. They also observed similar oscillations in adrenalectomized or hypophysectomized rats. Civen et al. (12) have also confirmed the existence of daily oscillations in rat liver tyrosine transaminase in normal and adrenalectomized rats and have suggested a correlation with plasma corticosterone. Similar studies in this laboratory had provided similar results.[3]

The present study was undertaken to further correlate diet, feeding schedule, adrenal status and other metabolic functions with changes in rat liver tyrosine transaminase activity, and incidentally to find possible improvements in the design of experiments involving measurements of enzyme induction in livers and hepatomas in laboratory rats, to be published elsewhere.

MATERIALS AND METHODS

Animals. Male rats of the Charles River [4] and Holtzman [5] strains were placed in a windowless room with an inverted and displaced lighting schedule in which lights were on from 2100 to 0900. Feeding dishes containing 12, 30 and 60% protein diets were placed in the cage just before lights were switched off, and removed after 8 hours. The diets contained glucose [6] as the carbohydrate, which was increased in proportion to the decrease in protein. The diet was made up according to specifications previously described (13) and supplied commercially in the form of pellets.[7] The composition of the diets follows: The constant ingredients were corn oil [8] 5% with oleum percomorphum added in the proportion of 0.5 ml/50 g corn oil before mixing, salt mix 4%, and vitamin-casein mixture 2%. Protein in the form of purified casein was added to give final protein concentrations of 12, 30 or 60%, and glucose was added at levels of 79, 61 or 31%, respectively. The salt mixture contained the following: (%) sodium chloride (USP), 4.36; magnesium sulfate (USP), 13.70; sodium biphosphate (USP), 8.74; potassium phosphate (K_2HPO_4), 23.97; cal-

[3] Watanabe, M., V. R. Potter and H. C. Pitot, unpublished experiment.
[4] The Charles River Breeding Laboratories, Wilmington, Massachusetts.
[5] Holtzman Rat Company, Madison, Wisconsin.
[6] Cerelose, Corn Products Company, Argo, Illinois.
[7] General Biochemicals, Inc., Chagrin Falls, Ohio.
[8] Mazola, Corn Products Company.

cium biphosphate [$CaH_4(PO_4)_2 \cdot H_2O$], 13.58; ferric citrate (USP reagent, 17.5% Fe), 2.98; calcium lactate (USP), 32.67. The vitamin-casein mix consisted of thiamine·HCl, 500 mg; riboflavin, 188 mg; pyridoxine·HCl, 500 mg; nicotinic acid, 1.88 g; Ca pantothenate, 1.3 g; vitamin K (2-methyl-1,4-naphthoquinone), 250 mg; folic acid, 50 mg; vitamin B_{12} (crystalline), 5 mg; biotin, 10 mg; choline·HCl, 15 g; and casein, 1000 g. The vitamins were mixed with the casein, and blended in a ball mill. The addition of 2% of this mix to the diet thus added 2% protein and was considered in the final composition of the diet. Vitamin mix and diets were stored in the cold.

Rats weighing 150 to 160 g of body weight were adapted to 8 hours' feeding in every 48-hour cycle ("8 + 40" regimen) (10) for more than 3 weeks, and also rats weighing 70 to 80 g were adapted to 8 hours' feeding in every 24-hour cycle ("8 + 16" regimen). Furthermore, some of the rats adapted to either the "8 + 40" regimen or the "8 + 16" regimen were adrenalectomized,[9] and were maintained on the same food regimen as previously noted and with 1% NaCl for drinking water.

L-Tryptophan, 5-hydroxytryptophan and serotonin[10] were dissolved in 0.9% NaCl, and were administered intraperitoneally to rats at 1000, namely 1 hour after the onset of food intake. Hydrocortisone acetate[11] was also administered by the same procedure.

Preparation of homogenate and supernatant fraction. At the clock times shown in each figure, rats were killed rapidly by means of a guillotine with no previous stress to the animal, and the livers were quickly removed and cooled in chilled buffer, blotted, weighed, and homogenized in 4 volumes of 0.2 M Tris-HCl buffer, pH 8.0, containing 10^{-4} M dithiothreitol by means of a Polytron homogenizer.[12] An aliquot of the homogenate was used to assay for glucose 6-phosphatase as soon as possible. The rest of the homogenate was centrifuged in a Spinco model L2 preparative ultracentrifuge at 104,000 × *g* for 3 hours. Each clear supernatant was stored in several small tubes at − 70° until the time of assay for each enzyme.

Enzyme assay. Tyrosine transaminase, serine dehydratase, glucose 6-phosphate dehydrogenase and citrate cleavage enzyme were assayed automatically with a combintion unit previously described by Pitot and Pries (14) and Pitot et al. (15). Glucose 6-phosphatase was assayed by the method of De Duve et al. (16).

Chemical determination. A sample of liver weighing approximately 500 mg wet weight was taken for the determination of glycogen, by the method of Carrol et al. (17) and Roe and co-workers (18). Plasma corticosterone was determined fluorimetrically by the Silber-Guillemin method as modified by Givner and Rochefort (19).

RESULTS

Rats adapted to 8 hours' feeding per 48-hour cycle. In a series of 5 experiments, 165 rats were adapted to 48-hour fasting and 8-hour feeding in each 48-hour cycle with 3 different levels of dietary protein, 12, 30 and 60%. Animals were killed at various time-points in an established 48-hour cycle and their livers were assayed for glycogen and enzyme activity for 4 different enzymes as shown in figures 1 A–E. In these figures, experimental groups are shown by points connected by a single curve and each point represents 3, or occasionally 2 or 4, animals. Each experiment with the 12 and 30% protein diets included an experiment with 60% protein except for two additional experiments with 60% protein as indicated. Figure 1A shows the results of tyrosine transaminase assays. The activity increased rapidly for 6 hours after the intake of 60% protein diet began and showed a sharp maximum 6 hours after feeding. This experiment did not include intermediate time-points between 0900 and 1500 during the intake of 12 and 30% protein diets, but other data indicate that because of an earlier decay the maximum value in animals fed these diets probably comes slightly earlier than 6 hours after food was taken. The most interesting feature of figure 1A is the secondary peak 16 hours into the fasting period, with a

[9] The Endocrine Laboratories of Madison, Madison, Wisconsin.
[10] Sigma Chemical Company, St. Louis.
[11] Merck Sharp and Dohme, Division of Merck and Company, Inc., West Point, Pennsylvania.
[12] Brinkman Instruments, Inc., Des Plaines, Illinois.

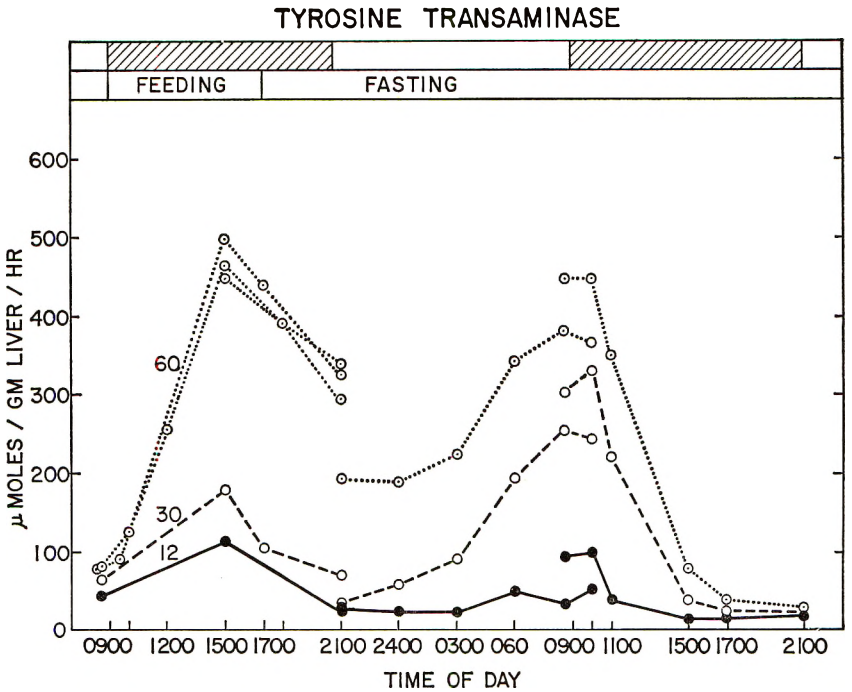

Fig. 1A–E Tyrosine transaminase, glycogen content, serine dehydratase, glucose 6-phosphate dehydrogenase, and citrate cleavage enzyme in the liver of Holtzman rats adapted to "8 + 40" regimen with 12, 30, and 60% protein diets. Darkness during alternate 12 hours as indicated by cross-hatched bars. Each closed circle connected with solid line represents a mean value of 2 to 4 rats fed 12% protein diets; open circle connected with dashed line, fed 30% protein diets; point within open circle connected with dotted line, fed 60% protein diets. Separate experiments are shown by groups of points connected by a single curve.

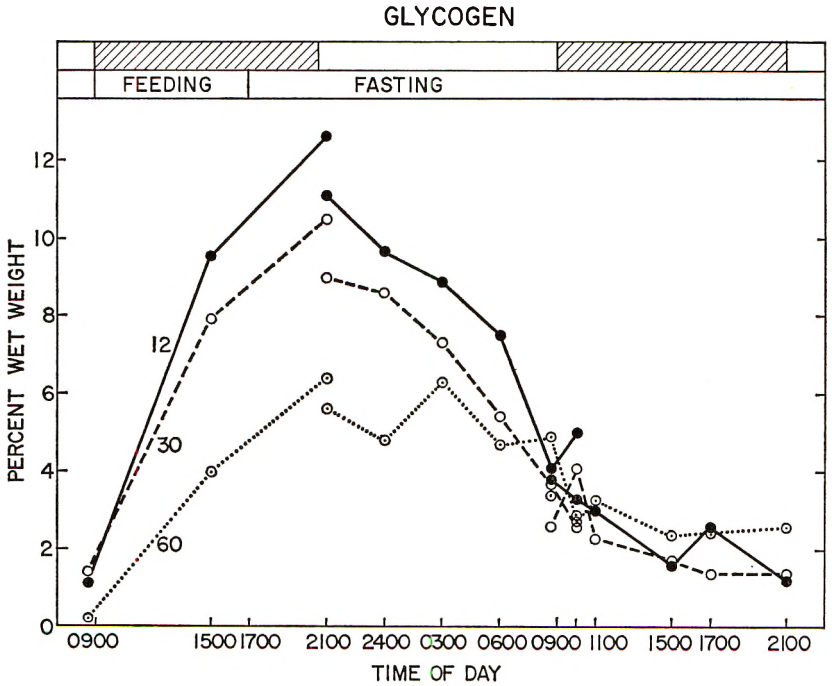

Figure 1B

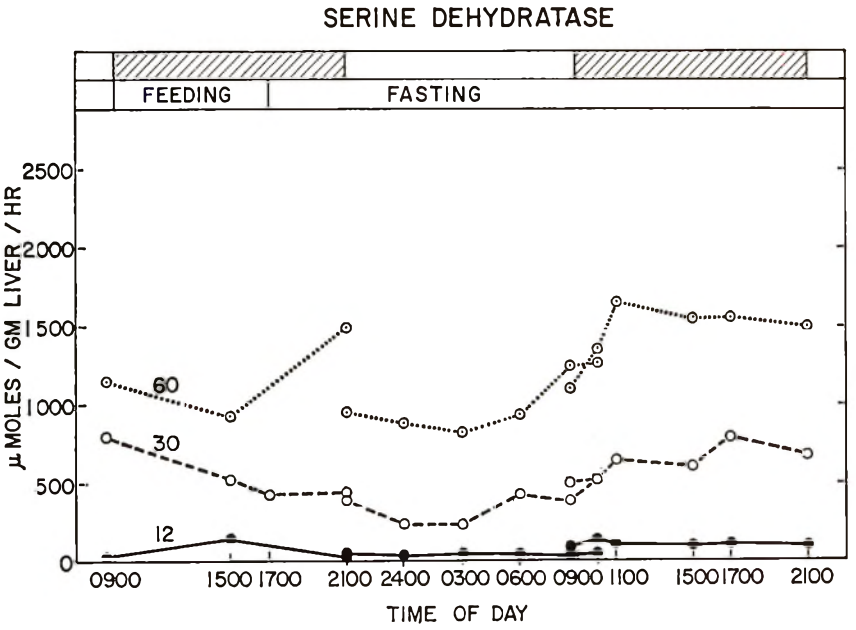

Figure 1C

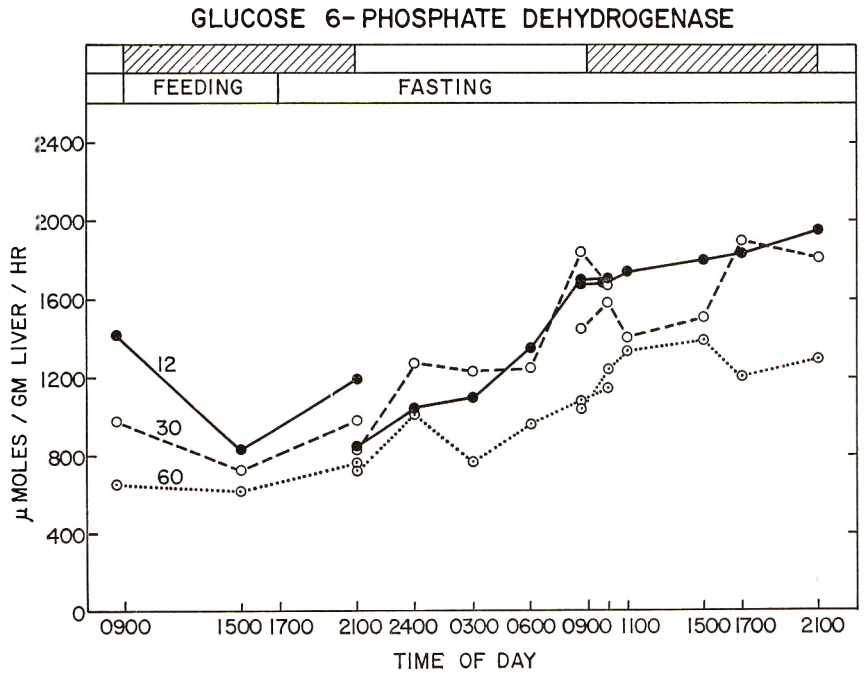

Figure 1D

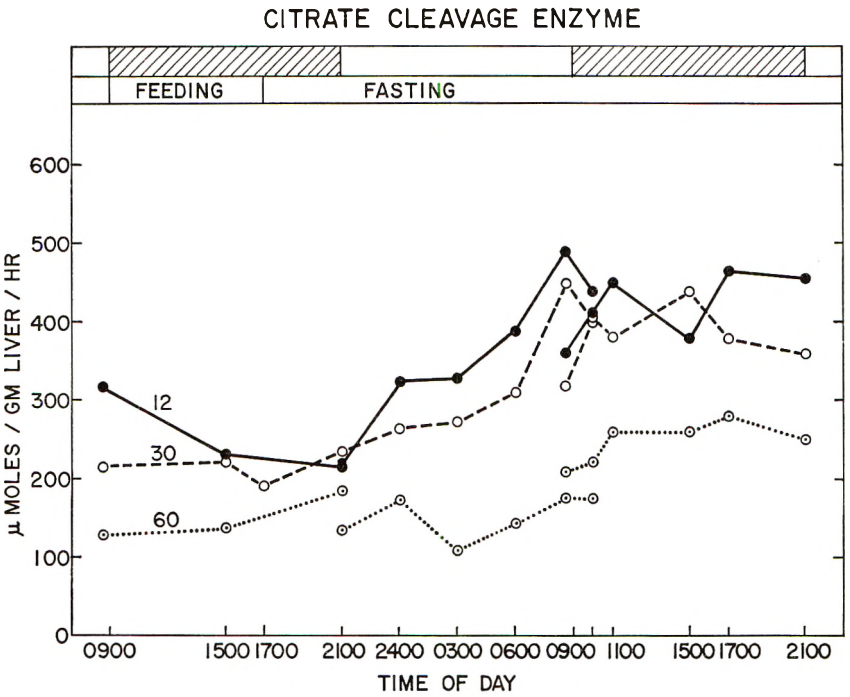

Figure 1E

sharp decline that coincides with the second dark period in the cycle. This secondary rise clearly is dependent upon *the protein content of the diet previously ingested.* It is also of interest that during the first dark period the enzyme activity rose for 6 hours, whereas during the second dark period the activity fell continuously following a rise during the light period. The decline in activity during the last half of the first dark period occurs while the livers are simultaneously continuing to lay down glycogen for the next 8 hours, as shown in figure 1B.

Figures 1C, D and E show, respectively, the activities of serine dehydratase, glucose 6-phosphate dehydrogenase and citrate cleavage enzyme, each of which shows a specific relationship to the protein content of the diet and to the time in the 48-hour cycle. In each case the level during the fasting period is a reflection of the previously ingested diet, but the decay phenomena are much less apparent than in the case of tyrosine transaminase. In these experiments approximately 45 rats were fed each of the 3 diets and the application of statistical methods may be made with reference to dietary protein or to time of day. It is apparent that the enzyme activities are a function of both variables but that in the case of the citrate cleavage enzyme and of glucose 6-phosphate dehydrogenase the differences are not significant at certain time-points, whereas the differences are highly significant at nearly all time-points in the case of the tyrosine and serine enzymes. It appears that statistically adequate treatment can best be accomplished by grouping various combinations of successive time-points to get values for n large enough to yield standard errors that are adequate for judging significance (table 1). Figure 1F shows the plasma corticosterone levels in the rats adapted to 30% protein diets, especially during the fasting periods in a 48–hour cycle. Since the corticosterone levels decreased during the feeding periods, as shown in the previous paper (10) the low levels are continued until 0600 in the second day, and then the increased levels of plasma corticosterone are found at 0830 when the

TABLE 1

Statistical treatment of data on variation in enzyme activities (figs. 1A, C, D, E) [1]

		Enzyme [2]	Protein in diet, %		
			12	30	60
				μmoles/g/hr	
A	Period 4–10 hours into fast (2100, 2400, 0300)	TAKG	26 ± 4 (9) [3]	62 ± 8 (9)	202 ± 14 (9)
		SDH	49 ± 10 (9)	280 ± 35 (9)	945 ± 47 (9)
		G6PDH	970 ± 68 (9)	1131 ± 76 (9)	837 ± 57 (9)
		CCE	292 ± 19 (9)	257 ± 9 (9)	140 ± 10 (9)
B	Period 22–28 hours into fast (1500, 1700, 2100)	TAKG [4]	54 ± 11 (12)	280 ± 13 (13)	407 ± 13 (13)
		SDH	102 ± 11 (11)	683 ± 54 (10)	1481 ± 72 (9)
		G6PDH	1862 ± 90 (10)	1717 ± 87 (10)	1290 ± 99 (9)
		CCE	431 ± 26 (11)	378 ± 15 (10)	262 ± 7 (9)

[1] All differences are highly significant ($P < 0.01$) except in the following cases:
TAKG: A vs B (12%) $P < 0.02$.
G6PDH: 12% vs 30% (A and B) $P > 0.1$, 12% vs 60% (A) $P > 0.1$.
CCE: 12% vs 30% (A and B) $P > 0.1$.
[2] Abbreviations are used: TAKG, tyrosine transaminase; SDH, serine dehydratase; G6PDH, glucose 6-phosphate dehydrogenase; CCE, citrate cleavage enzyme.
[3] SE, and in parentheses, number of animals.
[4] Each value presents the TAKG activities at period 15.5 to 17 hours into fasting (0830, 1000).

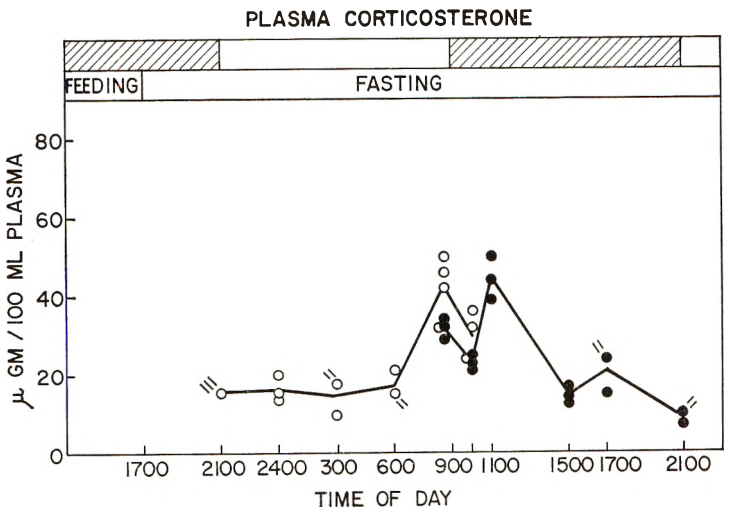

Fig. 1F Plasma corticosterone level in Holtzman rats adapted to "8 + 40" regimen with 30% protein diet. Two different experiments are shown by groups of points connected by a single curve. Each circle represents an individual rat. When open or closed circles coincide, such circles are indicated by 2 or 3 small bars, respectively, on the appropriate symbol.

secondary rise of tyrosine transaminase activity is observed (fig. 1A).

Rats adapted to 8 hours' feeding per 24-hour cycle. Previous reports by Potter et al. (10) presented data from rats on the "8 + 16" hour cycle but only the 60% protein diet was tested and only tyrosine transaminase, serine dehydratase, glycogen and plasma corticosterone were reported in comparison with animals fed ad libitum. In the present study glucose 6-phosphate dehydrogenase and citrate cleavage enzyme were included and the diets contained 12, 30 or 60% protein. Body weights did not differ much from those of ad libitum controls in comparable experiments, and the "8 + 16" regimen appears to be most useful as an approximation of ad libitum feeding on a 24-hour cycle when greater control over nutritional status is desired. Food was available from 0900 to 1700 in the dark and 2 or 3 animals were killed at 0830, 1500, 1700 and 2100. Data for glycogen and four different enzymes are

shown for individual animals in figures 2A–C. Parallel groups of animals assigned to each diet were housed in small drawer-type cages or in larger cages having access to voluntary exercise wheels, and the 2 groups were designated non-exercised and exercised, respectively (8–10). In the present experiment the effect of voluntary exercise was negligible and data from the 2 groups could probably be combined. These groups serve as controls for the data from the "8 + 40" regimen shown in figures 1A–E, wherein the data for tyrosine transaminase in figure 1A reach a high level at the onset of the second dark period which is unlike the behavior of the enzyme in livers of animals that are fed during every dark period. Otherwise the peak and decline of tyrosine transaminase during feeding periods is similar on both regimens. Further comparison of the 2 regimens is shown in figure 3, which presents data for stomach weight and liver weight as a function of time of day during a feeding cycle, and body weight minus stomach weight as a function of the percentage protein in the diet. The increased food intake during feeding on the "8 + 40" regimen was clearly unable to produce a body weight equal to that attained on the "8 + 16" regimen but the liver weights were proportional to body weight.

The enzyme data can best be compared by plotting all data obtained during the dark feeding periods as a function of protein in the diet, as shown in figure 4. This procedure had to be modified in the case of tyrosine transaminase since the data show marked changes according to time of day and only the maximum (1500) values were plotted as a function of dietary protein. These values are rendered more significant by the supporting data from the other time-periods even though the latter did not enter the average shown. Significant differences between the "8 + 16" regimen and the "8 + 40" regimen emerge in the case of glucose 6-phosphate dehydrogenase and serine dehydratase, with the "8 + 40" regimen yielding high values for the former and lowered values for the latter. Other differences are probably not significant and the noteworthy features of the data are the direct proportionality of activity to dietary protein level in the case of serine dehydratase and tyrosine transaminase and inverse proportionality in the case of glucose 6-phosphate dehydrogenase and citrate cleavage enzyme. There are no data for rats on the "8 + 40" regimen with exercise because the animals did not survive this combination of stresses for the duration of the experiment and died in from 5 to 20 days. The data summarized in figure

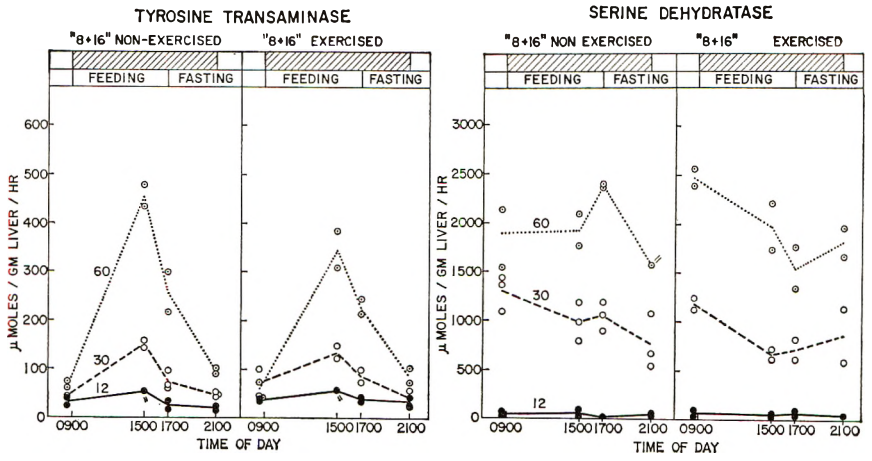

Fig. 2A–C Tyrosine transaminase, serine dehydratase, glucose 6-phosphate dehydrogenase, citrate cleavage enzyme, and glycogen content in the liver of Holtzman rats adapted to "8 + 16" regimen with 12, 30, and 60% protein diets under exercise and non-exercise conditions. Each closed circle connected with solid line represents an individual rat fed 12% protein diet; open circle connected with dashed line, fed 30% protein diet; point within open circle connected with dotted line, fed 60% protein diet. Other symbol is as in figure 1F.

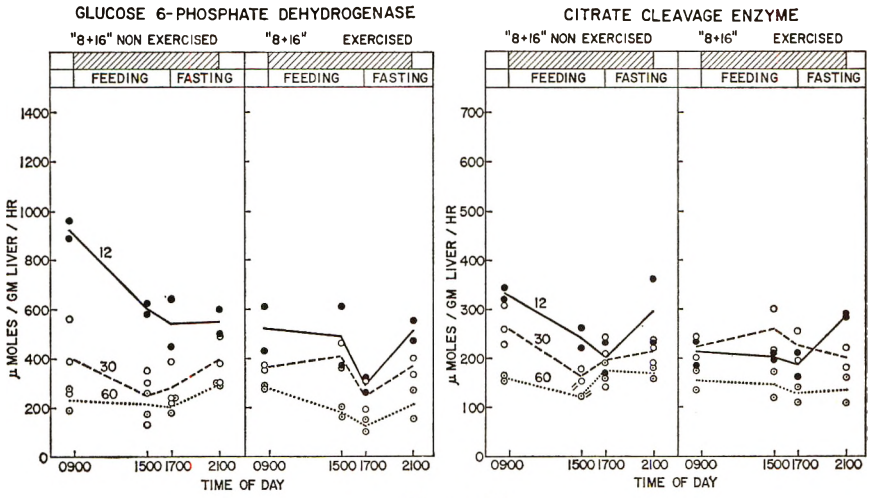

Figure 2B

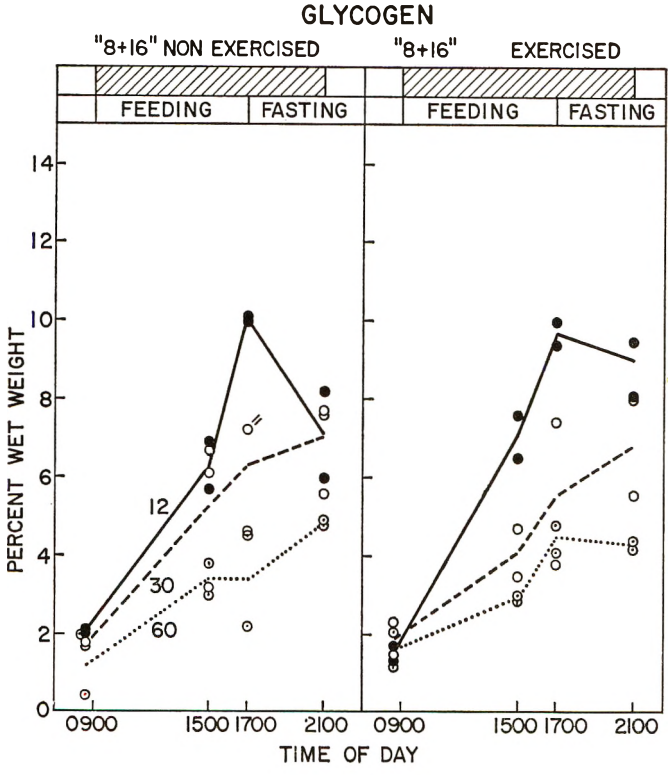

Figure 2C

4 provide a range of values for comparison with variations produced by other means.

Effect of adrenalectomy in rats shifted from 12% protein to 60% protein. Since the induction of tyrosine transaminase by hydrocortisone was well known (1, 3), several experiments were carried out with normal and adrenalectomized rats. In an

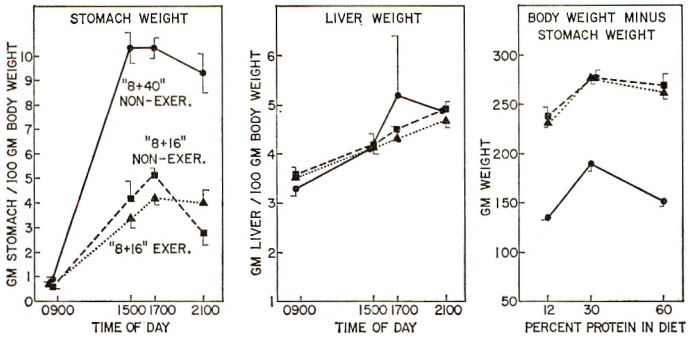

Fig. 3 Stomach weight, liver weight, and body weight minus stomach weight in Holtzman rats adapted to either "8 + 40" or "8 + 16" regimen with or without exercise. Rats in the group of "8 + 16" regimen are the same animals as in figure 2A–C. Each closed circle connected with solid line represents a mean value in rats adapted to "8 + 40" regimen under the non-exercise condition; closed square connected with dashed line, "8 + 16" regimen under the non-exercise condition; closed triangle connected with dotted line, "8 + 16" regimen under the exercise condition. Standard errors are indicated by brackets.

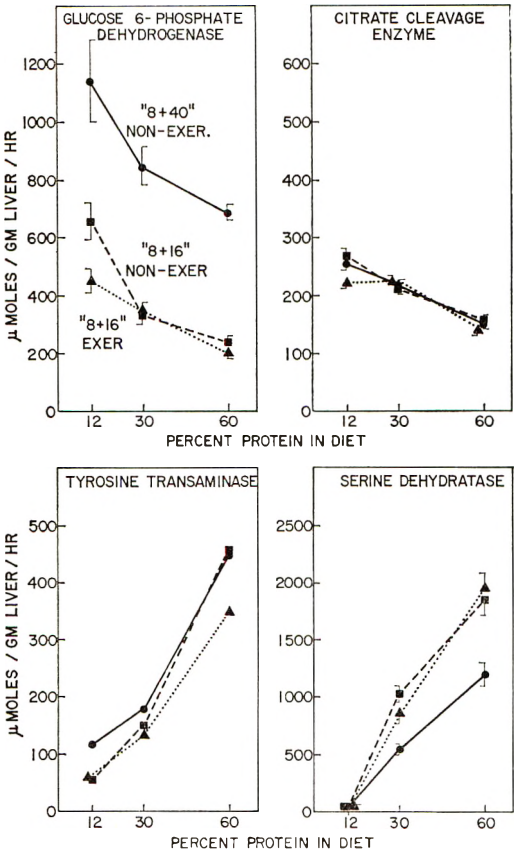

Fig. 4 Enzyme patterns of tyrosine transaminase, serine dehydratase, glucose 6-phosphate dehydrogenase and citrate cleavage enzyme in the liver of rats as a function of protein content in the diet. Symbols as in figure 3.

experiment of the type shown in figure 2A, rats adrenalectomized 5 or 10 days before they were killed showed the same rapid increases and decreases as the intact animals.[13] The following modification of the experiment was carried out in two different groups of rats to test whether adrenal function is required for the responses that occur when animals are shifted from a 12% to a 60% protein diet. Rats were adapted to 12 and 60% protein diets for 18 days on an "8 + 16" regimen. Half the animals in each group were then adrenalectomized and maintained with 1% NaCl for drinking water and all were continued with either 12% or 60% protein diets in the "8 + 16" regimen as previously. Three days later both the intact and adrenalectomized animals fed the 12% protein diet were presented for the first time with a 60% protein diet at 0900 while the other 2 groups were continued with the 60% protein to which they had been adapted. Pairs of animals from each of the 4 groups were killed at 0830 and at intervals for the next 12 hours, and 6 measurements were carried out on liver or blood from each animal as shown in figures 5A–D. Tyrosine transaminase showed the same increase in activity after intake of food in both intact and adrenalectomized rats, regardless of the previous diet, which shows that adrenal function is not required for the

[13] Watanabe, M., V. R. Potter and H. C. Pitot, unpublished experiment; cf. 11, 12.

short time response shown by tyrosine transaminase. The deposition of glycogen in liver was poor in both intact and adrenalectomized rats shifted from 12% protein to 60% protein because neither group of rats was able to develop gluconeogenesis from protein during the time-period shown, as demonstrated by the inability to raise the level of serine dehydratase. Thus no superiority of intact over adrenalectomized rats was evident. By contrast, in the groups of rats maintained on 60% protein diets, the intact rats stored glycogen in higher amounts than the adrenalectomized rats. The activity of serine dehydratase was not modified by adrenalectomy in the rats in either of the feeding schedules. Following adrenalectomy glucose 6-phosphate dehydrogenase and citrate cleavage enzyme showed slightly decreased activity in the rats shifted from the 12% protein diet to the 60% protein diet, but no change in activity in the rats fed 60% protein diets. Plasma corticosterone levels were reduced by adrenalectomy in both experimental groups.

Effect of adrenalectomy in rats adapted to 8 hours' feeding per 48-hour cycle. It was demonstrated that the secondary rise of tyrosine transaminase activity in the liver of rats adapted to the "8 + 40" regimen was dependent upon the protein content of the diets previously fed (fig. 1A). Further experiments were designed to show whether the secondary rise is modified by adrenalectomy. Rats were adapted to the "8 + 40" regimen with 60% protein diets for more than 3 weeks, and half of the animals were then adrenalectomized, and 3 days after the operation both the intact and the adrenalectomized rats were killed at midnight, 0600, 0830, and 1500 during the fasting periods, as shown in figure 6. At 0830 tyrosine transaminase in the liver of adrenalectomized rats showed lower activity than that in the liver of intact rats, but still maintained approximately the same level as that in the rats adapted to 30% protein diets (fig. 1A). Serine dehydratase and the other 2 enzymes studied in the liver of adrenalectomized rats did not show any differences compared with those of livers of intact rats during this period.

Another group of rats was fed the 30% protein diet instead of the 60% protein diet as in the previous experiment, and half of them were also adrenalectomized 3 days before they were killed. These rats

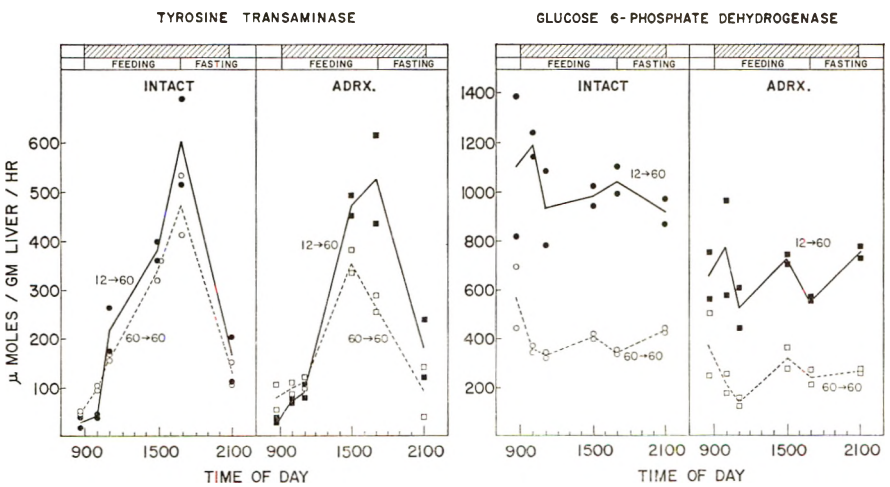

Fig. 5A–D Tyrosine transaminase, serine dehydratase, glucose 6-phosphate dehydrogenase, citrate cleavage enzyme and glycogen in liver, and corticosterone level in plasma in either intact or adrenalectomized Holtzman rats which were either shifted from 12% protein diet to 60% protein diet or maintained on 60% protein diet under the "8 + 16" regimens. Each closed circle or square connected with solid line represents an individual intact or adrenalectomized rat, respectively, which was shifted from 12% protein to 60% protein in the diet (12 → 60). Each open circle or square connected with dashed line represents an individual intact or adrenalectomized rat, respectively, that was maintained on a 60% protein diet (60 → 60).

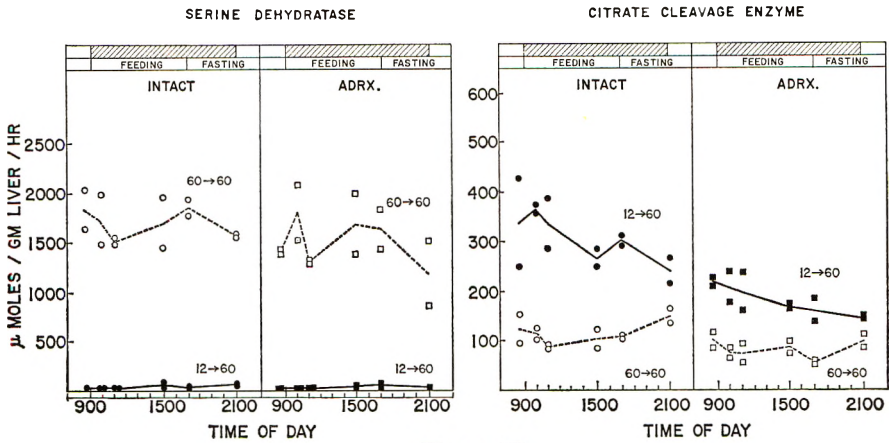

Figure 5B

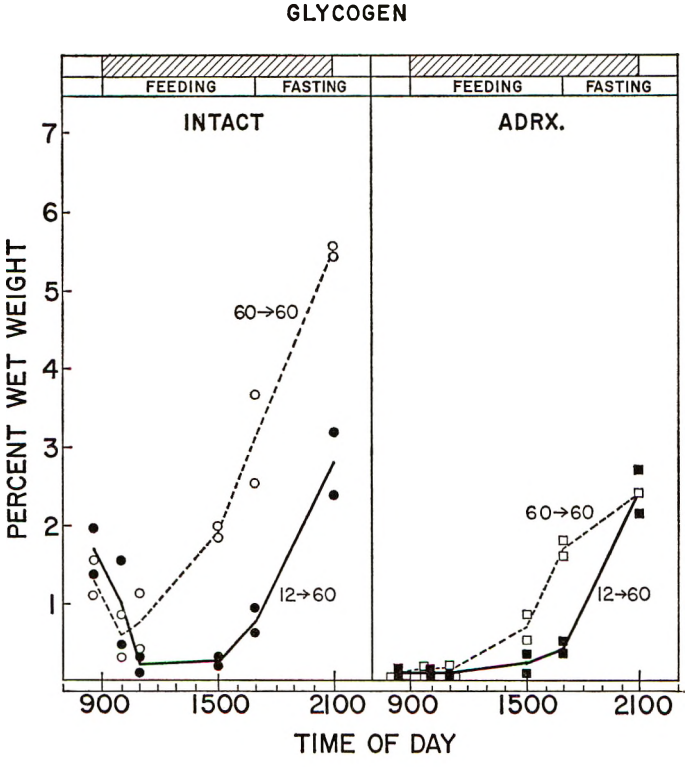

Figure 5C

were killed at the same intervals in the 48-hour cycle, as shown in figures 7A–C. Tyrosine transaminase activity in the adrenalectomized rats shows a significant increase 6 hours after feeding similar to that in intact rats, and shows the typical decline thereafter in both groups of rats. However, at 0830 in the second day of the 48-hour cycle the activity in the adrenalectomized rats is completely different from that of intact rats which show the typical secondary rise, and maintains at the low

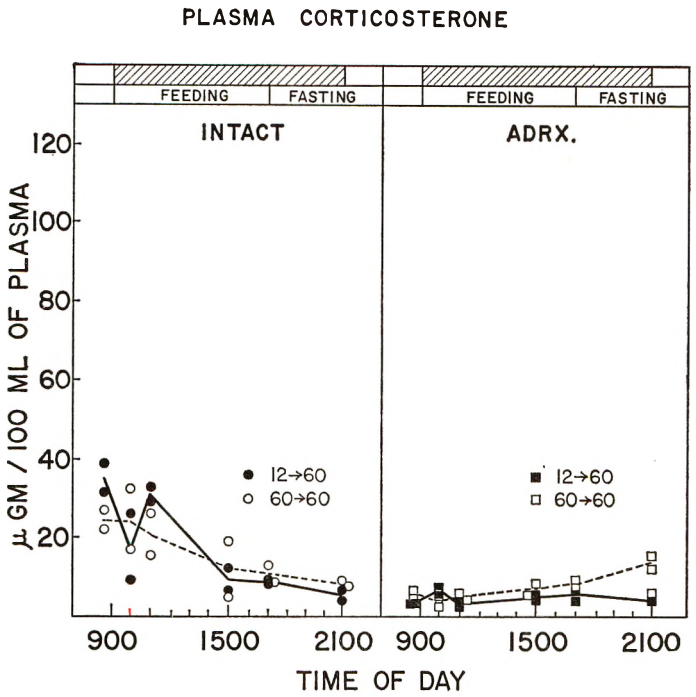

Figure 5D

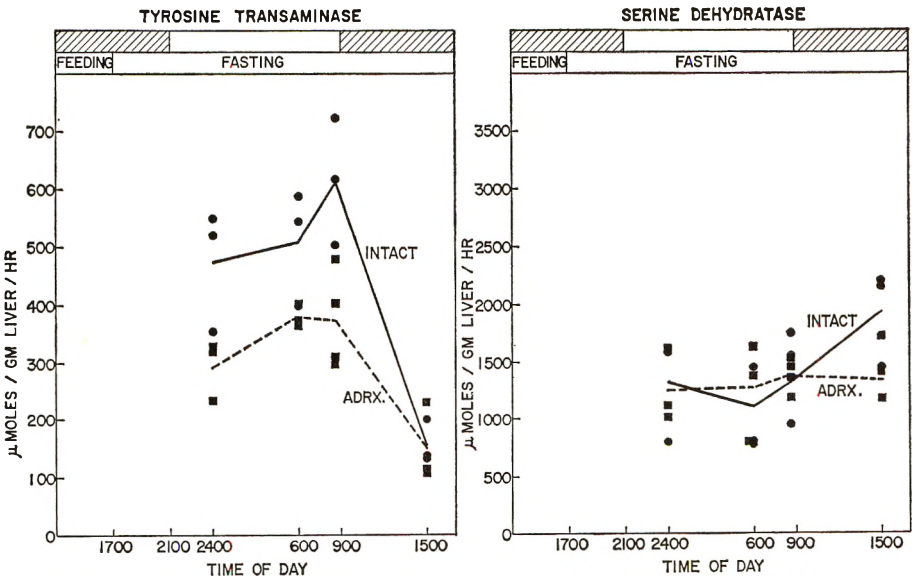

Fig. 6 Tyrosine transaminase and serine dehydratase in the liver of intact and adrenalectomized Charles River rats adapted to "8 + 40" regimens with 60% protein diets. Each closed circle connected with solid line represents an individual intact rat; closed square connected with dashed line, an adrenalectomized rat.

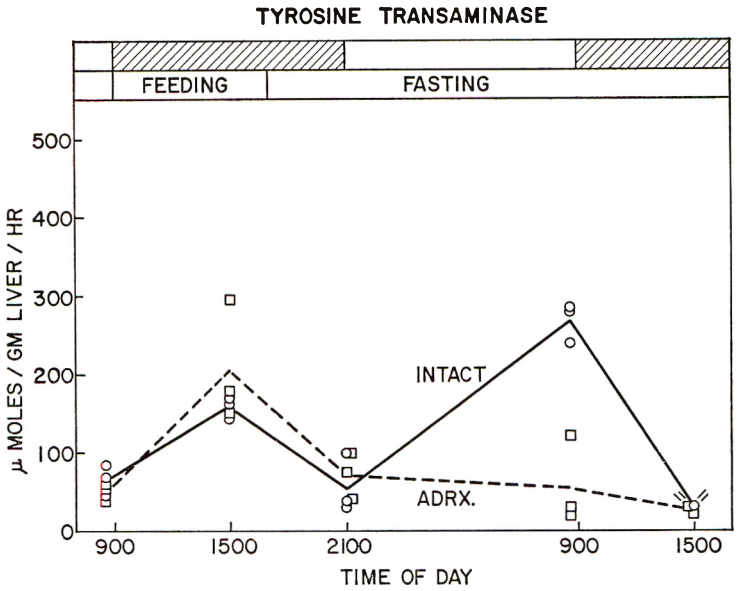

Fig. 7A–C Tyrosine transaminase, glycogen and glucose 6-phosphatase in the liver of intact and adrenalectomized Charles River rats adapted to "8 + 40" regimens with 30% protein diets. Each open circle connected with solid line represents an individual intact rat; open square connected with dashed line, an adrenalectomized rat. Other symbols as in figure 1F.

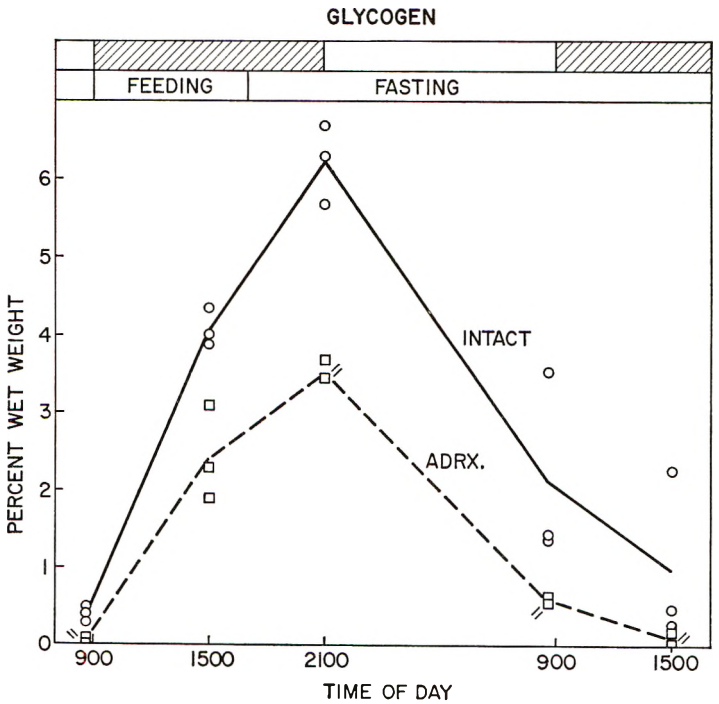

Figure 7B

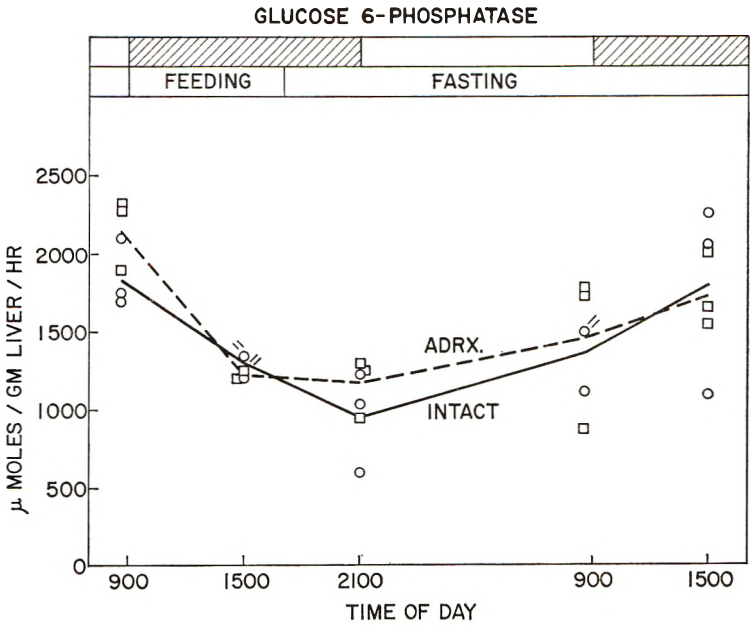

Figure 7C

levels shown in the preceding period. As shown in figure 7B, the deposition of glycogen in the liver of adrenalectomized rats is only about half as great as in intact rats even though the oscillation of the deposition occurs in the feeding and fasting cycles. The levels of activities of glucose 6-phosphate dehydrogenase, citrate cleavage enzyme and serine dehydratase were also measured but they showed no distinct difference between the intact and the adrenalectomized rats. It is of interest from this experiment, as shown in figure 7C, that glucose 6-phosphatase activity decreases gradually during feeding and again increases during the fasting periods in both intact and adrenalectomized rats.

Effect of serotonin in rats adapted to 12% protein diet and "8 + 16" regimen. Wurtman [14] has correlated the increase in tyrosine transaminase (8–12) with an increase of tryptophan in the liver, and there is an important issue as to whether the increase in this enzyme is a nutritional problem or a hormonal problem. If the role of the steroid is indirect and via tryptophan, is the tryptophan acting as such or only after it has been metabolically transformed to 5-hydroxytryptophan or further to serotonin, which cannot replace tryptophan as a nutrient? It was shown by Rosen and Nichol (20) that serotonin and 5-hydroxytryptophan stimulated increases in tryrosine transaminase activity and appeared to be more effective than L-tryptophan. The following modifications of their experiment were carried out. Rats were adapted to a 12% protein diet and the "8 + 16" regimen, and L-tryptophan, 5-hydroxytryptophan and serotonin were injected intraperitoneally into the rats at 1000, one hour after the onset of feeding, and the treated rats as well as the control rats were killed at 1500 and 1700, namely, 6 and 8 hours after the onset of the food intake. As shown in figure 8, although the deposition of glycogen in liver after feeding was completely blocked by the administration of the above 3 chemicals, tyrosine transaminase activity increased significantly as compared with the sustained low level of the activity in the control rats, which show no increase at this level of protein intake as shown earlier. The glycogen findings confirm the results of Foster et al. (21) who reported that tryptophan leads to both synthesis and activation of phosphoenolpyruvate carboxykinase but

[14] Personal communication.

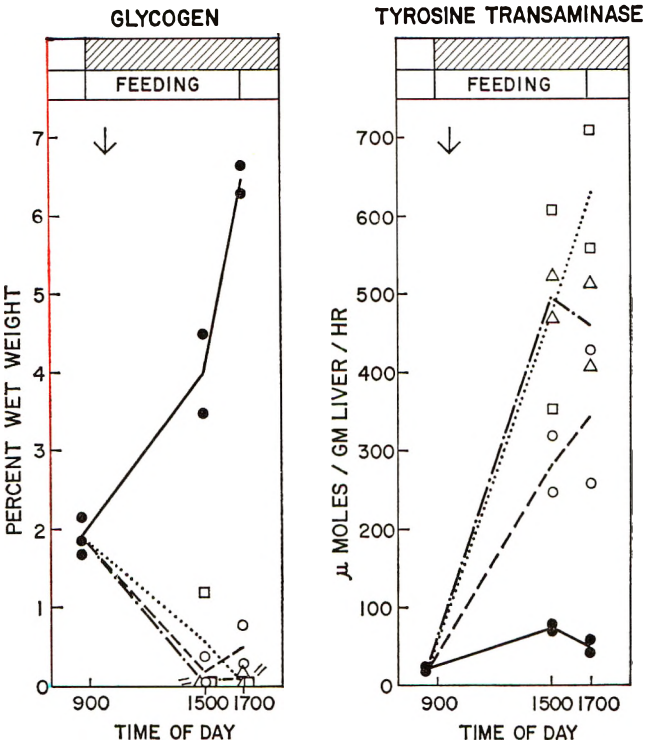

Fig. 8 Effects of L-tryptophan, 5-hydroxytryptophan and serotonin on tyrosine transaminase activity and glycogen deposition in the liver of Holtzman rats adapted to "8 + 16" regimen with 12% protein diets. Each closed circle represents an individual rat administered 0.9% saline solution as a control group; open circle, 3 mmoles of L-tryptophan per kg body weight; open triangle, 3 mmoles of 5-hydroxytryptophan per kg; open square, 1 mmole of serotonin per kg. Arrow shows the time of injection. Other symbols as in figure 1F.

paradoxically inhibits the activity of that enzyme in vivo.

Different doses of serotonin (0.01–1 mmole/kg of body weight) were administered at the same time as in the previous experiment and rats were killed at 1500 (fig. 9). In proportion to the increasing administered doses of serotonin the tyrosine transaminase activity is stimulated and glycogen deposition is reduced. There is apparently a reciprocal relationship between tyrosine transaminase activity and glycogen deposition in the liver under these conditions. At a dose of 0.1 mmole/kg, the results were intermediate and variable, and at 0.01 mmole/kg neither parameter was affected.

To determine whether treatment with hydrocortisone is modified by the serotonin stimulation of tyrosine transaminase activity, rats were administered hydrocortisone and serotonin simultaneously. As shown in figure 10, no additive effect of serotonin and hydrocortisone was observed either on deposition of glycogen or tyrosine transaminase activity, and the induction by serotonin was not increased by the presence of hydrocortisone. These experiments do not establish that injected tryptophan induces tyrosine transaminase directly or indirectly or whether degradation is affected, and the mechanism of the serotonin induction is yet to be determined.

DISCUSSION

Large changes in the content of tyrosine transaminase in liver are brought about by several factors, such as diet (2, 22–27), steroid hormones (1, 3, 28), glucagon (29, 30), coenzymes or their precursors (24) and others (20). Greengard (31) classified hormonal and substrate inductions of tyro-

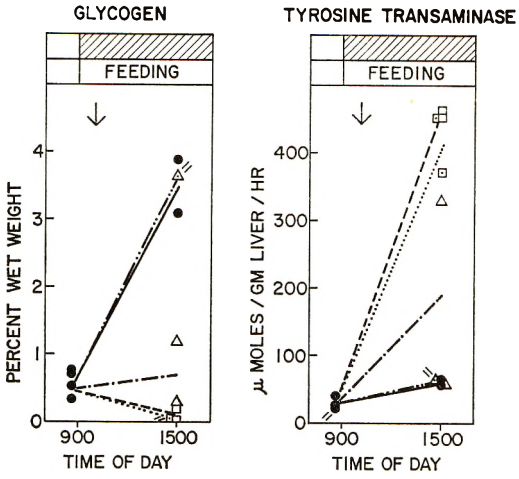

Fig. 9 Effect of different doses of serotonin on tyrosine transaminase activity and glycogen content in the liver of Holtzman rats adapted to the same regimen as in figure 8. Each open square represents an individual rat administered 1 mmole per kg body weight; point within open square, 0.5 mmole per kg; open triangle, 0.1 mmole per kg; point within open triangle, 0.01 mmole per kg; closed circles, saline controls. Arrow shows time of injection.

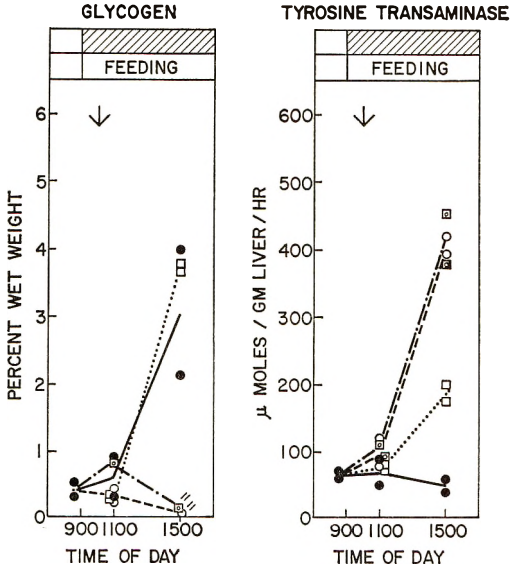

Fig. 10 Comparison between serotonin and hydrocortisone effects on tyrosine transaminase activity and glycogen content in the liver of Holtzman rats adapted to the same regimen as in figure 8. Each open circle represents an individual rat administered 1 mmole of serotonin per kg body weight; open square, 20 mg of hydrocortisone per kg; open circle within open square, serotonin and hydrocortisone simultaneously. Other symbols as in figure 8.

sine transaminase as two different mechanisms. However, the stimulation of tyrosine transaminase by the substrate or nonspecific agents required the intact adrenal gland or the presence of hydrocortisone (32, 33), and Singer and Mason (34, 35) also showed actinomycin-sensitive induction of tyrosine transaminase by the administration of non-hormonal substance, indicating "hormone type induction."

In the experiments on steroid hormone effect on tyrosine transaminase activity, hydrocortisone or cortisone was mostly used, indicating a steroid hormone induction, but the predominant corticosteroid in the rat is corticosterone, instead of hydrocortisone and cortisone (36, 37). However, the administration of deoxycorticosterone, as a precursor of corticosterone, did not show an increased activity, in contrast with a significant enhanced effect on the enzyme activity by hydrocortisone (25). The endogenous levels of corticosterone in rat plasma were easily modified by changing the environment of the rats (35, 38, 39). Considering the effects of endogenous corticosterone level of tyrosine transaminase activity in rat liver, Geller et al. (40) and Schapiro et al. (41, 42) showed that adult rats exposed to a stress did *not* respond with an increase in the tyrosine transaminase activity in liver despite marked corticosterone secretion, but enzyme activity was increased in stressed infant rats, as if the stress increased both synthesis and degradation in the former but only synthesis in the latter. Wurtman and Axelrod (11) and Civen et al. (12) noted the correlation between plasma corticosterone and tyrosine transaminase in unstressed animals on a regulated light schedule. The type of stress used by Geller et al. (40) partially inhibited the induction of tyrosine transaminase by hydrocortisone in adult rats, but not in infant rats.

Massive doses of steroid hormone administered parenterally may not be necessarily equivalent to the physiological level in the experimental animal (40, 43), and the pituitary function of the hypophysio-adrenal axis may be involved directly or indirectly in activation of induced tyrosine transaminase (44–46). Furthermore, some paradoxical effects of actinomycin on tyrosine transaminase induction in rat liver

were observed (47, 48) and these effects may be involved in the balance between rapid synthesis and degradation of an enzyme with a half-life of 1 to 2 hours (4, 49).

To create standard controlled physiological and nutritional environments for studying the relation of diet and hormone functions to enzyme activities, Potter et al. (8–10) have developed the concept of systematic adaptive challenge in terms of limited access to food under controlled lighting conditions, and found an apparent metabolic oscillation of tyrosine transaminase activity at more intensified levels in rats adapted to "8 + 16" or "8 + 40" regimens than in rats fed ad libitum. The data herein reported show that in rats adapted to the "8 + 16" regimen with 12, 30, and 60% protein diets tyrosine transaminase increased to a maximal level 6 hours after the onset of food intake, and thereafter gradually declined to the basal levels, and the maximal levels of the enzyme activity were observed in proportion to increasing protein content in the diet. Furthermore, if the rats were shifted from 12% protein diets to 60% protein diets on the killing day or were adrenalectomized 3 days before use, tyrosine transaminase activities showed the same oscillatory patterns as those of rats adapted to 60% protein diets. Sixteen hours after taking the food away a secondary rise in the activity in the liver of rats adapted to "8 + 40" regimen was also observed with a higher level of plasma corticosterone and lower deposition of liver glycogen, and the increased levels of tyrosine transaminase were completely dependent upon the adrenal status at that time.

The other enzymes studied serve as reference points for the peculiar case of tyrosine transaminase, but also have some intrinsic interest. Serine dehydratase activity in rat liver was modified intensively by protein content of diet (12, 13, 27, 50–52), by fasting (53) and by hormonal status (54, 55). Glucose 6-phosphate dehydrogenase was also changed by a different carbohydrate content of diets (27, 56–59). The activity of citrate cleavage enzyme in rat liver varies greatly with change in nutritional conditions (60–62). The changes of glucose 6-phosphatase activity by nutritional and hormonal status were mentioned by several authors (27, 56, 58, 63–65). As reported in the present paper, the activities of serine dehydratase, glucose 6-phosphate dehydrogenase and citrate cleavage enzyme, and the glycogen deposition in the liver showed specific relationships to the protein content of the diets and to the time during feeding and fasting in the 24- and 48-hour cycles, and glucose 6-phosphatase also showed an apparent oscillation in the liver of rats adapted to the "8 + 40" regimen.

In all the above situations the resolution of hormonal and nutritional contributions remains incomplete and both may conceivably affect simultaneously activation, inactivation, inhibition, synthesis or degradation (21). Their further interweaving is suggested by experiments using α-aminoisobutyric acid and 1-aminocyclopentane-1-carboxylic acid, which are probably transported across the cell membrane by a system that actively transports natural amino acids. Cortisone accelerates the uptake of these non-metabolizable amino acids by the liver and the uptake in adrenalectomized animals was reduced when compared with the intact animals (66, 67); hence the steroid may have indirect effects equivalent to an increased amino acid intake. Recently, Baril and Potter (68), using the new nutritional regimens herein described, found a systematic oscillation of the transport system of 1-aminocyclopentane-1-carboxylic acid by the liver and showed that the oscillations could not be demonstrated in rats fed ad libitum. Moreover, the initial and secondary rises in the activity of tyrosine transaminase coincided with the increased transport of the non-metabolized amino acid into the liver.[15] Thus, the action of steroid hormone may include an augmentation of the accumulation of amino acids by the liver (64, 69).

The available data thus suggest that previous interpretations (31) of the role of cortisone and of actinomycin in experiments involving induction of tyrosine transaminase may have to be modified to some extent to encompass the role of degradation and the possibility of action by way of the transport of amino acids into the liver.

[15] Watanabe, M., and E. F. Baril 1967 Oscillations in tyrosine transaminase activity and amino acid transport in liver of rats adapted to 24- or 48-hour cycles of feeding and fasting. J. Cell Biol., 35: 139A (abstract).

Changes in enzyme activity resulting from changing rates of synthesis and degradation of enzyme protein in rat liver occur in the course of the 24-hour cycle, as a result of feeding habits, as a result of changes in dietary composition and as a result of hormonal status (70) in the rat. The experimental use of rats that are adapted to special feeding regimens under controlled lighting conditions may lead to a clearer understanding of some of the factors involved in the regulation of enzyme activity under physiological environments. The data reported above suggest several ways in which the practice of ad libitum feeding might be modified to facilitate further studies on the change in the ratio between synthesis and degradation of specific enzymes.

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Systematic Oscillations of Amino Acid Transport in Liver from Rats Adapted to Controlled Feeding Schedules [1]

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ABSTRACT The model amino acid, cycloleucine, was used to study amino acid transport in the liver of rats fed ad libitum and rats adapted to 8 hours' feeding in either a 24-hour cycle or a 48-hour cycle under controlled lighting conditions. The rats were killed at various times in the cycle to determine whether oscillatory changes occurred in the amino acid transport system. Systematic oscillations of amino acid transport, with little individual variation, were observed in liver of rats adapted to controlled feeding and fasting periods. In contrast, no apparent oscillations and considerable individual variation were observed in amino acid transport in liver of rats fed ad libitum. The systematic oscillations of amino acid transport in liver appear to be related to the dietary state of the animal. Also, adrenal function contributed to a secondary oscillation observed during the early fasting period of the 48-hour cycle. The oscillations in the amino acid transport system of fasting-adapted rats correlate well with observed oscillations in the activity of liver tyrosine transaminase from the same rats.

The demonstration of a daily rhythm in some metabolic activities of rat liver and minimal deviation hepatomas by Potter et al. (1–3) indicates the need for controlled feeding and lighting schedules in experiments with the laboratory rat. A daily oscillation in the liver tyrosine transaminase activity of rats fed ad libitum was first observed by Potter et al. (1, 3) and later confirmed by other investigators (4, 5). However, Potter et al. (1–3) showed that the amplitude in the normal daily oscillation of some enzymes in liver was increased with animals on controlled feeding and fasting regimens.

The activity of rat liver tyrosine transaminase and other liver enzymes can be correlated with the diet, feeding schedule, adrenal status and other metabolic functions, as shown by Watanabe et al. (6). Data on the functional behavior of the amino acid transport system of liver are also required to obtain a clearer understanding of the sequence of metabolic events that give rise to the oscillations in enzyme activities. Changes in the level of free tyrosine in plasma and liver have been related to changes in the activity of rat liver tyrosine transaminase at different times of the day and different levels of dietary protein and hormonal activity (7). However, to unequivocally relate changes in the activity of the enzyme to variations in the amino acid transport system itself, it is necessary to separate transport from metabolism of the amino acids.

Use of model amino acids introduced by Christensen and co-workers permits investigation of amino acid transport in the absence of metabolic alteration of the amino acid itself (8, 9). Christensen and Jones (10) have shown that the synthetic amino acid 1-aminocyclopentane-1-carboxylic acid (ACPC, cycloleucine) is not metabolized by the rat and is almost completely reabsorbed by the kidney so that its rate of elimination from the body is extremely slow. Cycloleucine resembles the natural amino acids L-valine and L-methionine in transport properties (9) and is rapidly accumulated in vivo by liver, muscle (10), pancreas (11) and other tissues in the rat.

Simultaneous oscillations in amino acid transport and in the activity of tyrosine transaminase in liver from fasting-adapted rats were described in a preliminary report

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by Watanabe and Baril.[3] In the present investigation, the model amino acid 1-aminocyclopentane-1-carboxylic acid carboxyl-^{14}C was used to correlate diet, feeding schedule and adrenal status with changes in the activity of the amino acid transport system in liver and other tissues of the rat.

MATERIALS AND METHODS

Chemicals. 1-Aminocyclopentanecarboxylic acid-carboxyl-^{14}C (5 mCi/mmole) was dissolved in 1% NaCl before use.[4] The 30% and 60% protein diets (6) fed to the rats were made up as previously described and supplied in the form of pellets.[5]

Animals and regimens. Male rats of the Charles River strain[6] (150–160 g) were housed on arrival in a windowless room with lighting automatically regulated to provide a 12-hour period of light (9 PM – 9 AM) followed by 12 hours of darkness (9 AM – 9 PM). Food was available during the dark period only. Feeding dishes containing 30% or 60% protein diets were placed in the cage immediately before the lights were switched off and removed after 8 hours. Thus, the animals were fed for fixed periods of 8 hours in each 24-hour cycle ("8 + 16" regimen) or 48-hour cycle ("8 + 40" regimen) and subsequently fasted for fixed periods of 16 or 40 hours.

The rats were adapted to the desired regimens for more than 3 weeks before the start of the experiments, which were completed within 4 to 5 weeks of the regimen. Some of the rats adapted to the "8 + 40" regimen were adrenalectomized,[7] and maintained on the same regimen with a 1% NaCl solution in place of drinking water. Experiments were not performed with adrenalectomized animals until at least 24 hours after the operation.

The ^{14}C-cycloleucine was always injected subcutaneously near the lumbar region of the back at a concentration of 0.8 μmole (4 μCi in 0.1 ml of saline)/100 g body weight one hour after the start of a feeding period. In most of the experiments, the interval between injection and the first measurement of cycloleucine levels was at least 24 hours. The actual times are presented with the results for the respective experiments. Data from other experiments show that the blood level becomes constant within less than 2 hours (8).[8] The rats were decapitated with a guillotine without previous stress to the animal. Blood was collected in heparinized beakers and the livers were quickly removed, chilled and washed in several volumes of ice-cold 0.25 M sucrose-TKM (0.05 M Tris-HCl, pH 7.5 at 20°; 0.025 M KCl; 0.005 M $MgCl_2$). After weighing, each liver was homogenized in 2 volumes of ice-cold sucrose-TKM by means of a Polytron homogenizer.[9] Trichloroacetic acid (TCA) was added to aliquots of liver homogenates and blood to a final concentration of 10% and the TCA-insoluble fraction was removed by centrifugation at 600 × *g* for 10 minutes. The supernatant was decanted, the precipitate washed with 2 ml of 10% TCA, and the supernatants and washes were combined. One-half-milliliter aliquots of the TCA-soluble fractions were added to 10 ml ANPO scintillation fluid (13) and the radioactivity was counted in a Packard Tri-Carb liquid scintillation spectrometer.

In some experiments, the entire small intestine or kidney and spleen were removed along with the liver from the animal. The intestine was slit and washed in several volumes of ice-cold 0.25 M sucrose-TKM to remove its contents. The procedures used for homogenization, extraction and radioactivity counting were the same for these tissues as described for liver.

No radioactivity was found in the TCA-insoluble fractions of the homogenates and blood when aliquots were analyzed by the filter paper disc procedure of Mans and Novelli (14). Likewise, no radioactivity was found by the procedure of Whittle and Potter (15) in CO_2 expired over a 4-hour collection period.

RESULTS

^{14}C-cycloleucine levels in liver and blood from rats fed ad libitum or maintained on "8 + 16" regimen. The results presented

[3] Watanabe, M., and E. F. Baril 1967 Oscillations in tyrosine transaminase activity and amino acid transport in liver of rats adapted to 24- or 48-hour cycles of feeding and fasting. J. Cell Biol., 35: 139A (abstract).

[4] Calbiochem, Los Angeles.

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

[6] The Charles River Breeding Laboratories, Wilmington, Massachusetts.

[7] The Endocrine Laboratories, Madison, Wisconsin.

[8] Unpublished data, E. F. Baril and V. R. Potter, 1967.

[9] Brinkman Instruments, Inc., Des Plaines, Illinois.

in figure 1 demonstrate marked differences in the transport of ^{14}C-cycloleucine in liver of rats on controlled feeding regimens compared with rats fed ad libitum. For these experiments one group of rats was fed ad libitum at a dietary protein level of 30% for one month, and two other groups were adapted for one month to the "8 + 16" regimen at dietary protein levels of 30 and 60%. The rats in all 3 groups received a single injection of ^{14}C-cycloleucine 24 hours before the start of the experiment.

The level of ^{14}C-cycloleucine per gram of liver from rats on the "8 + 16" regimen oscillated over the 24-hour period. The values observed for individual rats at the same time-point were similar, as shown in figure 1, B and C. The highest level of ^{14}C-cycloleucine in the liver was observed during the feeding period and a gradual decline occurred during the fasting period. Between 0300 and 0600 of the cycle the level declined to one-half the value observed during the feeding period. A sharp rise in the level of ^{14}C-cycloleucine in the liver occurred between 0800 (one hour before feeding) and 1100 so that the level of ^{14}C-cycloleucine in liver during the feeding period of a second 24-hour cycle was approximately the same as that observed during the feeding period of the preceding 24-hour cycle. The oscillations in liver were not accompanied by complementary changes in the level of ^{14}C-cycloleucine in the blood. Qualitatively and quantitatively similar oscillations of the level of ^{14}C-cycloleucine in liver of rats on the "8 + 16" regimen at dietary protein levels of 30 and 60% were observed. However, the steady-state level of ^{14}C-cycloleucine in liver of rats fed a 60% protein diet was extended a few hours longer and the decline during the fasting period was sharper.

The observed changes in the amino acid levels in liver were not errors of the mode of expression since the decline in radioactivity per gram of liver was not caused by an increase in liver weight. Also, similar oscillations occurred when the data were represented on a per liver basis or as the distribution ratio of ^{14}C-cycloleucine in liver to blood.

Completely different results were obtained with rats fed ad libitum a 30% protein diet, as shown in figure 1A. Individual rats taken at the same time-point varied considerably in the values of ^{14}C-cycloleu-

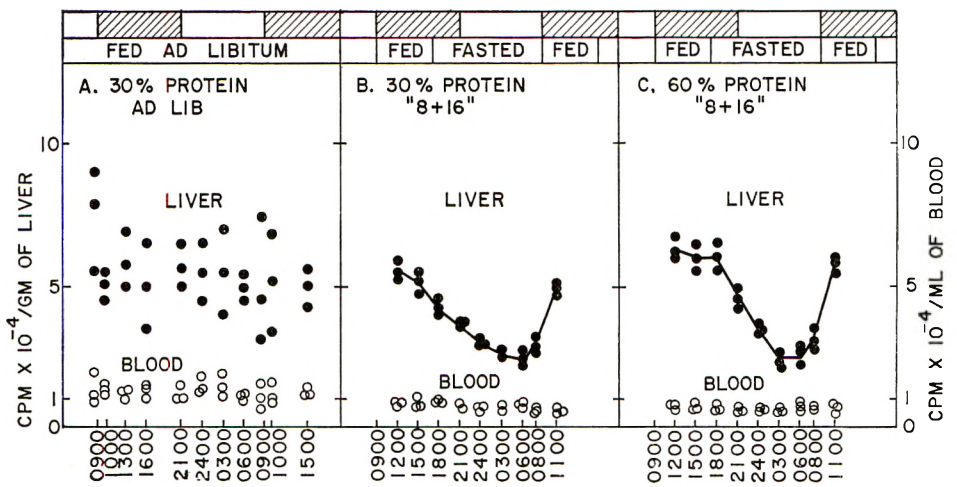

Fig. 1 Comparison of the level of ^{14}C-cycloleucine in liver and blood of rats fed ad libitum and adapted to the "8 + 16" regimen. Closed circles indicate liver values and open circles indicate blood values, with each circle representing the respective value from an individual rat. The open and hatch-marked areas indicate 12-hour intervals of light and dark, respectively. *A*, Rats fed ad libitum the 30% protein diet; *B*, rats adapted to "8 + 16" regimen on a 30% protein diet; and *C*, rats adapted to "8 + 16" regimen on a 60% protein diet. (Values for counts per minute shown in the figure have been derived by multiplying the actual values by 10^{-4}.)

cine in liver and blood over the 24-hour cycle. This same variation exists if the data are expressed as a distribution ratio of the ^{14}C-cycloleucine levels in liver and blood. Due to the wide variation in the level of ^{14}C-cycloleucine in liver of individual rats, oscillations were not apparent. However, oscillations in tyrosine transaminase activity in liver of rats fed ad libitum were demonstrated by Potter et al. (1) and later confirmed by other investigators (4, 5).

Systematic oscillations of amino acid transport with rats on an "8 + 40" regimen. The results of an experiment with rats adapted to an "8 + 40" regimen at a 60% dietary protein level are presented in figure 2 and show that oscillation of the amino acid transport system in liver of fasting-adapted rats is systematic. A single injection of ^{14}C-cycloleucine was given one hour after the start of a feeding period and 3 rats were killed at each interval during the 96-hour period, the first interval occurring 5 hours after the injection.

The highest level of the amino acid was maintained in liver during the feeding period, similar to the results obtained with rats on the "8 + 16" regimen. However, for rats adapted to the "8 + 40" regimen the steady-state level extended into the early fasting period.

A secondary rise in the level of cycloleucine in liver occurs during the first 5 to 16 hours of the 40-hour fasting period. However, the selected time-points did not permit its detection in the experiment for the data presented in figure 2. In this figure, the secondary rise is represented by a broken line which is based on data from experiments reported below. The level of the amino acid gradually declined as the fasting period continued and after 24 to 30 hours of fasting the level was approximately one-half that observed in liver during the feeding period. However, one hour after the start of feeding in the subsequent 48-hour cycle the level of the amino acid in liver increased about two-fold to the level observed during the feeding period of the preceding 48-hour cycle.

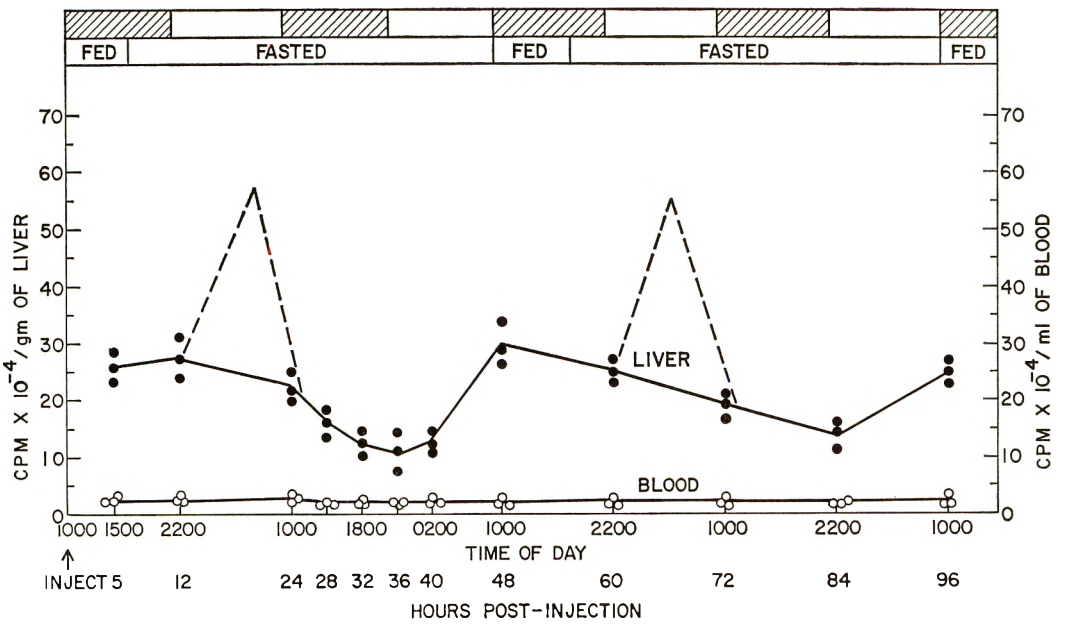

Fig. 2 Oscillations in the level of ^{14}C-cycloleucine in liver over a 96-hour period. Rats were adapted to an "8 + 40" regimen at a dietary protein level of 60%. The broken line represents an additional oscillation during the early fasting period which was not detected in this experiment but was consistently observed in several later experiments. Each circle represents the respective value from an individual rat. Other symbols are the same as in figure 1. (Values for counts per minute shown in the figure have been derived by multiplying the actual values by 10^{-4}.)

When the ^{14}C-cycloleucine level in liver and blood was followed through a second 48-hour cycle the level of the amino acid in liver again oscillated, whereas the blood level remained relatively constant.

The maximal and minimal levels of ^{14}C-cycloleucine observed in liver from rats on an "8 + 40" regimen were four- to five-fold greater than the values observed in liver from rats on an "8 + 16" regimen. At the present time, the basis for this difference is unknown and further investigation is required to determine whether the 2 regimens produced different rates of amino acid transport or whether other factors were responsible.

Increase in ^{14}C-cycloleucine level in liver of rats during feeding period on 30 and 60% protein, "8 + 40" regimen. Experiments performed with rats adapted to the "8 + 40" regimen at dietary protein levels of 30 and 60% suggested that the level of ^{14}C-cycloleucine in liver during the feeding and early fasting period was related to the dietary protein level. To further determine whether the correlation of cycloleucine level and protein diet could be explained by the transport of the amino acid during the feeding period, rats adapted to an "8 + 40" regimen at dietary protein levels of 30 and 60% received a single injection of ^{14}C-cycloleucine one hour after the start of the feeding period and 46.5 hours before the start of the experiment. The rats of both groups were then killed at various time-intervals beginning one-half hour before the start of and continuing 4 hours into the feeding phase of the subsequent 48-hour cycle. The results presented in figure 3 indicate that the rise in the level of ^{14}C-cycloleucine in liver during the feeding period is directly related to the dietary protein level. Although the level of ^{14}C-cycloleucine per gram of liver was about 50% higher in the rats maintained on a 60% protein diet, the level of ^{14}C-cycloleucine per milliliter of blood did not differ in rats fed a 30 or 60% protein diet.

These results also indicate that the observed oscillations are not due to competition with natural amino acids since one would expect the greater competition, and thus the lower level of cycloleucine, in liver from rats maintained on a 60%

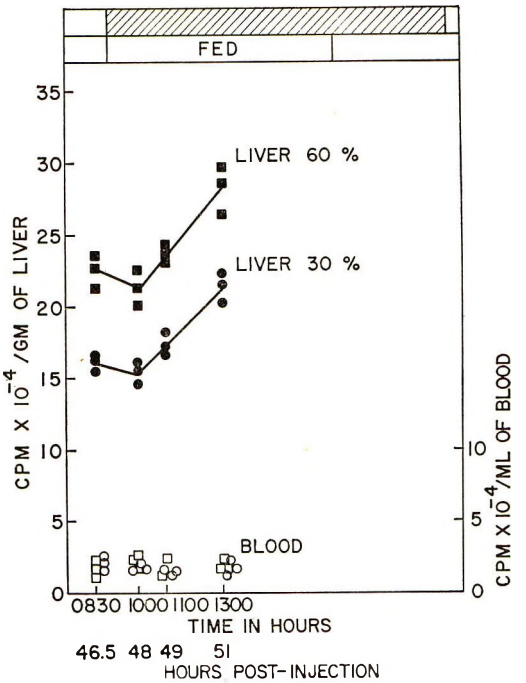

Fig. 3 Comparison of the level of ^{14}C-cycloleucine in liver and blood of rats during the feeding period with 30 and 60% protein, "8 + 40" regimen. Closed circles and squares indicate liver values for rats fed 30 and 60% protein diets, respectively. Open circles and squares indicate blood values for rats fed 30 and 60% protein diets, respectively. Each circle and square represents the respective value from an individual rat. Other symbols are the same as for figure 1. (Values for counts per minute shown in the figure have been derived by multiplying the actual values by 10^{-4}.)

protein diet. However, the level of ^{14}C-cycloleucine in liver was about 50% higher for rats on a 60% as compared with a 30% protein diet. Also, when rats adapted to the "8 + 40" regimen at a dietary protein level of 60% protein were shifted to a 12% protein diet, a corresponding decrease in the uptake of cycloleucine in liver occurred.[10] Thus, the rate of amino acid transport into liver appears to be related to the level of protein in the diet.

Effect of adrenalectomy on the level of ^{14}C-cycloleucine in liver and other tissues. The observation of systematic oscillations in the level of ^{14}C-cycloleucine in liver over the 96-hour period following the single injection and the absence of significant

[10] Unpublished data, M. Watanabe, E. F. Baril and V. R. Potter, 1967.

changes in the plasma level raise the question of a possible recycling of the amino acid between liver and other tissues or organs. The small intestine has been shown to actively concentrate cycloleucine in vitro and in vivo (9) and the level of cycloleucine in the small intestine was shown to decrease, whereas that in the liver increased following cortisone administration to the rat (16).

Variations in the plasma corticosteroid level have been observed in rats adapted to the "8 + 40" regimen (3). In view of these findings the effect of adrenalectomy on the uptake of ^{14}C-cycloleucine by liver and other tissues was examined. Adrenalectomy was performed on half of a group of rats adapted to a regimen of 8 hours of feeding at a dietary protein level of 30% and 40 hours of fasting. The remaining animals were used as intact controls. Twenty-four hours after the operation and one hour after the start of the feeding period, the adrenalectomized and control animals were injected with ^{14}C-cycloleucine. Control and adrenalectomized animals were killed at various intervals during the first 30 hours of a 48-hour cycle with the first interval occurring 46.5 hours following the single injection.

The data presented in figure 4 show that adrenalectomy has no effect on cycloleucine uptake by liver during the feeding period and the first 4 hours of fasting. However, adrenalectomy did prevent the increased uptake observed between 2100 and 1500 hours of the fasting period. During this period the level of ^{14}C-cycloleucine in liver from adrenalectomized animals remained at the same level as that observed for adrenalectomized and control animals during the feeding period.

The difference in the amino acid level of liver from adrenalectomized and intact, control animals was not due to the differences in the water content of the tissues since total tissue water measurements indicated a difference of less than 10%. Other investigators have reported that the reduced uptake of amino acids by the intestine of adrenalectomized rats was not observed when the rats were given saline in place of water for 7 days (17). However, in the present studies, replacement of drinking water by 1% NaCl for 9 days

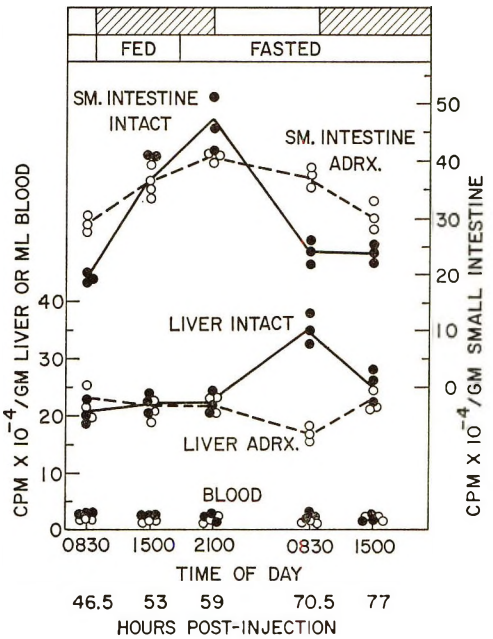

Fig. 4 Effect of adrenalectomy on the level of ^{14}C-cycloleucine in the liver, small intestine and blood of rats during the feeding and early fasting period of a 48-hour cycle. Rats were adapted to an "8 + 40" regimen at a 30% dietary protein level. Closed circles indicate values for control and open circles indicate values for adrenalectomized animals, with each circle representing the respective value from an individual rat. Light and dark periods as in figure 1. (Values for counts per minute shown in the figure have been derived by multiplying the actual values by 10^{-4}.)

failed to relieve the effect of adrenalectomy, which still resulted in reduction of the uptake of cycloleucine by liver at the time intervals described.[11] Experiments performed with sham-operated animals gave the same results as those for the intact, control rats when the experiments were performed five or more days after the operation.[12] Before this time, the results obtained with sham-operated animals were similar to results obtained with adrenalectomized animals. No explanation is given for these differences. However, the fact that adrenalectomy prevented the secondary rise in the cycloleucine level in liver without a recovery even 9 days after the operation suggests that the rise in the

[11] See footnote 10.
[12] See footnote 10.

amino acid level was most probably related to adrenal function. It may follow that the results obtained with sham-operated animals before 5 days post-operation were due in part to a taxing of adrenal function during the stress of the operation.

As shown in figure 4 the blood levels of cycloleucine in control and adrenalectomized animals were similar and showed little variation. However, the uptake of cycloleucine by the small intestine from control animals did vary over the time-period studied. The amino acid uptake by the small intestine was increased during the feeding period and the first 4 hours of the fasting period. This was followed by a rapid decline between 2100 and 0830 hours of the fasting period, the period during which the level of the amino acid in liver from control animals increased sharply. The level of ^{14}C-cycloleucine in the small intestine from adrenalectomized animals showed less variation over the same time-period.

The pancreas and the small intestine behaved similarly in the uptake of ^{14}C-cycloleucine measured under the same experimental conditions.[13] However, the uptake of the amino acid was greater in pancreas than that observed in liver or small intestine, confirming observations of other investigators (18, 19).

The comparison of data obtained from rats on the "8 + 40" regimen at a dietary protein level of 60% shown in figure 2 and data with rats fed a 30% protein diet, the control rats in figure 4, suggested that the secondary rise in the level of the amino acid in liver during early fasting is a function of the level of dietary protein, as well as adrenal status. The increase in the level of ^{14}C-cycloleucine in liver during this period was twofold higher for rats maintained on a 60% protein diet than for rats fed a 30% protein diet.

An experiment was performed in a similar manner to that described previously to determine the role of the adrenal function in the secondary oscillation. However, the rats used were adapted to an "8 + 40" regimen and a 60% protein diet. Control and adrenalectomized animals were injected with ^{14}C-cycloleucine at the start of a feeding period and 63 hours before the start of the experiment. Animals were killed during the early part of the fasting period, between 63 and 78 hours after the injection, when a secondary rise in the uptake of cycloleucine was observed (figs. 2 and 4). The level of ^{14}C-cycloleucine was measured at each time point in the liver, kidney, spleen and blood. As shown in figure 5A adrenalectomy prevented the oscillation in the level of ^{14}C-cycloleucine observed in liver of the control animals. The level of ^{14}C-cycloleucine in liver of the adrenalectomized animals was approximately the same as the maximal level observed during this time-period in the liver of control animals on a 30% protein diet (fig. 4). However, the level observed in liver of control animals fed the 60% protein diet was twice that of the control animals fed the 30% protein diet (figs. 4 and 5A).

The levels of ^{14}C-cycloleucine in the kidney, spleen and blood were not affected by adrenalectomy as shown in figures 5B–D. Also, no oscillation in the level of ^{14}C-cycloleucine in kidney, spleen or blood was apparent.

DISCUSSION

These results show that amino acid transport in liver varies systematically in rats maintained under controlled conditions of feeding and room lighting. The finding of an oscillation in the amino acid transport system extends the list of metabolic functions which have been shown by Potter and co-workers to oscillate daily in the liver of the laboratory rat (1–3,6,15).

The marked individual variation in the level of ^{14}C-cycloleucine in liver of rats fed ad libitum, in contrast with the results obtained with rats on the "8 + 16" regimen, provides additional support for the suggested use of controlled feeding schedules in experiments with the laboratory rat (2, 6). A wide individual variation in the transport of ^{14}C-cycloleucine into liver of mice, presumably fed ad libitum, was also observed by Christensen and Jones (10). These authors stated that "1-Aminocyclopentanecarboxylic acid showed hepatic levels varying all the way from 1.7 to 9.9 times the plasma level, with a mean value of 4.5 times, with sufficient instances

[13] See footnote 8.

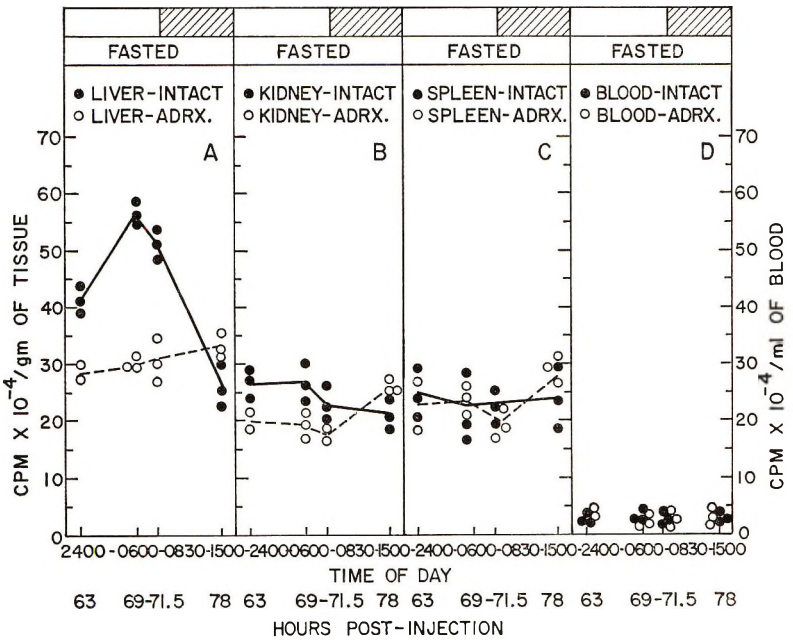

Fig. 5 The effect of adrenalectomy on the blood and tissue level of ^{14}C-cycloleucine. Rats were adapted to the "8 + 40" regimen at a 60% dietary protein level. Closed circles indicate values for control and open circles indicate values for adrenalectomized animals, with each circle representing the respective value from an individual rat. Light and dark periods as in figure 1. *A*, Liver; *B*, kidney; C, spleen; and *D*, blood. (Values for counts per minute shown in the figure have been derived by multiplying the actual values by 10^{-4}.)

throughout this range to indicate that the unusual variability was probably real."

The oscillations in the amino acid transport system of fasting-adapted rats correlate well with the observed oscillations in the activity of liver tyrosine transaminase from the same rats.[14] This conclusion is based on the following evidence presented in this paper and by Watanabe et al. (6): (a) the level of ^{14}C-cycloleucine and tyrosine transaminase activity oscillated together in liver of rats on the "8 + 16" and "8 + 40" regimens; (b) both the amino acid level and the activity of the enzyme were increased in liver by an increase in the level of dietary protein; (c) the response to the level of dietary protein was most apparent in both cases for rats on the "8 + 40" regimen; and (d) only the secondary oscillation, during the early fasting period, of the amino acid level and tyrosine transaminase activity in liver of rats on the "8 + 40" regimen was modified by adrenalectomy. The oscillations in the amino acid transport system of fasting-adapted rats also showed a correlation with the observed oscillations in liver ribonucleic acid content and orotic acid metabolism as reported by Whittle and Potter (15).

Our understanding of the factors responsible for this oscillatory behavior of the amino acid transport system in liver is incomplete at present. However, fasting-adapted rats may prove to be useful subjects for the study of the general control mechanisms for amino acid transport, which are also poorly understood (20).

The decline in the level of ^{14}C-cycloleucine in liver during the fasting period is apparently due to a change in the activity of the transport system during this period, rather than the result of competition with other amino acids. This is supported by the fact that the level of the amino acid increased during the feeding periods as the dietary protein level increased, a result which is not compatible with an alteration of the amino acid level by competition.

[14] See footnote 3.

Also, the oscillation does not appear in all tissues, as evidenced by the results obtained with kidney and spleen. Whether the decline in the ^{14}C-cycloleucine in liver was due to continued efflux by a simple diffusion process in the absence of influx by active transport or due to some other mechanism cannot be determined from this study. Although the mechanism of efflux of amino acids from liver has not been clearly defined, there is evidence from studies on Ehrlich ascites cells and other tissues that efflux does not involve active transport (11). The results obtained with small intestine and pancreas indicate that oscillations in these tissues were out of phase with the oscillations occurring in liver. These results might be expected since there was no detectable change in the blood level of cycloleucine and since this amino acid is non-metabolizable and almost completely retained by the body. Thus, it is possible that a recycling of this non-metabolizable amino acid over a 24- or 48-hour cycle involves reciprocal changes in the intracellular level of the amino acid in multiple tissues or organs. Oscillations in other metabolic functions in the liver correlate well with the observed oscillations in amino acid transport in this organ. These metabolic functions, however, have not been studied in other tissues of the fasting-adapted rat because the principal interest of this laboratory is to relate the biochemistry of hepatomas to that of normal liver.[15] Therefore, it is unknown whether the primary effect responsible for the observed oscillations in amino acid transport originates solely in liver.

The results of the experiments with the adrenalectomized animals on the "8 + 40" regimen indicate that a part of the secondary oscillation was related to adrenal function. However, it appears that factors other than adrenal function are also involved in maintaining the higher uptake of the amino acid during the feeding and early fasting period, as compared with the later fasting period, since adrenalectomy did not reduce the level of the amino acid in liver to that in the late fasting period. Actually, the lower level during the early fasting period for adrenalectomized animals was the same as the steady-state level observed during the feeding period, which was not altered by adrenalectomy. Thus, it is probable that the increased level of the amino acid during the feeding period is related to some non-adrenal function(s) and that the secondary oscillation observed with rats on the "8 + 40" regimen resulted from an additional increase in the rate of amino acid transport due to adrenal function(s) coming into play at this time. However, the nature of the factors involved remains unidentified and will require further biochemical and physiological analysis. Evidence has been presented that amino acid transport is modified by glucocorticoids (8, 16) and probably other hormones (21, 22). Thus, it is possible that the observed oscillations in the amino acid transport result from multihormonal influences and a rhythmicity in these systems (23).

The basis for the higher level of cycloleucine observed in liver for rats on the "8 + 40" regimen, in contrast with the "8 + 16" regimen, is being investigated. However, rats adapted to the "8 + 40" regimen show increased activity and lower body weights,[16] greater stomach weights (6, 15) and, in general, signs of greater stress than animals adapted to the "8 + 16" regimen or fed ad libitum (6). The amino acid transport system of rats adapted to an "8 + 40" regimen may be altered by increased activity in these animals. If such is the case, it lends support to the contention that rats adapted to the "8 + 16" regimen are similar to rats fed ad libitum but reflect more controlled environmental conditions, since the level of cycloleucine in liver of ad libitum-fed rats was in the same range but with greater scatter than for the "8 + 16" adapted rats.

The basis for the observed oscillations is being further investigated. However, the observed oscillations in amino acid transport complicate the interpretation of results from enzyme assays and amino acid incorporation studies conducted with tissue from animals whose daily activity is uncontrolled and whose feeding habits are ad libitum. The available data appear to warrant the recommendation that biochemical studies on laboratory rats should be

[15] See footnote 8.

[16] Reynolds, R. J., and V. R. Potter, 1967 (manuscript in preparation).

conducted with more careful attention to lighting, activity, and feeding schedules.

ACKNOWLEDGMENTS

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Systematic Oscillations in the Metabolism of Orotic Acid in the Rat Adapted to a Controlled Feeding Schedule [1]

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ABSTRACT Orotic acid labeled with ^{14}C in the 7-, 2- or 6-position has been used to obtain information on its metabolism in the rat adapted to a 48-hour cycle of 8 hours of feeding and 40 hours of fasting. The conversion of orotic acid to uridine monophosphate (UMP) did not vary with the nutritional state of the rat throughout the cycle. However, the further metabolism of UMP derived from injected ring-labeled orotic acid, as determined by its incorporation into liver RNA and degradation to CO_2, did show cyclic variations, corresponding with a cyclic variation of the total liver RNA content. The increase in degradation to CO_2 of UMP derived from injected orotic acid in the fasting period of the cycle suggests an increase in catabolic enzymic activity. A simple and efficient method for collecting and determining expired $^{14}CO_2$ is described.

The occurrence of a daily rhythm in various metabolic activities in the laboratory rat in association with cycles of light and darkness, food intake, and activity has led us to question the common practice of ad libitum feeding with unspecified killing times and light schedules (1,2). Some metabolic functions oscillate widely depending on the metabolic transitions associated with feeding and fasting even in the rat fed ad libitum, rats being nocturnal feeders (see references in (1)). The oscillations are much more marked, however, in animals fasted for 2 or 3 days and then refed as in our study (3) of the Tepperman experiment or in animals that are adapted to having food available for only 12 hours in each 48 hours (4) or for only 8 hours in each 48 hours (1,5). We have referred to rats in the latter 2 regimens as fasting-adapted rats. In addition to marked fluctuations in enzymatic activity (1) and amino acid transport in liver (2) we have observed increased voluntary exercise and much lower body weights in such animals as compared with controls fed ad libitum.[3]

The present work on some aspects of the metabolism of orotic acid in the fasting-adapted rat was carried out as an adjunct to more detailed studies on liver RNA metabolism in terms of labeling of specific cell fractions in this type of animal.[4] The general metabolism of orotic acid in the rat fed ad libitum was established by Hurlbert and Potter (6). Following injection of the ring-labeled compound, approximately 30% was excreted unchanged in the urine within 2 hours, a large proportion of the orotic acid was incorporated into pyrimidine derivatives in the liver, and labeled CO_2 as a degradation product was found in the expired air. Initially, the activity in liver was recovered almost quantitatively in the acid-soluble fraction as free uridine nucleotides and their derivatives (7). Subsequently, the radioactivity in the acid-soluble fraction decreased, and that in the RNA increased.

The experiments to be reported here were performed on rats that had been adapted to a 48-hour cycle comprising 8 hours of feeding and 40 hours of fasting. The conversion of orotic acid to uridine monophosphate (UMP) and its subsequent incorporation into liver RNA and catab-

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[3] Reynolds, R., and V. R. Potter, 1967 (manuscript in preparation).

[4] Unpublished studies, R. A. Gebert, G. Blobel and V. R. Potter, 1966.

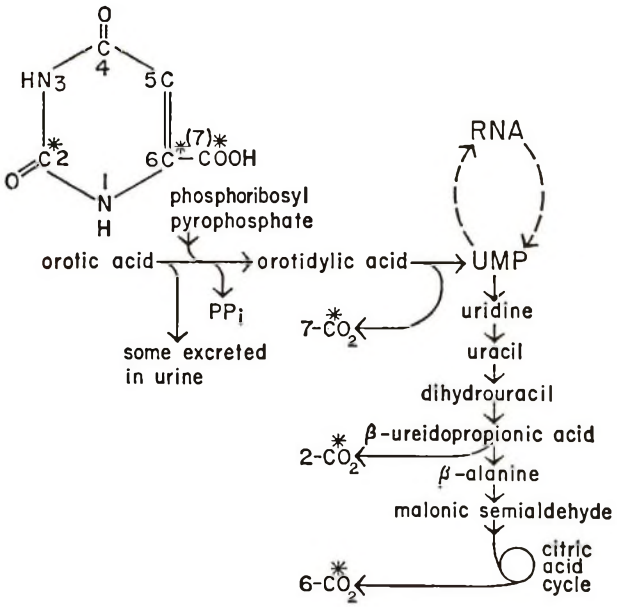

Fig. 1 Pathways for the metabolism of orotic acid, showing the fate of the label (*) in the case of the ^{14}C-carboxyl and ring-labeled compounds.

olism to CO_2 were studied in rats at different times in the 48-hour cycle by use of substrate labeled with ^{14}C in the 7-, 2- or 6-position (fig. 1).

MATERIALS AND METHODS

Chemicals. The orotic acid was labeled in 3 different positions and was available at somewhat different specific activities: Orotic acid-7-^{14}C (39.0 mCi/mmole),[5] orotic acid-2-^{14}C (22.0 mCi/mmole),[6] and orotic acid-6-^{14}C (34.7 mCi/mmole).[7] These preparations were diluted with nonradioactive orotic acid[8] to give specific activities of 1 μCi/μmole in all cases.

Animals and regimen. Male rats (140–150 g)[9] were housed on arrival in a windowless room with the lighting automatically regulated to provide 12 hours of light (9 PM to 9 AM) and 12 hours of darkness (9 AM to 9 PM). The animals were fed a 60% protein diet (1,8)[10] in alternative dark periods from 9 AM to 5 PM. The start of feeding was designated the beginning of the 48-hour cycle, and thus food was provided only during the first 8 hours of each cycle. The rats were kept on this strict regimen for 2 to 3 weeks before use in an experiment. In total, 58 rats were used.

Experiments were carried out at five different times in the 48-hour cycle, as listed in table 1.

The general experimental procedure was as follows: At the decided time, the rat was injected intraperitoneally with 1 ml

TABLE 1

Plan of experiments: the relation of rats in each experimental group to their nutritional state in the 48-hour cycle at the time of injection of orotic acid-^{14}C

Group	Time of injection	
	Time of day	Hour in cycle [1]
(Food provided at	9 AM)	
1	1 PM	4
2	4 PM	7
(Food removed at	5 PM)	
3	9 AM	24
4	3 PM	30
5	5 AM	44

[1] The beginning of the cycle was designated by the start of feeding.

[5] New England Nuclear Corporation, Boston 02118.
[6] Calbiochem, Los Angeles 90054.
[7] Schwarz BioResearch, Inc., Orangeburg, New York.
[8] Sigma Chemical Company, St. Louis 63118.
[9] Holtzman Company, Madison, Wisconsin.
[10] General Biochemicals, Inc., Chagrin Falls, Ohio.

of a solution containing 1 μCi of orotic acid-^{14}C labeled in the 7-, 2- or 6-position, diluted with nonradioactive orotic acid so that its specific activity was 1 μCi/μmole. The rat was immediately placed in a tube for determination of expired $^{14}CO_2$ for 4 hours.

Determination of $^{14}CO_2$. The rat was placed in a glass tube (6 cm internal diameter and 30 cm long) stoppered at both ends and fitted with air inlet and exit tubes. The exit tube was connected directly to a gas dispersion tube with a fritted cylinder at its end which dipped into a CO_2-absorbing solution contained in a long narrow cylinder (100-ml graduated cylinder elongated by 20 cm of plain tubing of the same diameter). A current of air was drawn through the system at the rate of 1.2 to 1.4 liters/minute. Two collecting cylinders were connected in parallel by means of 3-way taps to facilitate sampling of the expired $^{14}CO_2$ at regular intervals throughout the 4-hour collection period. The CO_2-absorbing solution was a mixture of ethanolamine [11] and ethylene glycol monomethyl ether [12] (1:2, v/v) (9), a volume of 25 ml being sufficient for complete absorption of the expired $^{14}CO_2$ up to a 30-minute interval. The absorbing solution maintained a constant volume throughout the collection interval, and 5-ml aliquots were transferred directly to 20-ml counting vials. Ten milliliters of scintillation solution (10) were added, and ^{14}C was determined with a Packard Tri-Carb scintillation counter by use of a large window and high amplification. The efficiency of counting was 75%, and quenching was corrected for by external standardization.

Analysis of liver tissue. Four hours after injection, CO_2 collection was discontinued and the rat was decapitated. The liver was quickly removed, chilled in ice-cold isotonic saline, cleaned, blotted, weighed, and frozen in liquid nitrogen. It was stored at − 25° until used for estimation. The stomach and its contents were weighed and discarded.

The liver was homogenized [13] in 4 volumes of ice-cold water and the homogenate was filtered through 2 layers of cheesecloth, and 0.5-ml aliquots were transferred to 12-ml centrifuge tubes for subsequent determination of total radioactivity in the acid-soluble fraction, radioactivity and content of RNA, and content of DNA.

Acid-soluble fraction. The homogenate was acidified with perchloric acid to give a concentration of 0.2 N. The mixture was allowed to stand in ice for 15 minutes, then centrifuged, and the supernatant fraction, together with two acid washes of the residue, was collected for counting. Ten milliliters of scintillation solution were added directly to a 0.5-ml aliquot of the acid-soluble fraction. The efficiency of counting was 86%, and quenching was corrected for by external standardization.

RNA fraction. The RNA fraction was obtained according to the method of Fleck and Munro (11). The acid-washed tissue residue was dissolved in 0.3 N KOH and incubated at 37° for 1 hour. The solution was then cooled in ice, acidified with perchloric acid to give a final concentration of 0.2 N, centrifuged, and aliquots of the supernatant solution were estimated for radioactivity and absorbancy. An absorbancy value of 1.000 at 260 mμ for a 1-cm light path was taken to be equivalent to 32 μg RNA/ml (12).

DNA. DNA in the residue was determined by the method of Ceriotti (13), slightly modified: 2.5 N HCl was used instead of concentrated HCl (14), and the reaction mixture was heated at 100° for 20 minutes instead of 10 minutes (15). Calf thymus DNA (containing 6.8% organic phosphorus, 6.1% sodium, and 20% moisture) was used as standard.[14] It was corrected for sodium and moisture content.

RESULTS

Fundamental data on the rats used in the experiments are given in table 2. For rats in group 2, after 7 hours of feeding followed by 4 hours in the apparatus without food, the weight of the stomach plus contents was about 24 g, i.e., nearly 12% of the weight of the whole animal. The rat had learned to compensate partially for the fasting period of the cycle by eating voraciously during the limited hours when food was available, so that at the time of killing its stomach was seen to be grossly

[11] Fisher Scientific Company, Chicago 60651.
[12] See footnote 11.
[13] Ultra-Turrax, made by Janke and Kunkel, K.G., Staufen, Breisgau, Germany.
[14] Sigma Chemical Company, St. Louis.

distended. The rats in group 3, killed 20 hours after the removal of food, were found still to have a small amount of solid matter in their stomachs.

Conversion of carboxyl-labeled orotic acid to UMP. Figures 2A and B show the cumulative percentage excretion of $^{14}CO_2$ following injection of 1 μmole of orotic acid-7-^{14}C (specific activity, 1 μCi/μmole) into rats in each of the 5 groups (table 1). The 4-hour values for each group were similar, approximately 74% of the injected dose then having been recovered as $^{14}CO_2$. Also, the time-course was similar, with the maximal rate of excretion occurring in the first 40 minutes after injection and the

TABLE 2

Fundamental data on rats used in the labeling experiments [1]

Orotic acid-^{14}C injected	Group	No. in group	Wt of rat	Wt of stomach	Wt of rat minus stomach	Wt of liver
			g	*g*	*g*	*g*
7-^{14}C	1	5	201 ± 2 [2]	17.3 ± 0.3	184 ± 2	7.14 ± 0.10
	2	4	208 ± 4	26.2 ± 1.3	182 ± 5	7.61 ± 0.50
	3	4	188 ± 2	5.6 ± 0.9	182 ± 1	7.63 ± 0.27
	4	4	189 ± 2	3.6 ± 0.4	185 ± 1	7.92 ± 0.59
	5	4	185 ± 1	1.7 ± 0.1	183 ± 1	6.31 ± 0.23
2-^{14}C	1	3	204 ± 1	18.8 ± 2.0	185 ± 2	7.43 ± 0.19
	2	4	208 ± 4	23.8 ± 1.2	184 ± 3	7.57 ± 0.25
	3	6	194 ± 1	5.7 ± 0.5	188 ± 1	8.06 ± 0.17
	4	5	194 ± 3	3.3 ± 0.6	191 ± 3	8.03 ± 0.25
	5	4	184 ± 2	1.6 ± 0.1	183 ± 1	6.18 ± 0.32
6-^{14}C	1	4	209 ± 3	15.4 ± 1.1	193 ± 4	7.77 ± 0.23
	2	4	209 ± 4	22.0 ± 1.2	187 ± 4	8.09 ± 0.41
	3	3	197 ± 3	4.9 ± 1.3	192 ± 3	8.17 ± 0.28
	4	4	184 ± 1	2.0 ± 0.2	182 ± 1	7.61 ± 0.17

[1] Rats were injected with 1 μmole of orotic acid-^{14}C at five different times in the 48-hour cycle (table 1). Each rat was killed 4 hours after injection.
[2] Mean ± SE of mean.

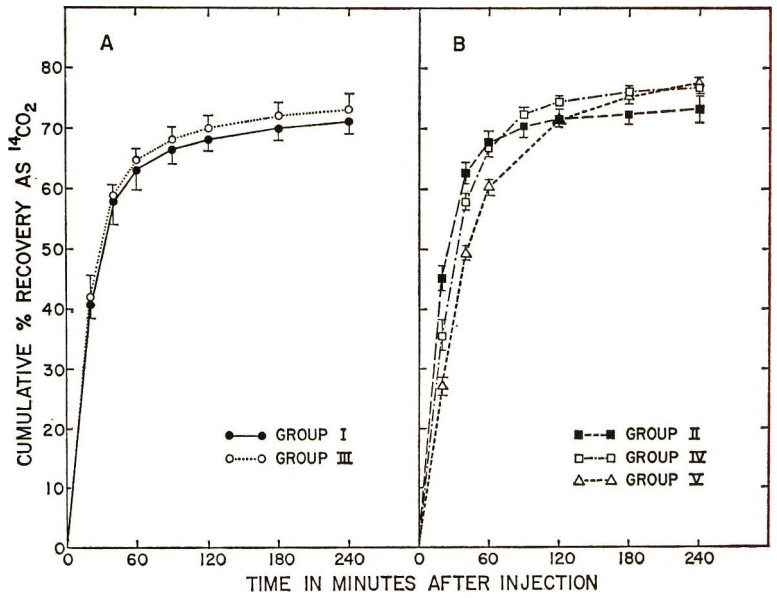

Fig. 2 Cumulative percentage recovery as $^{14}CO_2$ of injected dose of 1 μmole of orotic acid-7-^{14}C for rats in each of the five experimental groups (table 1). Number of rats in each group is given in table 2. The standard error of the mean is indicated by vertical bars.

cumulative excretion tending to reach a maximum by 2 hours. Figure 2B has been drawn separately to show more clearly that there appears to have been a slower initial rate of $^{14}CO_2$ excretion for rats of groups 4 and 5, which were injected after being fasted for 22 and 36 hours, respectively. For rats in groups 1, 2, and 3, the fraction of the initial dose which was recovered as $^{14}CO_2$ after 20 minutes was more than 40%, whereas it was 35% and 27% for groups 4 and 5, respectively.

The formation of uridine nucleotides from orotic acid has been shown to involve a two-step reaction (16,17) in which UMP is the product (fig. 1). The second step, the decarboxylation of orotidylic acid to form UMP, is an irreversible reaction which apparently takes place with such speed that orotidylic acid has not been detected in normal rat liver (17), although it can accumulate under appropriate conditions (18). Orotic acid is not degraded, at least in rat liver, via a reversal of its *de novo* pathway, i.e., via L-dihydroorotate and carbamyl-L-aspartate (19). Hence, it seems justifiable to assume that all the $^{14}CO_2$ that was recovered in the expired air following injection of orotic acid-7-^{14}C had arisen from the orotidylate decarboxylase reaction in which UMP was formed.

In the whole animal, the complexities of the processes involved in the transport and excretion of metabolically formed CO_2 have been investigated by several workers, e.g., Morris and Simpson-Morgan (20). The report of Shipley et al. (21) is especially pertinent to the present study. These workers injected $NaH^{14}CO_3$ intravenously to fed and fasted rats and collected the expired CO_2 from 2 to 120 minutes following injection. It was observed that, although the total CO_2 expired was the same, the rate of elimination of $^{14}CO_2$ was somewhat lower for the fasted rats. Thus, the difference observed between groups 2, 4 and 5 injected with orotic acid-7-^{14}C (fig. 2B) for recovery of $^{14}CO_2$ in the early time-intervals can most probably be accounted for by a difference in the initial rates of excretion of $^{14}CO_2$ once it had been formed by the orotidylate decarboxylase reaction, rather than by a difference in the rate of the reaction itself. As, moreover, the values for total $^{14}CO_2$ expired by 4 hours were close to maximum and so similar, it is strongly suggested that the rate of formation of UMP from injected orotic acid was at all times similar for rats in each of the five experimental groups.

For all 5 groups of rats, approximately 74% of the injected dose of orotic acid-7-^{14}C was recovered as $^{14}CO_2$. A small proportion of the metabolically formed $^{14}CO_2$ might have been incorporated into compounds having a relatively low turnover in the animal. Therefore, at least 74% of the 1 μmole of injected orotic acid was converted to UMP. The difference between the amount injected and the amount converted to UMP would represent orotic acid that was excreted unchanged in the urine (6,19).[15] Lipman and Potter [16] used dosages of 1, 3, 6, 12 and 18 μmoles of carboxyl-labeled orotic acid injected into rats fed laboratory ration ad libitum. At 1- and 3-μmole doses of orotic acid the CO_2 recovered was 70 and 75%, in close agreement with the recovery and time-course indicated in figure 2. At the higher dosages the recovery as CO_2 was 58, 48 and 39%, respectively, at 8 hours. The time-course was delayed, and the urinary output was increased. The data on carboxyl-labeled orotic acid provide a firm basis for the use of ring-labeled orotic acid for studies on RNA metabolism in rat liver.

Metabolism of ring-labeled ^{14}C-orotic acid. The labeled UMP that is formed from ring-labeled orotic acid is synthesized into acid-soluble derivatives of UMP and incorporated into RNA, and it is catabolized so that the ^{14}C appears as $^{14}CO_2$ in the expired air (fig. 1). Figure 3A shows the cumulative percentage recovery as $^{14}CO_2$ in the 4-hour period after injection of 1 μmole of orotic acid-2-^{14}C (specific activity, 1 μCi/μmole) into rats in each of the 5 groups. The rate of excretion of $^{14}CO_2$ in the first 20 minutes was low for all groups, but between 20 minutes and 4 hours the rate for rats in the fasting period of the cycle (particularly groups 4 and 5) was markedly higher than that for the fed rats (groups 1 and 2). There was no sig-

[15] Unpublished data, C. A. Lipman and V. R. Potter, 1961.
[16] See footnote 15.

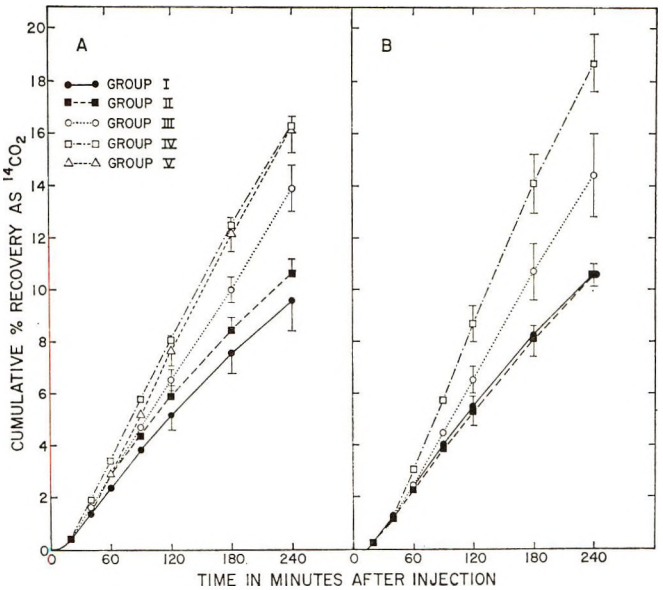

Fig. 3 Cumulative percentage recovery as $^{14}CO_2$ of injected dose of 1 μmole of orotic acid-2-^{14}C (A) and orotic acid-6-^{14}C (B) for rats in each of the five experimental groups (table 1). Number of rats in each group is given in table 2. The standard error of the mean is indicated by vertical bars.

nificant difference in the excretion rates between groups 1 and 2, or between groups 4 and 5. The rates of group 3 were intermediate. When the slight difference in rates of excretion of metabolically formed $^{14}CO_2$ for fasted and fed rats (21) is taken into account, the actual difference between rats in the fasting and feeding periods of the cycle with respect to the rate of formation of metabolic $^{14}CO_2$ from labeled UMP might be expected to be even slightly greater than the observed difference in their rates of $^{14}CO_2$ excretion.

Figure 3B shows the curves for $^{14}CO_2$ excretion obtained after 1 μmole of orotic acid-6-^{14}C (specific activity, 1 μCi/μmole) was injected. The results are similar to those in figure 3A, as would be expected if the further breakdown of β-alanine were rapid (fig. 1).

The results given in figure 2A and B and figure 3A and B thus show that, although the conversion of orotic acid to UMP was similar for rats at all 5 times of the cycle tested, the rate of breakdown of the UMP thereby formed was considerably greater for rats in the fasting period of the cycle.

Table 3 shows the corresponding results for incorporation of ^{14}C from orotic acid-2-^{14}C and orotic acid-6-^{14}C into the acid-soluble and total RNA fractions of liver 4 hours after injection. Fasting does not affect the DNA content of the average liver cell nucleus or the total number of cells in the liver (22). Therefore, in order to reveal possible changes in the metabolism at the cellular level, the activities in the acid-soluble and RNA fractions have been expressed per milligram of DNA.

The radioactivity in the acid-soluble fraction of the livers of rats in the 5 groups was remarkably similar, and its total value in each liver after 4 hours represented approximately 40% of the injected dose. Preliminary results of experiments performed under the same conditions have indicated that, for rats in both the feeding and fasting periods of the cycle, over 90% of the radioactivity in the acid-soluble fraction can be accounted for in pyrimidine nucleotides and nucleotide-containing compounds. The net incorporation of ^{14}C into RNA was higher for groups 1 and 2 than for groups 3 and 4, and bore an inverse

TABLE 3

Incorporation of ^{14}C from ring-labeled orotic acid into the acid-soluble and RNA fractions of liver 4 hours after injection of 1 μCi (specific activity, 1 μCi/μmole) [1]

Orotic acid-^{14}C injected	Group	^{14}C in acid-soluble fraction	^{14}C in RNA	RNA/DNA
		dpm/mg DNA	*dpm/mg DNA*	
2-^{14}C	1	59,900 ± 1,110 [2]	16,700 ± 3,300	3.47 ± 0.02
	2	62,200 ± 1,260	14,300 ± 950	3.53 ± 0.10
	3	55,900 ± 2,670	7,970 ± 1,160	3.98 ± 0.11
	4	52,800 ± 2,140	8,590 ± 620	3.88 ± 0.10
	5	54,600 ± 2,830	11,700 ± 950	3.24 ± 0.06
6-^{14}C	1	64,800 ± 1,560	17,300 ± 1,690	3.60 ± 0.17
	2	62,900 ± 1,770	16,400 ± 3,110	3.40 ± 0.10
	3	60,300 ± 1,000	7,300 ± 280	4.00 ± 0.25
	4	61,400 ± 1,620	8,730 ± 770	3.92 ± 0.09

[1] The relation of rats in each experimental group to their nutritional state in the 48-hour cycle at the time of injection is given in table 1.
[2] Mean ± SE of mean.

relationship to the amount of $^{14}CO_2$ recovered from these rats. However, for rats in group 5, at the extreme end of the fasting period, the net incorporation of ^{14}C into RNA gave an intermediate value, although these rats had a high rate of excretion of $^{14}CO_2$.

Cyclic variation in liver RNA content. Table 4 shows the average value of RNA/DNA for the total number of rats in each experimental group. The RNA/DNA ratio was at a minimum at the end of the fasting period (group 5); it increased during feeding (groups 1 and 2) and during the first part of the fasting period (group 3) to a value at least 21% higher than its minimum, and then it decreased. This variation in RNA content of the liver throughout the 48-hour cycle has been observed for a similar group of fasting-adapted rats, fed 12 hours out of 48 hours.[17] For rats in groups 1 and 2, the rate of liver RNA synthesis must therefore have exceeded its rate of degradation, whereas for rats in the second half of the fasting period, possibly including group 4, the rate of degradation was the greater. The relationship between the cyclic variation in the liver RNA/DNA ratio and the rates of labeling of respired CO_2 and liver RNA to the feeding and fasting periods is shown in figure 4.

TABLE 4

Variations in liver RNA/DNA ratio with the nutritional state of the rat [1]

Group	RNA/DNA
1	3.48 ± 0.06 (12) [2]
2	3.44 ± 0.06 (12)
3	3.92 ± 0.07 (13)
4	3.87 ± 0.06 (13)
5	3.24 ± 0.04 (8)

[1] The relation of rats in each experimental group to their nutritional state in the 48-hour cycle at the time of injection is given in table 1. The rats were killed after 4 hours without food (while $^{14}CO_2$ determinations were made).
[2] Mean ± SE of mean, with the number of rats in parentheses.

DISCUSSION

The studies with carboxyl-labeled orotic acid showed that the conversion of intraperitoneally injected orotic acid to UMP did not vary with the nutritional state of the rat throughout its 48-hour cycle of 8 hours of feeding and 40 hours of fasting. The liver RNA content did show a cyclic variation, and the studies with ring-labeled orotic acid showed there were correspondingly cyclic variations in the incorporation of labeled UMP into RNA and in the catabolism to CO_2 (fig. 4). The sum of the percentage radioactivity recovered in expired CO_2 and in total liver RNA was fairly constant throughout the cycle for a 4-hour labeling period, and is in agreement with the observation that the radioactivity remaining in the acid-soluble fraction also varied little. The inverse type of relationship found here between incorporation of labeled UMP into RNA and its degradation to $^{14}CO_2$ is similar to that observed by Canellakis (23) for the metabolism of uracil. He showed that tissues

[17] See footnote 4.

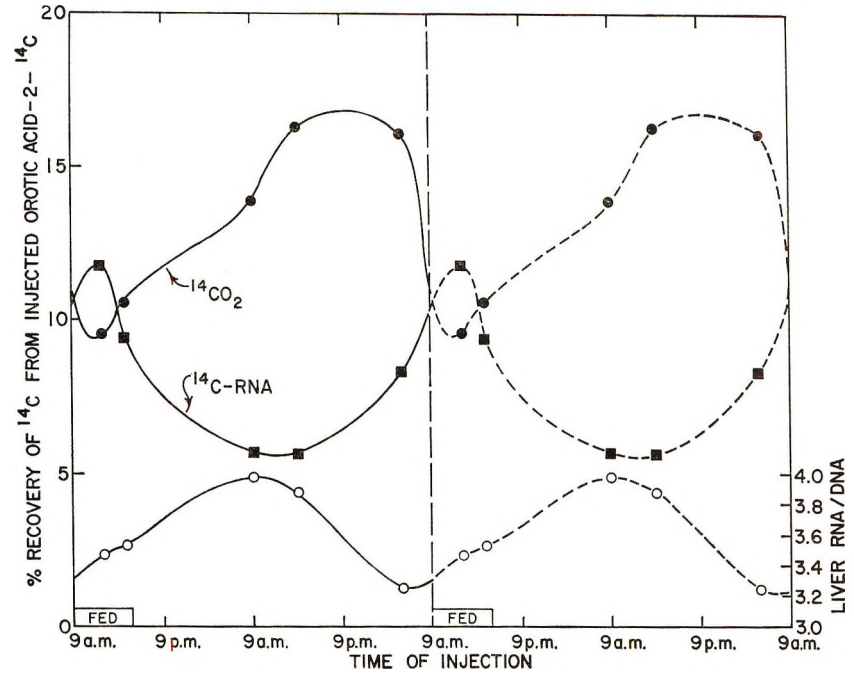

Fig. 4 Relationship between cyclic variations in the liver RNA/DNA ratio and rates of labeling of respired CO_2 and liver RNA to the feeding and fasting periods. The data are derived from figure 3A and table 3 for rats injected with 1 μmole of orotic acid-2-^{14}C, and killed 4 hours later. Two complete 48-hour cycles are shown, the data from the first cycle being repeated for the second cycle (dashed lines).

which incorporate uracil into RNA to an appreciable extent have a decreased ability to degrade it.

The incorporation of ^{14}C into liver RNA was lower in rats in the fasting period of the cycle, particularly in the middle of the fasting period when the RNA/DNA ratio was near maximum (fig. 4); however, without some information on the free nucleotide pool sizes, and hence their specific activities, it is considered that no firm conclusion can be drawn as to the relation of incorporation of labeled orotic acid into RNA to the rate of RNA synthesis throughout the cycle. Hirsch and Hiatt (24) have recently reported that there is a decrease in the rate of liver ribosomal RNA synthesis during fasting. Thus, the decrease observed in the incorporation of ^{14}C from ring-labeled orotic acid into liver RNA during the fasting period of the cycle may reflect such a decrease in the rate of RNA synthesis. In might also be caused, in part, by a dilution of free nucleotide pools resulting from an accelerated breakdown of RNA during fasting (25,26). Despite any possible dilution of the pyrimidine nucleotide pools resulting from increased RNA breakdown, there occurred an increase in the rate of degradation of UMP derived from injected ring-labeled orotic acid during the fasting period of the cycle. There must, therefore, have been a greater catabolic enzymic activity for UMP during the fasting period. These experiments add further support to the proposal that controlled feeding schedules should be maintained in nutritional experiments on animals that are to be used for biochemical studies (1,2).

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Acute Choline Deficiency in Germfree, Conventionalized and Open-Animal-Room Rats: Effects of neomycin, chlortetracycline, vitamin B_{12} and coprophagy prevention [1]

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ABSTRACT Acute choline deficiency was studied in germfree (GF), conventionalized (CONV) and open-animal-room (OAR) rats. With diet A (low cystine, low cholesterol), no rats died; nephropathy and liver fat were less in the GF rats. Liver cholesterol (measured only in OAR rats) increased. Neomycin was without effect in GF and OAR rats. Vitamin B_{12} lessened the nephropathy and liver fat in only OAR rats; cholesterol was not measured. GF rats fed diet B (high cystine, high cholesterol) showed much less nephropathy and mortality than CONV or OAR rats but increased liver fat. Prevention of coprophagy did not change the response of OAR rats to choline deficiency, nor did neomycin. Vitamin B_{12} and chlortetracycline decreased the nephropathy and mortality of OAR rats but not liver fat. Vitamin B_{12} and neomycin had no effect on choline-deficient GF rats. All rats had high liver cholesterol; the GF were the highest. The cholesterol levels were unaffected by choline deficiency, but chlortetracycline and neomycin increased liver cholesterol of choline-deficient rats. We ascribe the lessened nephropathy of GF rats fed choline-deficient diets to their lower metabolic rates, absent bacterial utilization of choline and methionine, greater liver choline synthesis, and lessened decrease in renal acetyl choline. We think the abrupt drop in renal acetyl choline in OAR rats fed such diets leads to nephropathy via renal vasospasm and ischemia.

The possible role of microorganisms on the liver and renal pathology resulting from acute choline deficiency (1–6) has interested us since one of us (S.M.L.) reported that germfree rats of the Lobund strain develop liver cirrhosis as a result of chronic choline deficiency earlier than open-animal-room (OAR) or conventionalized rats (7–8). Further, certain antibiotics have been reported by others to prevent many of the acute and chronic manifestations of choline deficiency (9–13). Our experiments indicate that the "germfree" state [5] diminishes and often prevents the renal manifestations of choline deficiency; its influence on the early accumulation of liver fat is dependent on the cholesterol and cystine concentrations in the diet. Vitamin B_{12} diminishes the nephropathy of acute choline deficiency in OAR or conventionalized rats [6] but has no added effect on the lessened renal lesions of germfree rats. The early liver fat accumulation is not affected by vitamin B_{12}. Similarly, neither neomycin nor chlortetracycline diminishes the early accumulation of liver fat but chlortetracycline (but not neomycin) diminishes the severity of the nephropathy in open-animal-room rats.

MATERIALS AND METHODS

The diets used were adopted from Salmon and Newberne (14) with the following modifications: (a) they were autoclaved at 126° for 25 minutes; (b) the amounts of certain vitamins were in-

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[5] The term "germfree" as used in this paper refers to rats free from viable bacteria, parasites, or fungi as determined by methods published elsewhere (8).

[6] Conventionalized rats were littermates of the germfree rats contaminated with cecal contents of OAR rats on the day after weaning, the day the experiments were started.

creased to compensate for their destruction by autoclaving. The amounts added were: thiamine, 10 mg/kg; calcium pantothenate, 20 mg/kg; pyridoxine, 10 mg/kg; niacin, 200 mg/kg; menadione, 5 mg/kg; riboflavin, 20 mg/kg; *i*-inositol, 200 mg/kg; (diet A);[7] and (c) in several experiments we added 0.4 g cystine and 5 g cholesterol for every 100 g of diet (diet B) [8] to enhance the renal pathology.

Male rats of the Fischer strain, 21 to 23 days old,[9] were used. The OAR rats were housed singly in stainless steel cages on a 2-mesh wire floor in a room where the temperature was controlled at 22° ± 1°. The germfree and conventionalized rats were housed in flexible plastic film isolators in rigid plastic cages on 2-mesh wire-raised floors, one rat per compartment in 3-compartmented boxes; the tops of the plastic cages were also 2-mesh wire. The conventionalized rats were littermates of the germfree rats and were purposefully contaminated with cecal contents of OAR Fischer rats the day after weaning, the day the experimental diets were started.

Within each group, some rats were given supplements of choline chloride in a concentration of 1.5 mg/ml in their drinking water; other groups of animals were given supplements of either neomycin sulfate (0.11 g/100 g in drinking water or 0.27 g/100 g diet) chlortetracycline·HCl [10] (0.20 g per 100 g in drinking water or 0.5 g/100 g diet), or vitamin B_{12} (50 μg/100 g drinking water). The rats were weighed 3 times each week between 1 and 3 PM. Diet and supplements were given at these times; the rats ate and drank ad libitum.

In some experiments the rats were bled via retro-orbital puncture, hematocrits were measured, and blood urea N (BUN), glucose, and uric acid and plasma sodium, potassium, chloride and total solids determinations made. All chemical determinations were made by autoanalyzer methods (15, 16); the total solids were determined by refractometry (17).

Rats that died were autopsied within 12 hours and the tissues placed in 10% buffered formalin. At 10 or 12 days, the surviving rats were anesthetized lightly with ether and bled by heart or aortic puncture. The livers were weighed and the caudate lobe was removed and placed in 10% buffered formalin together with the kidneys for later histological examination. Such histological examination of the livers provided only a gross approximation of their fat content; chemical methods were used for quantitative measurements of liver fat. The water content, total fat (18) and total nitrogen (15) determinations were performed on the liver. All kidney tissue sections were stained with hematoxylin and eosin (19) and the Mowry modification of Hale's colloidal iron (19); in some instances, Perl's (19), PAS (19) and Van Kossa (19) stains were used to evaluate, respectively, the degree of earlier hemorrhage, the status of the basement membranes and the presence of calcium deposits.

The following criteria were used for grading the acute renal damage: One plus (+) injury was not detectable grossly and was characterized microscopically by focal cortical tubular necrosis accompanied by small foci of hyperemic vessels and, less often, hemorrhage. Three plus (+++) injury was manifested grossly by diffusely hemorrhagic kidneys, principally cortical. Microscopically, a continuous subcapsular cortical rim of necrotic tubules, hyperemic vessels and fresh hemorrhages was seen. Many tubules contained fibrinous or hyaline casts; these were found at all levels extending to the collecting tubules. Scattered foci of amorphous hematoxylin-stained material were seen among the necrotic cortical tubules. Except for the casts, the pathologic changes were restricted to the cortex and were most severe and most common in the extreme outer portion. Two plus (++) injury was intermediate in intensity and could usually be diagnosed grossly. The microscopic changes were focal but more extensive than the one plus (+) lesion.

RESULTS

1. *Open-animal-room rats, diet A, experiments A and B (table 1).* In two 10-

[7] All dietary ingredients were purchased from General Biochemicals Inc., Chagrin Falls, Ohio.
[8] See footnote 7.
[9] Charles River Breeding Laboratories, Wilmington, Massachusetts.
[10] Chlortetracycline·HCl (Aureomycin·HCl) generously donated by the American Cyanamid Company (Lederle Laboratories), Pearl River, New York.

TABLE 1

Some effects of acute choline deficiency in rats [1] fed diet A [2]

Exp.	Group	Status [3] and supplement	No. rats	Fatalities	Non-fatal nephropathy				Liver fat	Hemato-crit	Plasma total solids	Body wt	
					+++	++	+	0				Initial	Final
									1	2	3		4
									%	%	*g/100 ml*	*g*	*g*
A	A	OAR, water	12	0	7	3	1	1	19 ± 1 [4]	43 ± 1	8.0 ± 0.4	29 ± 0	42 ± 1
	B	OAR, neomycin [5]	12	0	3	6	2	1	19 ± 2	47 ± 3	7.8 ± 0.3	29 ± 0	45 ± 1
	C	OAR, choline [6]	12	0	0	0	0	12	4 ± 0	41 ± 1	6.8 ± 0.2	29 ± 0	60 ± 2
B	D	OAR, water	8	0	4	0	4	0	21 ± 2	—	—	51 ± 1	77 ± 14
	E	OAR, neomycin	8	0	2	1	3	2	25 ± 1	—	—	51 ± 1	107 ± 3
	F	OAR, vitamin B_{12} [7]	8	0	0	0	0	8	16 ± 1	—	—	51 ± 1	104 ± 2
	G	OAR, choline	8	0	0	0	0	8	2 ± 0	—	—	51 ± 1	107 ± 3
	H	GF, water	8	0	0	0	0	8	13 ± 3	—	—	—	—
	I	GF, vitamin B_{12}	8	0	0	0	0	8	14 ± 3	—	—	—	—
	J	GF, neomycin	8	0	0	0	0	8	10 ± 2	—	—	—	—
	K	GF, choline	8	0	0	0	0	8	5 ± 1	—	—	—	—

[1] Male rats (Fischer strain) 21 to 23 days old (weanling) at start of the experimental diet.
[2] Salmon and Newberne (14) diet plus certain vitamins and autoclaving as described in the text.
[3] OAR indicates open-animal-room rats; GF, germfree rats.
[4] Mean ± SE; standard errors of zero were greater than 0.1 and less than 0.5.
[5] Neomycin sulfate, 1.09 mg/ml in drinking water.
[6] Choline chloride, 1.5 mg/ml in drinking water.
[7] Vitamin B_{12}, 0.5 μg/ml in drinking water.

Statistical analyses: (numerals preceding capital letters refer to numbered columns in table; letters refer to group). In each experiment, there were no statistically significant differences between the starting weights of the groups.

1A vs. 1B, P not significant
1A and 1B vs. 1C, $P < 0.001$
1D vs. 1E, P not significant
1D vs. 1F, $P < 0.05$
1E vs. 1F, $P < 0.02$
1D, 1E, or 1F, vs. 1G, $P < 0.001$
1H vs. 1I, P not significant
1H or 1I vs. 1J, P not significant
1H, 1I, or 1J vs. 1K, $P < 0.001$
2A vs. 2B vs. 2C, P not significant
3A vs. 3B vs. 3C, P not significant
4A vs. 4B, P not significant
4A or 4B vs. 4C, $P < 0.001$
4D vs. 4E, 4F, or 4G, $P < 0.001$

day experiments with diet A, the renal damage in the unsupplemented OAR rats was ++ to +++ in 14 of 20 animals, but none had died by the time they were killed. The incidence and degree of renal damage were similar in the 20 neomycin-treated rats. In contrast, none of 8 rats whose diet was supplemented with vitamin B_{12} (exp. B) had any gross or microscopic signs of renal damage. There were no statistically significant differences in the hematocrits and plasma total solids of the rats in the various groups.

The liver fat for the choline-supplemented group was 2 to 4% (wet weight), and the liver fat of the unsupplemented OAR rats was increased to 19 and 21% ($P < 0.001$). Neomycin did not affect the liver fat accumulation nor did vitamin B_{12} during the first 7 days, but by the tenth day the liver fat of the vitamin B_{12}-supplemented OAR rats (16%) was lower than that of the unsupplemented (21%, $P < 0.05$) and neomycin-supplemented rats (25%, $P < 0.001$).

The average weight gain of all groups was identical for the first 5 to 6 days; thereafter the unsupplemented rats grew much more slowly. In the first experiment, supplementation with neomycin was without effect on growth, but in the second, neomycin enhanced growth significantly ($P < 0.001$), so that it was identical to that of the choline- and vitamin B_{12}-supplemented groups.

2. *Open-animal-room rats, diet B, experiments C and D (table 2).* In experiments C and D, 86 OAR rats were divided into 4 groups and fed diet B for 12 days. Three of these groups were given supplements in their drinking water, either neomycin sulfate, vitamin B_{12} or choline chloride and one group was given just tap water. The results of these 2 experiments are presented together since the findings were similar.

Rats supplemented with choline grew normally and none died. At killing, their kidneys, hematocrits, plasma total solids and BUN concentrations were normal.

The unsupplemented rats gained weight at a normal rate for the first 5 to 6 days and then lost weight. Twenty of the 28 rats died in 7 to 10 days, with severe bilateral renal cortical hemorrhagic necrosis. One rat killed at 8 days had a BUN concentration of 200 mg/100 ml blood and + + + nephropathy. Five of the remaining 7 rats (killed at 12 days) had ++ and +++ hemorrhagic nephropathy, with BUN levels ranging from 61 to 187 mg/100 ml blood; the remaining 2 rats had + + + nephropathy but in the healing stage and their BUN concentrations were 10 and 25 mg/100 ml blood. The average hematocrit of the survivors was not different from that of the choline-supplemented groups, but the plasma total solids concentration of the former rats was significantly higher ($P < 0.02$). Random sections of the aorta and myocardiums of the rats were studied microscopically; no abnormalities were noted.

The growth of the 18 neomycin-supplemented rats was similar to that of unsupplemented rats. Ten of these rats died 8 to 10 days after the start of the diet with severe nephropathy. Two rats killed at 8 days had BUN values over 200 mg/100 ml blood and showed severe nephropathy. The 6 rats killed on day 12 had + + and + + + nephropathy and elevated BUN values ranging from 50 to 200 mg/100 ml blood. Their hematocrits were not altered but their plasma total solids concentrations (like those of unsupplemented rats) were significantly higher than those of choline-supplemented rats ($P < 0.02$).

The 10 vitamin B_{12}-supplemented rats also reached a plateau in their weights at 5 to 6 days; five of these rats recovered shortly and grew at a rate considerably faster than the unsupplemented rats ($P < 0.02$), though not as fast as the choline-supplemented groups. At killing, the 5 rats had + + and + + + nephropathy in the healing stage and their BUN concentrations averaged 22 mg/100 ml blood. Their hematocrits did not differ significantly from those of the choline-supplemented control rats; their plasma total solids concentrations were elevated and significantly higher than those of their choline-supplemented controls. The other 5 rats lost weight and died with severe renal disease. The 50% mortality of the vitamin B_{12}-supplemented rats was

TABLE 2

Some effects of acute choline deficiency in rats [1] fed diet B [2]

Group	Exp.	Status and supplement	No. rats	Fatal nephropathy	Non-fatal nephropathy			At death	At killing				Body wt	
				+++	+++	++	+	0	Liver fat	Liver fat	Blood urea N	Hemato-crit	Plasma total solids	Initial	Final
									1	2	3	4	5		6
									% wet wt	*% wet wt*	*mg/100 ml*	*%*	*g/100 ml*	*g*	*g*
C	A	OAR,[3] water	18	10	6	2	0	0	14 ± 1 [4]	27 ± 2	105 ± 17	42 ± 1	8.6 ± 0.3	29 ± 1	34 ± 2
	B	OAR, neomycin [5]	18	10	6	2	0	0	15 ± 1	23 ± 2	89 ± 18	43 ± 2	9.1 ± 0.3	29 ± 1	37 ± 2
	C	OAR, choline [6]	20	0	0	0	0	20	—	14 ± 0	10 ± 1	43 ± 1	6.8 ± 0.2	29 ± 1	50 ± 1
D	D	OAR, water	10	10	0	0	0	0	14 ± 1	—	—	—	—	27 ± 1	29 ± 1
	E	OAR, vitamin B_{12} [7]	10	5	4	1	0	0	13 ± 1	22 ± 2	24 ± 2	41 ± 2	7.8 ± 0.2	27 ± 2	40 ± 4
	F	OAR, choline	10	0	0	0	0	10	—	10 ± 1	18 ± 2	42 ± 0	6.7 ± 0.1	27 ± 1	51 ± 2

[1] Male rats (Fischer strain) 21 days old (weanling) at start of the experimental diet.

[2] The Salmon and Newberne (14) diet modified by the addition of 0.4 g cystine/100 g diet, 5 g cholesterol/100 g diet and certain vitamins as described in text, and autoclaving.

[3] OAR indicates open-animal-room rats.

[4] Mean ± SE; standard errors of zero were greater than 0.1 and less than 0.5.

[5] Neomycin sulfate, 1.09 mg/ml in drinking water.

[6] Choline chloride, 1.5 mg/ml in drinking water.

[7] Vitamin B_{12}, 0.5 μg/ml in drinking water.

Statistical analyses: (numerals preceding capital letters refer to numbered columns in table; letters refer to group). There were no statistically significant differences between the starting weights of the different groups.

1A vs. 1B, P not significant
1A vs. 2A, $P < 0.001$
1B vs. 2B, $P < 0.001$
2A vs. 2B, P not significant
2A or 2B vs. 2C, $P < 0.001$
3A vs. 3B, P not significant
3A or 3B vs. 3C, $P < 0.02$
4A vs. 4B vs. 4C, P not significant
5A vs. 5B, P not significant
5A or 5B vs. 5C, $P < 0.02$
6A vs. 6B, P not significant
6A or 6B vs. 6C, $P < 0.001$
2E vs. 2F, $P < 0.001$
2E vs. 1E, $P < 0.001$
1D vs. 2E, P 0.001
3E vs. 3F, P not significant
1D vs. 1E, P not significant
4E vs. 4F, P not significant
5E vs. 5F, $P < 0.02$
6D vs. 6E, $P < 0.02$
6E vs. 6F, P not significant
6D vs. 6F, $P < 0.001$

significantly lower than that of the unsupplemented group ($P < 0.001$).

An inverse correlation between weight gain and nephropathy and death was noted. All 40 rats fed diet B without any supplement or with vitamin B_{12} or neomycin grew at normal rates for the first 5 to 6 days. Thirty-two then lost weight, so that at death or killing they weighed 1 to 5 g less than their starting weights. Seventeen of these thirty-two died at 8 to 10 days and 15 were killed at day 12. All had severe nephropathy. In contrast, none of the 8 rats that gained weight after the short stationary period died, and none had severe nephropathy, one had + + nephropathy, and the remaining seven had either zero or + nephropathy when they were killed.

The liver fat of the choline-supplemented rats averaged 13% (usual for this high cholesterol-cystine diet); that of the unsupplemented animals that survived averaged 27% ($P < 0.001$) and neither neomycin nor vitamin B_{12} changed this. All rats that died with severe nephropathy (with or without vitamin B_{12} or neomycin supplements) had levels of liver fat which were significantly lower ($P < 0.001$) than those of the corresponding rats that survived.

3. *Open-animal-room rats — effects of cystine and cholesterol on acute choline deficiency (exp. E) (table 3).* This experiment was conducted to determine the extent to which cystine and cholesterol were responsible for the conversion of the mild nephropathy-producing diet A to the severe, lethal nephropathy-producing diet B.

Six groups of rats were studied. Group A: 10 rats fed diet A with 0.4 g cystine/100 g diet added; group B: 10 rats fed diet A with 5 g cholesterol/100 g diet added; and group C: 10 rats fed diet A with 0.4 g cystine and 5 g cholesterol/100 g diet added (diet B). The remaining 3 groups of 5 rats each (groups D, E, and F) were fed the above 3 diets, respectively, but in addition were given choline chloride in their drinking water.

All choline-supplemented rats gained weight but the 2 groups of rats fed diets containing cholesterol gained twice as much as the groups of rats without cholesterol ($P < 0.001$). At killing, none of the choline-supplemented rats showed any signs of choline deficiency. Their hematocrits and plasma total solids concentrations were similar and in the usual range.

After an initial 5 to 6 days of normal weight gain, all choline-deficient rats failed to gain for 1 to 2 days and then started to lose weight. The rats fed diet A with added cystine resumed growth shortly (though at a slowed rate), but the others did not. Each of these groups gained significantly less than the corresponding choline-supplemented rats. By the twelfth day, 30% of the choline-deficient rats fed diet A with cystine were dead, whereas 100% and 90% of the choline-deficient rats fed diet A with cholesterol were dead. These results were paralleled by the elevations in BUN and the severity of renal disease found at autopsy. There were no significant differences between the hematocrits and concentrations of plasma total solids in the choline-deficient cystine group and their controls; no statement in this respect can be made about the two cholesterol-supplemented groups because 19 of the 20 animals died. The one surviving animal had a low hematocrit and a high plasma total solids concentration.

The liver fat of the rats fed diet A with cystine was 4 to 5 times as much as that of their choline-supplemented counterparts. The liver fat of those rats fed the diets containing cholesterol with or without cystine was similar to and only slightly higher than the values for their choline-supplemented counterparts.

4. *Open-animal-room rats, diet B chlortetracycline and neomycin supplementation (exps. F, G and H) (table 4).* All rats in these 3 experiments gained weight for the first 5 to 6 days, but then all except the choline-supplemented rats failed to gain normally. In experiments F and G, the chlortetracycline-supplemented rats gained 13 to 14 g/rat in the 12 days of the experiment as contrasted with the zero-gram net weight gain of the unsupplemented groups ($P < 0.001$ for each). In experiment H, however, neither chlortetracycline nor neomycin influenced

TABLE 3

Effects of cystine and cholesterol on acute choline deficiency in open-animal-room (OAR) rats [1]

Exp.	Group	Diet supplement	No. rats	Fatal nephropathy	Non-fatal nephropathy				At death	At killing				Peak blood urea N	Body wt	
				+++	+++	++	+	0	Liver fat	Liver fat	Hematocrit	Plasma total solids	Blood urea N		Initial	Final
									1	2	3	4	5	6		7
									%	%	%	*g/100 ml*	*mg/100 ml*		*g*	*g*
E	A	Cystine [2]	10	3	4	1	0	2	20 ± 1 [3]	23 ± 1	41 ± 1	7.7 ± 0.2	44 ± 5	274 ± 19	28 ± 1	38 ± 2
	B	Cholesterol [4]	10	9	1	0	0	0	13 ± 1	19(1) [5]	31(1)	9.4(1)	155(1)	400 ± 20	24 ± 1	26(1)
	C	Cystine + cholesterol	10	10	0	0	0	0	11 ± 1	—	—	—	—	422 ± 23	27 ± 1	28 ± 2
	D	Cystine + choline [6]	5	0	0	0	0	5	—	5 ± 1	40 ± 1	7.4 ± 0.3	31 ± 4	31 ± 4	26 ± 1	44 ± 4
	E	Cholesterol + choline	5	0	0	0	0	5	—	9 ± 1	38 ± 1	7.0 ± 0.5	28 ± 4	29 ± 4	26 ± 1	61 ± 5
	F	Cystine + cholesterol + choline	5	0	0	0	0	5	—	10 ± 1	38 ± 1	7.2 ± 0.3	26 ± 2	33 ± 4	26 ± 1	65 ± 4

[1] Male (Fischer strain) open-animal-room rats 21 days old (weanling) at start of experimental diets.
[2] Cystine, 0.4 g/100 g added to the Salmon and Newberne (14) diet (with added vitamins).
[3] Mean ± SE.
[4] Cholesterol, 5 g/100 g added to the Salmon and Newberne diet (with added vitamins).
[5] Numbers in parentheses indicate number of rats.
[6] Choline chloride, 1.5 mg/ml added to drinking water.

Statistical analyses: (numerals preceding capital letters refer to numbered columns in table; letters refer to group). There were no statistically significant differences between the starting weights of the different groups.

1A vs. 1B or 1C, $P < 0.02$
1B vs. 1C, P not significant
2A vs. 2D or 2E, $P < 0.001$
2A vs. 1A, P not significant
3D vs. 3E vs. 3F, P not significant
3A vs. 3D, P not significant
4D vs. 4E vs. 4F, P not significant
4A vs. 4D, P not significant
5A vs. 5D, P not significant
6A vs. 6B, $P < 0.02$
6A vs. 6C, $P < 0.02$
6B vs. 6C, P not significant
6A or 6B or 6C vs. 6D or 6E or 6F, $P < 0.001$
7A vs. 7C, $P < 0.02$
7A vs. 7D, P not significant
7C vs. 7E or 7F, $P < 0.001$
7D vs. 7E or 7F, $P < 0.02$

TABLE 4

Effects of neomycin [1] and chlortetracycline [2] on rats [3] subjected to acute choline deficiency

Exp.	Group	Status and supplement	No. rats	Fatal nephropathy	Non-fatal nephropathy			At death	At killing				Peak blood urea N	Body wt	
				+++	+++	++	+	0	Liver fat	Liver fat	Hematocrit	Plasma total solids	Blood urea N		Initial	Final
									1	2	3	4	5	6		7
									%	%	%	g/100 ml	mg/100 ml		g	g
F	A	OAR,[4] water	3	3	0	0	0	0	13±2 [5]	—	—	—	—	373(1) [6]	29±1	29±1
	B	OAR, chlortetracycline	9	5	1	0	0	3	27±1	33±1	38±1	7.7±0.1	76±21	170±23	28±1	41±1
	C	OAR, choline [7]	9	0	0	0	0	9	—	10±1	39±1	6.8±0.1	15±2	14±1	28±1	68±4
G	D	OAR, water	12	12	0	0	0	0	14±1	—	—	—	—	400±63	30±1	30±1
	E	OAR, chlortetracycline	12	4	1	2	3	2	22±1	32±1	35±1	8.0±0.4	158±28	192±52	30±1	44±3
	F	OAR, choline	6	0	0	0	0	6	—	16±2	36±1	7.1±0.1	10±0	18±1	30±2	75±6
H	G	OAR, neomycin [1a]	10	8	2	0	0	0	14±2	21±0	30±0	9.3±0.4	100±10	367±29	25±1	30±1
	H	OAR, chlortetracycline [2a]	10	8	2	0	0	0	13±1	27±1	32±4	10.0±0.6	127±7	400±33	25±1	24±1
	I	OAR, neomycin [1b]	10	10	0	0	0	0	16±1	—	—	—	—	370±19	21±1	25±1
	J	OAR, chlortetracycline [2b]	10	5	1	0	3	1	25±2	36±1	41±0	7.7±0.1	108±20	267±34	24±1	29±1
	K	OAR, water	10	10	0	0	0	0	13±2	—	—	—	—	411±18	22±0	25±0
	L	OAR, choline	9	0	0	0	0	9	—	9±1	38±1	6.9±0.1	23±1	33±2	22±2	45±3
Summary																
A+D+K		OAR, water	25	25	0	0	0	0	13±1	—	—	—	—	380±14	26±1	29±2
B+E+H+J		OAR, chlortetracycline	41	22	5	2	6	6	21±1	33±1	37±1	8.3±0.3	124±13	247±7	27±1	39±4
G+I		OAR, neomycin	20	18	2	0	0	0	15±1	21±0	30±0	9.3±0.3	100±7	406±17	23±1	30±2
C+F+L		OAR, choline	24	0	0	0	0	24	—	11±1	38±1	7.0±0.1	20±1	22±1	26±1	67±6

[1] Neomycin sulfate, (a) 2.7 mg/g diet, or (b) 1.09 mg/ml in drinking water.
[2] Chlortetracycline, (a) 0.5 g/100 g diet, or (b) 0.2 g/100 ml in drinking water.
[3] Male (Fischer strain) 21 days old (weanling) at start of experiment.
[4] OAR indicates open-animal-room rats.
[5] Mean ± SE; standard errors of zero were greater than 0.1 but less than 0.5.
[6] Only one rat.
[7] Choline chloride, 1.5 mg/ml in drinking water.

Statistical analyses carried out on the summary, experiments F, G and H: (numerals preceding capital letters refer to numbered columns in table; letters refer to group). None of the initial weights of any of the groups were significantly different from one another.

1ADK vs. 1BEHJ, $P < 0.02$
1ADK vs. 1GI, P not significant
1BEHJ vs. 1GI, $P < 0.02$
1BEHJ vs. 2BEHJ, $P < 0.02$
1ADK vs. 2BEHJ, $P < 0.02$
2GI vs. 1GI, $P < 0.02$
2BEHJ vs. 2GI, $P < 0.02$
2BEHJ vs. 2CFL, $P < 0.001$
2GI vs. 2CFL, $P < 0.02$
3BEHJ vs. 3GI, $P < 0.02$
3BEHJ vs. 3CFL, P not significant
3GI vs. 3CFL, $P < 0.02$
4BEHJ vs. 4GI, P not significant
4BEHJ vs. 4CFL, $P < 0.02$
4GI vs. 4CFL, $P < 0.02$
5BEHJ vs. 5GI, P not significant
5BEHJ, 5GI vs. 5CFL, $P < 0.001$
6ADK or 6GI vs. 6 BEHJ, $P < 0.02$
6ADK, 6BEHJ, 6GI vs. 6CFL, $P < 0.001$
7ADK vs. 7BEHJ vs. 7GI, P not significant
7ADK, 7BEHJ, 7GI vs. 7CFL, $P < 0.001$

favorably the weight gain of the rats fed the choline-deficient diet.

All 25 unsupplemented rats died with severe nephropathy as did 18 of the 20 neomycin-supplemented rats. The two neomycin-supplemented rats killed at 12 days also showed signs of severe nephropathy. The incidence and severity of nephropathy was significantly less ($P < 0.001$) in the chlortetracycline-treated group. Only 22 of the 41 chlortetracycline-supplemented rats died ($P < 0.001$), each with severe nephropathy. Six of the 19 chlortetracycline-supplemented rats killed at 12 days had no renal damage, 6 had + nephropathy, and 7 had + + and + + + nephropathy. There was also a statistically significant difference in peak BUN among the groups. The BUN averaged 380 mg/100 ml blood and 406 mg/100 ml blood, respectively, for the unsupplemented and neomycin-supplemented groups and 247 mg/100 ml blood for the chlortetracycline-supplemented rats ($P < 0.02$ for both).

The liver fat of chlortetracycline-supplemented rats that died was 21%, a level significantly higher ($P < 0.001$) than that of the unsupplemented rats (13%) and that of the neomycin-supplemented rats (15%) that died. The liver fat of the chlortetracycline-supplemented rats killed at 12 days averaged 33%, a level significantly higher than that of the chlortetracycline-supplemented rats that died (21%) ($P < 0.001$), and of the choline-supplemented rats that were killed (11%) ($P < 0.001$).

At killing, the plasma total solids of the 7 chlortetracycline-supplemented rats with + + or + + + nephropathy averaged 9.1 g/100 ml plasma as compared with the average of 7.2 g/100 ml plasma for those 12 rats with mild or no nephropathy ($P < 0.02$). The choline-supplemented groups had plasma total solids concentrations which averaged 7.0 g/100 ml plasma. There were no significant differences in hematocrits among the chlortetracycline- and choline-supplemented rats. In experiment H, we found severe anemia in two of the rats receiving chlortetracycline and the 2 neomycin-supplemented rats (average hematocrits of 30 and 32%, respectively) when they were killed on day 12; these rats were moribund at killing. Their total plasma solids were also markedly elevated, averaging 9.3 and 10.0 g/100 ml plasma, respectively. Since none of the unsupplemented rats survived, hematocrits and plasma total solids determinations were not made in those groups.

In summary, although chlortetracycline lessened the severity of the choline deficiency, it was not as effective as choline when judged by growth, survival, liver fat, plasma total solids concentrations, BUN and renal pathology. Neomycin was ineffective.

5. *Effect of prevention of coprophagy on the response of open-animal-room rats to acute choline deficiency (exp. I) (table 5).* Barnes and Kwong (20)[11] found that when coprophagy was prevented, the hepatic synthesis of choline from methionine was enhanced in young rats and the nephropathy and death following acute choline deficiency was decreased. We carried out an experiment to extend these findings, using a different diet and a different strain of rats.

Sixty-three weanling rats receiving diet B were divided into groups as follows: group C, F (21 rats) were prevented from practicing coprophagy by application of tail cups; six of these rats were given supplements of choline chloride (group F) in their drinking water and the other 15 rats received no choline. Groups A, D and B, E were also composed of 21 rats each and again, six in each (groups D and E) were given supplements of choline chloride. The rats of groups A, B, D, and E were permitted to practice coprophagy; the rats of groups B and E wore sham tail cups, the rats of groups A and D wore no cups. The sham cups differed from the coprophagy-preventing cups in that the bottom of the sham cup was cut out and screwed to the top. The positioning, weight and attachment of all the cups were the same.

Of the 21 rats with sham cups, 6 unsupplemented rats and 2 choline-supplemented rats died within the first 3 days of the experiment; of the 21 rats with coprophagy-preventing cups, 3 choline-

[11] Personal communication, E. Kwong and R. H. Barnes.

TABLE 5

Coprophagy prevention and acute choline deficiency [1]

Exp.	Group	Status and supplement	No. rats	Fatal nephropathy	Non-fatal nephropathy				At death	At killing				Blood urea N at 8 days	Body wt	
				+++	+++	++	+	0	Dry liver fat/protein	Dry liver fat/protein	Hematocrit	Plasma total solids	Blood urea N		Initial	Final
									1	2	3	4	5	6		7
											%	*g/100 ml*	*mg/100 ml*		*g*	*g*
I	A	No tail cups, water	15	15	0	0	0	0	0.640 ± 0.047 [2]	—	—	—	—	170(2) [3]	26 ± 2	26 ± 2
	B	Sham tail cups, water	9 [4]	9	0	0	0	0	0.604 ± 0.050	—	—	—	—	355(1)	26 ± 2	26 ± 2
	C	Coprophagy-preventing cups, water	12 [4]	11	1	0	0	0	0.566 ± 0.058	—	21(1)	6.6(1)	—	351(3)	25 ± 2	24(1)
	D	No tail cups, choline [5]	6	0	0	0	0	6	—	0.395 ± 0.036	39 ± 2	6.7 ± 0.3	18(6)	22(2)	25 ± 1	50 ± 3
	E	Sham tail cups, choline [5]	4 [4]	0	0	0	0	4	—	0.197 ± 0.035	42 ± 2	7.4 ± 0.7	30(3)	22(2)	24 ± 1	38 ± 3
	F	Coprophagy-preventing cups, choline [5]	2 [4]	0	0	0	0	2	—	0.192 ± 0.051	37(2)	7.8(2)	35(2)	16(1)	29(2)	40(2)

[1] Modified Salmon and Newberne (14) diet as described in text (added cystine, cholesterol and vitamins) and autoclaved.
[2] Mean ± SE.
[3] Numbers in parentheses indicate number of rats.
[4] Each of the choline-deficient groups started with 15 rats. Six of the rats with sham cups and three of the rats with coprophagy-preventing cups died as a result of cupping. Each of the choline-supplemented groups started with 6 rats. Two of the sham-cupped rats and 4 rats with coprophagy-preventing cups died as a result of cupping.
[5] Choline chloride, 1.5 mg/ml in drinking water.

Statistical analysis: (numerals preceding capital letters refer to numbered columns in table; letters refer to group). There were no statistically significant differences in the starting weights of any groups.

1A vs. 2D, $P < 0.02$
1B vs. 2E, $P < 0.02$
1C vs. 2F, $P < 0.02$
1A vs. 1B vs. 1C, P not significant
2D vs. 2E or 2F, $P < 0.02$
3D vs. 3E, P not significant
4D vs. 4E, P not significant
7A vs. 7B, P not significant
7A, 7B vs. 7D, 7E, $P < 0.02$
7D vs. 7E, $P < 0.02$

deficient and 4 choline-supplemented rats died during the same time. We attribute these very early deaths to the cupping per se, not to choline deficiency.

Among the choline-supplemented rats, those without cups grew significantly faster (an average weight gain of 25 g in 12 days) than those with coprophagy-preventing cups (11 g weight gain on the average) or those with sham cups (an average weight gain of 14 g). None of these rats died or had any renal pathology at the time they were killed.

The choline-deficient rats lost weight after the first 5 to 6 days. All fifteen without cups, all nine with sham cups and eleven of the twelve with coprophagy-preventing cups died with severe nephropathy. The BUN concentrations of rats from each of these groups chosen at random correlated with the severity of nephropathy; choline-supplemented rats had normal BUN.

The ratio of liver fat to liver protein was 0.197 and 0.192 for choline-supplemented rats with coprophagy-preventing cups and sham cups, respectively. Rats without cups had a somewhat higher ratio, 0.395 ($P < 0.02$). Each of the choline-supplemented groups differed significantly from their respective choline-deficient counterparts ($P < 0.02$ for each) but not from one another. The choline-deficient rats with sham cups had a fat-to-protein ratio of 0.604, which was not significantly different from the ratio of 0.566 for the choline-deficient rats with coprophagy-preventing cups or the ratio of 0.640 for choline-deficient rats without tail cups.

6. *Germfree rats and open-animal-room rats, diet A (exp. B); germfree, conventionalized and open-animal-room rats, diet B (exps. J, K and L) (tables 1 and 6).* Experiment B included germfree and OAR rats fed diet A and included groups that were unsupplemented or supplemented with choline chloride, neomycin or vitamin B_{12} in their drinking water. No rats died. All eight unsupplemented OAR rats had morphologic evidence of renal damage at killing 10 days after the start of the diet, four with + + + and four with + nephropathy (table 1). None of the choline- or vitamin B_{12}-supplemented OAR rats had nephropathy, whereas six of the eight neomycin-supplemented rats did, two with + + +, one with + + and two with + nephropathy. In contrast, none of the germfree rats had any evidence of nephropathy. The liver fat of the unsupplemented germfree rats was significantly higher than that of the choline-supplemented germfree rats, but the increase in liver fat of the choline-deficient germfree rats was lower than that of the comparable OAR rats. Neither supplementation with vitamin B_{12} nor neomycin affected the increase in liver fat in the choline-deficient germfree rats. As mentioned earlier, supplementation with vitamin B_{12} appeared to lower the concentration of liver fat of OAR rats, but this difference was of borderline significance. Neomycin was without effect on liver fat in OAR rats.

Diet B was used in three other experiments (table 6). In two of these, experiments J and K, germfree rats and their conventionalized littermates were studied; in experiment L an additional group of OAR rats was studied. Diet B was used because it produces a more severe nephropathy than diet A.

The growth of all rats progressed normally for the first 5 to 6 days. At this point, growth of the conventionalized and OAR unsupplemented rats ceased and many started to lose weight, but the unsupplemented germfree rats continued to gain normally; this difference was statistically significant ($P < 0.001$).

None of the choline-supplemented rats died and none had nephropathy. Among the choline-deficient rats, 10 of 11 OAR rats and 19 of 33 conventionalized rats died with severe nephropathy by day 12, but only 1 of the 33 germfree rats developed fatal nephropathy ($P < 0.001$).

At killing of the choline-deficient rats, the BUN of the one surviving OAR rats was 426 mg/100 ml blood; the BUN of the 14 conventionalized rats averaged 92 mg/100 ml blood, whereas that of the 32 germfree rats averaged 25 mg/100 ml blood ($P < 0.001$, germfree vs. conventionalized). The peak BUN of the conventionalized rats at the eighth day averaged 170 mg/100 ml blood (6 rats), and that of the germfree rats at the peak averaged 83 mg/100 ml blood (4 rats) ($P < 0.02$) (exp. J). At killing, the plasma total solids

TABLE 6

Effects of acute choline deficiency [1] on germfree,[2] conventionalized [3] and open-animal-room rats [4]

Exp.	Group	Status and supplement	No. rats	Fatal nephropathy	Non-fatal nephropathy				At death	At killing				Peak blood urea N	Body wt	
				+++	+++	++	+	0	Liver fat	Liver fat	Hematocrit	Plasma total solids	Blood urea N		Initial [5]	Final
									1	2	3	4	5	6		7
									%	%	%	g/100 ml	mg/100 ml		g	g
J	A	CONV,[3] water	11	8	3	0	0	0	18±1 [6]	26±1	32±1	8.9±0.5	62± 8	170±12	40±2	50±3
	B	GF,[2] water	11	1	1	4	2	3	31(1) [7]	39±2	39±2	7.2±0.1	23± 1	83± 8	38±2	70±3
	C	CONV, choline [8]	4	0	0	0	0	4	—	16±1	43±1	7.0±0.2	15± 1	23± 1	46±3	90±5
	D	GF, choline	4	0	0	0	0	4	—	18±1	42±1	7.2±0.2	19± 1	20± 1	43±4	91±3
K	E	CONV, water	11	5	0	3	3	0	16±1	28±1	42±1	7.9±0.3	51± 1	—	39±1	62±3
	F	GF, water	11	0	0	0	0	11	—	36±1	41±1	6.8±0.1	19± 1	—	38±1	75±2
	G	CONV, choline	4	0	0	0	0	4	—	12±1	41±1	6.6±0.1	18± 1	—	39±2	72±7
	H	GF, choline	4	0	0	0	0	4	—	15±1	40±1	6.9±0.2	19± 1	—	38±3	80±6
L	I	OAR, water [4]	11	10	1	0	0	0	15±1	17(1)	32(1)	10.6(1)	426(1)	478(1)	46±1	52(1)
	J	CONV, water	11	6	3	2	0	0	14±1	23±2	33±2	7.9±0.4	157±30	—	42±2	47±3
	K	GF, water	11	0	0	0	0	11	—	34±2	41±1	7.2±0.4	27± 4	—	43±2	74±4
	L	OAR, choline	4	0	0	0	0	4	—	13±1	37±1	7.5±0.1	22± 1	—	45±1	97±4
	M	CONV, choline	4	0	0	0	0	4	—	12±0	38±0	6.6±0.1	24± 3	—	43±4	82±4
	N	GF, choline	4	0	0	0	0	4	—	15±2	38±0	7.0±0.2	17± 2	—	40±6	79±7
Summary																
		OAR, water	11	10	1	0	0	0	15±1	17(1)	32(1)	10.6(1)	426(1)	—	46±1	52(1)
		CONV, water	33	19	6	5	3	0	17±1	26±2	36±2	8.1±0.3	92±20	—	41±2	54±3
		GF, water	33	1	1	4	2	25	31(1)	36±1	39±1	7.1±0.1	25± 1	—	40±1	73±2
		OAR, choline	4	0	0	0	0	4	—	13±1	37±1	7.5±0.1	22± 1	—	45±1	97±4
		CONV, choline	12	0	0	0	0	12	—	13±1	41±1	6.7±0.1	23± 2	—	43±2	82±4
		GF, choline	12	0	0	0	0	12	—	16±1	39±1	7.0±0.1	23± 2	—	40±2	82±3

[1] Acute choline deficiency induced by the feeding of the modified Salmon and Newberne (14) diet, diet B.
[2] Germfree (GF) male rats (Fischer strain), 21 to 23 days old (weanling) at start of the experimental diet.
[3] Conventionalized rats (Fischer strain) 21 to 23 days old (weanling) at start of experimental diet and contaminated with cecal contents of open-animal-room rats at start of experiment. These rats were littermates of the germfree rats.
[4] Open-animal-room (OAR) rats, male (Fischer strain), 21 days old (weanling) at start of experimental diet.
[5] None of the starting weights of any of the groups at any given experiment were found to be statistically significantly different.
[6] Mean ± SE; standard errors of zero were greater than 0.1 but less than 0.5.
[7] Numbers in parentheses indicate number of rats.
[8] Choline chloride, 1.5 mg/ml in drinking water.

Statistical analyses: (numerals preceding capital letters refer to numbered columns in table; letters refer to group).

1A vs. 1E vs. 1J, P not significant
1A vs. 2A, $P < 0.02$
1E vs. 2E, $P < 0.02$
1J vs. 2J, $P < 0.02$
2A, 2E, 2J vs. 2B, 2F, 2K, $P < 0.02$
2A, 2E, 2J vs. 2C, 2G, 2M, $P < 0.02$
2B, 2F, 2K vs. 2D, 2H, 2N, $P < 0.02$
3A vs. 3B, 3C, 3D, $P < 0.02$
3B vs. 3C vs. 3D, P not significant
3E vs. 3F vs. 3G vs. 3H, P not significant
3J vs. 3K, 3L, 3M, 3N, $P < 0.02$
4A vs. 4B, 4C, 4D, $P < 0.02$
4B vs. 4C vs. 4D, P not significant
4E vs. 4F, 4G, 4H, $P < 0.02$
4F vs. 4G vs. 4H, P not significant
4J vs. 4K vs. 4N, P not significant
4J vs. 4M, $P < 0.02$
4J vs. 4L, P not significant
5A vs. 5B, $P < 0.02$
5B vs. 5D, P not significant
5A vs. 5C, $P < 0.02$
5E vs. 5F, $P < 0.02$
5F vs. 5H, P not significant
5J vs. 5K or 5M, $P < 0.001$
5K vs. 5N, P not significant
6A vs. 6B, $P < 0.02$
6A vs. 6C, $P < 0.001$
6B vs. 6D, $P < 0.02$
7A vs. 7B or 7C or 7D, $P < 0.02$
7B vs. 7C or 7D, $P < 0.02$
7C vs. 7D, P not significant
7F vs. 7G, P not significant
7E vs. 7F, $P < 0.02$
7E vs. 7H, $P < 0.02$
7F vs. 7G vs. 7H, P not significant
7J vs. 7K, 7L, 7M, 7N, $P < 0.02$
7K vs. 7L, $P < 0.02$
7L vs. 7M vs. 7N, P not significant

of the one OAR rat was 10.6 g/100 ml plasma; that of the fourteen conventionalized rats averaged 8.1 g/100 ml plasma, a figure significantly higher than that of the germfree rats, 7.1 ($P < 0.02$), and that of the choline-supplemented conventionalized and germfree rats, 6.7 and 7.0, respectively ($P < 0.02$). It was noted that in 2 of the 3 experiments (J and L), hematocrits of the killed conventionalized choline-deficient unsupplemented rats averaged 33%, a value which was significantly lower than that of the conventionalized supplemented or germfree rats supplemented or not ($P < 0.02$ in each case).

The histologic appearance of the kidneys of the rats killed on day 12 reflected the BUN findings; the OAR rat and 11 of the 14 conventionalized rats showed + + and + + + nephropathy; in contrast only 1 of 32 germfree rats revealed +++ nephropathy ($P < 0.001$). None of the conventionalized or OAR rats had normal kidneys histologically, but twenty-five of the germfree rats did. The kidneys of the 7 germfree rats that showed mild or moderate nephropathy showed focal cortical tubular atrophy and collapse microscopically. Such areas showed increased cellularity due to a mixture of tubular epithelium, lymphocytes, histiocytes and fibroblasts and there were scattered intra- and extra-cellular fat droplets and iron-positive pigment deposits. Occasional tubular fibrinous casts were encountered. Collapsed tubules could be traced by the PAS stain which showed basement membrane fraying, distortion and disruption. In many areas the tubules were normal or, at most, showed a slight dilatation. The glomeruli were not affected. The kidneys of all choline-supplemented rats were normal.

The liver fat content of the killed OAR and conventionalized choline-supplemented rats averaged 13% each; that of the choline-supplemented germfree groups averaged 16%, a difference which was not statistically significant. The liver fat of the choline-deficient conventionalized rats that died averaged 16% as contrasted with the 26% mean liver fat of those that lived ($P < 0.001$). Similarly, the mean liver fat of the OAR choline-deficient rats of experiment L that died was 15% as contrasted with the mean liver fat of 27% of those which lived in experiments C and D.[12] The liver, fat of the choline-deficient germfree rats at killing averaged 36%, a level significantly higher than that of the conventionalized or OAR rats at killing in all 3 experiments ($P < 0.001$ for each).

7. *Influence of vitamin B_{12} and neomycin on the response of germfree rats receiving diet B (exp. M) (table 7).* Twenty-eight germfree rats were fed diet B. Group A (8 rats) drank tap water; group B (8 rats) was given vitamin B_{12}; group C (5 rats) was given neomycin sulfate and the seven remaining rats, group D, were given choline chloride. There were 5 litters in the experiment. The 8 rats in groups A and B consisted of matched littermates (2 rats from each of 3 litters, 1 rat from each of 2 litters). The 7 rats of group D matched seven of those in each of groups A and B. The 5 rats in group C had one rat from each of the 5 litters.

The rats supplemented with choline chloride behaved as did the choline-supplemented rats in all our experiments; they grew normally, none died, and when killed on day 12 none showed any sign of renal disease, grossly or microscopically, or elevation in BUN.

One of the 8 unsupplemented germfree rats and two of the vitamin B_{12}-supplemented germfree rats died with severe nephropathy. These 3 rats had stopped gaining weight after 5 to 6 days and then lost weight. Of the 7 unsupplemented germfree rats killed at 12 days, one had + + + nephropathy, three showed + + nephropathy (with evidence of healing) and three were normal. At killing, 3 of the 6 vitamin B_{12}-supplemented rats showed + + renal injury with evidence of healing; the other three were normal. None of the 5 neomycin-supplemented rats died; at killing three had + + + nephropathy and one had + nephropathy and one was normal. The average BUN of all rats at 12 days was similar as were the hematocrits and plasma total solids concentrations.

[12] This comparison was made since only one OAR rat from the experiment lived.

TABLE 7

Effects of acute choline deficiency [1] on various blood chemistries in germfree rats [2]

Exp.	Group	Status and supplement	At killing: Fatal nephropathy	Non-fatal nephropathy				Blood: Urea N	Blood: Uric acid	Blood: Glucose	Plasma: Na	Plasma: K	Plasma: Cl	Liver fat	Hematocrit	Plasma total solids	Body wt: Initial	Body wt: Final
			+++	+++	++	+	0	1	2	3	4	5	6	7	8	9		10
								mg/100 ml blood			*mEq/liter*			%	%	*g/100 ml*	*g*	*g*
M	A	GF, water (8) [3]	1	1	3	0	3	23 ± 3 [4]	7.6	127 ± 5	153 ± 2	4.3 ± 0.1	82 ± 1	35 ± 2	42 ± 1	7.1 ± 0.4	26 ± 1	62 ± 6
	B	GF, vitamin B_{12} [5] (8)	2	0	3	0	3	16 ± 1	—	133 ± 12	158 ± 1	4.9 ± 0.2	82 ± 2	35 ± 2	41 ± 1	6.7 ± 0.2	29 ± 2	67 ± 4
	C	GF, neomycin [6] (5)	0	3	0	1	1	25 ± 5	—	143 ± 3	156 ± 2	4.3 ± 0.2	78 ± 1	33 ± 2	40 ± 1	7.6 ± 0.5	29 ± 2	61 ± 6
	D	GF, choline [7] (7)	0	0	0	0	7	15 ± 1	7.7	121 ± 9	159 ± 2	4.1 ± 0.2	82 ± 1	19 ± 1	40 ± 1	6.6 ± 0.2	29 ± 2	68 ± 4

[1] Acute choline deficiency induced by the feeding of diet B, the Salmon and Newberne (14) diet modified by addition of 0.4 g cystine/100 g diet, 5 g cholesterol/100 g diet, certain vitamins (see text) and autoclaving; diet fed for 12 days.

[2] Germfree rats (GF), male (Fischer strain) 21 to 23 days old (weanling) at start of experimental diet.

[3] Numbers in parentheses indicate number of rats.

[4] Mean ± SE.

[5] Vitamin B_{12}, 0.5 μg/ml in drinking water.

[6] Neomycin sulfate, 1.09 mg/ml in drinking water.

[7] Choline chloride, 1.5 mg/ml in drinking water.

Statistical analysis: (numerals preceding capital letters refer to numbered columns in table; letters refer to group). There were no statistically significant differences in the starting weights of any groups.

1A vs. 1B vs. 1C vs. 1D, P not significant
2A vs. 2D, P not significant
3A vs. 3B vs. 3C vs. 3D, P not significant
4A vs. 4B vs. 4C vs. 4D, P not significant
5A vs. 5B vs. 5C vs. 5D, P not significant
6A vs. 6B vs. 6C vs. 6D, P not significant
7A or 7B or 7C vs. 7D, $P < 0.001$
7A vs. 7B vs. 7C, P not significant
8A vs. 8B vs. 8C vs. 8D, P not significant
9A vs. 9B vs. 9C vs. 9D, P not significant
10A vs. 10B vs. 10C vs. 10D, P not significant

The liver fat of the unsupplemented group averaged 35%; the liver fats of the rats supplemented with neomycin or vitamin B_{12} were similar, averaging 33% and 35%, respectively. All differed significantly from the choline-supplemented group (19%) ($P < 0.001$).

Blood uric acid, blood glucose, plasma sodium, potassium and chloride were not altered in any of the animals killed at 12 days.

8. *Concentration of liver cholesterol in germfree, conventionalized, and open-animal-room rats fed either diet A, diet A with cholesterol or diet B (with or without neomycin, chlortetracycline, or choline supplementation (table 8)).* The livers of rats from most of the aforementioned experiments (whether the rats died or were killed after 10 or 12 days of choline deficiency) were assayed for cholesterol. All the rats in the study fed diet A lived until they were killed 10 days after the start of the diet; most of the rats (80–90%) fed diet B died 8 to 11 days after the start of the experiment, and therefore most of the cholesterol data in the averages represent data from rats that died of choline deficiency.

We found a three- to fourfold increase in the concentration and content of liver cholesterol ($P < 0.001$) in the unsupplemented choline-deficient (diet A) rats. The rise in liver cholesterol, however, was proportionately somewhat less than the increase in liver fat. These changes in liver cholesterol and liver fat were not affected by supplementation with neomycin (table 8).

The feeding of diet B or diet A plus 5% cholesterol caused marked increases in the concentration and content of liver cholesterol of choline-supplemented rats so that these were equal to the levels found in choline-deficient rats fed diet A; liver fat averaged 12%, a figure much higher than that for the rats fed diet A and choline but lower than that for choline-deficient rats receiving either diet A, diet A plus cholesterol or diet B. Choline deficiency had no further effect on the liver cholesterol of the rats eating the cholesterol-supplemented diets, but their liver fat increased still further. Neomycin and chlortetracycline given to the rats fed diet A plus 5% cholesterol or diet B without choline supplementation caused an additional (50%) increase in the concentration of cholesterol in the liver without altering the liver fat accumulation.

The concentration and content of liver cholesterol were similar in the choline-deficient and choline-supplemented conventionalized rats and in each case, these were higher than those of comparable OAR rats. Similarly, the liver cholesterol concentration and content of germfree rats were the same whether or not the rats were supplemented with choline chloride, but in each case these levels were higher than those of the conventionalized and OAR rats. Liver fat accumulation was the greatest in germfree rats and the least in OAR rats.

Neomycin did not modify either liver cholesterol or liver fat of the germfree rats fed the choline-deficient diet B.

DISCUSSION

Our investigations show that the growth-deterring, nephropathic and lethal effects of choline deficiency were considerably less severe in germfree rats. However, the liver fat of germfree rats was higher than that of conventionalized and OAR rats fed a low choline diet high in cholesterol (5%) and containing added cystine (0.4%), but lower in the diet without the added cholesterol and cystine. There were no differences in the liver fat of conventionalized and open-animal-room rats fed the various choline-deficient diets. Incidental findings in our experiments were a consistent significant elevation of the plasma total solids concentration and an occasional decrease in the hematocrits of rats with severe acute choline deficiency nephropathy.

These differences in response of germfree, conventionalized and OAR rats to acute choline deficiency are additional examples of the important influences of microorganisms on mammalian metabolism and nutrition. For example, germfree rats are less susceptible to ascorbic acid (21), vitamin E [13] and methionine [14] deficiencies, but more susceptible to vitamin K (22) and thiamine (23) deficiencies.

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[13] Unpublished observations, F. S. Doft and E. G. McDaniel.

[14] See footnote 13.

TABLE 8

Liver cholesterol in open-animal-room rats,[1] conventionalized rats [2] and germfree rats [3] subjected to choline deficiency

Diet		Supplement	Open-animal-room rats			Conventionalized rats			Germfree rats		
			Liver			Liver			Liver		
			Fat	Cholesterol		Fat	Cholesterol		Fat	Cholesterol	
				Total			Total			Total	
			1	2	3	4	5	6	7	8	9
			%	%	*mg*	%	%	*mg*	%	%	*mg*
A	a	Water (22) [4]	19 ± 1 [5]	2.1 ± 0.2	80 ± 11	—	—	—	—	—	—
	b	Neomycin [6] (8)	19 ± 2	2.0 ± 0.2	115 ± 13	—	—	—	—	—	—
	c	Choline [6] (17)	4 ± 0	0.6 ± 0.0	22 ± 6	—	—	—	—	—	—
A + 5% cholesterol or											
B	d	Chlortetracycline [6] (31)	26 ± 2	3.7 ± 0.3	140 ± 21	—	—	—	—	—	—
	e	Neomycin (18)	19 ± 2	3.2 ± 0.3	110 ± 15	—	—	—	33 ± 2(5)	4.7 ± 0.3	196 ± 27
	f	Water (72)	20 ± 2	2.3 ± 0.1	88 ± 12	26 ± 2(58)	3.8 ± 0.2	170 ± 24	36 ± 2(60)	4.5 ± 0.2	292 ± 36
	g	Choline (53)	12 ± 2	2.4 ± 0.2	96 ± 11	13 ± 1(20)	3.8 ± 0.2	152 ± 17	16 ± 2(25)	4.7 ± 0.3	244 ± 35

[1] Open-animal-room rats (Fischer strain), male (weanling) 21 days old at start of experiment.

[2] Conventionalized rats (Fischer strain), male (weanling) 21 to 23 days old at start of experiment; these rats were littermates of the germfree rats, contaminated with cecal contents of open-animal-room rats at start of experiment.

[3] Germfree rats (Fischer strain), male (weanling), 21 to 23 days old at start of experiment; these rats were free of viable bacteria as determined by methods described elsewhere (8).

[4] Numbers in parentheses indicate number of livers assayed; included are rats dying from acute choline deficiency and killed rats.

[5] Mean ± SE; standard errors of zero indicated a standard error less than 0.5 but greater than 0.1.

[6] Neomycin sulfate, 1.09 mg/ml in drinking water; choline chloride, 1.5 mg/ml in drinking water; chlortetracycline (Aureomycin·HCl), 0.2 mg/ml in drinking water.

Statistical analyses: (numerals preceding letters refer to numbered columns in table; letters refer to group.)

1a vs. 1b, P not significant
1a or 1b vs. 1c, $P < 0.001$
2a vs. 2b, P not significant
2a or 2b vs. 2c, $P < 0.001$
3a vs. 3b, P not significant
3a or 3b vs. 3C, $P < 0.001$
3d vs. 3f or 3g, $P < 0.05$
4f vs. 4g, $P < 0.001$
5f vs. 5g, P not significant
6f vs. 6g, P not significant
7e vs. 7f, P not significant
7e or 7f vs. 7g, $P < 0.001$
1d vs. 1e vs. 1f, P not significant
1d or 1e or 1f vs. 1g, $P < 0.001$
2d vs. 2e, P not significant
2f vs. 2g, P not significant
2d or 2e vs. 2f or 2g, $P < 0.02$
3d vs. 3e, P not significant
3f vs. 3g, P not significant
8e vs. 8f vs. 8g, P not significant
9e vs. 9f vs. 9g, P not significant
1f vs. 4f, P not significant
1f or 4f vs. 7e, or 7f, $P < 0.001$
1g vs. 4g vs. 7g, P not significant
2f vs. 5f vs. 8f, $P < 0.02$
2e vs. 8e, $P < 0.02$
2g vs. 5g vs. 8g, $P < 0.02$
3f vs. 6f vs. 9f, $P < 0.02$
3e vs. 9e, $P < 0.02$
3g vs. 6g vs. 9g, $P < 0.02$

The importance of choline in lipid metabolism and the prevention of fat accumulation in the liver has been recognized for 35 years (24). Most of the liver fat accumulated in choline deficiency is as triglycerides (25–28), and interference with the transport of triglycerides from the liver appears to be the main defect. The type of dietary fat, protein and carbohydrate in large measure determines the pattern of neutral fats accumulated in the liver in choline deficiency (3, 24, 26, 29–37). However, just which pathways fail, for example, the phosphatidylethanolamine (38, 39) or the cytidine diphosphocholine (40, 41), is unsettled.

Ashworth et al. (25) reported that the addition of 2% cholesterol to a choline-deficient diet enhanced the accumulation of liver triglycerides and led to a tenfold increase in liver cholesterol. Several other investigators have reported on the antilipotropic properties of cholesterol in choline deficiency and during choline supplementation (37, 42–46). As in the experiments of Ashworth (25) and Chaplin and Mulford (37), we found that a high cholesterol diet caused increases in liver fat whether choline was given or not, but the liver fat was higher in the choline-deficient rats. We also found that choline deficiency in rats fed a low cholesterol diet produced increases in the concentration and amount of liver cholesterol, but proportionately less than the increase in liver fat. Neomycin and vitamin B_{12} did not alter the liver cholesterol of OAR rats fed this type of diet. The rise in liver cholesterol of such choline-deficient rats has been considered a result in part of impaired mobilization of cholesterol from the liver since cholesterol is a precursor of bile salts (47), and choline-deficient rats produce significantly less bile than normal controls (48). When 5% cholesterol (with or without 0.4% cystine) was added to the diet, we found that the concentration and amount of liver cholesterol increased to the same levels whether choline was given or not, but the rise in liver fat was substantially greater in the choline-deficient rats. The germfree rats behaved in the same general way, but had a greater concentration and amount of liver cholesterol than OAR rats, confirming the findings of Wostmann and Weich (49). The conventionalized rats had liver cholesterol levels which were in between those of the germfree and OAR rats. Neomycin and chlortetracycline given to OAR rats fed the 5% cholesterol, 0.4% cystine diet, without choline, caused further increases in concentration and content of liver cholesterol so that these increases were then proportional to the increases in liver fat and approached the values for cholesterol of the conventionalized rats. The mechanisms underlying the effects of cholesterol diets on liver cholesterol have not been elucidated. The relationship between liver cholesterol and the gut flora was discussed in an earlier publication by Gustafsson and Norman (50).

Two studies have been reported on the increase in renal disease produced by adding cholesterol to a choline-deficient diet, Griffith and Mulford (51) with 0.5% cholesterol in the diet and Chaplin and Mulford (37) with 4.3% cholesterol in the diet. We found also that 5% cholesterol in the diet increased markedly the severity of the choline deficiency. One reason suggested by Chaplin and Mulford (37) for cholesterol enhancing the acute choline deficiency was that cholesterol causes a greater need for amino alcohols which are believed necessary for the formation of phosphatidylethanolamine and maintenance of the cholesterol-to-phospholipid ratio, which requires transmethylation (52). Another possibility which appears likely to us is that the cholesterol effect is mediated in part through its effects on growth. Rats fed small quantities of food during the period of acute choline deficiency show lower levels of liver fat and less nephropathy than rats allowed to eat ad libitum (42, 53). Hoffbauer and Zaki (54), using slow-growing primates, also showed that the requirement for choline is related to the rate of growth. In our experiments, rats fed the high cholesterol diet with choline supplementation grew faster than rats not fed cholesterol. Ashworth also reported an increase in growth rate for rats fed a 2% cholesterol choline-deficient diet (25).

The relationship between growth rate and the severity of choline deficiency has also been considered with respect to the

enhancing effect of cystine when added to choline-deficient diets (42, 51, 55). Mulford and Griffith (55) showed that the addition of 0.1 to 0.5 g cystine/100 g diet to 18% casein choline-deficient diets caused better growth, higher levels of liver fat and a higher incidence of bilateral renal cortical hemorrhagic necrosis. They felt the growth-promoting activity of cystine led to an increase in choline requirement. Kwong and Barnes (56), however, have shown that cystine enhances the severity of acute choline deficiency even in a situation where cystine does not affect growth. In their view, the cystine effect is due to its interfering with the endogenous synthesis of choline from methionine. The evidence they present for this is particularly dramatic for rats fed choline-deficient diets when coprophagy is prevented. We observed that germfree rats fed the choline-deficient diet without added cholesterol and cystine for 10 days had significantly less liver fat and nephropathy than their OAR counterparts, a reflection of the lesser susceptibility of the germfree rats to the nutritional choline deficiency. When 5 g cholesterol and 0.4 g cystine were added to each 100 g of the choline-deficient diet, the nephropathy was again less in the germfree rats but the levels of liver fat were higher than those of the conventionalized or OAR rats. At first glance it appears that there is a dichotomy in the responses of the germfree rats to this choline-deficient diet. We do not think so. We believe the higher liver fat of the germfree rats in this case does not reflect a greater choline deficiency, but rather that the nephropathic effects of the choline deficiency were more severe in the conventionalized rats, resulting in a decreased food intake and consequent less accumulation of liver fat. The influence of food intake on liver fat accumulation in choline deficiency has been discussed previously.

The renal lesions of acute choline deficiency are characterized by an increase in kidney weight and size due to hemorrhage (57, 58) and cortical necrosis. The renal damage grossly, histologically, as determined by the rise of the BUN, and by death, was considerably less in germfree rats and, when found at the time the rats were killed at 10 to 12 days, was usually in the healing stage. Among the possibilities for the lesser nephropathy in germfree rats are the following. (a) Many of the gastrointestinal microflora present in OAR and conventionalized rats metabolize choline to trimethylamine (59) a non-metabolizable compound which is probably not lipotropic. Since the diet as fed contains minimal amounts of choline, more would be available to the germfree rats. (b) Similarly, there is a greater requirement for methionine by OAR and conventionalized rats than by germfree rats because of the utilization of methionine by the intestinal bacteria.[15] (c) The livers of germfree rats on a choline-deficient diet synthesize more choline than the livers of conventionalized or OAR rats.[16] Kwong and Barnes [17] have shown an increased hepatic synthesis of choline from methionine when OAR rats are prevented from practicing coprophagy. (d) The less severe renal lesions of the germfree rats in choline deficiency may be due to their lower metabolic rate. We feel that this is a major factor. Levenson et al. (60) have shown that the oxygen consumption and carbon dioxide production by germfree rats is about 15 to 20% lower than by their conventionalized littermates. In this respect, Baxter (61) showed that thiouracil (2–3 μg/g diet) prevented the renal lesions of choline deficiency. He presented no data on the metabolic rates of these rats, but their thyroids were enlarged and their growth rates were slowed. The results could be partially reversed by feeding desiccated thyroid, but whether this was statistically significant is unclear.

The observation of Barnes and Kwong (20) that prevention of coprophagy minimizes the effects of a choline-deficient diet fed to weanling rats may also have bearing on the lessened acute choline deficiency nephropathy of germfree rats. Barnes and Kwong (20) reported that the rate of oxidation of methionine was greatly enhanced when coprophagy was prevented. This observation was interpreted to mean that transmethylation of methionine with consequent choline biosynthesis was in-

[15] See footnote 13.
[16] Unpublished data, E. Kwong, G. Fiala, R. H. Barnes, D. Kan and S. M. Levenson.
[17] See footnote 11.

creased under this condition. They substantiated this conclusion by finding that the fatty liver, nephropathy and lethality of acute choline deficiency are reduced by preventing OAR rats from practicing coprophagy. Furthermore, using ^{14}C methyl-labeled methionine injected into the portal vein, they found an increased specific activity of choline isolated from the livers of such rats (20). They believe that these effects of preventing coprophagy are a consequence of alteration in the gastrointestinal microbial flora because of decreased inoculation of the upper intestinal tract by certain fecal microorganisms.

The effects of prevention of coprophagy on the numbers of intestinal bacteria are controversial; Gustafsson and Fitzgerald (62) reported a marked decrease in the number of lactobacilli and an increase in coliforms and streptococci during the prevention of coprophagy, whereas Lev and his colleagues (63, 64) reported no significant differences. Chen et al.[18] have recently reported that the trimethylamine excretion following injection of choline by rats prevented from practicing coprophagy by tail cupping is considerably less than in rats permitted to practice coprophagy. They interpret this to mean that the intestinal bacterial flora must have been altered by the prevention of coprophagy.

Our experiments show no effect of preventing coprophagy on the development of acute choline deficiency by rats fed a diet low in choline but containing 5% cholesterol and 0.4% cystine. Barnes and Kwong repeated their coprophagy prevention experiments using a high cholesterol, choline-deficient diet and obtained the same results as we did.[19] It appears that preventing coprophagy influences favorably a mild choline deficiency, but not a very severe deficiency.

Baxter and Campbell (9) showed that neither chloromycetin, terramycin nor streptomycin were effective in protecting rats from acute choline deficiency. Other antibiotics, penicillin and sulfonamides, were tried by Salmon and Newberne (14) with little success. In the present experiments, we found that neomycin did not influence growth, the early liver fat accumulation, the renal disease or death from acute choline deficiency in germfree, conventionalized or OAR rats.

Baxter and Campbell (9) reported that chlortetracycline significantly lessened the acute renal damage and possibly the liver fat of rats fed choline-deficient diets. These experiments have been substantiated in part by our studies, although the extent of protection (as judged by the nephropathy and death) we found was far less striking than that reported by Baxter. Further, we found that liver fat increased in the chlortetracycline-supplemented rats, whereas Baxter reported a decrease in the liver fat of such rats; these differences may be related to the fact that our diet contained 5% cholesterol and 0.4% cystine and increased the choline requirement.

If the mechanism of chlortetracycline protection is antibacterial (and this is supported by the observations of Baxter that inactivation of chlortetracycline as an antibiotic by heat rendered it ineffective in choline deficiency (9)), there must be significant differences in the effects of chlortetracycline and the other antibiotics used on the intestinal flora of the rat. This would not be unexpected but there are few data about this (11, 65, 66). Weanling rats fed choline-deficient diets have a marked reduction in the fecal content of *Escherichia coli* during the first week of supplementation with chlortetracycline but not with penicillin (11). There are, however, two other studies showing that the *E. coli* content of the feces of rats fed normal diets is increased by both chlortetracycline and penicillin (65, 66) during the first week. These differences in bacterial response may be related to the diets used, and age and strain of the rats. There are no experiments reported which detail the effects of neomycin on the microflora of the OAR rat intestine. However, we observed that in dicontaminated rats fed normal diets, the *E. coli* and *Bacillus proteus* content of the gut falls quickly from 10^7 to 10^5 viable bacteria/g feces when neomycin is fed (60).

Baxter reported (9) that feces of chlortetracycline-treated animals collected every

[18] Chen, R., E. Kwong and R. H. Barnes 1967 Urinary trimethylamine following choline administration in the study of intestinal microflora of the rat. Federation Proc., 26: 526 (abstract).

[19] See footnote 11.

2 to 3 days during the 10 days they were fed a choline-deficient diet contained more choline (0.3 to 1.5 mg) than the feces of the choline-deficient rats not receiving chlortetracycline. This is consistent with the view that the flora of the gut are modified to lessen the conversion of choline to trimethylamine. DuVigneaud et al. (67, 68), however, reported that the total reineckate precipitate obtained from a 42-day feces collection of 2 ex-germfree rats fed a choline-deficient diet was less than 1 mg, whereas none was recovered from a 32-day feces collection of 2 germfree rats fed the same diet (67, 68). In addition, DuVigneaud et al. (67, 68) subjected adult germfree rats and ex-germfree rats reared under non-sterile conditions and drinking water containing D_2O to a choline-deficient diet for 2 to 3 weeks and assayed the rat tissues for labeled choline and creatine. They found that the levels of deuterium incorporated into the choline and creatine were slightly higher in the non-sterile rats than in germfree but indicated that the difference might not be significant. The authors (68) stated that "While it is possible that additional synthesis by intestinal bacteria of labile methyl groups may have taken place in the case of the non-sterile animals, it may be noted that the germfree animals were older and did not grow as much tissue during the experiment. Of ultimate importance, of course, is the fact that there was an enrichment of deuterium in the methyl groups of choline and creatine in the germfree animals."

Other investigators have shown (69, 70) that chlortetracycline, terramycin, and penicillin decrease the urinary excretion of trimethylamine by normal human subjects and patients without liver or intestinal disease. This decrease occurred very soon after antibiotic treatment and lasted as long as 2 weeks while the antibiotics were being given; subsequently the levels of trimethylamine in the urine started to rise despite the continued administration of the antibiotics.

While neomycin is ineffective in acute choline deficiency, its effects in the prevention of liver cirrhosis in certain strains of rats undergoing chronic choline deficiency are well-documented (12). Broitman et al. (10) have postulated that the beneficial effects of neomycin in preventing liver cirrhosis reflects a reduction of "normal endotoxemia," presumably by reducing the number of gram-negative intestinal bacteria. This was based on their findings that rats treated with neomycin during chronic choline deficiency develop liver cirrhosis much more slowly than controls. However, when given endotoxin orally (daily), the neomycin-treated rats developed cirrhosis to the same extent and within the same time as the controls. This appears to be in contradiction to the observation of Levenson et al. (8) that germfree Lobund rats develop cirrhosis earlier than OAR rats. It is unlikely that the germfree rats have more bacterial endotoxin in their intestinal tract than the neomycin-treated OAR rats. Furthermore, it has been well-documented that the germfree rat is somewhat more resistant to the acute lethal effects of injected endotoxin than OAR rats. One of us (S.M.L.) has postulated that the beneficial effects of neomycin in the prevention of chronic choline deficiency liver cirrhosis is due to an alteration in the intestinal bacterial flora and the subsequent increase in production of a protective nutrient or factor such as vitamin B_{12} by the surviving bacteria. We think that the lack of protection of neomycin in acute choline deficiency results from the time required for the establishment of the bacterial flora to produce this nutrient or factor being too long for the effects to be evident in 7 to 12 days. This theory also explains the increased susceptibility of germfree rats to chronic choline deficiency since some of these "beneficial" bacteria are present in the OAR rats and are producing this hypothetical factor, whereas in the germfree rats there are no viable bacteria, and thus no production of such a factor. This theory would also explain the lack of beneficial effects of neomycin in chronic choline deficiency of germfree animals.

Baxter (9) found that vitamin B_{12} reduced the incidence and severity of renal damage in acute choline deficiency. This has been confirmed by us and by others (53, 71). Since chlortetracycline has been shown to increase the vitamin B_{12} activity of the feces promptly (72) and to decrease the mortality of animals fed vitamin B_{12}-

deficient diets (73), Baxter postulated that the chlortetracycline effect in acute choline deficiency might be mediated in this way. However, we think it unlikely that this effect would come about quickly enough and suggest that the chlortetracycline effect is due to its influence on the conversion of choline to trimethylamine and the utilization of methionine by bacteria.

Wilson et al (74), using a regular commercial rat ration, found that formate and formaldehyde served as precursors of choline. Arnstein and Newberger (75), using rats fed a vitamin B_{12}-choline-deficient diet and Lust and Daniel (76), using a vitamin B_{12}-requiring protozoan found that vitamin B_{12} stimulated the synthesis of choline as measured by a greater incorporation of ^{14}C from labeled glycine, serine and formate (75) and from labeled formate and formaldehyde, but not from methionine (76), respectively. DuVigneaud et al. (67, 68) had found that the addition of 1.5 mg vitamin B_{12} to a choline-deficient diet of exgermfree rats did not increase the synthesis of methyl donor compounds. However, the diet used by them contained 10 μg vitamin B_{12}/100 g which may have been sufficient to prevent further stimulation by additional amounts of vitamin B_{12}.

In our experiments, vitamin B_{12} reduced the renal disease and to a lesser extent the liver fat accumulation resulting from acute choline deficiency in OAR rats but not in germfree rats. The lesser beneficial effect of vitamin B_{12} on the liver fat accumulation of acutely choline-deficient OAR rats than on the nephropathy is due, we think, to the likelihood that the production of methyl donor stimulated by vitamin B_{12} is limited and although enough is synthesized to prevent the acute renal damage of choline deficiency, not enough is made to prevent the accumulation of liver fat. Similar results were found in our experiments with chlortetracycline, in which the renal pathology and lethality of choline deficiency were prevented but not the increase in liver fat. In this respect, Griffith and Wade (77) found that weanling rats required 10 mg choline/day to protect them from liver damage but only 2 mg/day to protect them from renal damage.

If increased synthesis of methyl donors under the influence of vitamin B_{12} is related in part to the microflora, this would explain the lack of effect of vitamin B_{12} on acute choline deficiency in germfree rats. In other experiments Levenson and his coworkers [20] had found that vitamin B_{12} prevented cirrhosis in germfree rats. This may be a matter of the degree of labile methyl formation induced by vitamin B_{12}, less perhaps being required to prevent cirrhosis than the early accumulation of liver fat.

A question of prime importance is why choline deficiency causes renal damage. We have discussed this in detail elsewhere (78) and concluded that the nephropathy which occurs in young rats fed a choline-deficient diet reflects an abrupt and sustained fall in acetyl choline, which in turn alters the reactivity of the renal vasculature to vasopressor agents leading to vasospasm, ischemia, renal damage and hemorrhage. This was based on our finding in collaboration with Dr. S. Baez,[21] Albert Einstein College of Medicine, that the capillary flow of the mesoappendix of the acute choline-deficient rat was slower than normal and that there were fewer open capillaries. These differences were noted before there were any elevations of BUN or any histologic renal damage. We also noted that the precapillary arterioles and the precapillary sphincters of the mesoappendices of the choline-deficient rats were less responsive to topical epinephrine than the small (120–150 mμ) arteries, a reaction which is the reverse of the normal state. Vasomotor activity in the mesoappendix was increased in the choline-deficient rats. These changes are consistent with a deficiency of acetyl choline. We also noted a lessening of peristalsis of the choline-deficient rats, a finding also consistent with decreases in acetyl choline, but no direct measurements of peristalsis were made.

Coincident with these microcirculatory studies we performed several experiments in collaboration with Dr. W. Dettbarn, College of Physicians and Surgeons, Columbia University, assaying the level of acetyl choline in brain, intestine and kidneys of choline-deficient and normal OAR rats (78).

[20] Unpublished data, S. M. Levenson, F. S. Doft, L. Ashburn and E. F. Geever.

[21] Nagler, A. L., S. M. Levenson and S. Baez 1968 Microcirculation in choline deficiency. Federation Proc., 27: 1522 (abstract).

We have found marked decreases in the level of acetyl choline in the choline-deficient OAR rats. This decrease ranged from 70% in the kidneys to 30% in the brain and intestine. The cholinesterase levels in the same tissues were not increased.

We believe that the nephropathy which occurs in young rats fed a choline-deficient diet reflects an abrupt and sustained fall in kidney acetyl choline, which in turn increases the reactivity of the renal vasculature to vasopressors leading to vasospasm, ischemia, renal damage and hemorrhage. In this respect, Baez and Gordon [22] have noted that the mesoappendix vessels of the germfree rat are hyporesponsive to epinephrine. This, in addition to the aforementioned metabolic differences between germfree, conventionalized and OAR rats (metabolic rates, choline and methionine metabolism, and possible liver choline synthesis) affords adequate explanation for the markedly lessened response of germfree rats to acute choline deficiency.

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Effect of Alterations in Protein Intake on Liver Xanthine Dehydrogenase in the Chick [1,2]

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ABSTRACT Studies were conducted to investigate the influence of alterations in protein intake on body weight, liver weight, liver nitrogen and liver xanthine dehydrogenase (XDH) activity of chicks. After a 10-day period in which all chicks were fed a diet containing 25% isolated soybean protein, one-half of the chicks were changed to a diet containing 75% isolated soybean protein and one-half were continued with the 25% diet. The results of these studies showed no significant difference in body or liver weights at 20 days between birds fed the 25 and 75% isolated soybean protein diets. Fasting for 24 hours resulted in a similar decrease in body and liver weights for birds fed the two protein levels, but the decrease in liver N was greater for birds fed the high protein diet. Feeding a protein-free diet for 24 hours resulted in larger livers and lower total liver N as compared with control values. Regardless of the basis for expressing enzyme activity, liver XDH was significantly higher for birds fed the high protein diet ad libitum as compared with birds fed the 25% isolated soybean protein diet. Liver XDH levels of chicks that had been fed the high protein diet ad libitum and subsequently fed a diet devoid of protein, or fasted, for 24 hours were also significantly higher than those observed in chicks from similar treatments previously fed the 25% isolated soybean protein diet. In most cases, a 24-hour starvation period did not significantly lower total liver XDH as compared with ad libitum controls at each protein level, whereas feeding a protein-free diet for 24 hours resulted in significantly lower liver XDH levels.

Considerable attention has been given to the manner whereby animals adapt to dietary changes. Alterations in feed consumption, changes in organ size or composition, and changes in selected enzymes or enzyme systems are several responses observed in animals subjected to diverse dietary conditions. It appears that such responses arising through experimentally controlled changes in the environment represent an attempt to maintain homeostasis (1). Of the many reports concerned with metabolic adaptations in mammals, variations in dietary protein level have received considerable emphasis. Work with rats demonstrated that feeding high levels of protein resulted in increased levels of all the urea cycle enzymes compared with those observed when diets containing lower amounts of protein were fed (2–4). In addition, numerous other enzymes associated with amino acid metabolism per se are subject to extensive variation depending on the type and quantity of protein in the diet. Harper (1) has presented a review of this subject on work conducted with rats. Similar comprehensive studies with chicks subjected to wide alterations in dietary protein, however, are limited.

In the avian species the primary nitrogenous excretory product resulting from protein and amino acid catabolism is uric acid. The terminal reactions involved in the biosynthesis of this purine (hypoxanthine → xanthine → uric acid) are catalyzed by a NAD-dependent xanthine dehydrogenase. Richert and Westerfeld (5) were the first to demonstrate that xanthine dehydrogenase is not autoxidizable in avian tissue and in this respect differs from the more common xanthine oxidase of mammalian tissue. There are reports concerned with the embryonic and post-hatching development patterns and kinetic properties of xanthine dehydrogenase (6–10), as well as aspects of specific variations in dietary protein on xanthine dehydrogenase activity (11–16). The research presented in the present report was de-

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signed to investigate the responses of avian liver xanthine dehydrogenase to extreme alterations in protein intake. Liver xanthine dehydrogenase activities were studied in chicks fed normal or excessive levels of dietary protein and were compared with xanthine dehydrogenase levels of chicks that had been fed a normal or high protein diet ad libitum and subsequently fed a diet devoid of protein, or fasted, for a 24-hour period.

EXPERIMENTAL

The one-day-old White Rock cockerels used in each of the studies reported were initially fed a diet containing 25% isolated soybean protein (table 1) for the first 10 days. At this time, one-half of the chicks were transferred to a diet containing 75% isolated soybean protein and one-half were continued with the 25% diet. The protein content of the 25 and 75% isolated soy protein diets was 21.0 and 63.9%, respectively. At 20 days of age, groups of chicks from both protein levels were selected at random and divided into two additional subclasses: 1) starved for 24 hours, hereafter referred to as the 25% or 75% starved group; or 2) fed a diet devoid of protein for 24 hours, hereafter referred to as the 25% or 75% protein-free group. All chicks were killed at 21 days of age for liver xanthine dehydrogenase assays. Deionized water was provided ad libitum to all chicks.

TABLE 1

Composition of 25% isolated soy protein diet [1,2]

	g/kg
Isolated soy protein	250.0
Glucose monohydrate	587.39
Soybean oil	50.0
Cellulose	30.0
Mineral premix [3]	60.48
Vitamin premix [4]	15.0
DL-Methionine	5.0
Choline chloride (70%)	2.0
Butylated hydroxytoluene	0.13

[1] The 75% isolated soy protein diet contained 3 times the levels of isolated soy protein and DL-methionine used in the 25% isolated soy protein diet added at the expense of glucose monohydrate.

[2] The protein-free diet was identical to the 25% isolated soy protein diet with the exception that the isolated soy protein and DL-methionine were replaced with glucose monohydrate.

[3] The mineral premix provided the following reagent minerals in g/kg of diet: $CaCO_3$, 19.1; $Ca(H_2PO_4)_2 \cdot 2H_2O$, 21.15; K_2HPO_4, 11.2; NaCl, 6.0; $MgCO_3$, 2.08; $FeSO_4$, 0.2; $ZnCO_3$, 0.18; $CuSO_4 \cdot 5H_2O$, 0.015; $MnSO_4 \cdot H_2O$, 0.51; KI, 0.04; and $NaMoO_4 \cdot 2H_2O$, 0.0025.

[4] The vitamin premix provided the following units of vitamins per kg of diet: (in milligrams) thiamine·HCl, 6.0; riboflavin, 9.0; niacin, 50.0; Ca D-pantothenate, 20.0; pyridoxine·HCl, 8.0; biotin, 0.3; folic acid, 2.0; menadione sodium bisulfite, 2.0; inositol, 1000.0; and vitamin B_{12}, 20 μg; vitamin A palmitate, 25,000 USP units; vitamin D_3, 1200 ICU; *d*-α-tocopheryl acetate, 17.6 IU.

One procedure used to measure xanthine dehydrogenase activity in these studies generally follows the colorimetric method of Schwartz and Litwack (12) as later modified by Stirpe and Corte (15). Livers were removed from birds that had been decapitated and bled, and placed in cold 0.03 M Na-K phosphate buffer, pH 7.4. After blotting and weighing the livers, a portion was homogenized with 5 volumes of cold buffer in a Potter-Elvehjem homogenizer. The homogenate was then centrifuged for 20 minutes at 24,000 × *g* in a refrigerated centrifuge (0°). One milliliter of the clear supernatant was preincubated for 20 minutes at 41° in a water-bath shaker in 25-ml Erlenmeyer flasks containing 4.0 ml of buffer and 0.5 ml of 0.02 M methylene blue. After the preincubation period, 0.6 ml of 0.057 M xanthine (mono Na-salt) was added to the reaction mixture and 0.5 ml aliquots removed at zero and 10 minutes and immediately placed into centrifuge tubes containing 3.5 ml of a 40% Na-tungstate: 2 N H_2SO_4:H_2O solution (1:1:5, v/v). Final reaction mixture concentrations for methylene blue and xanthine were 1.63 mM and 5.61 mM, respectively. Xanthine remaining was determined colorimetrically as described by Litwack et al. (17). Control flasks containing all assay reagents with the exception of xanthine were assayed periodically and showed no endogenous xanthine dehydrogenase activity. The supernatant from the homogenized liver samples contained essentially all the xanthine dehydrogenase present since no activity was detected when an aliquot of resuspended particulate matter was assayed as described.

Where indicated, xanthine dehydrogenase activities were also assayed spectrophotometrically at 25° as the rate of formation of NADH at 340 mμ in 1-cm cells. In the final reaction mixture, 0.1 ml of suitably diluted enzyme preparation was suspended in 0.03 M Na-K phosphate buffer, pH 7.4, 0.38 mM xanthine and 0.816 mM NAD. As suggested by Stritt-

matter (6), KCN (0.20 mM) was added to prevent the reoxidation of NADH. The reaction was started by adding xanthine to both cells of a Beckman DB spectrophotometer, NAD being omitted from the reference cell.

Nitrogen was determined by the Kjeldahl method on duplicate liver samples.

Statistical procedures used for treating data obtained in these studies were performed by analysis of variance. Treatment means were tested for significant differences by the sequential method of Newman and Keuls (18).

RESULTS

The results of trial 1, which show the effects of alterations in protein intake on liver weight, liver nitrogen and liver xanthine dehydrogenase activity, are presented in table 2. Feeding either the 25% or 75% isolated soybean protein diets ad libitum did not result in significant differences in liver weights when expressed as total weight or liver weight per 100 g body weight in 3-week-old chicks. Liver nitrogen, however, expressed either on a per gram of liver or total liver basis was significantly greater ($P < 0.05$) for birds fed the high protein diet. Compared with the ad libitum groups within each protein level, fasting for 24 hours resulted in a significant reduction ($P < 0.05$) in liver weight and total liver nitrogen content. Although the livers from chicks fed the high protein diet contained significantly more nitrogen ($P < 0.05$) than those from chicks fed the 25% isolated soybean protein diet, similar values were observed in the 2 groups after fasting for 24 hours. These results indicate that a greater loss in liver protein occurred during starvation in birds previously fed the high protein diet. In contrast with the reduction in liver weight of the starved birds, feeding a protein-free diet for 24 hours resulted in increased liver weights when compared with the ad libitum groups. These results were not statistically different ($P > 0.05$) when expressed on a 100 g body weight basis. As compared with birds that had been starved for 24 hours, total liver nitrogen was depleted to a slightly greater extent in birds fed the protein-free diet.

Regardless of the basis for expressing xanthine dehydrogenase activity, birds fed the high protein diet ad libitum showed significantly higher enzyme levels when compared with birds fed the 25% isolated soybean protein diet (table 2). In these studies with chicks, a 24-hour starvation period has been observed to result in an 8% to 10% reduction in body weight and a 25% to 30% reduction in liver weight (compared with ad libitum controls). Birds fed a diet devoid of protein either maintain their weight or continue to gain in weight during this period. For these reasons, "total" enzyme activity (units per 100 g body weight) is expressed on a pre-starva-

TABLE 2

Effect of alterations in protein intake on liver weight, liver N and liver xanthine dehydrogenase activity (trial 1) [1]

	25% isolated soybean protein			75% isolated soybean protein		
	Ad libitum	Starved	Protein-free	Ad libitum	Starved	Protein-free
Liver wt,[2] g	12.6 [a]	9.7 [b]	14.0 [c]	13.3 [ac]	9.5 [b]	15.3 [d]
Liver wt [2]/100 g body wt, g	3.5 [a]	2.7 [b]	3.8 [ac]	3.7 [ac]	2.8 [b]	4.0 [c]
N/g liver,[2] mg	28.7 [a]	31.8 [b]	21.3 [c]	33.2 [b]	34.2 [b]	20.7 [c]
Xanthine dehydrogenase activity [3]						
Units/g liver	29.1 [a]	38.0 [ab]	16.2 [c]	61.6 [d]	91.7 [e]	42.7 [b]
Units/100 mg liver N	100.8 [a]	119.0 [a]	76.1 [b]	185.9 [c]	268.9 [d]	205.4 [e]
Units/liver	365.0 [a]	369.5 [a]	226.9 [b]	819.1 [c]	864.4 [c]	642.9 [d]
Units/100 g body wt	103.0 [a]	93.1 [a]	61.7 [b]	229.3 [c]	230.3 [c]	167.4 [d]

[1] Data obtained from 21-day-old chicks fed their respective protein-containing diets ad libitum, starved the preceding 24 hours, or fed a protein-free diet the preceding 24 hours. Values represent means of 7 observations each. Means with the same lettered superscript in a horizontal row are not statistically different ($P > 0.05$).

[2] Fresh tissue weight.

[3] Units represent enzyme activity catalyzing the disappearance of 1 μmole of xanthine/10 minutes at 41°.

tion weight because a decrease in total enzyme activity could otherwise be masked by a concomitant decrease in body weight. Similarly, if total liver enzyme did not change during the starvation period, expressing enzyme activity on a basis of starved body weight would erronously show it to increase.

Compared with the ad libitum controls, xanthine dehydrogenase activity within a protein level is higher for the starved groups when expressed on a per gram of liver or per gram of liver nitrogen basis. Enzyme activities of the starved birds were not greatly different from the ad libitum groups when expressed on a total liver or 100 g body weight basis, thus indicating that xanthine dehydrogenase protein had been spared during the 24-hour starvation period. Xanthine dehydrogenase activities of the protein-free groups were significantly lower ($P < 0.05$) than the respective ad libitum groups when expressed as activity per gram of liver, per liver or per 100 g body weight. Compared with the ad libitum values, enzyme activity per gram of liver nitrogen was significantly reduced ($P < 0.05$) for the 25% protein-free group but remained unchanged for the 75% protein-free group. These results suggest that the total amount of xanthine dehydrogenase available to the chick significantly decreases when a diet devoid of protein is fed for 24 hours. This is in contrast with birds starved for 24 hours, in which case total xanthine dehydrogenase remained at the ad libitum level.

The results of a second trial using the same 6 treatments as previously described are shown in table 3. The effects of alterations in protein intake on liver weight and liver nitrogen are in close general agreement with those described for trial 1. The extent of nitrogen depletion following a 24-hour starvation period was greater for birds previously fed the high protein diet in trial 2 as compared with trial 1.

Similar to the results in trial 1, liver xanthine dehydrogenase activities were significantly higher ($P < 0.05$) for birds fed the high protein diet as compared with birds fed a normal level of protein, regardless of the basis for expression of enzyme activity. Xanthine dehydrogenase activity was higher for the 25% starved group as compared with control birds fed the 25% isolated soybean protein diet ad libitum when expressed on the basis of per gram of liver or per gram of liver nitrogen, but similar when expressed on a basis of per

TABLE 3

Effect of alterations in protein intake on liver weight, liver N and liver xanthine dehydrogenase activity (trial 2) [1]

	25% isolated soybean protein			75% isolated soybean protein		
	Ad libitum	Starved	Protein-free	Ad libitum	Starved	Protein-free
Liver wt,[2] g	12.4 [a]	9.1 [b]	13.3 [cd]	12.8 [ad]	8.7 [b]	13.2 [ac]
Liver wt [2]/100 g body wt, g	3.2 [ad]	2.7 [b]	4.0 [c]	3.6 [ac]	2.9 [bd]	4.0 [c]
N/g liver,[2] mg	22.7 [a]	26.2 [b]	16.7 [c]	29.5 [d]	27.8 [bd]	19.1 [e]
N/liver,[2] mg	278.7 [a]	238.8 [b]	221.5 [b]	372.8 [c]	240.4 [b]	251.2 [ab]
Xanthine dehydrogenase activity: [3]						
Units/g liver	18.2 [a]	24.8 [b]	8.6 [c]	49.2 [d]	49.8 [d]	23.2 [b]
Units/100 mg liver N	81.0 [a]	95.1 [a]	51.8 [b]	169.0 [c]	180.9 [c]	120.6 [d]
Units/liver	225.6 [a]	226.9 [a]	114.7 [b]	629.4 [c]	432.7 [d]	303.6 [a]
Units/100 g body wt	59.3 [a]	67.8 [ab]	38.1 [c]	175.4 [d]	144.4 [d]	90.9 [b]
Xanthine dehydrogenase activity: [4]						
Units/g liver	0.58 [ab]	0.80 [b]	0.31 [a]	2.07 [c]	2.16 [c]	0.78 [b]
Units/100 mg liver N	2.56 [a]	3.06 [a]	1.65 [b]	7.11 [c]	7.74 [c]	4.05 [d]
Units/liver	7.16 [a]	7.35 [a]	4.17 [b]	26.37 [c]	18.45 [d]	10.19 [a]
Units/100 g body wt	1.84 [a]	2.18 [ab]	1.20 [a]	7.39 [c]	6.29 [c]	3.10 [b]

[1,2] See table 2, footnotes 1 and 2.

[3] Units represent enzyme activity catalyzing the disappearance of 1 μmole of xanthine/10 minutes at 41°, colorimetric assay of Schwartz and Litwack (12) as modified by Stirpe and Corte (15).

[4] Units represent enzyme activity catalyzing the reduction of 1 μmole of NAD/minute at 25° (spectrophotometric method of assay).

liver or per 100 g body weight. Liver xanthine dehydrogenase activity for the 75% starved birds responded differently in trial 2 than observed in trial 1. Enzyme activity was not changed when expressed on a per gram of liver basis and was significantly lower ($P < 0.05$) on the basis of total fresh liver weight. Expressing xanthine dehydrogenase activity per 100 g body weight, however, did not result in significant differences as compared with control levels. As observed in trial 1, feeding a protein-free diet for 24 hours resulted in a significant reduction in liver xanthine dehydrogenase activity compared with ad libitum control values.

A comparison of the spectrophotometric method with the colorimetric method as assays of xanthine dehydrogenase activity is also presented in table 3. Although absolute values for xanthine dehydrogenase activity obtained by these 2 methods cannot be compared because of differences in assay conditions, relative comparisons show close agreement. Because a continuous reaction rate is recorded with the spectrophotometric assay, this method is subject to less error than the colorimetric assay for xanthine disappearance. Omitting NAD, xanthine, or enzyme preparation from the reaction mixture resulted in no measureable xanthine dehydrogenase activity.

DISCUSSION

The results of these studies have shown a two- to threefold elevation in xanthine dehydrogenase activity in chicks fed a diet containing 75% isolated soybean protein as compared with chicks fed a diet containing 25% isolated soybean protein. This response was observed regardless of whether enzyme activity was expressed on a "unit" basis (per gram of liver or per gram of liver nitrogen) or as "total" activity (per liver or per 100 g body weight).

The manner chosen to express enzyme activity following a 24-hour starvation period could be misleading because body weight, liver weight, as well as liver composition were changed considerably during this time. In these studies, chick weight was reduced 8% to 10% and liver weight was reduced 25% to 30% following a 24-hour starvation period. Total liver N depletion paralleled the loss in liver weight for birds previously fed the high protein diet (25% in trial 1; 35% in trial 2) but was lower for birds previously fed the 25% isolated soybean protein diet (approximately 14% in both studies). In each study reported, xanthine dehydrogenase activity per gram of liver and per gram of liver nitrogen increased for the starved birds previously fed a normal level (25% isolated soybean protein) of protein. Expressing enzyme activity on a "total" basis resulted in similar values for these birds when compared with ad libitum controls, thus indicating that xanthine dehydrogenase had been spared during the 24-hour starvation period. Similar results were observed for the starved birds previously fed the high protein diet in trial 1, but in trial 2, xanthine dehydrogenase remained at the ad libitum level when expressed on a unit basis and decreased when expressed as "total" activity. An explanation for this difference in response between the 2 trials may be based on greater nitrogen depletion for the starved birds previously fed the high protein diet in trial 2. It is conceivable that some xanthine dehydrogenase protein was lost during this 24-hour starvation period.

As shown in these studies, the feeding of a protein-free diet for 24 hours resulted in increased liver weight, a decrease in total liver nitrogen similar to that observed during a 24-hour starvation period, and a significant reduction in liver xanthine dehydrogenase activity as compared with ad libitum controls. Although liver nitrogen expressed on the basis of per gram of liver was markedly higher for the starved birds as compared with birds fed the protein-free diet for 24 hours, the similar extent of total nitrogen depletion in both groups was unexpected in view of results of studies conducted with rats (19). Schimke (19) presented evidence for a lower degree of total protein depletion when rats were fed a protein-free diet, as compared with rats starved 4 or 7 days. The similar extent of total nitrogen depletion in chicks starved or fed a protein-free diet for 24 hours has been observed in subsequent studies conducted in this laboratory. No explanation can be offered for the apparent differences in response between the chick and the rat.

The elevated activities of liver xanthine dehydrogenase observed in the present studies with chicks fed diets high in protein closely resemble the changes observed in the urea cycle enzymes of rats under similar dietary conditions (2, 3). The question concerning whether the increased enzyme "activity" as measured in vitro represents the formation of additional enzyme or, alternatively, increased activity of the enzyme already present has been investigated by Stirpe and Corte (15) and Corte and Stirpe (16). Their studies using puromycin and actinomycin D have shown a reduction in xanthine dehydrogenase "activity" in chicks that had previously been stimulated by the administration of inosine. Strittmatter (6) has reported on patterns of xanthine dehydrogenase development in the chick and noted a large reduction in enzyme activity when puromycin was administered. The elevated liver and kidney xanthine dehydrogenase activities observed with inosine stimulation or during the early stages of chick development appear to be the result of increased enzyme synthesis.

Remy and Westerfeld (11) studied the effects of dietary protein on xanthine dehydrogenase in selected tissues in the chick and reported higher enzyme activity in the liver and pancreas with increased levels of protein intake. Low protein diets depleted liver xanthine dehydrogenase but did not affect intestinal or kidney xanthine dehydrogenase. In another report Westerfeld et al. (14) noted a linear increase in liver xanthine dehydrogenase activity in chicks and turkey poults with increasing levels of dietary protein intake. Stirpe and Corte (15) reported that xanthine dehydrogenase activity expressed on the basis of milligrams of liver or milligrams of nitrogen increased during a 24-hour starvation period in chicks reared with normal levels of protein. Litwack and Fisher (13) fed diets deficient in one or more specific amino acids or a diet low in protein content to young chicks and found a marked depression in liver xanthine dehydrogenase activity.

The present studies are in general agreement with those reported earlier and have shown that total liver xanthine dehydrogenase of starved birds remained at ad libitum levels in both experiments in birds fed the 25% isolated soybean protein diet and in 1 of the 2 experiments in birds fed the high protein diet. In addition, the effects of feeding a diet devoid of protein on liver xanthine dehydrogenase activity are presented. Although liver nitrogen was shown to decrease to a similar extent in birds that were starved or fed a protein-free diet, xanthine dehydrogenase levels were significantly higher for the starved birds. This difference in xanthine dehydrogenase activity between the 2 groups appears to be related to dietary differences in meeting energy needs as well as amino acid requirements under these conditions. During a 24-hour starvation period there appears to be a greater need for directing labile tissue protein toward fulfilling energy requirements. Since amino nitrogen would be liberated from elevated amino acid catabolism during the starvation period, xanthine dehydrogenase protein is spared to maintain levels sufficient to promote the formation of uric acid. For birds fed the protein-free diet for 24 hours, there is a reduced dependence on gluconeogenesis and amino acid catabolism since adequate energy is provided by the protein-free diet. That the effects of feeding a protein-free diet, or fasting, for 24 hours results in opposing influences on gluconeogenic activity has also been shown by the results of studies of 2 important gluconeogenic enzymes, glucose 6-phosphatase and fructose 1,6-diphosphatase.[3] In particular, glucose 6-phosphatase activity (units/100 g body weight) was reduced approximately threefold in birds fed a protein-free diet for 24 hours as compared with birds that were fasted or fed their respective protein-containing diets ad libitum.

Results of studies recently conducted in this laboratory suggest that the elevated response of xanthine dehydrogenase to a high level of dietary protein, as measured in vitro, has significance in vivo.[4] A direct relationship between increased xanthine dehydrogenase activity and elevated levels of uric acid production was observed dur-

[3] Unpublished data.

[4] Featherston, W. R., and R. W. Scholz. Changes in liver xanthine dehydrogenase and uric acid excretion in chicks during adaptation to a high protein diet (manuscript in preparation).

ing a 10-day experimental period when chicks were fed a high protein diet. These results are interpreted as adaptive responses and under conditions of diverse protein intake appear to represent attempts to maintain homeostasis.

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Biochemical, Skeletal and Allometric Changes Due to Zinc Deficiency in the Baby Pig [1]

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ABSTRACT Comparisons of biological measures made on baby pigs weaned shortly after birth to zinc-deficient or zinc-adequate purified diets show that zinc deficiency in the baby pig results in a reduced rate and efficiency of body weight gain, a reduced rate of food intake, parakeratosis, an alteration in leukocyte differentiation, a reduction in the levels of serum zinc, calcium and alkaline phosphatase, tissue zinc and liver alcohol dehydrogenase, an alteration of serum protein electrophoretic patterns, a reduction in size and strength of bone and changes in organ allometrics. Paired-feeding studies indicated that these changes with the exceptions of bone size and strength and certain allometric values were due to zinc deficiency and not due to reduced food intake. Thymus weight was greatly reduced in zinc-deficient pigs. Growth rate diminished in zinc-deficient pigs before food intake was reduced. Incorporation of 125 ppm of copper into the diet did not alleviate zinc deficiency or significantly influence the value of any of the measures taken.

Extensive studies of zinc deficiency in post-weanling pigs have been made since Tucker and Salmon (1) demonstrated that parakeratosis described by Kernkamp and Ferin (2) was due to zinc deficiency. Deficiency symptoms observed include parakeratosis (1–27) and a reduction in growth rate (1, 3–27), appetite (1, 3–27), efficiency of food conversion (1, 3–27), serum or plasma and tissue zinc concentration (3, 6, 8, 20, 28), zinc balance (3, 17, 22), serum albumin (19, 29) and serum alkaline phosphatase activity (5, 19, 27, 30, 31). Gilts receiving suboptimal dietary levels of zinc had a reduction in growth rate during gestation (25, 30), number and size of offspring (25, 31), rate of growth of offspring (25, 28), serum and tissue zinc levels of offspring (31, 32) and milk zinc concentration (32, 33). Roberts et al. (30) and Hoekstra et al. (31) found that many of the offspring from gilts receiving a low zinc, high calcium diet had parakeratosis at 9 weeks of age and these pigs had a greatly reduced serum alkaline phosphatase level. Studies of zinc deficiency produced in normal pigs weaned shortly after birth have not been published.

The purposes of this study were to determine biological changes due to zinc deficiency occurring in the baby pig weaned shortly after birth and to investigate the influence of type of protein and level of copper in the diet upon the development of zinc deficiency in the baby pig. Preliminary reports of this work have appeared previously.[4,5,6]

MATERIALS AND METHODS

Forty Yorkshire-Hampshire baby pigs were used in 3 trials. In all the trials the baby pigs were taken from their dams at 3 days of age and reared in stainless steel cages with stainless steel wire-mesh bottoms. All pigs received the soy basal, medium copper diet shown in table 1 during an initial 4-day adjustment period. Animals were then assigned at random to experimental lots at one week of age, balancing for sex and initial weight. Conditions of

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TABLE 1

Composition of purified diets

	Soy [1]	Casein [2]
Purified protein	30	30
DL-Methionine	0.3	—
Lard	5	5
α-Cellulose [3]	5	5
Glucose monohydrate [4]	50.7	51
Mineral mixture	6	6
Fat-soluble vitamins in corn oil [5]	1	1
Water-soluble vitamins in water [5]	2	2

Mineral mixture	
	%
KCl	10.0
KI	0.002
$FeSO_4 \cdot 2H_2O$	0.7
$CoCO_3$	0.1
$MnSO_4 \cdot H_2O$	0.1
$ZnSO_4 \cdot H_2O$	0.0 (0.4) [6]
$CuSO_4$	0.1 (0.625) [7]
$MgCO_3$	2.0
$NaHCO_3$	25.0
$CaHPO_4 \cdot 2H_2O$	36.0
$CaCO_3$	12.5
Glucose monohydrate	13.5

[1] Soya Assay Protein, General Biochemicals, Chagrin Falls, Ohio.
[2] Vitamin-Free Casein, Nutritional Biochemicals Corporation, Cleveland.
[3] Solka Floc, Brown Company, Chicago.
[4] Cerelose, Corn Products Company, Argo, Illinois.
[5] Vitamins supplied at levels as in Miller et al. (47) plus ergocalciferol to provide 12.5 μg/kg diet.
[6] Basal and zinc-supplemented diets analyzing 12 and 100 ppm, respectively.
[7] Medium and high copper diets containing 25 and 150 ppm, respectively.

the physical environment were similar to those of previous studies (34).

In the first trial, 4 pigs received the soy basal diet containing 12 ppm of zinc and 4 pigs received the same diet supplemented with $ZnSO_4 \cdot H_2O$ to make a diet containing 100 ppm of zinc. Animals in both groups were individually fed ad libitum. Food intake was accurately recorded and weights of the pigs were taken weekly during a 4-week period. At the conclusion of the trial, blood samples were taken from the anterior vena cava of all pigs for cellular determinations and for serum analyses. A small portion of the blood was quickly heparinized and from this, erythrocyte, hemoglobin, hematocrit and leukocyte determinations were made as described by Ullrey et al. (35). The remainder of the blood was placed in acid-washed centrifuged tubes, allowed to clot, centrifuged at 2500 × *g* and the serum was withdrawn and placed in acid-washed vials. Determinations of levels of serum calcium, zinc and inorganic phosphorus (36) and serum alkaline phosphatase activity [7] were made.

Electrophoretic separation of serum proteins was accomplished on agar gel in a modified Durrum cell (37). Quantitation was with a Beckman Spinco Analytrol densitometer. After the final weighing and blood sampling, all pigs were rendered insensible by intravenous administration of sodium pentobarbital and killed by exsanquination. Various organs, glands and the left femur were removed and weighed (38). Measurements of specific gravity (39) and strength of femurs (40) were made.

In the second trial 4 lots of 4 pigs each were group-fed in stainless steel cages. The four dietary treatments were 1) basal soy diet (12 ppm of Zn) ad libitum; 2) 100 ppm of zinc soy diet limited to intake of basal soy group; 3) 100 ppm of zinc soy diet ad libitum; and 4) basal casein diet (10 ppm of Zn) ad libitum. Measurements similar to those of the first trial were also made in this trial. In addition, alcohol dehydrogenase activity (41) was determined for livers of each pig immediately after autopsy.

The third trial was carried out to determine the influence of a high level of dietary copper upon the course of zinc deficiency. Four lots of 4 pigs each were group-fed ad libitum. The four dietary treatments were 1) basal soy diet (12 ppm of Zn and 25 ppm of Cu); 2) basal soy diet with an additional 125 ppm of Cu; 3) 100 ppm of zinc soy diet (100 ppm of Zn and 25 ppm of Cu); and 4) 100 ppm of zinc soy diet with an additional 125 ppm of Cu. Growth, blood and organ measurements were made as in the first 2 trials. In addition, determinations were made of the concentration of zinc in liver and pancreatic tissue. After tissue digestion with nitric and perchloric acid and appropriate dilution, zinc concentration was determined with a Perkin-Elmer Model 303 atomic absorption spectrophotometer using a single electrode zinc cathode tube with absorption mea-

[7] Sigma Technical Bulletin 104, Sigma Chemical Company, St. Louis, 1963.

sured at 2138 angstroms. Zinc concentration in the diets of each trial were determined similarly. Also liver alcohol dehydrogenase (41) and glutamic dehydrogenase (42) activities of all pigs were determined.

Data were examined by analysis of variance with statistical significance of treatment differences being determined by either the *F* test of Snedecor (43) or the multiple range test of Duncan (44).

RESULTS AND DISCUSSION

Results of measures taken during the first trial are presented in tables 2 and 3. All the zinc-deficient pigs gained weight during the first 2 weeks of the trial, but significantly less than the pigs receiving 100 ppm of zinc in diet and all the zinc-deficient pigs had slight-to-severe parakeratotic lesions. Growth stopped in all the zinc-deficient pigs during the third week of the trial and all these pigs lost weight during the fourth week of the trial at which time they all had severe parakeratotic lesions and were near death. Pigs receiving 100 ppm of zinc in the diet gained normally throughout the trial. Food intake and efficiency of food utilization for body weight gain were severely depressed after the first few days of the trial in the zinc-deficient pigs.

Measures of erythrocyte hematology were not significantly altered by zinc deficiency. These measures were erythrocyte population, blood hemoglobin concentration and hematocrit. Blood leukocyte population was not significantly affected by zinc deficiency but a relative and absolute reduction in lymphocyte population in zinc-deficient pigs were observed and there was

TABLE 2

Growth, food utilization and hematological effects of zinc deficiency in the baby pig (trial 1) [1]

	Dietary zinc, ppm	
	12	100
Initial wt, kg	2.1 ± 0.2 [2]	2.0 ± 0.2
Final wt, kg	2.6 ± 0.5 **	8.8 ± 1.7
Daily gain, g	18 ± 10 **	242 ± 36
Daily food intake, g	100 ± 15 **	360 ± 54
Gain/food	0.18 ± 0.01 **	0.67 ± 0.03
Pigs with parakeratosis, %	100 **	0
Final blood cellular values		
Erythrocytes, 10^6/mm^3	7.5 ± 0.3	6.8 ± 0.7
Hemoglobin, g/100 ml	12.0 ± 0.6	10.8 ± 0.8
Hematocrit, %	38.8 ± 1.9	35.9 ± 2.1
Leukocytes, 10^3/mm^3	7.9 ± 0.6	13.4 ± 2.9
Lymphocytes, %	49 ± 7 **	64 ± 5
Segmented neutrophils, %	27 ± 3	24 ± 6
Band neutrophils, %	14 ± 6	6 ± 1
Monocytes, %	9 ± 5	5 ± 2
Eosinophils, %	1 ± 1	1 ± 1
Basophils, %	0 ± 0	0 ± 0
Final serum values		
Serum Zn, μg/100 ml	17 ± 1 **	60 ± 1
Serum Ca, mg/100 ml	9.2 ± 0.4 **	10.9 ± 0.5
Serum inorganic P, mg/100 ml	6.8 ± 0.9	7.0 ± 1.1
Serum alkaline phosphatase, Sigma units	0.7 ± 0.1 **	9.1 ± 2.5
Total serum protein, g/100 ml	5.5 ± 0.2 **	4.8 ± 0.1
γ-globulin, %	10.0 ± 0.8 **	6.7 ± 0.8
β-globulin, %	12.2 ± 1.4	12.4 ± 0.4
α_3-globulin, %	3.6 ± 1.7	5.0 ± 2.4
α_2-globulin, %	25.0 ± 4.7 **	9.2 ± 1.8
α_1-globulin, %	4.7 ± 1.7	8.2 ± 0.7
Albumin, %	44.5 ± 5.2 **	58.5 ± 2.4

[1] Four pigs/group.
[2] Mean ± SE.
** Differs significantly from 100 ppm mean, $P < 0.01$.

TABLE 3

Skeletal and allometric effects of zinc deficiency in the baby pig (trial 1)

	Dietary zinc, ppm	
	12	100
Femur characteristics		
Weight, g	19.6 ± 1.5 [1] **	51.6 ± 1.1
Specific gravity	1.13 ± 0.01	1.15 ± 0.01
Breaking load, kg	25 ± 4 **	46 ± 3
Bending moment, kg-cm	34 ± 6 **	85 ± 5
Moment of inertia, cm^4	0.02 ± 0.003 **	0.07 ± 0.007
Breaking stress, kg/cm^2	660 ± 40	660 ± 48
Modulus of elasticity, kg/cm^2	12,100 ± 1270	15,500 ± 2300
Organ weights, g(%) [2]		
Liver	76 (2.8)	281 (3.2)
Lungs	39 (1.5)	115 (1.3)
Brain	48 (2.1) **	57 (0.8)
Kidneys	24 (0.9)	65 (0.8)
Stomach	26 (1.0) **	49 (0.6)
Heart	15 (0.58)	46 (0.52)
Thymus	1.7 (0.06) **	26 (0.28)
Pancreas	4 (0.15) *	23 (0.25)
Spleen	5 (0.18)	16 (0.17)
Testes	3.2 (0.12)	14.8 (0.15)
Thyroid	0.28 (0.011)	0.90 (0.010)
Adrenals	0.70 (0.031) **	0.79 (0.009)
Pituitary	0.044 (0.0018) *	0.074 (0.0008)

[1] Mean ± SE.

[2] Organ weights in grams and, in parentheses, percentage of body weight with statistical comparisons made of the latter.

* Differs significantly from 100 ppm mean, $P < 0.05$; ** $P < 0.01$.

a tendency for the percentage of nonsegmented neutrophils to be increased in these animals.

Serum zinc concentration was greatly reduced in the zinc-deficient baby pigs. Serum calcium level was also significantly reduced in these animals, whereas serum inorganic phosphorus level was unchanged. Serum alkaline phosphatase activity declined to a level below that observed in older zinc-deficient pigs (19, 27). Total serum protein and the percentage of γ- and α_2-globulins were increased in zinc-deficient pigs. These observations, along with a significant reduction in the percentage of serum albumin, suggest a diminution in ability of the zinc-deficient animal to utilize globulins and perhaps a diminution in its ability to synthesize serum albumin.

The data presented in table 3 indicate weak bones in zinc-deficient baby pigs. The strength of the bones, however, is in direct relation to their size, and the parameters of breaking stress and elasticity indicate that perhaps the basic modeling of the bone is unchanged by zinc deficiency. This view is further strengthened by the observation of similar values of femur specific gravity of zinc-deficient and zinc-adequate pigs.

All the measured organ and gland weights of zinc-deficient pigs were less than those of the zinc-adequate pigs. However, when organ and gland weights were determined relative to total body weight, only thymus and pancreas gave reduced values. Adrenals were enlarged relative to body weight in zinc deficiency, as also observed in other nutrient deficiencies of the baby pig (38).

Data from the second trial are presented in tables 4 and 5 and in figure 1. Growth of the zinc-deficient and ad libitum-fed, zinc-adequate baby pigs was similar to that observed in the first trial. Growth of the limited-fed, zinc-adequate baby pigs was significantly greater than that of zinc-deficient pigs with a similar quantity of dietary intake, but much less than that of ad libitum-fed zinc-adequate pigs. Pigs receiving the 10 ppm of zinc casein diet

TABLE 4

Comparison of measures from baby pigs receiving soy or casein diets not supplemented with zinc with measures from pigs receiving limited or ad libitum intake of soy diets supplemented with zinc (trial 2)

Type of protein	Soy	Soy	Soy	Casein
Dietary zinc, ppm	12	100	100	10
Dietary intake	Ad lib.	Limited	Ad lib.	Ad lib.
Initial wt, kg	2.7	2.6	2.7	2.7
Final wt, kg	3.4	5.3	10.8	10.4
Daily gain, g	25	88 [aa]	288 [bb]	273 [bb]
Daily food, g	170	170	430 [bb]	410 [bb]
Gain/food	0.14	0.57 [aa]	0.67 [bb]	0.67 [bb]
Pigs with parakeratosis, %	100	0	0	0
Final hematological values				
Serum Zn, μg/100 ml	22	98 [bb]	127 [cc]	34 [aa]
Serum alkaline phosphatase, Sigma units	0.4	5.5 [aa,b]	7.0 [bb,c]	3.8 [aa]
Leukocytes, 10^3/mm^3	17.0 [a]	12.9	11.8	10.6
Lymphocytes, %	50	68 [a]	70 [a]	67 [a]
Segmented neutrophils, %	30	22	21	26
Band neutrophils, %	11 [c]	3	4	1
Monocytes, %	6	4	2	3
Eosinophils, %	1	1	2	1
Basophils, %	2	2	1	2
Serum protein, g/100 ml	5.0 [a]	5.1 [aa,b]	4.9	4.8
γ-globulin, %	10.5	10.6	7.6	6.9
β-globulin, %	10.5	10.1	12.7	12.6
$\alpha_2+\alpha_3$-globulin, %	25.0 [cc]	17.5	17.0	18.7
α_1-globulin, %	5.6	5.3	7.1	7.4
Albumin, %	48.5	56.5 [a]	55.5 [a]	54.5 [a]
Liver alcohol dehydrogenase [1]	0.0602	0.0611	0.0632	0.0596

[1] $\triangle$ O.D./min/mg protein.
[a] Significantly greater than least value, $P < 0.05$; [aa] $P < 0.01$.
[b] Significantly greater than least two values, $P < 0.05$; [bb] $P < 0.01$.
[c] Significantly greater than all other values, $P < 0.05$; [cc] $P < 0.01$.

TABLE 5

Skeletal and allometric comparisons of zinc-deficient baby pigs with limited and ad libitum-fed zinc-adequate baby pigs (trial 2, soy diets)

Dietary intake	Ad lib.	Limited	Ad lib.
Dietary zinc, ppm	12	100	100
Femur characteristics			
Weight, g	17.8	24.8	43.4 [bb]
Shaft length, cm	5.6	6.5 [a]	7.2 [aa]
Shaft diameter, cm	0.95	1.00	1.14 [b]
Breaking strength, kg	30	35	47 [a]
Bending moment, kg-cm	42	58	85 [a]
Moment of inertia, cm^4	0.03	0.04	0.08 [bb]
Breaking stress, kg/cm^2	622	698	641
Modulus of elasticity, kg/cm^2 [2]	8,950	10,500	10,700
Organ weights, g (%) [1]			
Liver	105 (3.0)	158 (3.0)	345 (3.2)
Lungs	41 (1.2)	58 (1.1)	121 (1.1)
Brain	50 (1.5) [bb]	58 (1.1) [aa]	60 (0.6)
Kidneys	23 (0.6)	31 (0.6)	74 (0.7)
Stomach	33 (1.0) [aa]	50 (1.0) [aa]	68 (0.6)
Heart	19 (0.55)	27 (0.52)	57 (0.53)
Thymus	1.8 (0.05)	12.8 (0.23) [aa]	51.3 (0.47) [bb]
Pancreas	7.5 (0.22)	12.2 (0.24)	23.3 (0.22)
Spleen	5.1 (0.15)	10.8 (0.21)	15.9 (0.15)
Testes	6.5 (0.19)	9.9 (0.18)	27.5 (0.27) [bb]
Thyroid	0.62 (0.019) [b]	0.63 (0.012)	1.36 (0.012)
Adrenals	1.26 (0.037) [aa,b]	1.17 (0.023)	1.83 (0.017)

[1] Organ weights in grams and, in parentheses, percentage of body weight with statistical comparison made of the latter.
[a] Significantly greater than least value, $P < 0.05$; [aa] $P < 0.01$.
[b] Significantly greater than both other values, $P < 0.05$; [bb] $P < 0.01$.

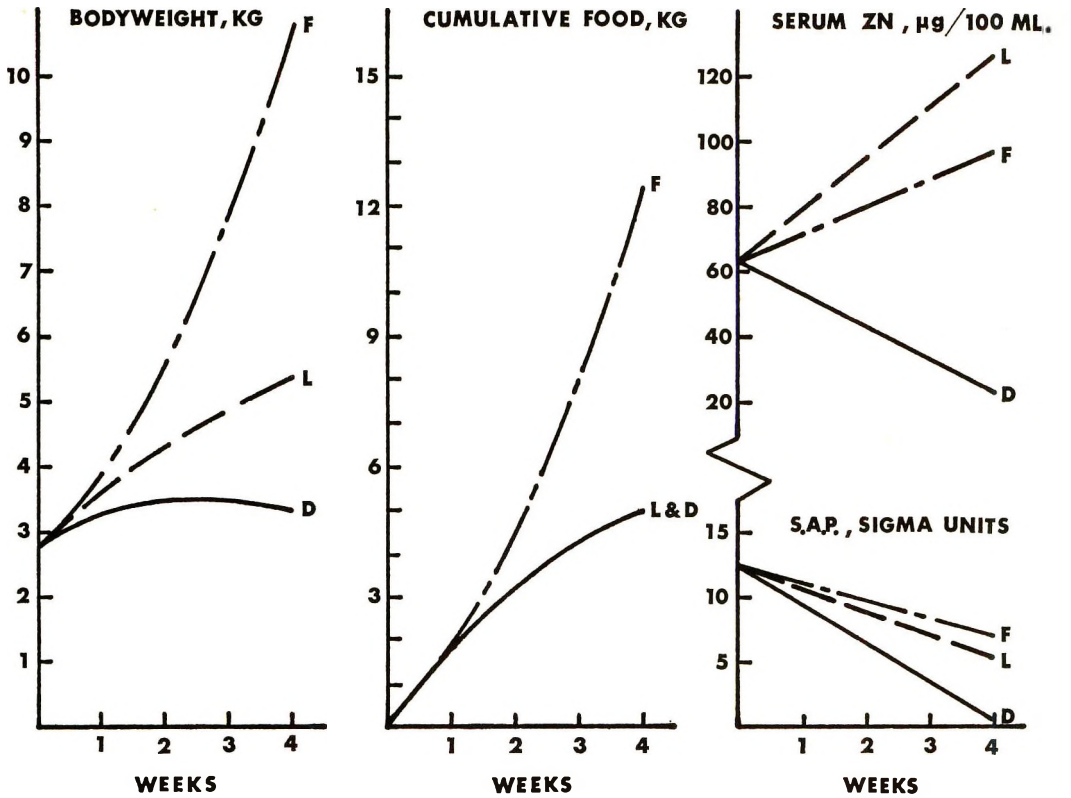

Fig. 1 Growth, food consumption, serum zinc concentration and alkaline phosphatase activity of zinc-deficient (D) and limited-fed (L) and full-fed (F) zinc-adequate baby pigs (trial 2, soy diets).

ad libitum showed growth similar to that of the pigs receiving the 100 ppm of zinc soy diet ad libitum. In this trial the pigs receiving the zinc-deficient soy diet consumed a quantity of diet similar to that of the pigs on the other dietary regimens during the first week, but their growth during that period was less (fig. 1), indicating that in zinc deficiency a diminution of growth rate occurs before voluntary food intake is affected. Voluntary food intake was diminished beginning in the second week of zinc deficiency and stayed at a low level for the remainder of the trial. Growth stopped during the second week of the trial. Parakeratosis was evident during the second week also and skin lesions became more severe as the trial progressed.

Serum zinc and alkaline phosphatase concentrations reached low levels in all the pigs fed the low zinc soy diet. Pigs receiving the low zinc casein diet had serum zinc and alkaline phosphatase levels which were significantly higher than those of the pigs receiving the low zinc soy diet, but significantly lower than those of the pigs receiving the soy diet containing 100 ppm of zinc with either limited or ad libitum intake. There were no clinical indications of zinc deficiency in the pigs receiving the casein diet. Serum zinc and alkaline phosphatase levels were similar for the limited- and ad libitum-fed, zinc-adequate soy diet pigs, with the values of the ad libitum-fed pigs somewhat higher.

Blood leukocyte population increased in the zinc-deficient pigs in this trial. This was possibly due to cutaneous infection which was observed following appearance of large fissures with the acute parakeratosis. Skin infections were not observed with parakeratosis in the first trial and leukocyte population was not increased

(table 2). Changes in leukocyte differential counts were observed in zinc-deficient pigs in this trial similar to those changes noted in the first trial, namely, a decrease in the percentage of lymphocytes and an increase in the young neutrophils. Serum protein changes with zinc deficiency which were observed in the first trial were again observed in this trial. These changes consisted of an increase in total serum protein concentration, a decrease in the albumin fraction and an increase in the α-globulins.

Data presented in table 5 indicate that bone growth was affected in direct proportion to the effect upon body growth by zinc deficiency, that maximum bending moment was directly related to bone size and that zinc deficiency does not exert a specific effect upon femur strength other than the general effect upon growth. This latter indication is inferred from the similar values of breaking stress and elasticity observed for all three soy diet treatments. Liver alcohol dehydrogenase activity was not significantly influenced by level of zinc in the diet, source of dietary protein or level of dietary intake.

The organ weights and allometric measures for the deficient, limited- and ad libitum-fed pigs (table 5) show that the allo-

TABLE 6

Comparisons of measures from baby pigs receiving soy diets supplemented or unsupplemented with zinc with medium or high levels of copper (trial 3) [1]

Dietary Zn, ppm Dietary Cu, ppm	12 25	12 150	100 25	100 150
No. of pigs	4	4	4	4
Initial wt, kg	2.5	2.5	2.5	2.5
Daily gain, g	30	40	316 **	276 **
Daily food intake, g	208	210	416 **	376 **
Gain/food	0.14	0.19	0.76 **	0.75 **
Pigs with parakeratosis, %	100	100	0 **	0 **
Final hematological values				
Serum Zn, μg/100 ml	22	19	86 **	64 **
Serum alkaline phosphatase, Sigma units	0.8	1.4	7.1 **	5.9 **
Leukocytes, $10^3/mm^3$	30.8	27.0	14.1 **	16.8 **
Lymphocytes, %	20	22	45 **	37 **
Segmented neutrophils, %	32	27	40	50
Band neutrophils, %	39	40	6 **	5 **
Monocytes, %	9	11	6	6
Eosinophils, %	0	0	2 **	2 **
Basophils, %	0	0	1	0
Serum protein, g/100 ml	5.1	5.1	4.9	4.9
Albumin, %	27.1	26.2	50.4 **	46.0 **
$\alpha_2+\alpha_3$-globulins, %	34.9	34.6	21.0 **	25.2 **
Organ weights, g (%) [2]				
Thymus	1.3 (0.03)	1.0 (0.03)	28 (0.28) **	27 (0.29) **
Adrenals	1.23 (0.030)	1.05 (0.028)	0.95 (0.10) **	0.90 (0.010) **
Pancreas	5.6 (0.16)	6.6 (0.19)	20 (0.20)	21 (0.22)
Tissue zinc				
Liver, μg/g dry matter	107	128	197 **	178 **
Pancreas, μg/g protein	102	95	196 **	199 **
Liver enzymes				
Alcohol dehydrogenase [3]	0.0406	0.0400	0.0576 **	0.0580 **
Glutamic dehydrogenase [4]	0.131	0.122	0.127	0.139

[1] Four pigs/group.
[2] Organ weights in grams and, in parentheses, percentage of body weight with statistical comparisons made of the latter.
[3] $\triangle$ O.D./min/mg protein.
[4] $\triangle$ O.D./min/mg protein.
** Significantly differs from values of both groups receiving 12 ppm zinc, $P < 0.01$.

metric increase of brain and stomach observed in zinc deficiency during the first trial was due to growth interruption. However, data from the limited-fed, zinc-deficient and zinc-adequate pigs of this trial indicate that the reduced allometric measure of thymus and the increased allometric measure of adrenals observed in zinc deficiency of both trials were a result of the nutrient deficiency.

In earlier studies Hoefer et al. (19) and Ritchie et al. (20) observed that supplementation of a natural corn-soybean meal diet for weanling pigs with 125 ppm or 250 ppm of copper from copper sulfate gave varying degrees of protection against parakeratosis. In some of their studies total protection against parakeratosis was afforded by inclusion of either level of copper supplementation. The third trial was conducted to inspect this relationship in the baby pig. Data from this trial are presented in table 6. Supplementation of 125 ppm of copper from copper sulfate did not provide any apparent effect upon the onset or severity of zinc deficiency using the criteria of incidence of parakeratosis, growth, food consumption and utilization, serum zinc and alkaline phosphatase levels, blood leukocyte population and differentiation, serum protein concentration and electrophoretic patterns, organ weights and allometric measures, liver and pancreas zinc concentration, and liver alcohol dehydrogenase and glutamic dehydrogenase activities. In this trial liver alcohol dehydrogenase activity, but not glutamic dehydrogenase activity, was positively influenced by dietary zinc level. All measures from pigs on the same dietary level of zinc (12 vs. 100 ppm) were similar regardless of dietary copper level (25 or 150 ppm). Hoekstra (45) and O'Hara et al. (46) were also unable to demonstrate any benefit of dietary copper supplementation in preventing or alleviating the consequences of zinc deficiency of weanling pigs.

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Maternal Dietary Zinc, and Development and Zinc, Iron, and Copper Content of the Rat Fetus [1,2,3]

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ABSTRACT High levels (0.2 and 0.4%) of zinc were fed to adult female rats beginning at either 0-day age of the fetus or 21 days before breeding to study the development and iron, copper, and zinc status of the 15- to 20-day-old fetus. Growth reduction in terms of dry matter or variable degrees of death and resorption occurred in fetuses from mothers fed 0.4% zinc; 100% resorption occurred in the 15- and 16-day-old fetus of mothers fed 0.4% zinc beginning at 21 days before breeding. Fetal development was normal in the fetus of mothers fed 0.2% zinc beginning at 21 days before breeding. No external anatomical malformations were observed in the fetus from zinc-fed mothers. Total zinc and concentration of zinc were significantly higher in the 15- to 20-day-old fetus of mothers fed 0.4% zinc. No significant elevation of zinc occurred in the fetus from mothers fed 0.2% zinc. Reduced total iron and concentration of iron was the trend found in the fetus from mothers fed excess zinc; the reduction was significant only in the 16- and 18-day-old fetus from mothers fed 0.4% zinc beginning at 0-day age of the fetus. Liver iron was not reduced in the 18-day-old fetus; the decrease in the fetus, therefore, was a reflection of the significant reduction of body iron. Copper was significantly reduced in the fetus from zinc-fed mothers except the 15- and 16-day-old fetus from mothers fed 0.4% zinc beginning at 0-day age of the fetus. No significant change was found in maternal liver, but trends showed liver iron to be increased in mothers fed 0.4% zinc for 15 and 16 days and decreased with the other zinc regimens. With the exception of mothers fed 0.4% zinc for 15 days of fetal age, copper content in the liver was significantly reduced by all zinc regimens.

Excess dietary zinc has been shown to cause a reduction in liver iron and copper in the rat (1–4) and liver iron in the pig (5). Although the mechanism of the zinc antagonism to iron and copper is obscure, available data suggest a decreased ability of ferritin and hemosiderin to store iron (6), a shortened life span of erythrocytes resulting in a faster turnover of iron (7), and an impairment of copper absorption as mediated primarily via the direct effects of zinc either in or on the intestine (8). The above studies were conducted with either weanling or adult rats. No data are available on the effect of excess zinc in the diet of the maternal animal on fetal zinc, iron, and copper, and scant information was found on the development of the fetus. Sutton and Nelson (9) noted stillbirths and reduced numbers of newborn from rats fed a diet containing 0.5% zinc for 14 to 17 weeks before and during gestation. Kinnamon (10) reported no resorption and no difference in the number per litter and average wet weight of rat fetuses from mothers fed a diet containing 0.5% zinc for 53 days before and during gestation. Although zinc accumulated in the liver of the fetal lamb, no adverse effect on development occurred in the fetuses of yearling ewes given a daily dosage of 5 mg zinc sulfate/kg body weight during the gestation period (11). The present investigation was designed to determine the effect of a high level of zinc in the diet of the female rat on fetal development; the iron, copper, and zinc relation in fetal tissue; and the iron, copper, and zinc relation in the maternal animal.

EXPERIMENTAL PROCEDURE

General. Nulliparous female rats,[4] ranging in weight from 160 to 190 g, were

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[3] Presented in part before the 51st Annual Meeting of the Federation of American Societies for Experimental Biology, Chicago, 1967. A portion of this paper was taken from a thesis submitted by the senior author to the Graduate School, Pennsylvania State University, in partial fulfillment of the requirements for the degree of Master of Science.

[4] CFE strain of Sprague-Dawley rats obtained from Carworth, New City, New York.

individually housed in wire cages and received feed and distilled water ad libitum. The composition of the basal diet was as follows: (in %) casein, 20; sucrose, 63; cellulose,[5] 2; corn oil, 10; salt mixture,[6] 4; and vitamin mixture,[7] 1. Excess zinc diets were made by the incorporation of either 0.2 or 0.4% zinc as zinc oxide into the basal diet. The diets contained 7.5 ppm of copper and 230 ppm of iron.

Mating was performed as previously described (12), and the day sperm was found in the vaginal smear was designated as 0-day age of the fetus. At the appropriate age of the fetuses, the mothers were stunned by a blow on the head, decapitated, exsanguinated, and maternal liver and fetuses removed via abdominal incision. Because of fetal size, the 15- and 16-day-old fetuses from individual mothers were pooled. For the 18- and 20-day-old fetuses, the liver was removed from each fetus and the livers and the bodies [8] of the fetuses from each mother were pooled separately. Therefore, the value for the older fetus is the mathematical sum of the determined values for liver and body.

All specimens were dried to a constant weight at 100°. The dried samples were wet-digested with nitric and sulfuric acid, and analyzed for zinc (13), copper (14), and iron (15).

Experiment 1. Ten female rats were fed the 0.4% zinc diet and ten were fed the basal diet beginning at 0-day age of the fetus. At each fetal age of 15 and 16 days, 5 experimental and 5 basal rats were killed.

Experiment 2. Twenty female rats were treated according to the same regimen as described in experiment 1 with the exception that the animals were killed at fetal ages of 18 and 20 days.

Experiment 3. Twenty female rats were subjected to the same regimen as stated in experiment 1 with the exception that the rats were fed the diets for 21 days before mating and continued to be fed the diets during the development of the fetus.

Experiment 4. Ten female rats were subjected to the same regimen as given in experiment 3 with the exceptions that the experimental diet contained 0.2% zinc, and the rats were killed only at a fetal age of 15 days.

RESULTS

Excess zinc (0.4%) in the diet of the maternal rat beginning at 0-day age of the fetus caused a significant reduction in growth (in terms of dry matter) of the 15- to 20-day-old fetus (table 1). Growth reduction was also reflected by a significantly smaller liver size of the 18- and 20-day-old fetus. Although variable degrees (4–29%) of fetal resorption occurred with this regimen, no external anatomical malformations were observed. Extension of the feeding of 0.4% zinc to 21 days before mating caused 100% resorption of the 15- and 16-day-old fetuses. In trials to determine at what fetal age resorption was initiated on the regimen of 0.4% zinc beginning at 21 days before mating, 36 and 40% resorption was found for the 12- and 14-day-old fetus, respectively. Feeding a 0.2% zinc diet beginning at 21 days before mating did not affect fetal growth in terms of dry matter, cause any significant degree of resorption, nor cause any anatomical malformations in 15-day-old fetuses.

Data illustrating the amount of zinc, iron, and copper expressed as total (μg) and concentration (ppm) of the individual fetus, liver, and body on a dry weight basis are given in tables 2, 3, and 4, respectively.

Total zinc and the concentration of zinc were significantly higher in the 15- to 20-day-old fetus of maternal rats fed the 0.4% zinc diet during pregnancy. The body of the 18- and 20-day-old fetus and the liver of the 20-day-old fetus from mothers fed zinc contained significantly higher total zinc and concentration of zinc. The liver of the 18-day-old fetus, however, did not contain a significantly increased total or concentration of zinc. Fetus from the maternal rat fed 0.2% zinc beginning at 21 days before mating contained about the

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[5] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

[6] Jones, J. H., and C. Foster, J. Nutr., 24: 245, 1942 (obtained from Nutritional Biochemicals Corporation).

[7] Vitamins in cornstarch, amount/kg of diet: vitamin A, 20,000 IU; vitamin D, 2,200; (in milligrams) ascorbic acid, 1017; vitamin E as α-tocopheryl acetate, 485; inositol, 110; choline dihydrogen citrate, 3715; menadione, 49.6; p-aminobenzoic acid, 110; niacin, 99.2; riboflavin, 22; pyridoxine·HCl, 22; thiamine·HCl, 22; Ca pantothenate, 66; biotin, 0.44; folic acid, 1.98; and vitamin B_{12}, 29.7 μg (obtained from General Biochemicals, Inc., Chagrin Falls, Ohio).

[8] In this report, the term body, such as body iron, is used to mean the fetus minus the liver.

TABLE 1

Effect of excess dietary zinc for the maternal rat on growth and viability of fetus

Diet	Fetal age [1]	Dry weight			Resorption [2]
		Fetus	Liver	Body	
	days	*mg*	*mg*	*mg*	*%*
		Experiment 1			
Basal	15	21.2± 0.7 [3]			0(12)
0.4% Zn	15	16.2± 1.1 **			29(9)
Basal	16	40.2± 1.7			0(12)
0.4% Zn	16	31.5± 1.5 **			11(10)
		Experiment 2			
Basal	18	131.8± 3.5	18.3±0.5	113.5± 3.3	0(12)
0.4% Zn	18	98.7± 8.0 **	11.5±1.7 **	87.2± 6.6 **	19(11)
Basal	20	421.9±11.2	49.3±3.7	372.6±11.1	0(11)
0.4% Zn	20	317.0±24.5 **	31.3±4.6 **	286.7±20.2 *	4(10)
		Experiment 3			
Basal	15(36)	22.9± 3.2			0(13)
0.4% Zn	15(36)				100(0)
Basal	16(37)	37.9± 1.4			0(11)
0.4% Zn	16(37)				100(0)
		Experiment 4			
Basal	15(36)	20.9± 0.1			1(12)
0.2% Zn	15(36)	20.7± 0.1			1(10)

[1] Day sperm found in vaginal smear counted as 0-day age of fetus; days pregnant animals were fed diets. Numbers in parentheses are total number of days females were fed diets.

[2] Criteria for resorption were fetuses in all stages of resorption and total resorption with implantation site evidence of fertilization. Numbers in parentheses are average number of viable fetuses.

[3] Average value per fetus, liver, or body and SE of mean.

* Significantly different from basal value, $P < 0.05$.

** Significantly different from basal value, $P < 0.01$.

same total zinc and concentration of zinc as the fetus from mothers fed the basal diet.

Reduced total iron and concentration of iron was the general trend in the fetus from mothers fed excess zinc. However, the reduction was significant in only the 16- and 18-day-old fetus from mothers fed 0.4% zinc. In this respect, no significant difference was found in total iron and concentration of iron in the liver of the 18-day-old fetus from mothers fed excess zinc. The reduction of iron in the fetus, therefore, was a reflection of the change in body iron; both total iron and concentration of iron were significantly reduced in the body of the 18-day-old fetus.

No significant reduction in total copper or concentration of copper occurred in the 15- and 16-day-old fetus from mothers fed a diet containing 0.4% zinc beginning at 0-day age of the fetus. The 18- and 20-day-old fetus from mothers fed zinc beginning at 0-day of the fetus had significantly reduced total copper and concentration of copper. Total copper and concentration of copper in the liver of these fetuses were also significantly reduced. Total copper and concentration of copper in the body, however, were only significantly reduced in the 18-day-old fetus; no change was found in the body of the 20-day-old fetus. Feeding the maternal rat a diet of 0.2% zinc beginning at 21 days before mating caused a significant reduction in total copper and concentration of copper in the fetus.

Data showing the amount of zinc, iron, and copper expressed as total (μg) and concentration (ppm) of the maternal liver on a dry-weight basis are tabulated in table 5.

A significant increase was found in total zinc and concentration of zinc in the liver of mothers on all zinc regimens. Lower total zinc and concentration of zinc were noted in the liver of the mothers fed 0.2% zinc as compared with those fed 0.4% zinc. No significant changes were observed in maternal liver iron. However, the liver of mothers fed 0.4% zinc for 15 and 16 days of fetal age contained a higher total and concentration of iron, whereas the other regimens resulted in a lower total iron and concentration of iron. With the

TABLE 2

Effect of excess dietary zinc for the maternal rat on zinc content of the fetus

Diet	Fetal age [1]	Fetus [2]		Liver [2]		Body [2]	
		Total	Concentration	Total	Concentration	Total	Concentration
	days	*μg*	*ppm*	*μg*	*ppm*	*μg*	*ppm*
				Experiment 1			
Basal	15	0.9 ± 0.2	43.1 ± 11.7				
0.4% Zn	15	1.7 ± 0.2 *	104.8 ± 15.3 *				
Basal	16	1.6 ± 0.3	38.7 ± 6.5				
0.4% Zn	16	4.1 ± 0.6 **	131.1 ± 21.2 **				
				Experiment 2			
Basal	18	4.4 ± 0.8	33.3 ± 5.8	3.0 ± 0.7	164.7 ± 38.4	1.4 ± 0.3	12.4 ± 3.1
0.4% Zn	18	6.6 ± 0.4 **	66.5 ± 6.7 **	3.4 ± 0.4	348.7 ± 100.4	3.2 ± 0.1 **	36.8 ± 2.0 **
Basal	20	11.6 ± 1.4	27.4 ± 3.3	5.5 ± 1.3	109.5 ± 23.8	6.1 ± 0.4	16.4 ± 1.5
0.4% Zn	20	28.3 ± 1.3 **	89.3 ± 7.4 **	7.7 ± 1.2 **	257.4 ± 33.0 **	20.6 ± 0.4 **	74.1 ± 7.1 **
				Experiment 4			
Basal	15(36)	1.1 ± 0.2	51.7 ± 8.1				
0.2% Zn	15(36)	1.4 ± 0.2	68.0 ± 11.1				

[1] See footnote 1, table 1.
[2] Average value of total content and concentration in fetus, liver, or body on dry-weight basis and SE of mean.
* Significantly different from basal value, $P < 0.05$.
** Significantly different from basal value, $P < 0.01$.

TABLE 3

Effect of excess dietary zinc for the maternal rat on iron content of the fetus

Diet	Fetal age [1]	Fetus [2]		Liver [2]		Body [2]	
		Total	Concentration	Total	Concentration	Total	Concentration
	days	*μg*	*ppm*	*μg*	*ppm*	*μg*	*ppm*
				Experiment 1			
Basal	15	3.6 ± 0.3	168.7 ± 6.9				
0.4% Zn	15	2.5 ± 0.5	155.0 ± 31.6				
Basal	16	7.6 ± 0.5	188.5 ± 5.4				
0.4% Zn	16	4.3 ± 0.4 **	138.6 ± 13.3 **				
				Experiment 2			
Basal	18	14.3 ± 2.2	108.5 ± 19.9	3.0 ± 0.7	161.5 ± 37.2	11.3 ± 1.8	101.1 ± 19.0
0.4% Zn	18	8.3 ± 2.1 *	84.1 ± 11.7 *	3.5 ± 1.1	316.5 ± 84.0	4.8 ± 0.4 **	54.5 ± 0.9 *
Basal	20	21.2 ± 4.1	50.3 ± 8.9	5.9 ± 0.9	118.8 ± 12.5	15.3 ± 3.3	40.9 ± 8.4
0.4% Zn	20	16.3 ± 3.4	51.4 ± 9.0	5.1 ± 1.4	168.2 ± 33.3	11.2 ± 2.2	39.5 ± 6.8
				Experiment 4			
Basal	15(36)	6.0 ± 2.4	284.4 ± 116.2				
0.2% Zn	15(36)	4.4 ± 1.1	208.4 ± 48.7				

[1] See footnote 1, table 1.
[2] See footnote 2, table 2.
* Significantly different from basal value, $P < 0.05$.
** Significantly different from basal value, $P < 0.01$.

TABLE 4

Effect of excess dietary zinc for the maternal rat on copper content of the fetus

Diet	Fetal age [1]	Fetus [2]		Liver [2]		Body [2]	
		Total	Concentration	Total	Concentration	Total	Concentration
	days	*μg*	*ppm*	*μg*	*ppm*	*μg*	*ppm*
				Experiment 1			
Basal	15	0.9 ± 0.2	43.5 ± 10.2				
0.4% Zn	15	0.7 ± 0.3	44.4 ± 21.9				
Basal	16	0.8 ± 0.2	18.9 ± 4.2				
0.4% Zn	16	0.6 ± 0.2	18.7 ± 7.7				
				Experiment 2			
Basal	18	1.9 ± 0.1	14.0 ± 0.9	1.2 ± 0.1	61.7 ± 6.4	0.7 ± 0.1	6.5 ± 1.2
0.4% Zn	18	0.5 ± 0.1 **	5.3 ± 1.6 **	0.2 ± 0.1 **	23.3 ± 9.6 *	0.3 ± 0.1 **	3.7 ± 1.0 *
Basal	20	2.9 ± 0.4	6.9 ± 0.9	1.5 ± 0.4	31.3 ± 3.0	1.4 ± 0.3	3.8 ± 0.7
0.4% Zn	20	1.5 ± 0.3 *	5.0 ± 1.1 *	0.3 ± 0.1 **	10.2 ± 2.8 **	1.2 ± 0.3	4.5 ± 1.0
				Experiment 4			
Basal	15(36)	0.4 ± 0.05	19.9 ± 2.6				
0.2% Zn	15(36)	0.2 ± 0.04 **	8.8 ± 1.8				

[1] See footnote 1, table 1.
[2] See footnote 2, table 2.
* Significantly different from basal value, $P < 0.05$.
** Significantly different from basal value, $P < 0.01$.

exception of the liver of mothers fed 0.4% zinc for 15 days of fetal age, total copper and concentration of copper were significantly reduced by all zinc regimens.

DISCUSSION

Since proper nutrition before and during pregnancy has such marked influences on physical and mental potential of the individual, increased research is being carried out in this area. Although much information has been obtained concerning the influence of the maternal diet in terms of an excess or a deficiency of a nutrient on such parameters as malformation, fetal resorption, and ultimate development of the individual, the basic metabolic lesions are poorly understood.

A normal estrous cycle and normal mating with fertilization and implantation were observed in female rats fed a diet containing either 0.2 or 0.4% zinc. During the development of the embryo or fetus from mothers fed 0.4% zinc, however, changes occurred in the amount of zinc, iron, and copper, and probably other metabolites, and death and resorption or reduced growth ensued.

Abnormal fetal development occurred coincident with the level of zinc in the maternal diet and the length of time the diet was fed. In addition to reduced growth, small percentages of fetal resorption at fetal age of 15 to 18 days were noted in rats fed 0.4% zinc beginning at 0-day age of the fetus. However, feeding 0.4% zinc to the female rat beginning at 21 days before breeding, resulted in 36, 40, and 100% resorption for 12-, 14-, and 15- and 16-day-old fetuses, respectively. Lowering the level of zinc in the diet to 0.2%, and feeding it beginning at 21 days before breeding, had no adverse effect on fetal development. Although the abnormal fetal development was similar to that reported by Sutton and Nelson (9), it is difficult to make direct comparisons since they fed a zinc diet to weanling rats instead of to the mature female. Why Kinnamon (10) found no adverse fetal development from mature females fed 0.5% zinc for 53 days cannot be explained.

Zinc was significantly elevated in the viable fetus of mothers fed 0.4% zinc.

Although the zinc content in the liver of the 18-day-old fetus from the mothers fed zinc was the same as in the liver of the fetus from mothers fed the basal diet, a significantly higher zinc content was present in the body. This variation in zinc uptake between liver and body, which was not observed in the 20-day-old fetus, suggests a difference in tissue uptake or retention of zinc. As a possible explanation, Kinnamon (10) reported a variation in the distribution of ^{65}Zn in 12- to 17-day-old fetuses. The highest activities, in order of decreasing ^{65}Zn concentration, were in calcifying bones; liver, spleen, and lumen of the large blood vessels; and in kidneys. The lack of an accumulation of zinc in the fetus of mothers fed 0.2% zinc suggests a placental barrier to the transfer of abnormal amounts of zinc to the fetus, which becomes ineffective at higher concentrations of zinc.

Changes observed for the iron and copper content of the fetus from mothers fed excess zinc resembled the reduction previously reported (1–4) for the weanling and adult rat fed excess zinc. Although the general trend was reduced fetal iron with all zinc regimens, the only significant reduction was in the 16- and 18-day-old fetus from mothers fed 0.4% zinc beginning at 0-day age of the fetus. In contrast, a significant reduction occurred in the copper content of the 18- and 20-day-old fetus, but not in the 15- and 16-day-old fetus. Copper was also significantly reduced in the fetus of mothers fed 0.2% zinc; however, the zinc content in the fetus was not elevated. These findings suggest that the antagonism of zinc with copper is mediated in the mother or the placenta rather than in the fetus. An iron reduction in the body, but not in the liver of the 18-day-old fetus, indicates that whereas iron in the liver of the weanling rat was lowered in 3 to 5 days after initiating the feeding of zinc (1), fetal liver was relatively stable to the antagonism of zinc. However, liver copper was reduced to a greater degree than body copper in the 18-day-old fetus.

The reduction in content of iron and copper suggests an altered synthesis of important iron and copper metabolites in the fetus, which may have precipitated the abnormal fetal development. Reduced activity of liver cytochrome oxidase and catalase (17), heart cytochrome oxidase (3), and liver xanthine oxidase (6) have been noted in rats fed excess dietary zinc. Since the activity of fetal liver and heart cytochrome oxidase does not peak until days 18 and 19 of fetal age, respectively (18), and since hepatic xanthine oxidase activity is absent in the fetus (16), ascribing fetal death and resorption to possible changes in these enzymes is tenuous. Changes in the activity of the enzymes may, however, play a role in the reduced growth of the fetus from mothers fed zinc. These aspects of the problem are being investigated in this laboratory.

The increase in the amount of zinc in the fetus suggests the possibility of abnormal fetal development, particularly death and resorption, as the result of a toxic condition due to zinc per se. Rather than a zinc toxicity, the elevation of zinc may cause abnormal metabolism by adversely affecting tissue or organ systems. An increased osmotic fragility observed (19) for erythrocytes incubated in vitro with solutions containing zinc has been reported (7) to occur in rats fed excess zinc.

With the exception of the liver of mothers fed 0.4% zinc beginning at 0-day age and extending to day 15 of fetal age, the copper content was significantly reduced in the liver of mothers on all zinc regimens as similarily reported (1–4) for the nonpregnant animal. In contrast with this earlier work, and, although statistically insignificant, the values for liver iron indicate that trends of either an increase occurred when 0.4% zinc was fed for the short period of 15 to 16 days or a decrease occurred when 0.2 or 0.4% zinc was fed for longer periods of time. The lack of more positive agreement with the earlier work may be explained by the difference in animal age, as the adult rat was used in the present study rather than the weanling animal and consequently the diet was fed for a shorter period of time.

ACKNOWLEDGMENT

The authors acknowledge the assistance of Gerald H. Meyer in preparation of diets and in care and feeding of animals.

TABLE 5

Effect of excess dietary zinc on total and concentration of zinc, iron, and copper in maternal liver

Diet	Fetal age [1]	Total [2]			Concentration [2]		
		Zinc	Iron	Copper	Zinc	Iron	Copper
	days	*μg*	*μg*	*μg*	*ppm*	*ppm*	*ppm*
				Experiment 1			
Basal	15	125.5 ± 20.9	419.0 ± 155.6	42.9 ± 5.6	36.2 ± 5.3	119.2 ± 40.3	12.4 ± 1.3
0.4% Zn	15	495.0 ± 87.6 **	543.6 ± 145.1	26.5 ± 4.9	144.4 ± 24.5 **	157.6 ± 36.3	7.9 ± 1.6
Basal	16	112.7 ± 15.1	302.0 ± 65.4	56.0 ± 6.7	33.3 ± 5.6	84.4 ± 14.0	16.1 ± 1.4
0.4% Zn	16	521.2 ± 25.6 **	479.0 ± 80.1	27.5 ± 3.4 **	162.4 ± 8.9 **	149.3 ± 24.7	8.5 ± 1.0 **
				Experiment 2			
Basal	18	163.0 ± 6.6	464.3 ± 53.4	68.9 ± 4.2	40.1 ± 2.3	113.0 ± 12.2	16.8 ± 0.5
0.4% Zn	18	724.4 ± 38.7 **	384.6 ± 27.6	36.2 ± 4.9 **	187.1 ± 8.6 **	99.9 ± 8.5	9.4 ± 1.3 **
Basal	20	179.9 ± 14.4	410.0 ± 37.3	64.8 ± 4.9	47.4 ± 3.2	107.0 ± 6.1	16.9 ± 0.7
0.4% Zn	20	766.0 ± 67.1 **	334.0 ± 28.8	39.5 ± 4.3 *	211.8 ± 20.5 **	92.4 ± 8.6	11.0 ± 1.4 *
				Experiment 3			
Basal	15(36)	125.8 ± 13.3	294.0 ± 44.8	45.8 ± 4.5	40.3 ± 4.7	93.6 ± 14.7	14.5 ± 1.3
0.4% Zn	15(36)	456.8 ± 79.8 **	237.6 ± 18.8	16.3 ± 4.8 **	165.8 ± 35.8 **	84.7 ± 11.6	5.4 ± 1.5
Basal	16(37)	101.2 ± 6.1	409.8 ± 146.5	44.6 ± 2.6	31.4 ± 2.8	124.1 ± 42.8	13.7 ± 0.8
0.4% Zn	16(37)	691.4 ± 97.2 **	380.6 ± 95.0	21.1 ± 1.5 **	212.0 ± 38.1 **	112.5 ± 25.5	6.4 ± 0.5 **
				Experiment 4			
Basal	15(36)	123.0 ± 15.5	507.0 ± 113.9	68.8 ± 3.0	37.8 ± 4.0	154.0 ± 32.5	21.2 ± 0.7
0.2% Zn	15(36)	268.0 ± 11.6 **	274.4 ± 18.4	55.6 ± 4.6 *	85.8 ± 4.2 **	87.8 ± 6.1	17.7 ± 1.2 *

[1] See footnote 1, table 1.
[2] Average value of livers from 5 maternal rats; values for total content and concentration on dry-weight basis and include SE of mean.
* Significantly different from basal value, $P < 0.05$.
** Significantly different from basal value, $P < 0.01$.

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Effect of Imbalanced Diets Containing Natural Proteins on Appetite and Body Composition in the Rat [1,2]

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ABSTRACT Experiments were undertaken to determine some biochemical changes that occur in protein-depleted rats fed imbalanced diets for 24 hours. The diets used contained only natural proteins—wheat gluten and defatted whole egg—mixed in different amounts. Basal diets contained only defatted whole egg having the same level of "complete protein" as the corresponding imbalanced ones. Animals fed imbalanced diets showed a typical consumption curve, but their total intake was lower than that of the control animals. After a subsequent 4-hour fast, the imbalanced rats lost a significantly higher percentage of weight than the corresponding groups fed the balanced diet. Body water content was increased in imbalanced rats, but that value returned to a more normal figure after fasting subsequent to the feeding period. Hematocrit value was also higher in rats fed the imbalanced diets than in control animals, but the values tended to be closer after the fasting period.

In previous studies we have reported that concurrently with variations in the plasma amino acid pattern, some changes occur in weight and in the composition of organs in rats fed imbalanced diets (1–2). The imbalanced diets used in those studies were obtained by adding to a basal diet (containing 10% of wheat gluten supplemented with 0.1% of lysine) 3.1% of a synthetic essential amino acid mixture lacking threonine.

As some of the effects observed were similar to biochemical and clinical signs characteristic of kwashiorkor, we suggested that in this condition these symptoms could be related not only to a low protein intake or to a low protein-to-calorie ratio but also to the imbalanced amino acid pattern of the diet (2).

In the present work we studied the food consumption and changes in body composition that occur in protein-depleted rats after feeding for 24 hours imbalanced diets containing a mixture of animal and vegetable proteins resembling that commonly used in areas in which malnutrition is prevalent. The imbalanced and the corresponding basal diets used in these experiments contained the same percentage of "complete" or "utilizable protein" or, according to Platt et al. (3), the same "protein value," expressed as NDpCals%, but with the imbalanced diets having a large excess of vegetable protein not available for synthetic purposes.

The results reported here indicate that coincidently with the characteristic changes in appetite and weight, rats fed this type of imbalanced diet showed marked shifts in body water distribution that significantly altered their body composition. These changes also showed some similarity to those observed in protein malnutrition, thus supporting the hypothesis previously stated (2).

The biochemical mechanisms that could be responsible for the changes reported in this paper are also discussed.

EXPERIMENTAL

Male rats of the Wistar strain, having an average weight of 63 g, were used in these experiments. All rats were depleted by feeding them a protein-free diet for 10 days; after that they were fasted for 24 hours. Animals that lost approximately 14 to 16 g were selected.

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The rats were housed in individual suspended cages with screen bottoms. The animals were grouped as indicated below and were fed the experimental diets ad libitum for 24 hours. Food intake was recorded periodically to establish the consumption curve throughout the experimental period.

In all experiments rats were weighed after the 24-hour feeding period. The diets were then withdrawn and the animals were weighed again 4 hours later (fasting period).

Water was offered ad libitum during both the feeding and fasting periods.

Diets. Two series of diets, A and B, were used in these experiments. The composition of the diets is outlined in table 1. Lysine was the limiting amino acid for growth in both series of diets.

Diets of series A (A_1, A_2 and A_3) constituted basal or balanced diets and contained as the only source of protein defatted whole egg in different amounts to provide increasing levels of available lysine. Diet A_3 also contained a supplement of 0.2% L-lysine.

Diets of series B (B_1, B_2 and B_3), the imbalanced diets, contained as the source of protein a mixture of defatted whole egg and wheat gluten, in different amounts. The imbalance was produced by a large excess of vegetable protein over egg protein.

Accordingly, the values for the ratio of complete protein to total protein were markedly higher for diets of series A than for those of series B (table 1).

The diets of both series were grouped in 3 pairs. Each pair included a diet of series A and another of series B, both containing the same level of lysine, for example, 0.20, 0.30 and 0.40%, respectively, in the diet. Therefore the quantity of "complete protein" was the same for each pair, with the imbalanced diets of series B having an excess of amino acids provided by the wheat gluten, not available for anabolic purposes.

Experiments with pairs 1 and 2, were performed with groups of 6 rats each. For pair 3, two groups of 12 animals were used.

Available lysine was determined in all diets by the procedure of Carpenter (6) as modified by Rao et al. (7).

Chemical analysis. Body water content and hematocrit values were determined in the rats of pair 3: 6 rats of each group — basal and imbalanced — were killed after the 24-hour feeding period, and the remaining 6 rats were killed after the subsequent

TABLE 1

Composition of diets

	Series A (balanced)			Series B (imbalanced)		
	A_1	A_2	A_3	B_1	B_2	B_3
	%	%	%	%	%	%
Defatted whole egg [1]	5.80	8.75	6.20	3.0	4.0	6.20
Wheat gluten [2]	—	—	—	9.0	13.0	16.0
L-Lysine [3]	—	—	0.20	—	—	—
Minerals [4]	5.0	5.0	5.0	5.0	5.0	5.0
Vitamin mixture [4]	0.25	0.25	0.25	0.25	0.25	0.25
Choline chloride	0.15	0.15	0.15	0.15	0.15	0.15
Corn oil	5.0	5.0	5.0	5.0	5.0	5.0
Dextrin [5]	83.80	80.85	83.20	77.60	72.60	67.40
Total available lysine	0.20	0.30	0.40	0.20	0.30	0.40
Total protein content	4.00	6.00	4.46	9.47	13.56	17.56
Complete protein content [6]	2.20	3.30	4.40	2.20	3.30	4.40
Complete protein as % of total protein	55.0	55.0	98.6	23.2	24.30	25.10

[1] Containing 10.8% N (68.8% protein) and 3.43% available lysine.
[2] Containing 13.2% N (83.0% protein) and 1.20% available lysine.
[3] As L-lysine·HCl.
[4] Harper (4).
[5] Moist cornstarch autoclaved at 121° for 3 hours.
[6] Munro et al. (5).

4-hour fasting period, for the corresponding analysis. Zero-time values were determined in a group of 6 rats killed after a 4-hour fasting period following protein depletion. Before killing, blood samples were taken for the determination of hematocrit. The rats were anesthetized with ether, and blood was withdrawn by heart puncture. Hematocrit values were determined on the heparinized blood of each group according to the micro-hematocrit technique of Dacie and Lewis (8).

Water in animal carcasses was determined by heating at 105° for 48 hours as described by Bender and Miller (9). The composition of gained tissue was calculated by the difference between the individual final values and the mean carcass composition at zero time.

RESULTS

The food intake and weight gain of protein-depleted rats fed the different diets ad libitum are summarized in table 2.

As shown, for each experiment rats fed the diets of series B had a lower consumption than those receiving the corresponding diet of series A, despite the percentage of complete protein being the same in each pair of diets.

The consumption curves shown in figure 1 are similar for the 3 experiments. The food intake of rats fed imbalanced diets B was higher during the first hours than that for animals fed the corresponding balanced diets A; but after a few hours, the food consumption of groups fed diets of series B decreased, and at the end of a 24-hour feeding period the total intake was significantly higher for animals fed the balanced diets than for those fed the corresponding imbalanced diets.

Weight gain of rats after the 24-hour experiment was lower for groups fed diets B than for those fed the corresponding diets A. The difference was statistically significant ($P < 0.01$) for pairs 1 and 2 (table 2).

Weight loss after the subsequent fasting period (table 2), determined in pairs 2 and 3, was markedly higher for groups fed the diets of series B, than for groups fed the corresponding diets of series A. Rats fed the imbalanced diet B_2 lost, after the 4-hour fasting period, nearly 90% of the

TABLE 2

Food consumption and changes in weight of rats fed the experimental diets ad libitum for 24 hours followed by a 4 hour fasting period

Paired diet no.	Total available lysine	Diet	Complete protein in diet	Food intake	Wt gain		Weight loss during fasting period [1]
					At end of feeding period	At end of fasting period	
	%		%	g/24 hr	g	g	%
1	0.20	A_1 (5.8 defatted whole egg)	2.20	10.1 ± 0.6 [2]	5.7 ± 0.6	—	—
	0.20	B_1 (3% defatted whole egg + 9% wheat gluten)	2.20	7.4 ± 0.5 [3]	3.0 ± 0.6 [3]	—	—
2	0.30	A_2 (8.75% defatted whole egg)	3.30	12.0 ± 0.6	7.1 ± 0.5	5.0 ± 0.1	33.6 ± 6.4
	0.30	B_2 (4% defatted whole egg + 13% wheat gluten)	3.30	9.0 ± 0.3 [3]	4.2 ± 0.9 [3]	0.5 ± 0.7 [3]	88.1 ± 10 [3]
3	0.40	A_3 (6.2% defatted whole egg + 0.2% lysine)	4.40	12.9 ± 0.3	7.6 ± 0.5	4.6 ± 0.4	38.7 ± 3.0
	0.40	B_3 (6.2% defatted whole egg + 16% wheat gluten)	4.40	9.9 ± 0.3 [3]	6.7 ± 0.6	2.6 ± 0.3 [3]	63.1 ± 4.4 [3]

[1] Expressed as % of the weight gain during the 24-hour feeding period.
[2] Mean ± SE of mean.
[3] Highly significantly different ($P < 0.01$) from group fed the corresponding balanced diet.

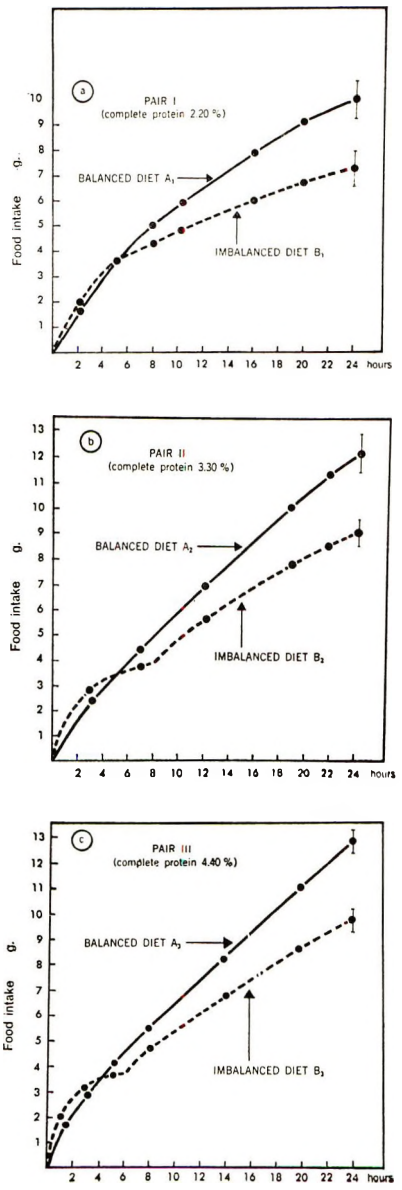

Fig. 1 Food consumption of protein-depleted rats fed the experimental diets for 24 hours: a) pair 1; b) pair 2; and c) pair 3. Vertical lines represent SE of mean.

weight they had gained during the 24-hour feeding period, weighing at that time only 0.5 g more than at zero time. On the contrary, rats fed the balanced diet A_2 lost, after fasting, approximately only 30% of the gained weight, their weight at that time being still 5 g more than at zero time. A similar result was observed in pair 3.

Table 3 shows that at the end of the 24-hour feeding period body water content was significantly higher for animals fed the imbalanced diet B_3 than for those fed the balanced diet A_3 and was similar to that in the zero-time group (table 3); but after 4 hours of subsequent fasting no significant differences were observed for either group.

Changes in hematocrit values are shown in figure 2. In group A_3 the zero-time value — higher than normal as a consequence of depletion — decreased at the end of the feeding period to a value close to normal showing only a small change after the following fasting period. On the contrary, in rats fed imbalanced diet B_3 the hematocrit value tends to be slightly higher after 24 hours of feeding when compared with the zero-time value, decreasing after the subsequent fasting period to a value that is still higher than normal and significantly different than that observed in group 5 fed balanced diet A_3.

DISCUSSION

Diet and food intake. In all experiments carried out in this study, rats fed diets of series B — in which imbalances were created by adding wheat gluten to egg protein — had a lower food intake than those fed the corresponding balanced diets containing only egg protein. These results are in agreement with others reported in previous papers (2,10), confirming that depression in food intake could be considered an initial and distinctive effect of imbalance.

Curves of the progressive food consumption show a similar and characteristic picture in the 3 experiments that deserves further consideration.

During the first 4 hours of the feeding period, food intake was higher for groups fed imbalanced diets than for those fed the corresponding balanced diets. However, by the fifth hour a change of behavior was observed in imbalanced animals and the intake decreased markedly. Thereafter and in the remaining experiments their rate of consumption diminished when compared with control animals. As a con-

TABLE 3

Body water content of rats fed diets A_3 and B_3 (pair 3) ad libitum for 24 hours followed by a 4-hour fasting period

Paired diet no.	Total available lysine	Diet	Complete protein in the diet	Body water content		
				Zero time [1]	At end of feeding period	At end of fasting period
	%		%	% of body weight		
3	0.40	A_3 (6.2% defatted whole egg + 0.2% lysine)	4.40	70.5 ± 0.4 [2]	68.3 ± 0.4	68.0 ± 0.5
	0.40	B_3 (6.2% defatted whole egg + 16% wheat gluten)	4.40	70.5 ± 0.4	71.2 ± 0.5 [3]	69.2 ± 0.4

[1] After a 4-hour fasting period following protein depletion.
[2] Mean ± SE of mean.
[3] Highly significantly different ($P < 0.01$) from the group fed the corresponding balanced diet A_3.

sequence, the total food intake at the end of 24-hour feeding period was significantly lower for rats fed imbalanced diets than for those fed the corresponding balanced diets.

This fact can be clearly observed in rats fed imbalanced diet B_3 (fig. 3). In this group, food intake was recorded every hour during the first 5 hours of the feeding period together with that of the group fed the corresponding balanced diet.

These findings showed marked similarity to some previously reported observations when protein-depleted rats were fed imbalanced diets, created by adding synthetic mixtures of amino acids to diets with a low protein content. In those experiments the depression in food intake was also observed after a feeding period in which the amount of diet eaten was slightly higher for the imbalanced than for the basal groups (10).

This particular behavior could be ascribed to a high ability of depleted rats to efficiently utilize imbalanced diets, whether the imbalance was semisynthetic or natural, during a short period of time.

It is possible that this "high intake period" may be curtailed when some biochemical changes occur that alter the body homeostasis; these changes would trigger an appetite-depressing mechanism causing the decrease in food intake. The time in which this effect is produced appears to be variable and possibly dependent on the type of imbalance.

In previous papers we assumed that the appetite-depressing mechanism could be related to changes in relative proportions of amino acids between the plasma and tissues that occur concurrently with the

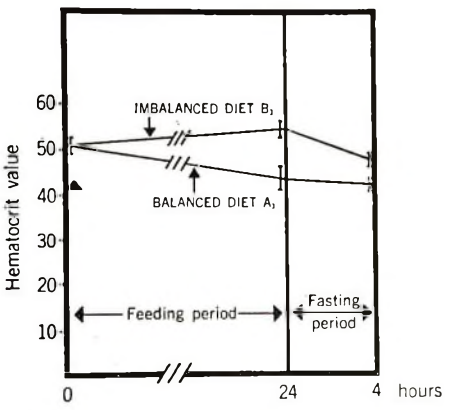

Fig. 2 Hematocrit values for protein-depleted rats of pair 3, after the 24-hour feeding period and the subsequent 4-hour fasting period. ▲ normal value for nondepleted rats. Vertical lines represent SE of mean.

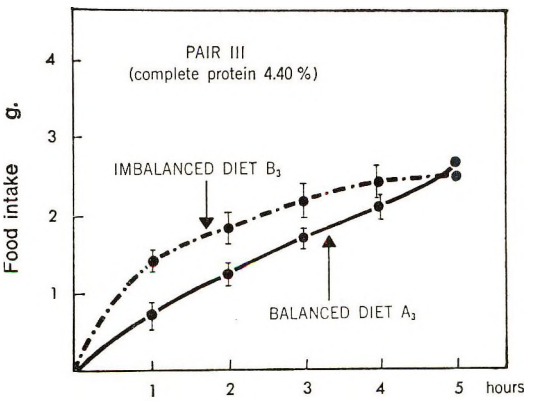

Fig. 3 Food consumption of protein-depleted rats of pair 3 during the first 5 hours of the 24-hour feeding period. Vertical lines represent SE of mean.

alteration of the plasma amino acid pattern (11). Protein-depleted rats fed for 24 hours imbalanced diets of the same composition used in this study showed an abnormal distribution of amino acids between plasma and tissues.[3] These results would support our assumption.

Imbalanced diet and body composition. The effect of an altered dietary pattern on body composition has been the subject of numerous studies during the last years. Weil et al. (12), working with diets containing 12.5 and 25% of protein, pointed out that the only alterations in body composition which could be ascribed to the dietary treatment ". . . were those changes which would be expected to accompany the different rates of total growth produced by the two levels of protein intake." According to these authors, diets of very low protein content do not appear to modify the extremely narrow limits of compositional homeostasis to any appreciable extent. These statements are in agreement with the concept of lean body "constancy" that has been postulated previously by Pace et al. (13).

Miller and Payne (14) have reported that weight increments in weanling rats fed diets of the same NDpCals%, but containing different proteins, should be similar. However, Tagle et al.[4] noted different growth responses in rats fed diets containing wheat gluten and casein at the same NDpCals% level, and which could be considered therefore nutritionally equivalent; but the authors did not report significant differences for the animals carcass composition.

Our results show that the body composition of protein-depleted rats fed diets of identical "protein value," but having a different total protein content, is markedly altered. As shown in table 3, the percentage of water, higher than normal, observed in rats at zero time, which could be considered the consequence of the protein depletion, diminished in animals fed balanced diet A_3 after the 24 hours of feeding, reaching a value close to normal. On the contrary, in the group fed imbalanced diet B_3, body water percentage increased after the feeding period, being significantly different from that of group A_3.

These changes are reflected in the values of some ratios derived, showing the markedly different effects of the balanced and imbalanced diets on body composition of the rats (fig. 4). The ratio of total body water to dry body weight was increased over the zero-time value for the group fed the imbalanced diet, indicating that water retention accounted for the largest part of the weight gained by these animals. On the contrary, in the group fed diet A_3 this ratio showed a lower value than at zero time, indicating a relative increment in the dry body weight of the rats after feeding the balanced diet (fig. 4a).

These differences are still more marked when the body water gain-to-body dry weight gain ratios were compared (fig. 4b).

The data obtained after the fasting period subsequent to the 24 hours of feeding are worthy of note. During this period rats fed imbalanced diets B_2 and B_3 lost a greater percentage of weight gained during the feeding period than the animals fed the corresponding balanced diets A_2 and A_3. The study of body composition showed that after fasting, the water con-

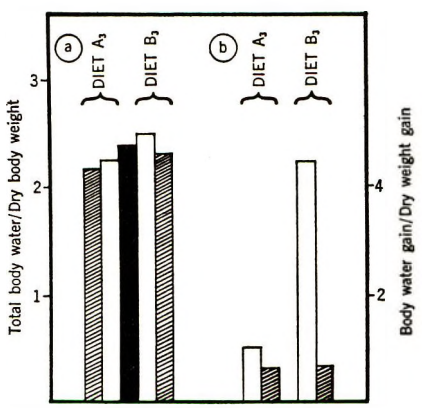

Fig. 4 Ratios showing changes in body composition of protein-depleted rats fed the balanced diet A_3 and the corresponding imbalanced diet B_3. a) Total body water-to-dry body weight ratio; b) body water gain-to-dry weight gain ratio. White bars: values after the 24-hour feeding period. Slanted bars: values after the subsequent 4-hour fasting period. Black bar: zero-time value.

[3] Rio, M. E., S. Closa and J. C. Sanahuja 1967 Biochemical changes in natural amino acid imbalance in the rat. Federation Proc., 26: 521 (abstract).

[4] Tagle, M. A., and G. Donoso 1967 Long-term effects of feeding rats on casein and gluten diets of the same protein value (submitted for publication).

tent of rats previously fed imbalanced diet B_3 was significantly lower than at the end of the feeding period. Thus it could be assumed that an increase in water excretion occurred during fasting in imbalanced rats that lost in this way some of the water accumulated during the feeding period. This phenomenon was not observed in rats fed the corresponding balanced diet A_3 in which the water carcass showed no differences at the end of both periods. As a consequence, after fasting, the values for the ratios previously noted were similar for both diets (fig. 4a,b).

From these observations that correlate variations in weight with changes in body water content, it might be assumed that changes concerning body composition in depleted rats, under conditions of dietary protein imbalance, appear in the following progressive sequence. First, the excess of amino acids provided by the imbalanced diet, which cannot be utilized for protein synthesis, would accumulate in rat tissues. In a previous paper we have reported the high level of free amino acids that appear in the livers of rats after feeding an imbalanced diet (2). Therefore an overhydration could be expected due to an osmotic flow of fluid from the extracellular space. The rise in hematocrit value at the end of the 24-hour feeding period agrees with such an assumption. Second, during fasting following the feeding period, the increased water loss observed in imbalanced animals may be related to the excretion of the excess of amino acids or its metabolites. Albanese (15) reported that the excessive renal loss of free amino acids provided by diets containing wheat gluten, which cannot be utilized for tissue synthesis, caused an increased water loss through an electrolytic diuresis.

This high water excretion in rats fed the imbalanced diet would explain the fact that after fasting, body water content and hematocrit values reached values closer to normal than at the end of the feeding period.

These changes in body composition produced by feeding imbalanced proteins indicate that the compositional homeostasis — which according to Weil et al. (12) appears to achieve an independence from the nutritional environment — could be markedly affected by the dietary imbalance of proteins.

However, the effect observed with balanced diets of low protein content, such as the diets of series A_3, is in agreement with the results reported by Weil et al., indicating that those diets do not modify the homeostasis to any appreciable extent.

On the basis of these results we can assume that changes observed in the body composition of rats fed imbalanced protein could be considered specific for the imbalance, as they did not appear when the animals were fed balanced diets considered nutritionally equivalent to the imbalanced ones on the basis of complete protein content.

Disturbances in water and electrolyte metabolism are consistent characteristics of protein-calorie malnutrition. In fact, the malnourished child shows overall body tissue overhydration, generally considered a consequence of a decrease in some plasma protein fractions subsequent to a low protein intake.[5]

However, the results obtained by feeding depleted rats imbalanced diets containing natural proteins support our assumption (2) concerning the possible role of dietary amino acid imbalance in kwashiorkor development.

An altered amino acid pattern in the diet might be a factor capable of influencing per se the degree of tissue hydration, and this effect can be superimposed on that produced by the low protein content of diet, as can be inferred from our findings in the present study.

Therefore the complete protein-to-calorie ratio, commonly used to establish the adequacy of diets to prevent protein malnutrition, does not appear to be a reliable index when the diets contain an excess of amino acids, not available for tissue synthesis, supplied by a protein of low nutritive value. In those cases, such an imbalance could aggravate the metabolic impairment resulting from a low ratio value.

[5] Frenk, S. 1960 Some aspects of protein malnutrition in childhood. Vth International Congress on Nutrition, Panel II, Washington, D. C., p. 35.

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Effect of Source of Carbohydrate as Influenced by Dietary Fat: Carbohydrate ratio and forced exercise in rats [1]

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ABSTRACT The effects were studied of changing the carbohydrate source in diets high or low in fat and fed to sedentary or force-exercised rats. Eighty mature, male rats were either force-exercised by treadmill-running every day or allowed voluntary activity in an activity cage. The 40 animals receiving each exercise treatment were then fed ad libitum either a high fat-low carbohydrate diet or a diet low in fat and high in carbohydrate. Equicaloric intakes of the 2 diets provided equal intakes of protein, vitamins and minerals. The 20 animals receiving each diet at each exercise level were divided into 4 groups of 5 animals each to receive their dietary carbohydrate as: 1) cornstarch, 2) lactose, 3) sucrose, or 4) a mixture approximating the carbohydrate composition of an average U. S. "market basket" diet. The animals fed lactose as the only carbohydrate in a low fat-high carbohydrate diet had diarrhea and weighed less than all other animals, even when diets were corrected to equicaloric intakes by analysis of covariance. Metabolism of 1-^{14}C- and 6-^{14}C- glucose to $^{14}CO_2$ and U-^{14}C-glucose to citrate-^{14}C indicated greater activity of the HMP shunt pathway in animals fed lactose. The moderate level of forced exercise used in this study significantly lowered the ingesta-free body weights of animals fed equicaloric amounts of the high fat-low carbohydrate diets, but had no effect on the body weights of animals fed equicaloric amounts of the high carbohydrate-low fat diets. This appeared to indicate a greater muscular efficiency in rats fed the high carbohydrate-low fat diets than in those fed the high fat-low carbohydrate diets. Body composition was altered by all three of the main experimental variables. Animals fed the high fat-low carbohydrate diet had more body fat than animals of the same weight fed the high carbohydrate-low fat diet. Force-exercised animals had less body fat than animals of the same weight allowed voluntary activity only. Among rats of the same weight, those fed starch as the only source of carbohydrate had more body fat than those fed the other carbohydrate sources. However, rats fed starch had a lower carcass concentration of cholesterol than rats fed the other carbohydrate sources. Rats fed the mixture of carbohydrates at either a high or a low level in the diet increased in body cholesterol more rapidly as they gained weight than rats fed the single carbohydrate sources. Weight control was of primary value in limiting the cholesterol content of the animals fed the carbohydrate mixture, regardless of fat:carbohydrate ratio of the diet.

Investigators have generally found that high fat-low carbohydrate diets result in lower body weights than diets high in carbohydrate and low in fat (1–4). The claim has been made that a high fat-low carbohydrate diet can be used for weight reduction without restricting caloric intake.[3] However, most weight loss has occurred a short time after adoption of a high fat-low carbohydrate diet (5)[4] and has been most marked when caloric intake was restricted, though equal to the intake of rats that lost less weight when fed a high carbohydrate-low fat diet (6). The greater loss of weight in rats fed a high fat-low carbohydrate diet could be completely accounted for by the reduced bulk of the gastrointestinal contents, possibly due to a shift in water balance. Rats fed the high fat-low carbo-

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[2] Data are from a thesis by the author submitted to the Graduate School, University of Maryland, in partial fulfillment of the requirements for the M.S. degree.

[3] Alsop, J. 1965 The diet that finally did it. McCalls, 92 (8): 138.

[4] Anderson, J. T., H. L. Taylor and A. Keys 1956 Serum cholesterol in subjects on reducing diets high and low in fat. Federation Proc., 15: 542 (abstract).

hydrate diet had more body fat ($P < 0.01$) than rats of the same size consuming a high carbohydrate-low fat diet (6).

It is not known whether the effect of carbohydrate restriction is different when the carbohydrate source in the diet is different. Wells and Anderson (7) reported that cholesterologenesis in rabbits was accelerated when lactose replaced cornstarch in the diet. Several studies have shown that animals fed dextrin had a higher body fat content than those fed sucrose (8–10) or those fed lactose (8, 11). Studies involving the feeding of a mixture of carbohydrates such as that reported by Eheart and Mason (12) to be used in U. S. "market basket" diets have not been made. Forced exercise has been observed by Konishi (13) to reduce the activity of the hexose monophosphate (HMP) shunt in rats.

The present study compares cornstarch, sucrose, lactose, and a mixture of carbohydrates simulating that used in U. S. "market basket" diets (12). Each carbohydrate source was fed in both a high fat-low carbohydrate diet and a high carbohydrate-low fat diet to rats that were either force-exercised or allowed voluntary activity in an activity cage.

EXPERIMENTAL

Eighty-five male rats,[5] obtained at 140 days of age, were housed individually and fed a stock diet[6] ad libitum for a week in order to compensate for any lack of uniformity in the previous dietary history. Five rats were then selected at random and killed for initial carcass composition analysis. During the adjustment period, forty of the animals were trained to exercise on a treadmill at increasing speeds and times. The lights of the animal laboratory were kept lighted for 12 hours each day. All animals, whether force-exercised or not were placed on alternate weeks in rotating treadmill activity cages which allowed voluntary activity but did not require activity of any kind. The housing of experimental rats in activity cages whenever possible provides a more natural basis of physical activity effects on which to study nutritional variables. The investigation of nutritional phenomena on rats restricted in activity to individual wire cages may limit the applicability of the data obtained. The voluntary activity of rats housed in rotating treadmill cages can be monitored to determine whether different dietary regimens predispose the rats to different patterns of physical activity (14).

The 80 experimental rats were assigned at random to one of 16 groups of 5 rats each for 8 weeks. An analysis of variance showed no significant differences in the initial weights of the 16 groups. Eight groups of rats were subjected to a regimen of daily forced exercise and 8 groups were allowed only the voluntary activity they exhibited while in the activity cages. The forced exercise consisted of a brief run on a treadmill at a speed sufficient to stimulate the cardio-respiratory system. Four groups of animals in each exercise treatment were fed a high fat diet, and 4 groups a high carbohydrate diet designed to provide similar amounts of vitamins, protein, and minerals when an equal calorie intake was consumed (table 1).[7]

Approximately 24% of the added fat was provided by a polyunsaturated fatty acid source (corn oil), which is representative of U. S. "market basket" diets. The high carbohydrate-low fat diet contained 54% of its calories as carbohydrate, which is again representative of some U. S. "market basket" diets. The high fat-low carbohydrate diet contained only 12% of its calories as carbohydrate, which is approximately the degree of carbohydrate restriction recommended by those advocating low carbohydrate-high fat diets for reducing purposes (1–6).

The carbohydrate source in the diets fed to the 4 groups at each exercise and carbohydrate intake level was either cornstarch, lactose, sucrose, or a mixture of carbohydrates approximating that in U. S. "market basket" diets (12). This is a $2 \times 2 \times 4$ factorial experiment designed to be analyzed by analysis of variance and covariance, to aid in interpreting data (15). Although 5 rats per group is not a large number, the second-order interaction (carbohydrate level $\times$ carbohydrate source $\times$ exercise treatment) was significant ($P < 0.05$) only in the case of serum cholesterol,

[5] Sprague-Dawley strain from Rockland Barns, Gilbertsville, Pennsylvania.
[6] Wayne Lab-Blox, Allied Mills, Inc., Chicago.
[7] Prepared by General Biochemicals, Chagrin Falls, Ohio.

TABLE 1

Composition of high fat-low carbohydrate and high carbohydrate-low fat diets fed ad libitum to rats [1]

	High fat	High carbohydrate
	%	%
Lactalbumin	36.70	27.50
Carbohydrate source [2]	16.17	56.75
Corn oil [3]	9.30	2.30
Beef tallow	29.50	6.90
Vitamin A and D conc [4]	0.07	0.05
Salt mix [5]	5.40	4.00
Cellulose [6]	2.00	2.00
Vitamin mix	0.86 [6]	0.50 [7]
Total	100.00	100.00
Gross calories, kcal/g	6.45	4.60

[1] Lactalbumin, mineral, and vitamin content of the high fat-low carbohydrate diet was elevated so that equicaloric amounts of the 2 diets provide an equal intake of these nutrients.

[2] Either 1) cornstarch; 2) lactose; 3) sucrose; or 4) a mixture of 40% cornstarch, 20% lactose, 10% dextrin, 10% sucrose, 10% glucose, and 10% fructose.

[3] Mazola, Corn Products Company, Argo, Illinois.

[4] Percomorph Oil, Mead Johnson Company, Evansville, Indiana.

[5] Jones and Foster (16).

[6] CelluFlour, Chicago Dietetic Supply House, Chicago.

[7] The following vitamins were added per kilogram of ration: thiamine·HCl, 7 mg; pyridoxine·HCl, 7 mg; niacin, 7 mg; riboflavin, 13 mg; Ca D-pantothenate, 34 mg; *p*-aminobenzoic acid, 405 mg; *α*-tocopheryl acetate, 34 mg; 2-methyl-1,4-naphthoquinone, 3 mg; folic acid, 3 mg; biotin, 135 μg; vitamin B_{12}, 40 μg; choline chloride, 3 g; and inositol, 1.3 g.

[8] The following vitamins were added per kilogram of ration: thiamine·HCl, 5 mg; pyridoxine·HCl, 5 mg; niacin, 5 mg; riboflavin, 10 mg; Ca D-pantothenate, 25 mg; *p*-aminobenzoic acid, 300 mg; *α*-tocopheryl acetate, 25 mg; 2-methyl-1,4-naphthoquinone, 2 mg; folic acid, 2 mg; biotin, 100 μg; vitamin B_{12}, 30 μg; choline chloride, 2 g; and inositol, 1 g.

and hence in the case of all other parameters studied, groups could be combined for comparison purposes. Except in the case of serum cholesterol, all other statistically significant differences involved the comparison of groups containing at least 10 rats each.

Food and water were provided for all animals ad libitum and calorie intake was monitored. Calorie intake differences were equalized between groups by an analysis of covariance, which was used as a tool to determine the probability that differences observed were explainable through differences in caloric intake. This procedure for equalizing intake differences has been outlined by Steel and Torrie (15). During the experimental period, the 5 rats in each of the 16 experimental groups received intraperitoneal injections of 1 μCi each of U-^{14}C-, 1-^{14}C-, and 6-^{14}C- glucose in physiological saline at 96-hour intervals. Animal, food and water were placed in a metabolism chamber and $^{14}CO_2$ was recovered and counted; total activity was determined, and data are expressed as a percentage of the dose injected as suggested by Wang (17). Urinary citrate was isolated for the first 12 hours after injecting U-^{14}C-glucose by use of a Dowex 50 column and paper chromatography using a 4:1:5 (v/v) upper phase *n*-butanol:acetic acid:water solvent system. Citrate was identified by the use of standards and spraying with a solution of 0.04% bromophenol blue in 95% ethanol solution. Citrate spots were cut from the paper, ignited in an O_2 bomb, and the $^{14}CO_2$ was counted and expressed as percentage of dose injected (14).

At the end of 8 weeks, all rats were anesthetized with sodium amytal, blood was removed by heart puncture for cholesterol analyses, the gastrointestinal tract removed, flushed of its contents and returned to the carcass. The animals were grouped according to their dietary source of carbohydrate and killed over a 4-day period, in the same order they had been placed on the study. The animals in each carbohydrate source grouping were fed as usual on the morning they were killed and were killed by blocks (groups) over the next 8 hours so that any influence of time of last feeding would show up as a block effect in subsequent statistical analyses. The ingesta-free carcasses were weighed and homogenized in a Waring Blendor by dilution with added water and ice at a ratio of 2:1 (18). Carcass homogenates were analyzed for moisture (9), fat according to the micro-Soxhlet method,[8] ash (19), and nitrogen according to the semi-micro-Kjeldahl method of Hiller et al. (20). Serum samples and carcass homogenates were analyzed for cholesterol content by the method of Koval (21).

RESULTS AND DISCUSSION

Among the main experimental effects studied, only changing the source of carbo-

[8] Molander, A. L. 1956 A comparison of quantitative determinations of fat in foods analyzed by four different methods. M.S. thesis, College of Home Economics, University of Maryland.

hydrate in the diet significantly affected final ingesta-free weight. Table 2 reports the mean ingesta-free weights of the 20 rats fed each carbohydrate source, their body composition and the recovery of ^{14}C-glucose in these animals as $^{14}CO_2$ and urinary citrate-^{14}C. The finding that the ingesta-free body weight of animals fed lactose was significantly lower ($P < 0.01$) than that of all other animals was to be expected since the rats consuming lactose as the sole source of carbohydrate had diarrhea and undoubtedly digested their diets less efficiently. However, Womack et al. (11), in an earlier report, observed that differences in digestible calorie intakes could account for only part of the superior weight gains of animals fed cornstarch as compared with those fed lactose. They postulated that a metabolic effect due to feeding lactose accounted for part of the poor weight gains of animals fed lactose as the sole source of dietary carbohydrate. Our data on the metabolism of ^{14}C-glucose appear to agree with this postulate (table 2).

Although the $^{14}CO_2$ recovery from rats injected with 6-^{14}C-glucose was not affected by changing the carbohydrate source in the diet, rats fed lactose as the only carbohydrate source exhaled more $^{14}CO_2$ from 1-^{14}C-glucose in 12 hours than rats fed the mixture of carbohydrates simulating that used in the U.S. "market basket" diets ($P < 0.05$). During the 12 hours following the administration of U-^{14}C-glucose the smallest percentage of recovery as citrate-^{14}C in urine was obtained from animals fed lactose ($P < 0.05$). The mean citrate excretion in the urine of rats consuming the 4 carbohydrate sources was nearly the same, ranging from 0.86 ± 0.10 mEq/day for the rats fed lactose to 0.92 ± 0.09 mEq/day for those fed sucrose. The specific activity of urinary citrate following glucose-U-^{14}C administration was, therefore, less ($P < 0.05$) for rats fed lactose than for rats fed the other three carbohydrate sources (table 2). Assuming that urinary citrate is representative of the TCA cycle intermediates throughout the body, this agrees with the radiorespirometry data and it appears that less of the ^{14}C from U-^{14}C-glucose was passing through the glycolytic scheme and into the TCA cycle for the rats

TABLE 2

Effects on body weight, body composition, and ^{14}C-glucose metabolism of changing carbohydrate source in the diet [1,2,3]

Carbohydrate source	Ingesta-free body					^{14}C recovered after 12 hours			
	Wt	Water	Protein	Fat	Ash	1-^{14}C-glucose as $^{14}CO_2$	6-^{14}C-glucose as $^{14}CO_2$	U-^{14}C-glucose as $^{14}CO_2$	U-^{14}C-glucose as urinary citrate
	g	*g*	*g*	*g*	*g*	*% dose*	*% dose*	*% dose*	*% dose* [4]
Cornstarch	366.6 [a]	236.6 [a]	77.8 [a]	41.1 [a]	12.9 [a]	30.6 [ab]	18.8 [a]	25.3 [a]	2.9 [a]
Lactose	322.2 [b]	210.8 [b]	70.2 [b]	25.1 [b]	12.1 [b]	33.2 [a]	20.2 [a]	25.4 [a]	1.1 [b]
Sucrose	358.2 [a]	226.8 [a]	75.5 [a]	27.7 [b]	12.8 [a]	32.3 [ab]	20.9 [a]	25.8 [a]	3.3 [a]
Mixture	369.2 [a]	241.9 [a]	78.1 [a]	27.0 [b]	13.5 [c]	29.9 [b]	20.3 [a]	23.9 [a]	3.0 [a]

[1] The effect of changing the carbohydrate source was not different in rats fed high fat-low carbohydrate or high carbohydrate-low fat diets and was not different in rats that were force-exercised instead of allowed voluntary activity only.

[2] Twenty rats/group.

[3] Means in each column identified by a different superscript letter are significantly different ($P < 0.05$).

[4] Specific activities (μCi/mEq) were: cornstarch $= 0.033 \pm 0.010$; lactose $= 0.013 + 0.004$; sucrose $= 0.036 + 0.008$; mixture $= 0.034 \pm 0.008$; rats fed lactose had a lower specific activity in their urinary citrate ($P < 0.05$) than rats fed the other 3 carbohydrate sources.

fed lactose than for those fed the other carbohydrate sources. This agreement in results of two independent routes of investigation indicates greater activity of the hexosemonophosphate (HMP) shunt in rats fed lactose than in the rats fed the other carbohydrate sources.

Rats fed cornstarch as the only source of carbohydrate had a greater relative proportion of body fat ($P < 0.01$) than rats fed the other carbohydrate sources (table 2). This agrees with earlier reports that animals fed dextrin had a higher body fat content than those fed sucrose (8–10) or those fed lactose (8, 11). The higher rate of fat accretion in animals fed starch may be the result of more efficient utilization of the starch-containing diets due to slower absorption controlled by the digestive release of its monosaccharide units. This would be a similar postulate to that of Harper et al. (23) who observed a growth reduction in rats when casein in a dextrin diet was replaced by a mixture of amino acids. These workers postulated that the rate of release or absorption of amino acids from casein in relation to the carbohydrate source has an important effect on growth.

Table 3 shows the final ingesta-free body weights and body composition of rats either force-exercised or allowed only voluntary activity with diets either low in fat and high in carbohydrate or diets high in fat and low in carbohydrate. The effects of forced exercise on body weight was different with the 2 diets, but the picture is complicated by the caloric intakes with the 2 diets not being the same. Table 4 reports the daily caloric intakes of the 16 groups in this study. When an analysis of covariance was used to correct all groups to an equal calorie intake as recommended by Steel and Torrie (15), then it became apparent that forced exercise of rats

TABLE 3

Effects on body weight and body composition of force-exercised rats fed high fat-low carbohydrate and high carbohydrate-low fat diets [1,2,3]

Carbohydrate level-activity combination	Ingesta-free body				
	Wt	Water	Protein	Fat	Ash
	g	*g*	*g*	*g*	*g*
HC-LF-FE [4]	354.6 [ab]	231.5 [ab]	77.0 [a]	23.2 [a]	13.2 [a]
HC-LF-VOL	340.3 [a]	219.6 [a]	74.2 [a]	27.1 [a]	13.0 [a]
HF-LC-FE	350.8 [ab]	229.6 [ab]	76.0 [a]	29.2 [a]	12.8 [a]
HF-LC-VOL	370.6 [b]	235.6 [b]	74.5 [a]	41.5 [b]	12.4 [a]

[1] Twenty rats/group.
[2] The effect of changing the exercise treatment within high fat-low carbohydrate and high carbohydrate-low fat diets was not different with any of the different carbohydrate sources.
[3] Means within each column identified by a different superscript letter are significantly different ($P < 0.05$).
[4] HC = high carbohydrate; HF = high fat; LC = low carbohydrate; LF = low fat; FE = force-exercised; VOL = allowed only voluntary activity in an exercise wheel.

TABLE 4

Comparison of mean caloric intakes and serum cholesterol levels as influenced by dietary carbohydrate source, fat:carbohydrate ratio, and forced exercise

Treatment group [1]	Caloric intake		Serum cholesterol	
	HC-LF	HF-LC	HC-LF	HF-LC
	kcal/day	*kcal/day*	*mg/100 ml*	*mg/100 ml*
Starch, VOL	86.2	93.2	44.7	48.7
Starch, FE	92.4	92.8	49.8	56.8
Lactose, VOL	90.5	102.1	52.3	58.1
Lactose, FE	88.9	103.7	77.7	59.9
Sucrose,VOL	92.3	98.8	87.0	96.6
Sucrose, FE	91.9	99.9	91.9	93.3
Mixture, VOL	94.5	95.5	65.4	105.4
Mixture, FE	93.1	92.6	83.5	81.3

[1] HC = high carbohydrate; HF = high fat; LC = low carbohydrate; LF = low fat; FE = force-exercised; VOL = allowed only voluntary activity in an exercise wheel.

consuming equicaloric amounts of the high fat-low carbohydrate diet reduced ($P < 0.05$) their final ingesta-free body weights (343.2 g vs. 362.7 g for rats performing only voluntary activity). The effect of the same amount of moderate forced exercise in rats fed equicaloric amounts of the high carbohydrate-low fat diet was not enough to cause a statistically significant change in body weight (349.0 g vs. 361.4 g). This appears to indicate greater muscular efficiency in rats fed the high carbohydrate-low fat diets than in those fed the high fat-low carbohydrate diets. This finding agrees with the earlier reports of Marsh and Murlin (24) and Krogh and Lindhard (25). This phenomenon is thought to be due to increased protein catabolism for energy when subjects receiving a high fat-low carbohydrate diet are forced to exercise. This could increase the specific dynamic effect of this diet and reduce its efficiency of utilization.

Despite the report of Konishi (13) that HMP shunt activity was inhibited by forced exercising, we could find no significant differences in ^{14}C-glucose metabolism caused by exercise. Therefore, we have not reported the $^{14}CO_2$ and citrate-^{14}C recovery data pertaining to exercise effects. The difference of our findings from those of Konishi (13) is probably due to the differences in experimental conditions. We used a very moderate level of forced exercise and allowed free voluntary activity to those rats not forced to exercise. The sedentary rats of Konishi were severely restricted in activity.

It is of interest that despite the carbohydrate level × exercise treatment interaction in final body size response to equicaloric intakes, when an analysis of covariance was used to correct all groups to an equal body size, rats of the same size had less body fat ($P < 0.05$) when they were force-exercised instead of allowed only voluntary activity. The use of covariance to correct for body size differences in comparing body composition between groups is preferred to comparing percentage composition figures since percentage data are not normally distributed. Steel and Torrie (15) outline the comparison of composition data in this way. Forced exercising by moderate treadmill-running of these ad libitum-fed animals significantly changed their relative body composition, which is in agreement with the earlier work of Hanson et al. (6) who used swimming. An analysis of covariance correcting for body weight differences also showed that rats of the same size fed high fat-low carbohydrate diets have more body fat ($P < 0.01$) than similar-size rats fed high carbohydrate-low fat diets. This is in agreement with the earlier report of Hanson et al. (6).

The serum cholesterol levels reported in table 4 are higher for rats fed high fat-low carbohydrate diets than for rats fed high carbohydrate-low fat diets ($P < 0.01$). However, when an analysis of covariance corrected for differences in caloric intake this "effect" completely disappeared. Therefore, the higher serum cholesterol levels in rats fed the high fat-low carbohydrate diets appear to have been due entirely to the fact that the caloric intakes of rats consuming these diets were higher than for rats fed the high carbohydrate-low fat diets.

The serum cholesterol levels were changed by all three experimental variables in this study (carbohydrate level × carbohydrate source × exercise treatment). Despite the variation in the reported values (table 4), rats fed diets containing cornstarch or lactose as the only source of carbohydrate tended to have lower serum cholesterol values than rats fed the other two carbohydrate sources. Figure 1 shows the regression lines representing the relationship between total cholesterol in the carcass and carcass weight. Carbohydrate level and exercise treatment did not affect carcass cholesterol levels in this study. However, rats fed cornstarch as the only source of carbohydrate in their diets had much lower levels of cholesterol in their carcasses ($P < 0.01$) than observed in the carcasses of rats fed the other carbohydrate sources. This effect was no different when the carbohydrate sources represented only 12% of the caloric intake instead of 54%. It is of interest that the starch-fed animals, which had the lowest levels of body cholesterol, had the highest levels of body fat (table 2). Dupont (26) reported increased cholesterologenesis when fat was used for energy, and hence lipogenesis and cholesterologenesis may be independent of one another. The dietary supply of cholesterol

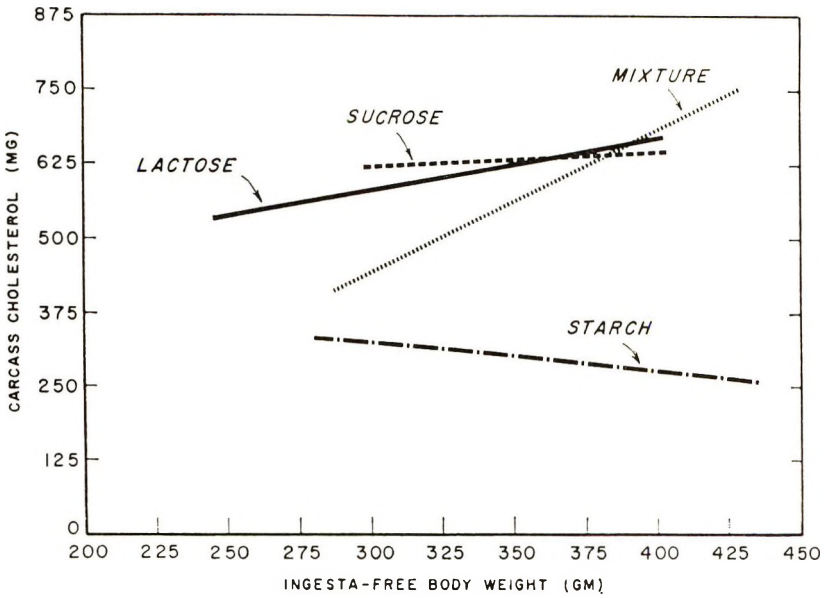

Fig. 1 Regression lines of body cholesterol vs. ingesta-free body weight as influenced by dietary carbohydrate source. The fat-to-carbohydrate ratio of the diet and moderate forced exercising had no effect on this regression, and hence each line represents 20 observations. Means of each regression were as follows: lactose, + 0.844; starch, − 0.489; sucrose, + 0.294; and mixture, + 2.384.

was not different in the carbohydrate sources in this study since all other components of each diet were identical (table 1).

Rats fed the mixture of carbohydrates simulating that in U. S. "market basket" diets (12) increased in body cholesterol more rapidly ($P < 0.01$) as they gained in weight than rats fed the single carbohydrate sources. This effect was just as evident when the carbohydrate sources accounted for 12% of caloric intake as at 54%. This indicates that a mixture of carbohydrates such as that fed in this study predisposed rats to lay down body cholesterol at a more rapid rate as they gained in weight. Thus, weight control was of primary importance in limiting body cholesterol content in these animals. The lightest animals fed the mixture of carbohydrates did not have a greatly different carcass cholesterol content from rats of the same weight fed cornstarch. However, the heaviest rats fed the mixture of carbohydrates had a carcass cholesterol concentration over 3 times that in rats of similar weight but fed cornstarch. The importance of this finding in human nutrition will require further study.

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Effect of Linoleic Acid Deficiency on the Fertilizing Capacity and Semen Fatty Acid Profile of the Male Chicken

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ABSTRACT To determine the effect of a linoleic acid deficiency on the fertilizing capacity of the chicken, White Leghorn males were fed a vitamin-free casein-gelatin basal diet practically devoid of linoleic acid from 4 weeks of age. Corn oil was used as a source of linoleic acid. On the basis of one insemination (0.05 cm^3 undiluted semen) per hen per 3-week period, a linoleic acid deficiency significantly decreased the average fertility of males. No differences in fertility were observed between the groups fed the linoleic acid-supplemented diet and a practical diet. There were no differences in percentage of dead germs, pipped eggs, or chicks hatched among the dietary treatments. The fertilizing capacity, as measured by the decline in number of fertilized eggs after a single insemination, indicated that the linoleic acid deficiency resulted in a decrease of fertility below 90% on day 4 after insemination as contrasted with day 9 for the linoleic acid-supplemented diet. Linoleic acid deficiency had no effect on body weight maintenance, feed consumption, semen volume, sperm motility, and sperm count. Fatty acid determinations of semen and blood indicated a much lower value for linoleic acid in the linoleic acid-deficient group than for the supplemented groups. Two fatty acids tentatively identified as docosadienoic and docosatetraenoic were found in semen lipid. The percentages of docosadienoic and docosatetraenoic were 6 times larger and 6 times less, respectively, in the deficient semen than in the nondeficient semen. The significance of these findings remains to be elucidated.

The comprehensive review of Aaes-Jørgensen (1) on the role of essential fatty acids on the reproductive performance of animals did not include any data on the male fowl. Although Edwards (2) conducted essential fatty acid deficiency studies with the growing domestic cock, he did not report fertility data with the deficient males. Initial studies conducted by Lillie and Menge [1] showed that a linoleic acid deficiency significantly reduced the fertilizing capacity of the male fowl, as measured by the decrease in number of fertilized eggs after a single insemination. These results prompted further investigation in greater detail and scope, the results of which are presented here.

EXPERIMENTAL PROCEDURE

One-day-old May-hatched White Leghorn cockerels were maintained in electrically heated batteries and fed a practical-type starter diet (21% protein). At 4 weeks of age, 45 males were distributed at random into 3 groups of 15 each and continued in batteries.

The dietary treatments from 4 to 23 weeks of age were as follows: group 1 was fed a vitamin-free casein-gelatin basal diet practically devoid of linoleic acid; group 2, the same diet supplemented with corn oil (1.7%) to supply one percent of linoleic acid; group 3, a corn-soy diet calculated to contain one percent of linoleic acid. The grower diets for groups 1 and 3 are presented in table 1. From 23 to 59 weeks of age, the grower diets were changed as follows: corn oil was increased to 6.9% and 5% in group 2 and 3 diets, respectively, to supply 4% of linoleic acid, and the protein content was decreased to 20% in group 1 and 3 diets (table 1). Adjustments were made with cellulose and dextrose so that all diets were equal in protein and energy content.

At 20 to 22 weeks of age, semen from individual males was obtained by abdominal massage as described by Burrows and Quinn (3). Because some of the males did not respond to abdominal massage after

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[1] Unpublished data.

TABLE 1

Composition of diets

	Grower diets		Adult diets	
	Linoleic acid-deficient (group 1)	Corn-soy (group 3)	Linoleic acid-deficient (group 1)	Corn-soy (group 3)
	%	%	%	%
Casein, vitamin-free	18.0	—	18.0	—
Gelatin	11.0	—	5.0	—
Ground yellow corn	—	40.0	—	47.0
Soybean meal, 50%	—	36.00	—	32.0
Coconut oil (hydrogenated)	2.0	2.0	2.5	2.5
Methionine hydroxy analogue	0.34	0.25	0.3	0.23
Choline, 70%	0.35	0.35	0.35	0.35
Vitamins	0.50 [1]	0.50 [1]	0.50 [2]	0.50 [2]
Minerals	6.00 [3]	6.00 [3]	12.2 [4]	12.2 [4]
Antioxidant [5]	0.015	0.015	0.015	0.015
Cellulose, nonextracted	—	—	—	0.205
Cellulose, extracted	2.0	—	9.11	—
Dextrose	59.795	14.885	52.025	—
Corn oil	—	—	—	5.0
Total	100.0	100.0	100.0	100.0
Metabolizable energy, kcal/kg	3410	2932	3000	3000
Protein, %	25.8	21.5	20.1	20.1

[1] Vitamin mix no. 1 supplied the following: (mg/kg of diet) thiamine·HCl, 100; niacin, 100; riboflavin, 16; Ca D-pantothenate, 25; pyridoxine·HCl, 10; vitamin B_{12} (0.1%), 20; folacin, 4; menadione, 5; biotin, 0.6; dry vitamin A palmitate (500,000 USP/g), 36; vitamin D_3 (200,000 ICU/g), 7; *d*-α-tocopheryl acetate (1360 IU/g), 26; dextrose to 0.5% of diet.

[2] Vitamin mix no. 2 is identical to vitamin mix no. 1 with 3 exceptions: vitamin B_{12} (0.1%), 15; vitamin D_3 (200,000 ICU/g), 8; *d*-α-tocopheryl acetate (1360 IU/g), 40; dextrose to 0.5% of diet.

[3] Mineral mix no. 1 supplied the following: (in %) $CaCO_3$, 0.75; $CaHPO_4 \cdot 2H_2O$, 3.44; KH_2PO_4, 1; NaCl, 0.3; $MgCO_3$, 0.13; $FeC_6H_5O_7 \cdot 5H_2O$, 0.06; $MnSO_4 \cdot H_2O$, 0.03; $ZnCO_3$, 0.01; H_3BO_3, 0.001; KI, 0.004; $Cu(CH_3COO)_2$, 0.0032; $CoSO_4 \cdot 7H_2O$, 0.0002; $Na_2MoO_4 \cdot 2H_2O$, 0.0009; $Na_2SeO_4 \cdot 10H_2O$, 0.00005; glucose monohydrate (Cerelose, Corn Products Company, Argo, Ill.), to 6.0% of diet.

[4] Mineral mix no. 2 is identical to mineral mix no. 1 with 7 exceptions: $CaCO_3$, 6.6; $CaHPO_4 \cdot 2H_2O$, 3.75; $MgCO_3$, 0.425; $ZnCO_3$, 0.0125; $Cu(CH_3COO)_2$, 0.004; $Na_2MoO_4 \cdot 2H_2O$, 0.0006; glucose monohydrate to 12.2% of diet.

[5] 1,2-Dihydro-6-ethoxy-2,2,4-trimethylquinoline.

several weeks and also because a few deaths occurred during the grower period, the number of males was reduced from 15 to 10 per group at 22 weeks of age. Semen collections were made on a weekly basis by the same person for artificial insemination and observations on semen volume, motility, sperm counts, and fatty acid analysis. Semen volume was measured to the nearest 0.01 cm^3 with a tuberculin syringe at the time of collection. The semen was tested for motility and sperm count, according to the method of Parker et al. (4). The fatty acid analysis of semen and blood was determined by the method of Miller et al. (5). All measurements were made of samples obtained from individual males.

White Leghorn pullets, maintained in individual cages and fed a standard practical-type breeder diet, were used for insemination studies. From one to five females were assigned at random to each male, depending on the quantity of semen collected. Inseminations were made every 21 days with 0.05 cm^3 of undiluted semen per hen. All eggs laid on the third day through the sixteenth day after insemination were pedigreed and dated for hatchability studies. The pedigreed eggs were set on day 17 after insemination. After 7 days of incubation, those eggs that showed no signs of life were removed, broken open and examined macroscopically for classification as infertile or dead germs. At the end of incubation period, those eggs that did not hatch were examined and tabulated as dead germs or pipped eggs. All the percentage data obtained with fertility, embryonic mortality, and hatchability represented the mean based on averaging the data of individual males per group.

Since no differences were observed on the effect of linoleic acid deficiency on

TABLE 2

Effect of linoleic acid (corn oil) on hatchability and semen characteristics

	Diet		
	Linoleic acid-deficient (group 1)	Linoleic acid-supplemented (group 2)	Corn-soy (group 3)
Hatchability (6 inseminations):			
Males tested, no.	10	10	10
Eggs set, no.	1,512	1,418	1,572
Fertility, % [1]	64.2 [a 2]	77.9 [b]	82.3 [b]
Dead germs 0–7 days,% [3]	5.9	5.2	5.6
Dead germs 8–21 days, % [3]	7.2	6.0	6.6
Pipped eggs, % [3]	7.6	7.2	5.5
Chicks hatched,% [3]	79.2	81.6	82.3
Semen characteristics:			
Volume/male, cm^3	0.14	0.13	0.15
Motility [4]	2.0	2.4	2.2
Sperm count, millions/cm^3	4.3	4.0	4.4

[1] Data represent a mean fertility value based on averaging the fertility of individual males.
[2] Means on the same line bearing different superscript letters are significantly different ($P \leq 0.01$).
[3] % of fertile eggs.
[4] 0 = no motility; 1 = sluggish motility; 2 = moderate motility; and 3 = vigorous motility.

TABLE 3

Effect of linoleic acid (corn oil) on the decline in percentage of fertile eggs laid by hens inseminated with 0.05 cm^3 undiluted semen once every 3 weeks

Day of ovi-position after insemination	Linoleic acid-deficient males (group 1)	Linoleic acid-supplemented males (group 2)	Corn-soy males (group 3)
3	95	96	100
4	87	94	99
5	82	96	100
6	82	94	99
7	84	91	95
8	77	91	95
9	70	87	93
10	69	85	86
11	56	80	85
12	54	68	83
13	45	63	65
14	33	55	51
15	27	43	53
16	17	33	38

body weight gains and feed consumption of males fed the three dietary treatments from 4 to 59 weeks of age, the data are not presented.

RESULTS

The fertility data of males, as summarized in table 2, substantiated the initial studies of Lillie and Menge [2] in that there was a significant reduction in the average fertility of males fed the linoleic acid-deficient diet (group 1).

As shown in table 3, the percentage of fertile eggs laid by hens inseminated with semen from group 1 deficient males fell below ninety on day 4 as contrasted with day 9 for group 2 and day 10 for group 3. The fertilizing capacity was twice as great for the group 2 and 3 males as for the group 1 males by day 16.

Analysis of semen lipids from group 1 exhibited a fatty acid profile indicative of an essential fatty acid deficiency (table 4). This was noted in the increased percentages of palmitoleic, oleic and eicosatrienoic acids with a concomitant decrease in linoleic and arachidonic acids when com-

[2] See footnote 1.

TABLE 4

Fatty acid analysis of semen

Fatty acid	Diet		
	Linoleic acid-deficient (group 1)	Linoleic acid-supplemented (group 2)	Corn-soy (group 3)
	%	%	%
Unknown	1.76	2.02	1.45
14:0	0.33	0.29	0.29
Unknown	3.37	3.85	3.95
16:0	12.09	13.25	14.00
16:1	1.18	0.51	0.72
Unknown	2.37	3.30	2.35
18:0	18.28	20.19	19.39
18:1	20.77	13.32	14.25
18:2	0.15	4.18	4.09
Unknown	0.36	0.47	0.11
18:3	5.48	3.15	3.28
Unknown	0.75	0.94	1.25
20:3 [1]	8.24	0.00	0.00
20:4	2.14	8.61	8.97
22:2 [2]	18.91	2.52	2.99
22:4 [2]	3.85	23.03	22.43

[1] The 20:3 acid is tentatively identified as the 5,8,11-isomer of eicosatrienoic acid.
[2] Tentative identification.

TABLE 5

Fatty acid analysis of blood plasma

Fatty acid	Diet					
	Linoleic acid-deficient (group 1)		Linoleic acid-supplemented (group 2)		Corn-soy (group 3)	
	23 weeks	47 weeks	23 weeks	47 weeks	23 weeks	47 weeks
	%	%	%	%	%	%
16:0	22.04	20.63	26.00	16.31	21.34	17.99
16:1	6.20	4.57	1.71	tr	0.57	tr
Unknown	1.45	1.47	1.78	1.80	2.57	2.94
18:0	14.49	14.44	17.87	16.71	18.71	17.40
18:1	41.45	41.18	22.07	12.99	16.85	10.76
18:2	0.25	0.13	17.39	36.83	26.59	37.66
18:3	0.19	0.29	tr	tr	0.71	0.13
20:3 [1]	7.78	10.06	—	—	—	—
20:4	0.32	tr	6.89	8.28	6.89	7.80

[1] The 20:3 acid is tentatively identified as the 5,8,11-isomer of eicosatrienoic acid.

pared with the analyses of groups 2 and 3. Unsaturated fatty acids tentatively identified as docosadienoic and docosatetraenoic were found in the semen lipids from all 3 groups. The docosadienoic was found in group 1 deficient semen in a concentration approximating 6 times that noted in the nondeficient groups 2 and 3. In contrast, the percentage of docosatetraenoic in the semen lipid of the deficient group was approximately one-sixth of that observed in the nondeficient groups. The significance of these observations cannot be explained at this time.

Fatty acid analysis of plasma lipids (table 5) indicated that the males in group 1 were effectively depleted of linoleic acid when compared with the analysis of the plasma lipids from groups 2 and 3.

DISCUSSION

The data clearly demonstrate that the fertilizing capacity of the male, as measured by the decline in number of fertile eggs after a single insemination, was decreased by linoleic acid deficiency. The reduction in fertility was due to factors other than semen volume, sperm motility

and sperm count since these characteristics were essentially the same for all groups. If weekly inseminations had been used, the effect of dietary treatment on the decrease in fertilizing capacity of the sperm would not have been established. A plausible explanation for the decrease in fertilizing capacity may be attributed to the low linoleic acid content of the sperm from deficient males. Further research is needed to explore the effect of this deficiency on sperm metabolism within the oviduct of the hen.

The overall average of 1.34 and 0.37 cm^3 of semen volume reported by Parker and McCluskey (6) and Crawford and Proudfoot (7), respectively, was higher than the semen volume obtained for all groups in this study. Edwards (2) was unable to obtain semen from deficient males at 24 weeks of age, even though the linoleic acid-supplemented males responded in a normal manner.

The data of Ahluwalia et al. (8) indicated an increase of eicosatrienoic and a decrease in the members of the linoleate family in testes of rabbits fed an essential fatty acid-deficient diet. These results substantiate our data with semen of the chicken. This paper is believed to be the first to present a complete fatty acid profile of the semen of any species of animal fed different diets.

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ERRATUM

Tollenaar, D. 1966 Effects of mineral and vitamin supplementation on swimming times and other parameters related to performance of rats on a low calorie regimen. J. Nutr., *90:* 441. On page 444, column 1, line 16, the statement, "Analysis of variance (29) indicated that the nonsupplemented groups . . . ," should have read: "Analysis of variance (29) indicated that during period 1 the nonsupplemented groups . . ."

To correct this omission in your copy of volume 90, number 4, please cut along lines of reprinted section below and paste at the bottom of page 444.

Column 1, lines 16 and 17, should read: Analysis of variance (29) indicated that during period 1 the nonsupplemented groups . . .

ERRATUM

Miller, W. J., Y. G. Martin, R. P. Gentry and D. M. Blackmon 1968 ^{65}Zn and stable zinc absorption, excretion and tissue concentrations as affected by type of diet and level of zinc in normal calves. J. Nutr., *94:* 391. The unit of measurement in table 5 should have read, *% of absorbed ^{65}Zn dose/kg fresh tissue,* instead of *% of absorbed ^{65}Zn dose/g of Zn.*

To correct this error in your copy of volume 94, number 3, page 397, table 5, please cut out the unit of measurement printed below and paste over *% of absorbed ^{65}Zn dose/g of Zn.*

% of absorbed ^{65}Zn dose/kg fresh tissue
